UNIVERSITE DE LA ROCHELLE

UFR «Sciences fondamentales et sciences pour l'ingénieur»

HABILITATION A DIRIGER DES RECHERCHES

Mémoire présenté par

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«Tenter de maîtriser les maladies infectieuses chez les mollusques : une clé pour une aquaculture durable»

Jury

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Tenter de maîtriser les maladies infectieuses chez les mollusques : une clé pour une aquaculture durable

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Remerciements

Ce mémoire cherche à être une synthèse de vingt ans de vie professionnelle. L'exercice est ardu dans la mesure où les travaux de recherche ne peuvent exister que par le fait de rencontres et d'échanges. Ils ne sont en aucun cas le fruit d'un seul individu. Ce que je rapporte ici n'a été possible que grâce à l'implication d'un grand nombre de personnes qu'il me sera impossible de toutes remercier. Cependant, je ne peux pas laisser dans l'ombre certaines d'entre elles qui au hasard des rencontres m'ont permis de faire le chemin de manière très agréable et surtout avec passion. Ne jamais avoir l'impression de travailler, de faire ce que l'on aime, est une chance inouïe.

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Maurice Héral qui connaît mon activité de longue date au sein de l'Institut et qui a accepté de faire partie du jury malgré ses très nombreuses responsabilités ;
Jean-Robert Bonami qui lui aussi suit mon activité avec intérêt et qui est un exemple à suivre en terme de virologie chez les invertébrés marins.

A Carole et à Juliette, mes compagnes de chemin.

A mes parents sans qui rien de tout cela ne serait.

A Hélène et Olivier, ma fratrie.

1. Présentation du candidat

1.1. Etat civil

RENAULT Tristan Charles

Né le 15 juin 1962 à Ivry sur Seine (France)
Nationalité française
Vie maritale - Un enfant

1. 2. Diplômes et formations complémentaires

1. 2. 1. Diplômes

♦ Docteur Vétérinaire (1988) (obtention d'une allocation de spécialisation attribuée par le Ministère de l'Agriculture), lauréat de l'Université P. Sabatier de Toulouse et lauréat de l'Académie Vétérinaire de France

♦ Complément de formation, Maîtrise de Biochimie (Université Paris VII) (1987)

♦ Titulaire du DEA d'Immunologie d'Ile de France (Université Paris VII) (1988)

♦ Diplômé du Cours d'Immunologie Approfondie (1988), du Cours de Génie Génétique (1993) et du Cours de Microscopie Electronique (1994) de l'Institut Pasteur (Paris)

♦ Diplômé du Cours de Méthodologie Statistique (1998) de l'Université Louis Pasteur, (Strasbourg)

1. 2. 2. Formations complémentaires

♦ Formation à l'utilisation du microscope électronique à transmission JEOL JEM 1200 EX, 1992 (Société JEOL, 2 jours)

♦ Formation à l'utilisation de la PCR, Institut Pasteur, Paris, 1993 (2 semaines)

♦ Formation interne « Gestion à l'Ifremer », Ifremer, Centre de Nantes, juin 1993

♦ Hybridation *in situ* en microscopie photonique, Institut Pasteur, Lyon, février 1996 (1 semaine)

♦ Formation Expérimentation Animale niveau II, Ecole Nationale Vétérinaire de Nantes, décembre 1995 (1 semaine)

♦ Formation « Compétence à la radioprotection », APAVE de l'Ouest, Saint Herblain, juin 1996 (1 semaine)

♦ Formation Expérimentation Animale niveau I, Ecole Nationale Vétérinaire de Nantes, janvier 1997 (1 semaine)

♦ PCR *in situ*, Université Claude Bernard Lyon I, Lyon, 1997 (17 au 21 novembre)

♦ Stage de formation aux risques chimiques, Ifremer, Centre de Nantes, 1998 (1 journée)

♦ Cours de Méthodologie Statistique (3 modules), Université Louis Pasteur, Strasbourg (10 au 12 décembre 1997, 2 au 5 février 1998 et 27 au 30 avril 1998)

♦ Analyse d'image et microscopie quantitative en biologie et en médecine, Université Claude Bernard Lyon I, Centre Commun de Quantimétrie, Lyon, 1999 (11 au 15 octobre)

♦ Formation à l'utilisation du logiciel Expo Analyse et acquisition pour la cytométrie en flux, Beckman Coulter France, Formation Groupe Diagnostics, Villepinte, 1999 (21 au 23 décembre)

♦ Formation au montage de projets européens, 2001 (3 jours)

♦ Formation interne en Démarche Qualité, Ifremer, La Tremblade, 2003 (1 journée)

♦ Formation des responsables de thèmes et de programmes Ifremer, Centre de Nantes, 2004 (1 journée, Collège de Polytechnique)

♦ Formation à l'entretien annuel d'appréciation, Ifremer, Centre de Nantes, 2004 (1 journée, Cohérence)

♦ Formation en gestion de projets, Ifremer, Centre de Nantes, 2005 (3 jours, Collège de Polytechnique)

♦ Formation en épidémiologie, Ifremer, La Tremblade, 2006 (3 jours)

♦ Formation à l'utilisation du microscope électronique à transmission JEOL JEM 1011, décembre 2006 (1 journée, Société JEOL)

♦ Formation à l'utilisation de l'ultramicrotome Leica EM UC6, 15 décembre 2006 (Société LEICA, 3 heures)

1. 3. Cursus professionnel

1. 3. 1. Emploi occupé aujourd'hui

Employeur	Ifremer (Institut Français de Recherche pour l'Exploitation de la Mer)
Emploi occupé	♦ Cadre de recherche II ♦ Directeur du Département « Amélioration Génétique, Santé Animale et Environnement » ♦ Responsable du Laboratoire Génétique et Pathologie ♦ Chef du projet « Pathogènes en aquaculture et interactions hôte/environnement »
Adresse professionnelle	Ifremer, Laboratoire de Génétique et Pathologie, 17390 La Tremblade, France, tél: 33 (0)5 46 76 26 49, fax: 3 (0)5 46 76 26 11, E-mail: trenault@ifremer.fr

1. 3. 2. Expériences professionnelles

♦ *1985-1986:* Moniteur de travaux pratiques en chaire d'anatomie-pathologie - histologie, Ecole Nationale Vétérinaire de Toulouse

♦ *1987:* Recrutement au titre de main d'oeuvre occasionnelle, Laboratoire d'Ichthyopathologie, station INRA Thiverval-Grignon

♦ *Décembre 1988 à mars 1990:* Volontaire à l'Aide Technique (VAT), Laboratoire de Pathologie, Ifremer, Centre Océanologique du Pacifique, Tahiti, Polynésie Française

♦ *Mai 1990 à août 1991:* Contrat à durée déterminé en tant que chercheur, responsable du Laboratoire de Pathologie, Ifremer, Centre Océanologique du Pacifique, Tahiti, Polynésie Française

♦ *1992-1996:* Responsable d'une unité de recherche (Unité de Recherche en Pathologie et Immunologie Générales, Laboratoire de Génétique, Aquaculture et Pathologie, Station Ifremer de Ronce les Bains, Charente Maritime, France)

♦ *1996-2001:* Chercheur au sein du Laboratoire de Génétique et Pathologie (Station Ifremer de Ronce les Bains, Charente Maritime, France)

♦ *2001-2004:* Responsable du pôle Recherche en Pathologie du Laboratoire de Génétique et Pathologie (Station Ifremer de Ronce les Bains, Charente Maritime, France)

♦ *2003-2004:* Animateur thématique Pathologie du Département Ressources Aquacoles (Direction des Ressources Vivantes, Ifremer)

♦ *Depuis janvier 2004:* Responsable du projet « Pathogènes en aquaculture »

♦ *Depuis janvier 2005:* Directeur du Département « Amélioration Génétique, Santé Animale et Environnement » et Responsable du Laboratoire Génétique et Pathologie (Station Ifremer de La Tremblade, Charente Maritime, France)

1. 4. Compétences

1. 4. 1. Domaines de compétence

Santé animale, pathologie générale, pathologie des invertébrés marins, virologie, immunologie, bactériologie, protistologie, microscopies, biologie moléculaire, cytométrie en flux, écotoxicologie

1. 4. 2. Compétences techniques

♦ ***Histologie.*** Techniques d'histologie, d'histochimie et d'immunohistochimie

♦ ***Microscopie électronique à transmission.*** Techniques classiques de préparation des échantillons et de marquage à l'or colloïdal

♦ ***Biologie moléculaire.*** Purification et clonage, dot et Southern-blot, PCR, séquençage d'ADN, analyse de séquences, hybridation *in situ*, hybridation soustractive

♦ ***Culture cellulaire.*** Cultures cellulaires (lignées de mammifères, poissons, insectes et mollusques), production et titration de virus, tests de polluants

♦ ***Méthodes immunologiques.*** Production et caractérisation d'anticorps polyclonaux et monoclonaux, immunodiffusion, immunofluorescence, immunohistochimie, western-blotting, marquage à l'or colloïdal (technique d'immunogold), électrophorèse en gel d'agarose, caractérisation de glycoprotéines à l'aide de lectines

♦ ***Purification de parasites protozoaires et de virus***

♦ ***Pathologie expérimentale*** (bactéries, virus et parasites)

♦ ***Cytométrie en flux***

1. 5. Principales réalisations

♦ Implication dans de nombreux programmes de recherche nationaux et internationaux

♦ Participation à différents contrats européens (Contrat CEE 1530 PCF (Study to be carried out in the aquaculture sector on the transmission of the pathogenic agents *Bonamia ostraea* and *Marteilia refringens*), ROMEO, DISENV, VINO, PLUDAMOR, AVINSI, PANDA, AQUAFIRST, EROCIPS et WOPER[1]) avec la fonction de coordinateur pour trois d'entre eux : VINO, AVINSI et PLUDAMOR

♦ Encadrement d'équipes et encadrement scientifique (plus de 50 étudiants dont 13 doctorants)

♦ Gestion de la recherche (responsabilité de laboratoire et de département)

♦ Publications (plus de 75 dans des revues à comité de lecture) et présentations des travaux de recherche (plus de 110) à des congrès nationaux et internationaux

♦ Développement d'outils de diagnostic utilisés aujourd'hui dans différents laboratoires

♦ Implication dans la mise en place (1992) et le fonctionnement du Réseau Pathologie des Mollusques (REPAMO) de l'Ifremer

♦ Expertises dans le domaine de la pathologie et de l'immunologie des invertébrés marins (expert pour le Conseil International pour l'Exploration de la Mer (CIEM), expert auprès de l'Afssa (Agence Française de Sécurité Sanitaire des Aliments), expert auprès de l'European Food Safety Authority (EFSA), expert auprès de la Commission Européenne pour l'évaluation de projets de recherche (FP5, FP6 et FP7), expert pour l'Agence d'Evaluation de la Recherche et de l'Enseignement Supérieur (AERES)), revues d'articles et de projets de recherche, …

♦ Enseignement (Ecoles Nationales Vétérinaires, Affaires Maritimes, Master, DEA, Maîtrise, Licence, IUT, …)

1. 6. Encadrement/co-encadrement de doctorants et de stagiaires

Depuis 1990, encadrement scientifique de nombreux étudiants ayant suivi des cursus de formation différents (thèses, DEA, maîtrise, Master, EPHE, IUT, …)

1. 6. 1. Thèses : 8 *(entre parenthèses est indiqué le nombre de publications issus du travail de thèse auxquelles mon nom est associé)*

◆ LE DEUFF Rose-Marie 1992/1995 (bourse Conseil Général Charente Maritime). « Contribution à l'étude de virus de mollusques marins apparentés aux *Iridoviridae* et aux *Herpesviridae »* - Soutenance le 21 décembre 1995 *(6 publications)*

1: ROMEO: **R**esearch **O**bjective: **M**ortality in **E**uropean **O**ysters / E. C. n° FA-S2 9052 ; DISENV : Environmental Factors and Shellfish Diseases / FAIR-CT98-4129 ; VINO: **V**irus **I**nfections in **O**ysters : Diagnosis of oyster herpes-like virus infections : development and validation of molecular, immunological and cellular tools / FAIR-CT986434 ; PLUDAMOR : **Plu**ridisciplinary **A**pproach for **M**ollusc **R**esearch / QLK5-CT-2000-60036 ; AVINSI: Antiviral innate immnity in cultured aquatic species / QLK2-CT2002-01691 ; PANDA: Permanent network to strengthen expertise on infectious diseases of aquaculture species and scientific advice to EU polcy, FP6, contrat n° 502329 ; AQUAFIRST: Combined and functionnal genomic approaches for stress and disease resistance marker-assisted selection in fish and shellfish, FP6, contrat n° 513692 ; EROCIPS: Emergency Response to Coastal Oil, Chemical and Inert Pollution from Shipping, FP6 ; WOPER: **Wo**rkshop for the analysis of the impact of **Per**kinsosis to the European shellfish industry, FP6, contrat n° 044442

◆ XUE Qinggang, 1995/1998 (bourse de l'Ambassade de France, Chine). « Caractérisation morphologique et fonctionnelle des hémocytes chez l'huître plate, *Ostrea edulis* », Université de Bretagne Occidentale - Soutenance le 14 décembre 1998 *(5 publications)*

◆ ARZUL Isabelle, 1998/2001 (bourse 100% Ifremer). « Herpès virus infectant les bivalves marins : détection, génome et transmission », Université de Montpellier II - Soutenance le 29 novembre 2001 *(5 publications)*

◆ OLICARD Cécile, 2000/2003 (bourse 100% Région Poitou-Charentes). « Mécanismes anti-viraux développés par les huîtres lors d'infections à virus de type herpès », Université de La Rochelle (co-encadrement Pr. N. BOURGOUGNON, Université de Vannes) - Soutenance le 25 mars 2004 *(2 publications)*

◆ GAY Mélanie, 2001/2004 (bourse co-financée 50% Ifremer / 50% Région Poitou-Charentes). « Vibrions pathogènes de *Crassostrea gigas :* agonisme et antagonisme ». Direction de thèse assurée par le Dr A. M. Pons (Université de La Rochelle) - Soutenance le 10 décembre 2004 *(1 publication)*

◆ GAGNAIRE Béatrice, 2002/2005 (bourse co-financée 50% Ifremer / 50 % Région Poitou-Charentes). « Etude des effets de polluants sur les paramètres hémocytaires de l'huître creuse, *Crassostrea gigas* / Interactions entre environnement, mécanisme de défense et maladies infectieuses » - Soutenance le 9 décembre 2005 *(5 publications)*

◆ BADO-NILES Anne (bourse co-financée 50% Cèdre / 50% Région Poitou-Charentes, co-encadrement avec H. Thomas-Guyon de l'Université de La Rochelle et S. Le Floch du CEDRE/Brest). « Etude des effets induits de pollutions chroniques et accidentelles par des hydrocarbures aromatiques polycycliques sur le système immunitaire de l'huître creuse et du bar commun » - Soutenance prévue fin 2008

◆ MORGA Benjamin, 2006/2009 (bourse co-financée 50% Ifremer / 50 % Région Poitou-Charentes) - Directeur de thèse

1. 6. 2. Doctorants étrangers : 5 *(entre parenthèses est indiqué le nombre de publications issus du travail de thèse auxquelles mon nom est associé)*

◆ SMOLARZ Kasia, 2002 (6 mois). « Détection d'une néoplasie branchiale chez *Macoma balthica* au moyen de la cytométrie en flux », étudiante en 3^{e} année de thèse, Université de Gdansk, Pologne (étudiante polonaise accueillie au Laboratoire de Génétique et Pathologie (LGP, Ifremer, La Tremblaie, France) dans le cadre du site Marie Curie PLUDAMOR, co-encadrement avec le Pr. M. Wolowicz) *(4 publications)*

◆ BARBOSA SOLOMIEU Valérie, 2002/2004 (18 mois). « Infections virales des coquillages d'intérêt économique », étudiante en 2^{e} et 3^{e} années de thèse, CIBNOR, La Paz, Mexique *(2 publications)*

◆ BATISTA Frédérico, 2004/2005 (12 mois). « Etude de l'huître portugaise, *Crassostrea angulata* : approche en génétique et approche en pathologie ». Etudiant portugais accueilli au LGP dans le cadre du site Marie Curie PLUDAMOR *(2 publications)*

◆ FOSCHI Jurgen, 2006 (3 mois) « Etude des interactions entre polluants et système immunitaire chez l'huître creuse, *Crassostrea gigas* et l'huître plate, *Ostrea edulis* ». Etudiant italien accueilli au LGP dans le cadre du site Marie Curie PLUDAMOR

◆ DIAZ Seila, 2006 (1 mois) « Etude des cellules néoplasiques chez la coque au moyen de la cytométie en flux ». Etudiante en 3e année de thèse, Saint Jacques de Compostelle, Espagne

1. 6. 3. DEA et Master : 6

◆ ARZUL Isabelle, 1998. « Transmission expérimentale du virus de type herpès chez l'huître creuse, *Crassostrea gigas* ». DEA Océanologie Biologique, Université de Bretagne Occidentale

◆ GAGNAIRE Béatrice, 2001. « Etude de l'impact de micro polluants sur les capacités immunitaires de l'huître creuse, *Crassostrea gigas*, *in vivo* et *in vitro* ». DEA Océanologie Biologique et Environnement Marin, Université Pierre et Marie CURIE, Paris VI (co-encadrement Dr. H. THOMAS-GUYON, Université de La Rochelle)

◆ VIGNERON Vassilia, 2002. « Détection et étude de la stabilité de l'ADN de virus de type herpès infectant les huîtres dans des échantillons d'eau ». DEA Chimie et Microbiologie, Université de Nancy (co-encadrement Dr. H. MONTANIE, Université de La Rochelle)

◆ ATON Emilie, 2003. « Etude des mouvements calciques dans les hémocytes de l'huître creuse, *Crassostrea gigas* : premières approches expérimentales ». DEA Interactions cellulaires et transferts membranaires, Université de Poitiers (co-encadrement Dr. N. IMBERT, Université de La Rochelle)

◆ AMBERT Xavier, 2005. « Etude de l'homéostasie calcique dans les hémocytes chez l'huître creuse, *Crassostrea gigas*, par cytométire en flux ». DEA National de Toxicologie et d'Ecophysiologie, Université Paris V (co-encadrement Dr. N. IMBERT, Université de La Rochelle)

◆ MOREAU Kévin, 2005. « Approche moléculaire des effets de polluants sur l'infection à virus OsHV-1 (Ostreid Herpes virus 1) chez l'huître creuse, *Crassostrea gigas*. Master du Muséum d'Histoires Naturelles, Parcours Toxines et Ecosystèmes, Paris

◆ ORY Pascaline, 2006. « Clonage du gène de la prophénoloxidase chez l'huître creuse, *Crassostrea gigas* », Master 2 EDEL, Université de La Rochelle (co-encadrement Dr. C. BARTHELEMY et Dr. H. THOMAS-GUYON, Université de La Rochelle)

1. 6. 4. Maîtrise : 5

◆ HUBERT Cyrille, 1992. « Techniques histologiques ». Maîtrise de Biologie des Organismes et des Populations, Université de Rennes I. Août 1992

◆ BIRAUD Valérie, 1996. « Application de reconnaissance et de comptage par imagerie numérique des différents types hémocytaires présents dans l'hémolymphe de l'huître plate, *Ostrea edulis »* Maîtrise de Génie Informatique, Institut Universitaire Professionnalisé, La Rochelle. Février-juin 1996

◆ SEGRETIN Angélique, 1997. « Caractérisation des types hémocytaires dans l'hémolymphe de l'huître plate, *Ostrea edulis*, par classification robuste ». Maîtrise de Génie Informatique, Institut Universitaire Professionnalisé, La Rochelle. Février-juin 1997

◆ AMBERT Xavier, 2004. « Première approche des mouvements calciques dans les hémocytes chez l'huître creuse, *Crassostrea gigas*, par cytométrie en flux ». Maîtrise de Biologie des Populations, Université de La Rochelle. Mai-juin 2004

◆ MOREAU Kevin, 2004. « Etude de l'immunité antivirale chez l'huître creuse, *Crassostrea gigas :* identification de gènes viro-induits par la technique de SSH ». Maîtrise de Biologie des Populations, Université de La Rochelle. Mai-septembre 2004

1. 6. 5. Licence : 2

◆ EPAUD Cécilia, 2000. « Recherche dans des échantillons d'eau de claires ostréicoles d'ADN du virus de type herpès de l'huître ». Université de La Rochelle

◆ BIGNON Nicolas, 2006. « Etude de l'homéostasie calcique dans les hémocytes d'huître creuse, *Crassostrea gigas*, en cytométrie de flux ». Université de La Rochelle

1. 6. 6. EPHE : 4

◆ COCHENNEC Nathalie, 1995/1996. « La bonamiose : étude du parasite *Bonamia ostreae* et des mécanismes cellulaires de défense mis en jeu par l'huître plate vis à vis de ce parasite ». Professeur EPHE responsable : Y. BENYAMIN

◆ DENIAU Stéphane, 1998/2000. « Essais de propagation *in vitro* de virus de type herpès infectant les mollusques marins et contribution à l'étude des interactions hôte-pathogène ». Professeur EPHE responsable : A. VAN WORMOUTH

◆ DIDIER Yohann, 2000/2002. « Mécanismes anti-viraux développés par l'huître creuse, *Crassostrea gigas*, vis à vis des virus de type herpès ». Professeur EPHE responsable : A. VAN WORMOUTH

◆ SOLLIEC Gaëlle, 2001/2003. « Recherche dans l'eau de mer d'ADN d'herpès virus infectant les bivalves ». Professeur EPHE responsable : M. BERGOUIN

1. 6. 7. DES : 2

◆ GAGNAIRE Béatrice, 2002. « Environnement et immunomodulation : étude d'activités hémocytaires chez l'huître creuse, *Crassostrea gigas* », Université Pierre et Marie CURIE, PARIS VI (Pr. P. NIVAL)

◆ ORY Pascaline, 2006. « Essais de clonage du gène de la prophénoloxidase chez l'huître creuse, *Crassostrea gigas,* par une approche de PCR ». Université de Bordeaux II (Pr. CAPDEPUY)

1. 6. 8. IUT (La Rochelle, Génie Biologique, 2e année, stage de trois mois) : 7

◆ TIBI Isabelle, 2000. « Mise en évidence d'une activité de type phénoloxydase chez l'huître creuse, *Crassostrea gigas* », (co-encadrement Dr. H. THOMAS-GUYON, Université de La Rochelle)

◆ DIDIER Yohann, 2000. « Apoptose et infection à virus de type herpès chez les bivalves marins : détection de séquences codant pour des protéines inhibitrice de l'apoptose », (co-encadrement Dr. I. ARZUL, Ifremer, LGP La Tremblade)

◆ SOLLIEC Gaëlle, 2001. « Recherche dans des échantillons d'eau de claires d'ADN de virus de type herpès infectant les bivalves marins », (co-encadrement Dr. H. MONTANIE, Université de La Rochelle)

◆ MOREAU Kévin, 2002. « Etude en cytométrie en flux de l'influence de la température et de l'émersion sur les fonctions hémocytaires de l'huître creuse, *Crassostrea gigas* »

◆ BRUNETIERE Claire, 2004. « Etude de l'immunité antivirale chez l'huître creuse, *Crassostrea gigas :* production de larves axéniques, culture d'hémocytes et extraction d'ARN, clonage et Dot Blot »

◆ COLLAS Lucille, 2005. « Essais de reproduction d'infection expérimentale à OsHV-1 (Ostreid Herpes virus 1) chez l'huître creuse, *Crassostrea gigas* »

◆ ASSANY Mamoun, 2006. « Caractérisation moléculaire d'échantillons archivés d'huître creuse, *Crassostrea gigas*, et d'huître portugaise, *C. angulata* : étude génétique et de pathologie »

1. 6. 9. BTS : 1

◆ GUICHARD Sonia, 1994. « Analyse électrophorétique de lysat de protozoaire, *Bonamia ostreae*, parasite des hémocytes de l'huîte plate, *Ostrea edulis* ». BTS Biochimie 2ème année, Lycée Polyvalent R.-J. Valin, La Rochelle

1. 6. 10. Post BTS : 5

◆ LIPART Cécile, 1993. « Contribution à l'étude d'agents pathogènes de l'huître creuse, *Crassostrea gigas* - Histologie classique, microscopie électronique à transmission et culture cellulaire ». Post BTS Lycée Saint Louis Bordeaux. Mars-juin 1993

◆ FLAUJAC Géraldine, 1994. « Mise au point d'une méthode de primoculture de cellules cardiaques d'huître plate, *Ostrea edulis* ». Post BTS Lycée Saint Louis Bordeaux. Mars-juin 1994

◆ PERROQUIN Magalie, 1995. « Mise au point d'une méthode de primoculture d'hémocytes d'huître plate, *Ostrea edulis* ». Post BTS Lycée Saint Louis Bordeaux. Mars-juin 1995

◆ PARTHUISOT Nathalie, 1996. « Infestation des hémocytes par le parasite *Bonamia ostreae* : cytologie comparative *in vitro* ». Post BTS Lycée Saint Louis Bordeaux. Mars-juin 1996

◆ DENOIX Mikaël, 1997. « Approche *in vitro* et *in vivo* des interactions hémocytes/parasites *Bonamia ostreae*, chez l'huître plate, *Ostrea edulis* ». Post BTS Lycée Saint Louis Bordeaux. Mars-juin 1997

1. 6. 11. Ecoles Nationales Vétérinaires et Faculté des Sciences Pharmaceutiques: 5

◆ DESCHANDELLIERS Séverine, 1993. « *Bonamia ostreae*, parasite de l'huître plate : connaissances générales et techniques de purification ». Ecole Nationale Vétérinaire de Nantes, 2ème année

◆ SORDET Fabien, 1994. « Anatomie et physiologie de l'huître - Bonamiose et *Bonamia ostreae* ». Faculté des Sciences Pharmaceutiques et Biologiques, 4ème année, Université R. Descartes, Paris V. Juillet-août 1994

◆ VIEILLARD Sebastien, 1994. « Analyse électrophorétique de lysat de protozoaire, *Bonamia ostreae*, parasite des hémocytes de l'huîte plate, *Ostrea edulis* ». Ecole Nationale Vétérinaire de Nantes. Avril-mai 1994

◆ SEVERIN Patricia, 1996. « Technique de PCR appliquée à la détection du virus de type herpès de l'huître creuse, *Crassotrea gigas* : diagnostic et applications expérimentales ». Ecole Nationale Vétérinaire de Nantes - Formation en Aquaculture et Pathologie Aquacole. Mars-juin 1996

◆ ARZUL Isabelle, 1997. « Essais de transmission de l'infection à virus de type herpès chez les huîtres creuses, *Crassotrea gigas »*. Ecole Nationale Vétérinaire de Nantes - Formation en Aquaculture et Pathologie Aquacole. Mars-juillet 1997

1. 7. Productions sous forme d'articles, de rapports, de valorisation ou de vulgarisation

1. 7. 1. Diplômes

◆ ***1998 :*** Doctorat de Médecine Vétérinaire. « La thermodépendance de la cinétique de synthèse de l'interféron *in vivo* chez la truite arc en ciel, *Salmo gairdneri* Richardson »

◆ ***1998 :*** DEA. « Structure, expression cellulaire, activité et spécificité des immunoglobulines de truite arc en ciel, *Salmo gairdneri* Richardson »

1. 7. 2. Publications dans des revues à comité de lecture (78 listées par ordre alphabétique)

1. Arzul I. & **T. Renault** (2002). Herpèsvirus et bivalves marins. *Virologie* 6: 169-174.
2. Arzul I., **Renault T.** & C. Lipart (2001). Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. *Diseases of aquatic Organisms* 46(1): 1-6.
3. Arzul I., Nicolas J. L., Davison A. J. & **T. Renault** (2001). French scallops: a new host for Ostreid herpes virus 1. *Virology* 290: 342-349.
4. Arzul I., **Renault T.**, Lipart C. & A. J. Davison (2001). Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *Journal of General Virology* 82: 865-870.
5. Arzul I., **Renault T.**, Thébault A. & A. Gérard (2002). Detection of oyster herpes virus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Research* 84: 151-160.
6. Aton E., **Renault T.**, Gagnaire B., Thomas-Guyon H., Cognard C. & N. Imbert (2006). A flow cytometric approach to study intracellular-free Ca^{2+} in *Crassostrea gigas* haemocytes. *Fish & Shellfish Immunology* 20: 493-502.
7. Barbosa-Solomieu V., Miossec L., Vazquez-Juarez R., Ascencio-Valle F. & **T. Renault** (2004). Diagnosis of Ostreid herpes virus 1 in fixed paraffin-embedded archival samples using PCR and *in situ* hybridization. *Journal of Virological Methods* 119: 65-72.
8. Barbosa-Solomieu V., Dégremont L., Vazquez-Juarez R., Ascencio-Valle F., Boudry P. & **T. Renault** (2005). Ostreid Herpes virus 1 (OsHV-1) detection among three successive generations of Pacific oysters (*Crassostrea gigas*). *Virus Research* 107: 47-56.
9. Batista F. M., Taris N., Boudry P. & **T. Renault** (2005). Detection of Ostreid Herpes virus 1 (OsHV-1) by PCR using a rapid and simple method of DNA extraction from oyster larvae. *Diseases of Aquatic Organisms* 64 (1): 1-4.
10. Batista F. M., Taris N., Boudry P. & **T. Renault** (2007). Detection of Ostreid Herpes virus 1 DNA in bivalve molluscs: a critical review. *Journal of Virological Methods* 139(1): 1-11.

11. Bouilly K., Bonnard M., Gagnaire B., **Renault T.** & S. Lapègue (2007). Impact of diuron on aneuploidy and hemocyte parameters in Pacific oyster, *Crassostrea gigas*. *Archives of Environmental Contamination and Toxicology* 52(1): 58-63.
12. Bouilly K., Gagnaire B., Bonnard M., Thomas-Guyon H., **Renault T.,** Miramand P. & S. Lapègue (2006). Effects of cadmium on aneuploidy and hemocyte parameters in the Pacific oyster, *Crassostrea gigas*. *Aquatic Toxicology* 78: 149-156.
13. Boussaïd B., Grippari J. L., **Renault T.**, Tigé G. & G. Dorange (1999). *Trichodina sp.* infestation of *Crassostrea gigas* oyster gills in Brittany, France. *Journal of Invertebrate Pathology* 73: 339-342.
14. Burge C. A., Judah L. R., Conquest L. L., Griffin F. J., Cheney D. P, Suhrbier A., Vadopalas B., Olin P. G., **Renault T.** & C. S. Friedman (2007). Summer seed mortalities of the Pacific oyster, *Crassostrea gigas* Thunberg, grown in Tomales Bay, California, USA: the influence of oyster stock, planting time, pathogens and environmental stressors. *Journal of Shellfish Research* 26 (1): 163-172.
15. Burgeot T., Quirion S., Quiniou F., Knoery J., Gagne F., Gagnaire B., **Renault T.**, Haure J., Moraga D., Osada M., Geret F. & P. Soletchnik (2006). Environmental stress of the Pacific oyster *Crassostrea gigas* during summer mortalities. *Marine Environmental Research* 62: S380-S381.
16. Choi D.-L., Sohn S.-G., Park M.-A., Heo M.-S. & **T. Renault** (1996). Detection of a mollicute-like organism in Kuruma shrimp, *Penaeus japonicus*. *Journal of fish Pathology* 9(1): 33-40.
17. Cochennec N., **Renault T.**, Boudry P., Chollet B. & A. Gérard (1998). *Bonamia*-like parasite found in the Suminoe oyster *Crassostrea rivularis* reared in France. *Diseases of aquatic Organisms* 34: 193-197.
18. Cochennec-Laureau N., Auffret M., **Renault T.** & A. Langlade (2003). Changes in circulating and tissue-infiltrating hemocyte parameters of the European flat oyster, *Ostrea edulis*, naturally infected with *Bonamia ostreae*. *Journal of Invertebrate Pathology* 83: 23-30.
19. Davison A. J., Trus B. L., Cheng N., Steven A. C., Watson M. S., Cunningham C. Le Deuff R. L. & **Renault T.** (2005). A novel class of herpesvirus with bivalve hosts. *Journal of General Virology* 86: 41-53.
20. Faucet J., Maurice E., Gagnaire B., **Renault T.** & T. Burgeot (2003). Isolation and primary culture of gill and digestive gland cells from the common mussels *Mytilus edulis*. *Methods in cell Science* 23: 177-184.
21. Gagnaire B., H. Thomas-Guyon & **T. Renault** (2004). *In vitro* effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas (Thunberg)*, haemocytes. *Fish & Shellfish Immunology* 16: 501-512.
22. Gagnaire B., Thomas-Guyon H., Burgeot T. & **T. Renault** (2006). Pollutant effects on Pacific oyster, *Crassostrea gigas* (Thunberg), hemocytes : screening of 23 molecules using flow cytometry. *Cell Biology and Toxicology* 22: 1-14.
23. Gagnaire B., Frouin H., Moreau K., Thomas-Guyon H. & **T. Renault** (2006). Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg*). Fish & Shellfish Immunology* 20: 536-547.
24. Gagnaire B., Gay M., Saulnier D. & **T. Renault** (2007). Combination of a pesticide exposure and a bacterial challenge: *In vivo* effects on immune response of Pacific oyster, *Crassostrea gigas* (Thunberg). *Aquatic Toxicology* 84: 92-102.

25. Gagnaire B., **Renault T.**, Bouilly K., Lapègue S. & H. Thomas-Guyon (2003). Study of atrazine effects on Pacific oyster, *Crassostrea gigas*, haemocytes. *Current Pharmaceutical Design* 9: 193-199.
26. Gagnaire B., Soletchnik P., Faury N., Kerdudou N., Le Moine O. & **T. Renault** (2007). Analysis of hemocytes parameters in Pacific oyster, *Crassostrea gigas*, reared in the field - Comparison of hatchery diploids and diploids from natural beds. *Aquaculture* 264: 449-456.
27. Gagnaire B., Soletchnik P., Madec P., Geairon P., Le Moine O. & **T. Renault** (2006). Diploid and triploid Pacific oyters, *Crassostrea gigas* (Thunberg), reared at two heights above sediment in Marennes-Oleron Basin, France: difference in mortality, sexual maturation and hemocyte parameters. *Aquaculture* 254: 606-616.
28. Gay M., **Renault T.**, Pons A.-M. & F. Le Roux (2004). Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. *Diseases of Aquatic Organisms* 62: 65-74.
29. Hellio C., Bado-Niles A., Gagnaire B., **Renault T.** & H. Thomas-Guyon (2007). Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) *in vitro*. *Fish & Shellfish Immunology*, 22 (4): 433-440.
30. de Kinkelin P., Dorson M. & **T. Renault** (1992). Interferon and viral interference in viroses of salmonid fish. Proceedings of the OJI International Symposium on Salmonid Diseases, Hokkaido University Press, Sapporo, Japan, 241-249.
31. Le Deuff R.-M. & **T. Renault** (1993). Lymphocystis outbreaks in farmed sea bream, *Sparus aurata*, first report on French Mediterranean coasts. *Bulletin of the European Association of Fish Pathologists* 13(4): 130-133.
32. Le Deuff R.-M. & **T. Renault** (1999). Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. *Journal of General Virology* 80: 1317-1322.
33. Le Deuff R.-M., Lipart C. & **T. Renault** (1994). Primary culture of Pacific oyster, *Crassostrea gigas* heart cells. *Journal of Tissue Culture Methods* 16(1): 67-72.
34. Le Deuff R.-M., **Renault T.** & N. Cochennec (1995). Antibodies specific for Channel Catfish Virus (CCV) cross-react with Pacific oyster, *Crassostrea gigas*, herpes-like virus. *Veterinary Research*, Special Issue: "Third International Symposium on Viruses of Lower Vertebrates " 26: 526-529.
35. Le Deuff R.-M., **Renault T.** & A. Gérard (1996). Thermal effects on herpes-like virus detection among hatchery-reared larval oyster, *Crassostrea gigas*. *Diseases of Aquatic Organisms* 24: 149-157.
36. Le Deuff R.-M., Nicolas J.-L., **Renault T.** & N. Cochennec (1994). Experimental transmission of herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. *Bulletin of the European Association of Fish Pathologists* 14(2): 69-71.
37. Lipart C. & **T. Renault** (2002). Herpes-like virus detection *in Crassostrea gigas* spat using DIG-labelled probes. *Journal of Virological Methods* 101: 1-10.
38. Naciri-Graven Y., Martin A.-G., Baud J.-P., **Renault T.** & A. Gérard (1998). Selecting the falt oyster *Ostrea edulis* (L.) for survival when infected with the parasite *Bonamia ostreae*. *Journal of Experimental Marine Biology and Ecology* 224: 91-107.
39. Mortensen S., Harkestad L. S., Stene R.-O. & **T. Renault** (2005). Picoeucaryot alga infecting blue mussel *Mytilus edulis* in Southern Norway. *Diseases of Aquatic Organisms* 63: 25-32.

40. Olicard C., Didier, Y., Marty C., Bourgougnon N. & **T. Renault** (2005). *In vitro* research of anti-HSV-1 activity in different extracts from Pacific oysters *Crassostrea gigas*. *Diseases of Aquatic Organisms* 67: 141-147.
41. Olicard C., **Renault T.**, Torhy C., Benmansour A. & N. Bourgougnon (2005). Putative antiviral activity in hemolymph from adult Pacific oysters, *Crassostrea gigas*. *Antiviral Research* 66: 147-152.
42. Pilet C. & **T. Renault** (1988). Thermodependance of synthetic kinetic of in vivo interferon in rainbow trout (*Salmo gairdneri* Richardson). *Bulletin de l'Académie Vétérinaire de France* 61 (2): 217-218.
43. **Renault T.** (1996). Appearance and spread of diseases among bivalve molluscs in the northern hemisphere in relation to international trade. *Revue scientifique et technique de l'Office International des Epizooties* 15(2): 551-561.
44. **Renault T.** (1998). Infections herpétiques chez les invertébrés : détection de virus de type herpès chez les mollusques bivalves marins. *Virologie* 2: 401-403.
45. **Renault T**. (2006). Les virus infectant les bivalves marins. *Virologie* 10: 35-41.
46. **Renault T.** & I. Arzul (2001). Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: specific viral DNA detection by PCR. *Journal of Fish Diseases* 24: 161-167.
47. **Renault T.** & N. Cohennec (1994). Rickettsia-like organisms in the cell cytoplasm of gill epithelial cells of the Pacific oyster *Crassostrea gigas*. *Journal of Invertebrate Pathology* 64: 160-162.
48. **Renault T.** & N. Cochennec (1995). Chlamydia-like organisms in ctenidia and mantle cells of the Japanese oysters, *Crassostrea gigas* from the French Atlantic coast. *Diseases of Aquatic Organisms* 23: 153-159.
49. **Renault T.** & C. Lipart (1998) Diagnosis of herpes-like virus infections in oysters using molecular techniques. *EAS Special Publication* 26: 235-236.
50. **Renault T.** & Novoa B. (2004). Viruses infecting bivalve molluscs. *Aquatic Living Resources* 17: 397-409.
51. **Renault T.**, Arzul I. & Lipart C. (2004). Development and use of an internal standard for oyster herpes virus 1 detection by PCR. *Journal of Virological Methods* 121: 17-23.
52. **Renault T.**, Cochennec N. & B. Chollet (1995). Marteiliosis in American oyster *Crassostrea virginica* reared in France. *Diseases of Aquatic Organisms* 23: 161-164.
53. **Renault T.**, Cochennec N. & H. Grizel (1995). *Bonamia ostreae*, parasite of the European flat oyster, *Ostrea edulis*, does not experimentally infect the Japanese oyster, *Crassostrea gigas*. *Bulletin of European Association of Fish Pathologists* 15(3): 78-80.
54. **Renault T.**, Flaujac G. & R.M. Le Deuff (1995). Cultivation of cells from the heart of the European flat oyster, *Ostrea edulis*. *Methods in Cell Science* 17: 199-205.
55. **Renault T.**, Lipart C. & I. Arzul (2001). A herpes-like virus infects a non-ostreid bivalve species : virus replication in *Ruditapes philippinarum* larvae. *Diseases of Aquatic Organisms* 45: 1-7.
56. **Renault T.**, Lipart C. & I. Arzul (2001). A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. *Journal of Fish Diseases* 24: 369-376.

57. **Renault T.**, Torchy C. & P. de Kinkelin (1991). Spectophotometric method for titration of trout interferon, and its application to rainbow trout fry experimentally infected with viral haemorrhagic septicemia virus. *Diseases of Aquatic Organisms* 10: 23-29.
58. **Renault T.**, Xue Q. G. & S. Chilmonczyk (2001). Flow cytometric analysis of European flat oyster, *Ostrea edulis*, haemocytes using a monoclonal antibody specific for granulocytes. *Fish & Shellfish Immunology* 11: 269-274.
59. **Renault T.**, Le Deuff R.-M., Lipart C. & C. Delsert (2000). Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. *Journal of Virological Methods*, 88: 41-50.
60. **Renault T.**, Chollet B., Cochennec N. & A. Gerard (2002). Shell disease in eastern oysters, *Crassostrea virginica*, reared in France. *Journal of Invertebrate Pathology* 79: 1-6.
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◆ Note d'information sur les infections à virus de type herpes pour le Tribunal de Grande Instance de Saint Nazaire (1997) - RG : 96 001 697, 5 pages

◆ Thébault A., Robert M., Renault T., Dumais M. & P. Goulletquer (2001). Impact de l'Erika sur la pathologie de bivalves d'intérêt commercial : résultats préliminaires. Rapport interne Ifremer (nov-2001-R.INT.DRV/RA/LGP/), 51 pages.

◆ Faucet J., Gicqueau A., Maurice M., Gagnaire B., Renault T. & T. Burgeot (2004). Etude de l'application du concept de TEQ (toxicité équivalente) chez la moule *Mytilus sp* par la mesure d'altérations primaires de l'ADN sur des primo cultures exposées aux hydrocarbures aromatiques polycycliques. Rapport interne Ifremer (avril-2004-R.INT.DEL/PC/2004.03/Nantes), 56 pages.

1. 7. 8. Sites Web

◆ Viral DNA detection - the key to healthy oysters (Detection of herpes-like viral DNA by PCR in European bivalve larval samples, Programme E. C. no FA-S2 9052, Cordis Technology Marketplace, http://www.cordis.lu/marketplace)

◆ Herpes viruses in Marine Bivalves (http://www.ifremer.fr/latremblade/, IFREMER laboratory located in La Tremblade, Charente Maritime, France)

◆ Site du projet européen AVINSI. «Anti-viral innate immunity in cultured aquatic species» (*A*nti *V*iral *I*nfection *N*on-*S*pecific *I*mmunity) - Contrat QLK2-CT-2002-01691, http://www.ifremer.fr/drvlgp/en/europeanporjects/Avinsi/avinsi.htm

◆ Newsletter 21. Geographical distribution of Oyster herpes virus (OsHV), Miossec L & **Renault T.**, 2006, http://www/dipnet

◆ Site web du Département "Amélioration génétique, Santé animale et Environnement", mis en place en 2006, http://www.ifremer.fr/agsae

◆ Mise à jour du site web du Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade) en 2006, http://www.ifremer.fr/drvlgp

1. 8. Avis et expertises

1. 8. 1. Participation à des groupes d'experts

- Membre du Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) dans le cadre du Conseil International pour l'Exploitation de la Mer (CIEM)

- Membre du Comité d'experts spécialisés Santé Animale (CES SA) de l'Agence Française de Sécurité Sanitaire des Aliments (Afssa) de 2003 à 2006

- Experts auprès de l'Agence Française de Sécurité Sanitaire des Aliments (Afssa) depuis 2006

- Membre du Comité de Programme du Département Ifremer « Ressources Aquacoles » (1997-1999)

- Membre du Comité de Programme « Durabilité des systèmes de productions aquacoles » (Ifremer) depuis 2004

- Membre du Comité du Thème « surveillance et optimisation des ressources aquacoles» (Ifremer), depuis 2005

- Membre de la Commission de Spécialistes CSE 08 Biologie de l'Université de La Rochelle depuis 2005

- Membre du Conseil Scientifique de l'Ecole Vétérinaire de Nantes depuis 2007

- Expert auprès de la Commission Européenne pour évaluation de projets dans le cadre du 5e PCRD, Quality of Life and Management of Living Resources (Key Action 2 : "Control of Infectious Diseases" et Key Action 5 : "Sustainable Agriculture, Fisheries and Forestry, du 6^{e} PC et du 7^{e} PC

- Groupes de travail, en présence ou non de professionnels, concernant les problèmes de mortalités d'huîtres dans les bassins conchylicoles et dans les écloseries

- Aide à la conception de protocoles expérimentaux du Syndicat Mixte pour le Développement de l'Aquaculture et de la Pêche des Pays de Loire (SMIDAP)

1. 8. 2. Rapports d'expertise

- Renault T., François C. & Azul I. (2006). Note d'information sur les infections à virus de type herpes pour le compte de la DGAl. « Virus de type herpès et bivalves marins ».

- Renault T. (2006). Rapport pour le compte de l'Afssa. Consultation sur un projet de décision d'application de la future directive relative aux conditions de police sanitaire applicables aux animaux d'aquaculture.

- Renault T. (2006). Rapport pour le compte de l'Afssa. Consultation sur un projet de modification d'un arrêté ministériel concernant l'allègement des conditions sanitaires d'importation des crustacés tropicaux d'ornement.

- Renault T. (2006). Rapport pour le compte de l'Afssa. Consultation sur l'annexe IX du projet d'arrêté relatif à la surveillance sanitaire et aux soins régulièrement confiés au vétérinaire pris en application du projet de décret relatif aux conditions de prescription et de délivrance au détail des médicaments vétérinaires.

1. 8. 3. Revue d'articles (56)

- Acta Zoologica Sinica : 3
- Aquaculture: 6
- Aquatic Toxicology: 3
- Aquatic Living Resources: 4
- Archives of Environmental Contamination and Toxicology: 2
- Comparative Immunology, Microbiology and Infectious Diseases: 1
- Developmental and Comparative Immunology: 11
- Diseases of Aquatic Organisms: 3
- Environmental Research: 1
- Fish & Shellfish Immunology: 9
- Journal of Fish Diseases: 8
- Journal of General Virology: 1
- Marine Biology: 1
- Microbes and Infections: 1
- Virologie: 1
- Water Research: 1

1. 8. 4. Evaluation de projets de recherche

- Evaluation d'un projet de recherche pour le compte de la Région Languedoc Roussillon (Direction de l'Enseignement Supérieur, de la Recherche et des Techniques de la Communication) en 1999
- Evaluation d'un projet de recherche dans la cadre de l'appel d'offre PNEC 2000 (Action de Recherche Thématique)
- Evaluation de projets de recherche pour le Woods Hole Oceanograpohic Institution Sea Grant Program (Woods hole, USA) (1 projet en 2001 et en 2007)
- Evaluation d'un projet de recherche pour le National Sea Grant College Program (University of Maine) en 2001, 2002 et en 2007
- Evaluation d'un projet de recherche pour l'AFSSA (Direction de la programmation des laboratoires) en 2001
- Evaluation du travail de thèse de V. Barbosa Solomieu, étudiante mexicaine en thèse au CIBNOR (La Paz, Mexique) en 2000 et 2001
- Evaluation d'un projet de recherche pour le compte de l'OFIMER (2003)
- Evaluation d'un projet de recherche pour le compte du Centre Franco-Indien pour le Promotion de La Recherche Avancée en 2003
- Evaluation de projets pour le GIP ANR (1 projet en 2005 et 2006)
- Evaluation d'un projet en virologie pour le compte de The Israel Science Foundation en 2006

1. 9. Formations dispensées

Une activité d'enseignement a été effectuée avec la réalisation de cours (DEA, IUT, Ecoles Vétérinaires). Les enseignements prodigués ont essentiellement porté sur les maladies chez les invertébrés (mollusques et crustacés) et leur contrôle ainsi que sur l'immunologie.

- Ecole Nationale Vétérinaire de Nantes, Option Aquaculture et Pathologie (4e année), 1996 à 2004 :
  - « Aperçu des maladies majeures des élevages et des méthodes de lutte en pénéiculture - Eléments pratiques de diagnostic pathologique », cours 3 heures
  - « Maladies majeures des élevages conchylicoles - Agents pathogènes, pathogénie, épidémiologie et méthodes de lutte », cours 3 heures
  - « Conduite du diagnostic pathologique en conchylicultue », TP 3 heures
- DEA Biologie et Productions Animales, Option Biologie Aquacole, Université de Rennes I, 1996 à 2003 : « Pathologies des mollusques bivalves », cours 3 heures
- DEA Exploitation Durable des Ecosystèmes Littoraux, Option Dynamique et Résistance des Ecosystèmes Littoraux et Côtiers : approches cellulaires et moléculaires des interactions environnement/hôtes/pathogènes. Université de La Rochelle, depuis 2002, cours : 3 heures.
- IUT de La Rochelle, cours et TD d'Immunologie (1ère et 2e année), 1997 à 2004 (18 heures de cours et 30 heures de TD)
- Université de La Rochelle, DEUG 2 Biotechnologies et Bio-industrie (IUP), cours d'immunologie, 2002 (8 heures de cours et 16 heures de TD)
- Stage de formation des techniciens de Cultures Marines (Groupe Ecole des Affaires Maritimes, Centre d'Instruction et de Documentation Administrative Maritime), Bordeaux, novembre 1996 et novembre 1999, « Pathologie des mollusques », cours 3 heures
- Organisation d'une formation interne Ifremer en pathologie, intitulée « Pathologie des mollusques », les 6 et 7 octobre 1999 au Laboratoire de Génétique et Pathologie de la station Ifremer de La Tremblade (Charente Maritime). Participation de 7 personnes
- Université de Teramo, Ecole de Médecine Vétérinaire, Italie, 1998 et 1999. « Pathogens in molluscs », cours de 4 heures
- Session de formation interne Ifremer au diagnostic de l'infection à virus de type herpès par PCR. La Tremblade, 23 et 24 avril 1996.
- Complément de formation à la technique PCR pour G. Le Mouroux et R. Le Chanjaur (Ifremer La Trinité), La Tremblade du 28 au 30 mai 1996.
- Formation de P. Lévy (Ifremer, Centre Océanologique du Pacifique, Tahiti, Polynésie Française) aux techniques de diagnostic des maladies infectieuses chez les invertébrés, du 3 au 25 novembre 1995.

2. Synthèse de l'activité scientifique

2. 1. Contexte général

La production halieutique mondiale a atteint un nouveau record de 133 millions de tonnes en 2002, en grande partie grâce à l'accroissement de la production dans les exploitations aquacoles. En effet, la part de la production halieutique mondiale attribuable à l'aquaculture a progressé de 25,8% à 29,9% entre 1998 et 2002 (FAO, 2006). Durant la même période, si la production des pêches a augmenté de 6,3%, la production aquacole a réalisé une progression de 30%. Selon les projections de la FAO, la consommation mondiale de poissons, mollusques et crustacés (pour l'alimentation humaine et animale) pourrait s'établir à 179 millions de tonnes d'ici à 2015, soit un relèvement de 47 millions de tonnes par rapport à 2002. L'essentiel de cette nouvelle demande devra être satisfait par l'aquaculture, qui pourrait assurer 39% de la production halieutique totale en 2015.

Les maladies infectieuses peuvent influer sur la survie, mais également sur la croissance et les performances zootechniques des animaux en élevage. Elles sont de ce fait des aléas qu'il est indispensable de prendre en compte et qu'il faut tenter de maîtriser. L'aquaculture comme toutes les autres activités d'élevage doit y faire face. La forte croissance, ces dernières décennies, des productions aquacoles, des espèces exploitées et de leurs échanges à des fins commerciales s'est accompagnée d'une augmentation du nombre et de la répartition des maladies infectieuses. En effet, la libre circulation des animaux et de leurs produits est un élément favorisant la dissémination et l'émergence d'agents pathogènes. Il est cependant nécessaire de concilier le contrôle des maladies infectieuses par le biais de la mise en place de réglementations en terme d'échanges avec la libre circulation des animaux (et de leurs produits) dans le cadre de l'Organisation Mondiale du Commerce.

Les risques en terme de maladies infectieuses induits par la dérégulation des transferts commerciaux internationaux et par l'augmentation de l'activité économique globale qui en résulte sont une réalité. Ils sont assez bien identifiés et impliquent autant les transferts d'animaux vivants que les produits d'origine animale et les structures et matériels servant à leur transport. L'évolution des agents pathogènes eux-mêmes et les effets des activités humaines sur l'environnement (pollution, changement global et réchauffement climatique) sont aussi des facteurs de première importance à prendre en considération. Parmi les facteurs directement liés aux activités d'élevage, la complexité des cycles de production et les risques particuliers liés aux systèmes de recyclage jouent enfin un rôle notable. Par ailleurs, dans le milieu aquatique (marin en particulier), il est indispensable de prendre en compte la difficulté, voire l'impossibilité d'empêcher les déplacements des animaux sauvages. Dans ces conditions, il est important de mesurer les risques respectifs représentés par l'importation d'animaux vivants pour l'aquaculture ou le repeuplement d'une part, et par les mouvements des espèces sauvages d'autre part.

Considérant que l'aquaculture doit être tournée toute entière vers le **principe de développement durable** en fournissant des produits sains de qualité, en intégrant des mesures en matière de **santé et de bien-être des animaux**, tout en respectant l'environnement et en créant des emplois à long terme, **les maladies infectieuses** restent une préoccupation de tout premier plan.

Différentes approches et/ou outils sont envisageables pour tenter de réduire l'impact économique que peuvent avoir les maladies infectieuses sur les élevages aquacoles. Elles passent par :

- l'identification et la connaissance des agents infectieux,
- la surveillance des cheptels et le contrôle des transferts des animaux et de leurs produits dans un cadre réglementaire,
- la compréhension des interactions entre l'hôte (en particulier les mécanismes de défense), l'agent infectieux et l'environnement,
- la modélisation de l'apparition et de la persistance des maladies infectieuses,
- la mise en place de traitements raisonnés,
- la sélection d'animaux résistants ou tolérants aux maladies infectieuses.

L'identification et la connaissance des agents infectieux sont les premières étapes indispensables pour initier une réflexion sur la maîtrise des maladies en aquaculture. Si le danger (les agents infectieux) n'est pas identifié et connu, il reste difficile de mettre en place des mesures de lutte.

Dans un second temps, si des agents infectieux sont identifiés et considérés comme pouvant perturber les productions aquacoles, il est possible de surveiller les cheptels et de contrôler les transferts d'animaux et de produits animaux. La surveillance et les contrôles doivent être réalisés dans un cadre réglementaire dans un but d'efficacité et d'harmonisation nationale et internationale. Cependant, cette approche de surveillance et de contrôle est une approche « passive ». Elle a pour objectif majeur d'éviter la dissémination des maladies et de préserver ainsi des zones indemnes. Il faut souligner que la réglementation doit pouvoir aussi s'adapter à des besoins parfois mouvants.

L'étude des interactions entre hôtes, agents pathogènes et milieux est une approche indispensable et complémentaire des points précédents. Par ce biais, il est possible de définir comment le milieu peut agir sur les agents infectieux eux-mêmes (les variations de facteurs environnementaux peuvent induire des modifications dans la virulence et la répartition géographique des agents pathogènes) et sur l'animal (les fluctuations du milieu peuvent faire varier la sensibilité à un agent infectieux). En associant cette approche à une analyse de risque, il peut être possible de prévoir quand et où une maladie va se développer.

Concernant les traitements et la protection (incluant la vaccination et les milieux contrôlés), les filières ne sont pas à égalité. En effet, si pour la filière piscicole (poissons), il est possible de développer une stratégie de protection des cheptels par la vaccination et l'utilisation de médicaments, la conchyliculture, essentiellement opérée sur l'estran (milieu ouvert), ne peut pas se prévaloir d'utiliser ces outils pour la maîtriser les maladies infectieuses.

Enfin, il est clair qu'une meilleure compréhension des mécanismes de défense mis en place par les espèces aquacoles doit permettre de développer des stratégies de lutte adaptées contre les maladies infectieuses. Parmi, celles ci, l'obtention d'animaux résistants apparaît comme une voie à privilégier. Cette approche en terme de pathologie demande la maîtrise des maladies au laboratoire (pression de sélection) et/ou l'identification de gènes impliqués dans les mécanismes de défense (marqueurs de sélection).

Cependant, dans le domaine de l'aquaculture et tout particulièrement en conchyliculture, un certain nombre de difficultés majeures existe.

La première de ces difficultés réside dans un manque de connaissances de base sur :

- les agents infectieux eux-mêmes,
- les mécanismes de défense mis en jeu par les animaux pour lutter contre ces agents infectieux,
- les divers facteurs environnementaux favorisant ou conditionnant l'apparition d'une maladie.

Le nombre restreint d'outils d'investigation (outils de diagnostic) peut aussi être un frein à la lutte contre les maladies infectieuses.

Par ailleurs, le milieu aquatique et ses caractéristiques (diffusion facilitée des agents infectieux dans et par l'eau, difficulté de mettre en place des traitements dans certains cas et difficulté, voir impossibilité d'agir sur les conditions de milieu, …) induisent des contraintes particulières.

Enfin, en France, comme dans d'autres pays, les moyens humains et les ressources financières mobilisés en terme de recherche et développement pour combattre les agents infectieux en aquaculture sont relativement limités et dispersés par rapport à d'autres filières de production.

Dans ce contexte, l'objectif du pathologiste est de pouvoir **fournir des outils** et de proposer des **stratégies permettant de réduire les effets néfastes des maladies infectieuses sur les exploitations** tout en **préservant l'environnemen**t. En effet, le développement d'une **aquaculture durable** passe aujourd'hui par la connaissance des capacités des systèmes de production à protéger et améliorer les milieux et à préserver les ressources. Les espèces exploitées peuvent représenter des sentinelles d'une grande utilité pour suivre les modifications de l'environnement. Les maladies infectieuses peuvent avoir un effet négatif sur les productions et être interprétées comme la résultante des pratiques d'élevage et de la qualité de l'environnement, avec de plus un risque accru pour les ressources non exploitées environnantes (transferts d'agents infectieux des espèces exploitées vers les espèces non exploitées). Cependant, en étudiant les **maladies infectieuses chez les espèces en élevage**, il est possible de **générer des connaissances** utiles pour mieux **gérer les ressources naturelles et les milieux** et **entretenir la biodiversité** dans laquelle il sera alors possible de puiser pour des **développements futurs de l'aquaculture**.

2. 2. Thématiques de recherche

Les travaux que j'ai développés depuis 1986 peuvent être regroupés autour de trois axes principaux en terme de pathologie aquacole :

(1) l'identification du danger,

(2) la caractérisation du risque,

(3) la gestion du risque.

Il a été choisi de développer ces trois points autour de publications ciblées (au nombre de 13).

2. 2. 1. Identifier le danger : Outils de diagnostic et émergence

Le diagnostic est une étape cruciale dans la lutte contre les maladies, en particulier dans le cas d'émergences. L'émergence est un phénomène infectieux inattendu en référence à ses propriétés intrinsèques. Une maladie infectieuse émergente peut être aussi définie comme une maladie grave récemment reconnue qui est susceptible de se propager au sein d'une population ou entre populations, à l'occasion d'échanges d'animaux et/ou de produits d'origine animale. Les causes de l'émergence sont multiples comme l'apparition d'un nouvel agent infectieux, l'évolution d'un agent existant, l'introduction d'un agent pathogène exotique, la présence de vecteurs et de transferts, l'évolution des techniques de diagnostic ou les effets de facteurs environnementaux. Pour répondre de manière efficace au problème de l'émergence, il est nécessaire de disposer de réseaux de surveillance possédant un maillage assez fin et d'outils de diagnostic efficaces et rapides à mettre en oeuvre.

Dans ce contexte, j'ai consacré une part importante de mon activité au cours de mon cursus professionnel à la détection et à l'identification d'agents infectieux chez différentes espèces d'intérêt en aquaculture (poissons, crustacés et mollusques).

Différentes techniques intégrant l'histologie, la microscopie électronique à transmission, la culture cellulaire, les marquages immunologiques et la biologie moléculaire ont ainsi été utilisées et ont permis la mise en évidence de différents types d'agents pathogènes :

- des parasites (**Renault *et al.*, 1995*** ; **Cochennec *et al.*, 1998** ; **Boussaid *et al.*, 1999** ; **Renault *et al.*, 2000**);
- des procaryotes (**Renault & Cohennec, 1994** ; **Renault *et al.*, 1994** ; **Renault & Cochennec, 1995** ; **Choi *et al.*, 1996**) ;
- des virus (**Renault *et al.*, 1991** ; **Renault *et al.*, 1994** ; **LeDeuff *et al.*, 1995** ; **Arzul *et al.*, 2001** ; **Renault *et al.*, 2001**) ;
- des algues (**Mortensen *et al.*, 2005**).

L'apparition d'une maladie est le plus généralement associée à des conditions d'élevage données. De ce fait, le recueil des commémoratifs est absolument indispensable pour orienter le diagnostic et choisir les outils adaptés. Le choix des techniques à mettre en œuvre dépend du stade de développement de l'animal et de la forme de l'infection. Il apparaît aussi indispensable pour un même agent infectieux de disposer de plusieurs outils de diagnostic complémentaires.

Certaines des méthodes de diagnostic bien que présentant une spécificité réduite et une sensibilité limitée sont largement utilisées en pathologie chez les animaux aquatiques : c'est le cas de l'histologie, en particulier chez les mollusques. Un besoin urgent se faisait sentir dans les années 90 pour le développement de techniques alternatives, plus sensibles et plus spécifiques et surtout permettant d'obtenir une réponse rapide, pour la détection d'agents infectieux chez les bivalves. C'est dans ce contexte que **différentes techniques pour diagnostiquer des infections à virus de type herpès chez les coquillages ont été développées sur la base de données moléculaires**. La première étape pour le développement d'outils de diagnostic spécifiques correspond à l'acquisition de connaissances sur le génome et la structure de l'agent infectieux ciblé.

*: Les références en caractères gras dans le texte correspondent à des travaux réalisés et valorisés au cours de mon cursus professionnel

Les résultats obtenus sont détaillés dans le Chapitre 3. - Identifier le danger Infections à herpès virus chez les coquillages, vraie émergence ou effort d'analyse ciblé -.

2. 2. 2. Caractériser le risque : Environnement et facteurs de risque d'apparition ou d'émergence de maladies infectieuses

Les risques induits par les transferts commerciaux internationaux sont bien identifiés et impliquent autant les transferts d'animaux vivants que les produits d'origine animale et les structures et matériels servant à leur transport. La mondialisation est un facteur d'émergence et de réémergence de maladies animales. La globalisation conduit en effet à des mouvements sans précédent des marchandises et des animaux et donc d'agents pathogènes.

En revanche, il est beaucoup plus difficile de cerner les moteurs de l'évolution des agents infectieux, de la sensibilité des espèces hôtres ou des effets environnementaux (pollution et réchauffement climatique). Ce dernier peut en effet avoir des effets considérables sur le statut infectieux chez les animaux.

C'est dans ce contexte que des travaux ont été entrepris au sein du LGP (Ifremer, La Tremblade, France) afin d'**étudier le système immunitaire** chez différentes espèces de **bivalves comme l'huître plate, *Ostrea edulis*, et l'huître creuse, *Crassostrea gigas***. Dans une première étape, l'acquisition de connaissances apparaissait indispensable afin de développer dans un second temps des recherches sur les effets de divers facteurs environnementaux sur les mécanismes de défense. Il a ainsi été obtenu des informations sur différents éléments du système immunitaire comme les hémocytes (**Xue & Renault, 2001**) ou certains effecteurs présent dans l'hémolymphe comme le système prophénol oxydase/phénol oxydase (**Hellio *et al.*, 2007**). Des expérimentations ont ensuite été réalisées *in vitro* et *in vivo* chez l'huître creuse, *C. gigas*, afin de définir les effets de différents facteurs physico-chimiques comme la température et la salinité ainsi que les effets de divers xénobiotiques sur différents paramètres hémocytaires et sur l'activité phénol oxydase (**Gagnaire *et al.*, 2003** ; **Gagnaire *et al.*, 2006a** ; **2006b)**. Enfin, dans une dernière étape, ce sont les interactions entre polluants, système immunitaire et agents pathogènes qui ont été explorées (**Gagnaire *et al.*, 2007**).

Les informations concernant ces travaux sont en partie rapportées au Chapitre 4. - Relations hôtes, agents pathogènes et environnement -.

2. 2. 3. Gérer le risque : Variabilité génétique pour la réponse aux agents pathogènes chez les espèces d'intérêt en aquaculture

L'amélioration génétique apparaît comme une **stratégie de choix pour lutter contre les maladies infectieuses chez les invertébrés**, et tout particulièrement **chez les mollusques**. En effet, chez ces espèces, les moyens de lutte restent limités et la sélection avec le contrôle des transferts d'animaux est une des voies les plus réalistes.

Afin d'illustrer cet aspect de la pathologie chez les espèces d'intérêt en aquaculture, il a été choisi de rapporter dans le Chapitre 5 - Amélioration génétique et maladies infectieuses chez les huîtres - des travaux réalisés chez l'huître plate*, O. edulis*, en vue d'obtenir **des animaux résistants à une maladie parasitaire, la bonamisose** ainsi que des recherches initiées chez **l'huître creuse, *C. gigas*, afin de définir les bases génétiques de la réponse au virus OsHV-1** chez cette espèce.

3. Identifier le danger: Infections à herpès virus chez les coquillages, vraie émergence ou effort d'analyse ciblé

3. 1. Des virus, des virus et encore des virus

La première description d'un virus de type herpès chez un bivalve a été réalisée chez l'huître américaine, *Crassostrea virginica,* aux USA par Farley *et al.* en 1972. C'est une découverte fortuite. En effet, c'est au cours d'une étude concernant les effets de la température sur la croissance chez cette huître que les auteurs ont observé des mortalités plus élevées chez des animaux maintenus à 28°C-30°C (52%) que chez des huîtres placées à 12°C-18°C (18%). Des analyses en histologie ont permis de détecter des anomalies nucléaires (présence d'inclusions positives à la réaction de Feulgen) uniquement chez des individus conditionnés à 28°C-30°C (Farley *et al.,* 1972). Des examens complémentaires effectués en microscopie électronique à transmission ont montré la présence de particules virales chez ces animaux. Sur la base de ses caractéristiques structurales (capsides de forme hexagonale de 70 à 90 nanomètres de diamètre, présence d'une enveloppe) et de sa localisation, le virus détecté a été apparenté aux herpès virus. Les auteurs ont ensuite entrepris une étude épidémiologique. En étudiant des échantillons prélevés à différentes périodes de l'année, il leur a été possible de montrer que l'infection virale se développait préférentiellement à des températures élevées. Ces auteurs ont émis l'hypothèse que le virus se trouvait à l'état latent ou était faiblement exprimé à températures basses et qu'une augmentation de la température favorisait la propagation de la maladie, en faisant passer le virus d'une phase de latence à une phase d'expression clinique.

A la suite de cette première description (première description d'un virus de type herpès, mais aussi première description d'un virus chez un bivalve), aucun travail faisant état d'infections à virus de type herpès chez les mollusques n'a été publié au cours des vingt années qui ont suivi.

Ce n'est qu'en 1991 que des virus apparentés à la famille des *Herpesviridae* ont été à nouveau décrits chez des larves d'huître creuse, *C. gigas,* en France (Nicolas *et al.,* 1992) et en Nouvelle Zélande (Hine *et al*., 1992). Depuis cette date, de fortes mortalités sporadiques de larves de *C. gigas* sont régulièrement observées dans des écloseries en France. Ces mortalités surviennent le plus généralement durant l'été et sont associées à la détection de virus de type herpès (**Renault *et al.,* 1994a et b**). Des mortalités importantes ont également été observées à partir de 1993 dans des lots de juvéniles (naissain) d'huître creuse, *C. gigas*, dans différents sites en France. Un virus de type herpès (Figure 1) a pu également être détecté (**Renault *et al.,* 1994b**). Des épisodes de mortalité ont été rapportés au stade larvaire dans des écloseries commerciales en Espagne et au Royaume Uni. Dans ce cas, aucune analyse permettant de visualiser des particules virales (microscopie électronique) n'a été réalisée. Cependant, des analyses effectuées en PCR ont permis de détecter de l'ADN viral dans des échantillons provenant de certaines de ces écloseries (**Renault & Arzul, 2001**). De fortes mortalités de naissain de *C. gigas* ont aussi été rapportées en Irlande en 1994 et 1995. La cause de ces mortalités n'a pas été identifiée (Culloty & Mulcahy, 1995). Néanmoins, des analyses en microscopie photonique ont montré la présence d'anomalies cellulaires (noyaux hypertrophiés et marginalisation de la chromatine) observées lors des infections à herpès virus chez cette espèce. Ces résultats semblent indiquer qu'un virus de type herpès est présent au moins dans un site sur la côte sud de l'Irlande (Culloty & Mulcahy, données non publiées). Enfin, plus récemment, ce type de virus a été décrit chez la même espèce en Californie (Friedman *et al.*, 2005).

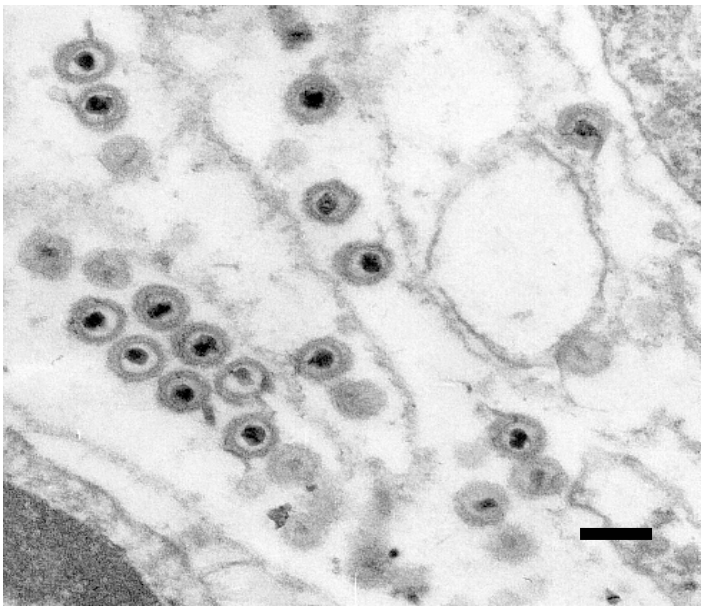


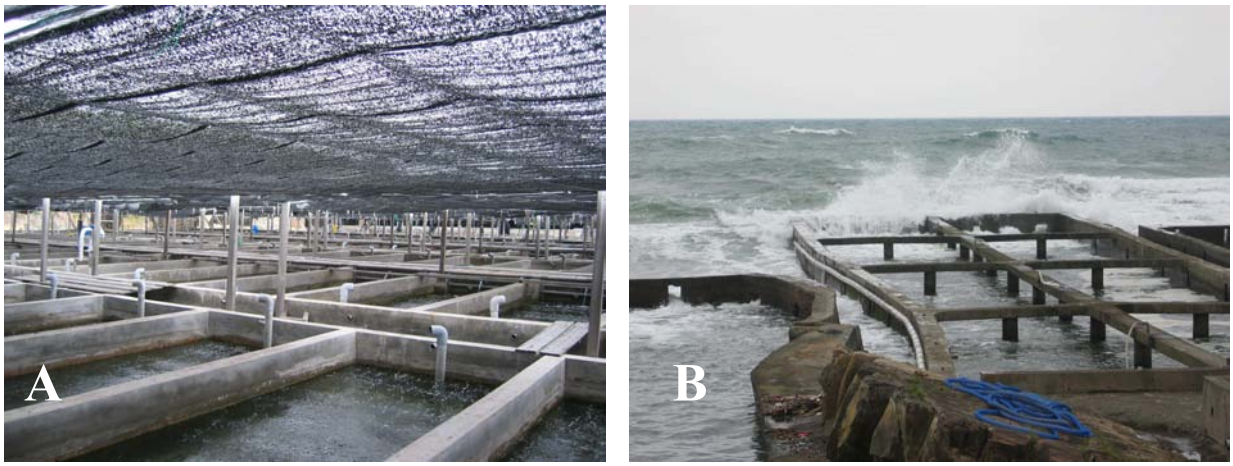
Figure 1. *Particules virales extracellulaires ; naissain d'huître creuse C. gigas ; barre = 200nm*

Des infections impliquant des virus de type herpès ont aussi été rapportées en France chez l'huître plate, *Ostrea edulis,* chez le naissain et les larves (Comps & Cochennec, 1993 ; **Renault *et al.*, 2000a**). Il est à noter qu'au cours des années 1994 et 1995, des mortalités concomitantes ont été observées chez des huîtres des deux espèces, *C. gigas* et *O. edulis*, au sein d'écloseries commerciales. Des particules virales de type herpès ont pu être détectées en microscopie électronique à transmission (**Renault *et al.*, 2000a**).

D'autres espèces de coquillages comme les huîtres des espèces *O. angasi* et *Tiostrea chilensis*, la palourde, *Ruditapes philippinarum,* et la coquille Saint Jacques, *Pecten maximus* apparaissent sensibles à des infections à virus de type herpès. En effet, des particules virales ont été observées chez des huîtres adultes appartenant à l'espèce *O. angasi* en Australie (Hine and Thorne, 1997), chez les larves de l'espèce *T. chilensis* en Nouvelle Zélande (Hine *et al.*, 1998), de palourde japonaise, *R. philippinarum* in France (**Renault, 1998** ; **Renault *et al.*, 2001a** et **b**) et de coquille Saint Jacques, Pecten maximus (Arzul *et al.*, 2001). Un virus de type herpès a aussi été rapporté chez l'huître perlière, *Pinctada margatifera,* en Polynésie Française (Comps *et al.*, 1999).

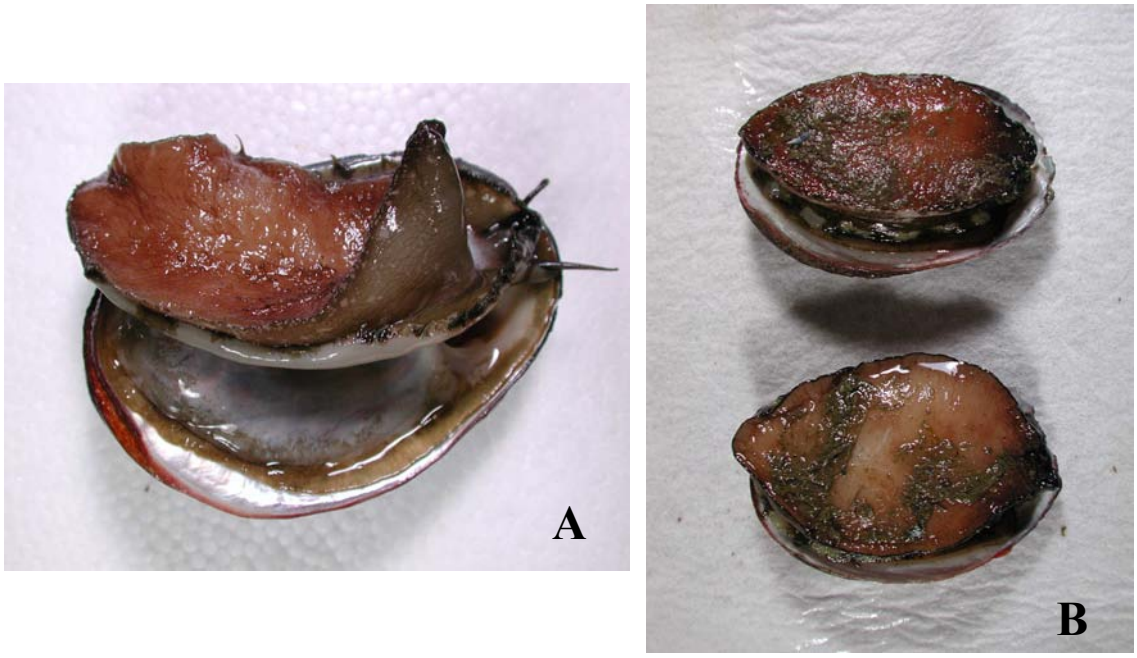
Très récemment, des virus de type herpès ont été détectés chez différentes espèces d'ormeaux, *Haliotis diversicolor supertexta* (Chang *et al.,* 2005), *H. laevigata*, *H. rubra rubra* et leurs hybrides (Hardy Smith *et al.*, 2007).

Ayant débutées en janvier 2003, des mortalité massives affectant l'ormeau *H. diversicolor supertexta* ont été observées dans le nord est de Taiwan. Ces mortalités touchaient aussi bien des animaux élevés dans des bassins à terre que dans des systèmes directement alimentés en eau de mer (Figures 2A et 2B).



Figures 2A et 2B. *Systèmes d'élevage d'ormeaux à Taiwan. A : système à terre. B : système en bord de mer directement alimenté en eau à marée haute*

La mort des ormeaux survenait rapidement, dans une période de trois jours après l'apparition des signes cliniques (animaux n'adhérant plus au substrat, Figures 3A et 3B), les mortalités cumulées pouvant atteindre 70% à 80% des effectifs. Les adultes et les juvéniles étaient affectés de la même manière. Au cours des épisodes de mortalité, la température de l'eau variait de 16°C à 19°C, ces valeurs étant anormalement basses pour la saison (climat sub-tropical).



Figures 3A et 3B. A : *Ormeau (H. diversicolor supertexta) sain ; l'animal posé sur la coquille est capable de se retourner. B : Ormeaux (H. diversicolor supertexta) infectés ; l'animal posé sur la coquille reste inactif*

Des analyses en histologie ont révélé la présence de lésions au niveau du tissu nerveux et des branchies (infiltration et nécrose) chez les animaux malades (Chang *et al.,* 2005). Des particules virales présentant les caractéristiques ultra structurales des herpès virus ont été observées en microscopie électronique à transmission. Des essais de transmission de la maladie ont été réalisés par injection (intra-musculaire) et par balnéation. Ils ont permis de démontrer le pouvoir pathogène du virus. De très fortes mortalités peuvent ainsi être induites en quelques jours. Des fortes mortalités associées à cette maladie virale ont été observées les années suivantes en 2004 et en 2005 (Dr Chang, communication personnelle).

Des fortes mortalités d'ormeaux ont également été observées en décembre 2005 dans trois fermes dans le sud est de l'Australie (Victoria). Dans ce cas, les jeunes stades étaient plus affectés que les animaux plus âgés avec des mortalités variant de 5% à 90% (Hooper *et al.*, 2007). Dans l'une des fermes affectées, un taux de mortalité journalière de 5000 a pu être enregistré (Figure 4).

Figure 4. *Bassins d'élevage vides suite à un épisode de mortalité dans une ferme d'ormeaux australienne*

Le rôle de facteurs de stress comme les fortes densités d'animaux en élevage, la température de l'eau (au dessus de 17,5°C-18°C) et la maturation sexuelle a été fortement suspecté. La maladie se traduisait par un élargissement de la zone buccale et une protrusion de la radula. Par ailleurs, les animaux affectés n'adhéraient plus au substrat. Les ormeaux des deux espèces, *Haliotis laevigata* and *H. ruba rubra*, ainsi que leurs hybrides étaient atteints. Comme cela a été rapporté à Taiwan, des lésions du système nerveux ont été observées chez les ormeaux australiens et un virus de type herpès a pu être détecté en microscopie électronique à transmission. Les essais d'infection expérimentale réalisés ont démontré que le virus pouvait être transmis d'animaux malades à des individus sains par injection intra-musculaire, mais aussi par balnéation. Le pouvoir pathogène du virus a été ainsi démontré, ce virus étant capable de tuer les ormeaux expérimentalement infectés en quelques jours (3 à 5 jours).

Une approche épidémiologique a permis de montrer que l'infection virale touchait également les stocks naturels de coquillages dans la même région. Plus de 40 sites ont été surveillés dans l'état du Victoria à partir de mai 2006. En juin de cette même année, l'infection virale a pu être observée sur la base d'analyse en histologie classique (lésions des tissus nerveux) pour 19 des sites suivis. L'origine exacte du virus reste inconnue. Cependant, pour les fermes, il est fortement suspecté que ce soit l'introduction de géniteurs provenant de stocks naturels qui soit à l'origine des épisodes infectieux observés.

La production annuelle d'ormeaux pour l'Etat du Victoria est d'environ 1500 tonnes (exploitation de stocks sauvages en plongée et production des fermes) pour un chiffre d'affaire de 50 millions d'euros. Les fermes produisent environ 130 tonnes annuellement. Plus de 99% de la production est destinée à l'exportation, en particulier vers les pays d'Asie. Les pertes enregistrées fin 2005 et en 2006 aussi bien dans les fermes et que parmi les stocks naturels ont compromis fortement ce secteur d'activité et expliquent le très fort intérêt des administrations et des professionnels pour le sujet.

De plus, la détection d'un virus de type herpès chez des ormeaux à Taiwan et en Australie soulève la question sur les relations taxonomiques qui peuvent exister entre les virus observés en Europe, aux USA et dans la zone Pacifique.

Des **infections à virus de type herpès** ont ainsi été rapportées dans **différentes régions du globe** et affectent **différentes espèces de coquillages** (huître creuse américaine *C. virginica,* huître creuse japonaise *C. gigas*, huîtres plates *O. edulis* et *T. chilensis*, palourde européenne *R. decussatus*, palourde japonaise *R. philippinarum*, coquille Saint-Jacques *P. maximus*, et ormeaux, *H. diversicolor supertexta, H. laevigata, H. rubra rubra)*. Le caractère ubiquitaire des virus de type herpès chez les mollusques marins apparaît clairement, des particules ayant la morphologie des herpès virus étant détectées chez différentes espèces de coquillages, à différents stades de développement (larves, juvéniles et adultes) et dans différents pays. Ces **infections sont associées à des épisodes de fortes mortalités**, plus particulièrement chez les larves et les juvéniles. Les bivalves adultes semblent moins sensibles que les stades plus jeunes. Des observations comparables ont été rapportées pour les herpès virus de vertébrés, en particulier chez les poissons (Kimura *et al.*, 1981). Cependant, chez les ormeaux il apparaît clairement que les animaux adultes sont aussi sensibles à l'infection que les jeunes stades. L'ensemble de ces données a incité et incite à développer des travaux sur ces virus. En effet, ils apparaissent largement distribués et impliqués dans des phénomènes de mortalité anormale. Une meilleure connaissance de ces agents infectieux passe par une caractérisation précise.

Pour l'ensemble des descriptions réalisées (Tableau I), les particules virales détectées présentent des caractéristiques morphologiques similaires à celle des virus appartenant à la famille des *Herpesviridae* (capsides et nucléocapsides circulaires ou polygonales contenues dans le noyau des cellules infectées et particules extracellulaires possédant une enveloppe). Cependant, les **critères morphologiques ne sont pas suffisants pour assigner avec certitude un virus au sein d'une famille**. Un travail **d'identification plus poussée** a été mené pour un de ces virus, le **virus infectant les larves et les juvéniles d'huître creuse, *C. gigas* (OsHV-1)** (Cf. Chapitre 3. 2. Le virus OsHV-1 ou comment établir une carte d'identité).

Tableau I - Comparaison des tailles de virus de type herpès rapportées chez les mollusques

Espèce / Stade	**Taille des nucléocapsides**	**Taille des virions enveloppés**	**Références**
Crassostrea virginica (adultes)	70-90 nm	/	Farley *et al.,* 1972
Crassostrea gigas (larves)	70 ± 2 nm	90 ± 5 nm	Nicolas *et al.,* 1992
Crassostrea gigas (larves)	97 ± 4 nm	131 ± 9 nm	Hine *et al.,* 1992
Crassostrea gigas (larves)	75 ± 3 nm	122 ± 8 nm	Renault *et al.,* 1994a
Crassostrea gigas (larves)	75 ± 4 nm	117 ± 5 nm	Renault *et al.,* 2000a
Crassostrea gigas (naissain)	75 ± 3 nm	121 ± 7 nm	Renault *et al.,* 1994a
*Crassostrea gigas (*naissain)	77 ± 3 nm	116 ± 5 nm	Renault *et al.,* 2000a
Ostrea edulis (larves)	76 ± 4 nm	118 ± 8 nm	Renault *et al.,* 2000a
Ostrea edulis (naissain)	80 nm	140-150 nm	Comps & Cochennec, 1993
Ostrea edulis (naissain)	75 ± 3.0 nm	109 ± 8 nm	Renault *et al.,* 2000a
Ostrea angasi (adultes)	98 ± 4 nm	135-140 nm	Hine & Thorne, 1997
Tiostrea chilensis (larves)	101 ± 9 nm	130 ± 11 nm	Hine *et al.*, 1998
Ruditapes philippinarum	74 ± 4 nm	111 ± 5 nm	Renault *et al.*, 2000a; 2000b
Pecten maximus (larves)	74-86 nm	110 nm	Arzul *et al.*, 2001
Haliotis diversicolor supertexta (adultes, juvéniles)	90-110 nm	/	Change *et al.*, 2005

3. 2. Le virus OsHV-1 ou comment établir une carte d'identité

En 1991, des virus de type herpès ont été décrits associés à des phénomènes de mortalités massives chez des larves d'huître creuse, *Crassostrea gigas,* en France (Nicolas *et al.* 1992). Puis à partir de 1992, des épisodes de fortes mortalités de larves de cette même espèce ont été régulièrement observés dans des écloseries en Europe (**Renault *et al.* 1994a**, **Renault & Arzul 2001**). Des mortalités massives sporadiques ont également été régulièrement observées à partir de 1993 dans les lots de juvéniles (naissain) d'huître creuse, *C. gigas*, dans différents sites en France (**Renault & Arzul 2001**). Enfin, le pouvoir pathogène du virus a été confirmé au stade larvaire par des essais d'infection expérimentale (**Le Deuff *et al.,* 1994a**).

3. 2. 1. Un premier pas: la purification du virus (Le Deuff R.-M. & T. Renault, 1999, Journal of General Virology, 80: 1317-1322)

Dans ce contexte, il est apparu indispensable de caractériser plus précisément le virus infectent les huîtres creuses en France. Par ailleurs, se posait la question du risque pour les consommateurs. Le virus détecté pouvait-il être d'origine anthropique et représenter un danger pour la consommation humaine ?

Afin de pourvoir disposer de quantités suffisantes de virus au laboratoire (LGP, Ifremer, La Tremblade, France) pour tenter de le caractériser, des essais de culture *in vitro* ont été réalisés. Du fait, de l'absence de lignées établies de cellules de mollusques marins, ce sont des cellules de poissons, d'insectes et de mammifères qui ont été utilisées dans un premier temps. L'ensemble des essais a été infructueux. Dans un second temps, la lignée Bge (*Biomphalaria glabrata* embryo*)*, la seule lignée de mollusque existante, a été testée. Cette lignée a été établie à partir de cellules embryonnaires du gastéropode d'eau douce, *Biomphalaria glabrata*, hôte intermédiaire du parasite *Schistosoma mansoni*. Les essais réalisés en utilisant cette lignée n'ont pas permis d'observer de multiplication virale. L'ensemble de ces résultats pourrait s'expliquer en particulier par la spécificité d'hôte du virus et l'absence de récepteurs à la surface des cellules testées.

Enfin, il a été tenté de cultiver le virus sur des primo-cultures de cellules cardiaques d'huîtres creuses. Des travaux ont ainsi été réalisés pour mettre en culture des cellules à partir de ventricules cardiaques d'huîtres (**Le Deuff *et al.,* 1994** ; **Renault *et al.,* 1995**). La technique de primo-culture mise au point, plusieurs essais d'infection ont été effectués. Cependant, cette dernière approche a été aussi infructueuse que les précédentes. Dans ce cas, il est possible d'expliquer les résultats observés par le fait que les primo-cultures obtenues sont constituées d'un mélange de types cellulaires (**Le Deuff *et al,.* 1994b** ; **Renault *et al.,* 1995**) et que les cellules cibles du virus sont peu ou ne sont pas représentées.

Devant ces résultats décevants, il a été décidé en 1995 de tenter de purifier le virus à partir de matériel biologique infecté. L'une des difficultés majeures rencontrées pour mettre en œuvre cette approche, était de pourvoir disposer de quantités suffisantes d'animaux. Dans ce cadre, une démarche d'information et de sensibilisation a été entreprise auprès de plusieurs écloseries commerciales françaises. En effet, ces structures de production semblaient les plus à même de fournir des huîtres infectées, larves ou naissain (juvéniles) en grandes quantités. Cependant, si la démarche a permis un approvisionnement en matériel biologique adapté, le laboratoire Ifremer de La Tremblade (Charente Maritime, France) a du rester très disponible dans la mesure où il était totalement impossible de prévoir la survenue des épisodes infectieux

dans les structures de production. Par ailleurs, définir un protocole de purification a été une tâche ardue. En effet, de nombreux travaux concernant la purification d'herpès virus sont basés sur l'utilisation de surnageant de cultures cellulaires infectées. Dans ce contexte, les protocoles sont relativement simples : le virus d'intérêt est extrait à partir d'un milieu liquide contenant essentiellement des particules virales. Dans notre cas, ce sont plus particulièrement les travaux réalisés sur des virus infectant les poissons qui ont été utilisés (Kimura *et al.*, 1981).

Les essais de purification ont été menés en parallèle sur des larves et des juvéniles infectés, et par ailleurs sur du matériel frais et du matériel congelé (conservé à -20°C ou -80°C). Dans un premier temps, il a été nécessaire de définir un milieu adapté aux différentes étapes. L'eau de mer filtrée à 0,22 µm supplémentée avec du phénylméthylsulfonyl fluoride (PMSF, inhibiteur de protéases) a été sélectionnée sur la base des résultats obtenus. Le principe général du protocole de purification retenu reposait sur l'utilisation de gradients de « sucrose ». Cette approche a permis d'obtenir une fraction très riche en capsides, nucléocapsides et particules virales enveloppées intègres à l'interface 40%-50% , lorsque la purification était réalisée à partir de larves fraîches infectées. La démarche entreprise auprès des écloseries professionnelles a porté ses fruits dans la mesure où la quantité de larves utilisée était de 10 à 20 grammes par essai, une telle quantité ne pouvant être obtenue qu'à partir de structures de production.

Il est à noter que seuls les essais réalisés à partir de larves fraîches infectées ont permis d'obtenir des particules purifiées. Les tests effectués à partir de matériel biologique congelé n'ont pas abouti à l'obtention de virions. Ce résultat peut s'expliquer par la fragilité du virus et un protocole de conservation non adapté. De plus, les essais réalisés avec du naissain infecté (juvéniles) n'ont permis l'observation que de très rares particules dans certaines fractions de purification. Il est apparu ainsi que les larves consistaient le matériel biologique privilégié. La présence du virus dans l'ensemble des tissus des individus à ce stade de développement (confirmée par des observations en microscopie électronique à transmission) peut expliquer ce résultat. De plus, il est possible de suspecter une plus grande abondance de protéases et d'enzymes de toutes sortes dans les extraits de tissus préparés à partir de naissain que dans ceux obtenus à partir de larves.

Il a été choisi de suivre l'efficacité des différentes étapes de purification en réalisant des observations en microscopie électronique à transmission sur des culots ultra-centrifugés et fixés en glutaraldéhyde. En effet, les analyses effectuées directement en utilisant la technique de contraste négatif n'ont pas permis d'obtenir de résultats probants et sûrs. Cependant, la technique sélectionnée, du fait du temps nécessaire à sa mise en œuvre (fixation, préparation des blocs, réalisation, contraste et observation des coupes), présentait l'inconvénient d'une vérification *a posteriori*. En d'autres termes, les essais de purification ont été réalisés en aveugle.

La **purification de particules virales à partir de larves d'huître creuse** a permis **d'extraire le matériel génétique** de cet agent infectieux et de réaliser une première **caractérisation moléculaire du virus (Le Deuff & Renault, 1999)**. Les virions contiennent un ADN double brin de grande taille (environ 180 kpb). Cette observation était aussi en faveur de l'intégration du virus dans la famille des *Herpesviridae*. Par ailleurs, le clonage de fragments de restriction a permis un travail préliminaire de **séquençage** et de développer des premiers **outils de diagnostic moléculaire**.

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Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*

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First observed in 1972 in *Crassostrea virginica*, herpes-like viruses of bivalves were more recently found to be associated with high mortality rates in other cultured oyster species, such as *Crassostrea gigas* and *Ostrea edulis*. The diagnosis of herpes-like virus infections is performed currently by laborious histological and transmission electron microscope examinations. Preparation of specific reagents for use in more amenable diagnostic techniques prompted purification of virus particles and investigation of the viral genome. This paper is the first description of the purification of a virus pathogen from a bivalve mollusc. A procedure was developed which facilitated purification of large amounts of virus particles on the 40–50% interface of sucrose gradients. Transmission electron microscopy showed that a purified virus suspension contained capsids and enveloped virus particles. High molecular mass viral DNA was extracted, and the genome size was estimated by the summation of the sizes of restriction endonuclease fragments to be approximately 180 kbp. Partial cloning of the virus genome was achieved and the specificity of certain cloned fragments was established by dot blot hybridization.

Introduction

More than 100 different herpesviruses have been described in higher and lower vertebrates (Roizman & Baines, 1991). Concerning invertebrates, viruses morphologically related to the *Herpesviridae* have been identified in different oyster species (Renault *et al.*, 1994 *a*). The first was observed in *Crassostrea virginica* (Farley *et al.*, 1972). Subsequently, herpes-like viruses in other cultivated oyster species, such as *Crassostrea gigas* (Hine *et al.*, 1992; Nicolas *et al.*, 1992; Renault *et al.*, 1994 *b*) and *Ostrea edulis* (Comps & Cochennec, 1993), have been found to be associated with high mortality rates. More recently, other herpes-like virus infections have been described in *Ostrea angasi* adults (Hine & Thorne, 1997) and in *Tiostrea chilensis* larvae (Hine, 1997).

Herpes-like virus infections in bivalves seem to be ubiquitous and are associated with substantial mortality levels. Since 1991, sporadic high mortality rates of larval *C. gigas* have been regularly observed in some French private hatcheries, occurring each year during the summer period in association with the detection of a herpes-like virus (Renault *et al.*, 1994 *b*). Three to four days after the spond, reduction in larval feeding and swimming activities are observed. Significant mortality occurs by day 6, with 100% mortality by day 8–12 in most affected batches (Renault *et al.*, 1994 *b*).

As seen by electron microscopy, infected cells in infected bivalves exhibit intranuclear and intracytoplasmic virus particles. The nuclei contain spherical or polygonal particles, 70–100 nm in diameter, depending on the infected oyster species (Farley *et al.*, 1972; Hine *et al.*, 1992; Nicolas *et al.*, 1992; Comps & Cochennec, 1993; Renault *et al.*, 1994 *a*; Hine, 1997; Hine & Thorne, 1997). Some particles appear empty and consist of structures assumed to be capsids; other contain an electron-dense core and are interpreted as being nucleocapsids. Enveloped particles are detected in cytoplasmic vesicles and extracellular spaces. These particles consist of a capsid with an electron-dense nucleoid that is in turn surrounded by a unit-membrane-like structure. The enveloped virions, 90–180 nm in size, exhibit spike-like protrusions on the surface, and sometimes a tail (Farley *et al.*, 1972; Hine *et al.*, 1992; Nicolas *et al.*, 1992; Comps & Cochennec, 1993; Renault *et al.*, 1994 *a*; Hine, 1997; Hine & Thorne, 1997).

To date, studies of viruses of bivalve molluscs have been

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The GenBank accession number of the sequence reported in this paper is HJ223507.

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limited to descriptive aspects. Moreover, diagnosis of herpes-like virus infections is currently performed by laborious histological and transmission electron microscope (TEM) examinations. Also, marine mollusc cell lines for virus cultivation are not available. Thus, specific probes are needed to aid the development of rapid diagnostic techniques capable of processing large sample numbers.

Most progress has been made with the herpes-like virus described in *C. gigas* (Nicolas *et al.*, 1992). Experimental reproduction of the disease in larval *C. gigas* has been achieved (Le Deuff *et al.*, 1994), thus establishing the pathogenicity of the virus for individuals at this stage of development and enabling further studies of the virus *in vivo*. The effect of temperature on the development of the virus in larvae has been demonstrated (Le Deuff *et al.*, 1996). Attempts to propagate the virus in fish and insect cell lines and in primary cultured *C. gigas* cells were unsuccessful (Le Deuff, 1995). In this report, purification of *C. gigas* herpes-like virus has been achieved. This paper is the first describing the purification of a virus pathogen from a bivalve mollusc.

Methods

■ **Biological material.** Although both larval and juvenile *C. gigas* can be affected by herpes-like virus infections, observations by TEM revealed that larvae exhibit generalized infections, whereas only focal infections were generally detected in spat (Renault *et al.*, 1994*a*). For this reason, several batches of *C. gigas* larvae (60–250 µm) were used as a source of infected material. Larvae were provided by private French hatcheries suffering mortalities associated with herpes-like viruses. They were stored at 4 °C for a maximum of 48 h until processed. Infection by herpes-like virus in these batches of larvae was confirmed by examination of specimens using TEM. Several batches of juvenile and larval *C. gigas*, healthy or infected with herpes-like virus, and several healthy adult *C. gigas* were also stored at −20 °C for DNA extraction.

■ **Virus purification.** Purification of virus particles was achieved using sea water as 'buffer'. Sea water was filtered through 0·2 µm membranes to remove particulate contaminants and autoclaved. Just before use, it was filtered again through 0·2 µm membranes and PMSF was added to 5 ng/ml. Initial amounts of 10–20 g of *C. gigas* larvae were used for each purification, and subsequent additions were scaled appropriately. A 10 g batch of larvae was mixed with 50 ml sea water–PMSF and 20 g of 400 µm sand. The sand had been washed previously with 5% SDS, rinsed with tap water and then distilled water and baked at 450 °C for 1 h. The mixture was homogenized using an Ultra-Turrax tissue homogenizer. The suspension was diluted by adding 100 ml sea water–PMSF and homogenized again. The resulting suspension was sieved successively through 250 µm and 60 µm nylon meshes to remove sand and larger shell debris, and was then clarified in three steps at 250 ***g***, 1000 ***g*** and 4000 ***g*** for 30 min each. The resulting supernatant was centrifuged for 1 h at 200 000 ***g***. The pellet was resuspended in 50 ml sea water–PMSF and mixed at 4 °C using a magnetic stirrer until it had completely resuspended (30–60 min). The suspension was layered on discontinuous sucrose gradients composed of five fractions, 60% (6 ml), 50% (5 ml), 40% (6 ml), 30% (6 ml) and 10% (5 ml) sucrose (w/w) prepared in sea water without PMSF. Volumes of 5–6 ml of virus suspension were layered onto each gradient tube. The gradients were centrifuged for 30 min at 80 000 ***g***. Fractions (1–2 ml) were collected at each interface, and corresponding fractions were pooled from different gradient tubes. The fractions were diluted four times by adding sea water drop by drop, and the virus was pelleted for 90 min at 300 000 ***g***.

■ **Transmission electron microscopy.** Virus pellets from sucrose gradients were treated in two ways. For negative staining, a small piece of pellet was resuspended in filtered (0·2 µm) sea water, particles were adsorbed on collodion-coated 200 mesh copper grids and negatively stained using filtered (0·2 µm) 2% phosphotungstic acid (PTA). A small piece of each pellet was also fixed in 3% glutaraldehyde in 0·2 M cacodylate buffer (pH 7·2), post-fixed with 1% osmium tetroxide in the same buffer and embedded in Epon resin using methods already described (Renault *et al.*, 1994*b*). Ultrathin sections were contrasted with uranyl acetate and lead citrate, using conventional methods. *C. gigas* larvae were treated as above, but a decalcification step in a solution of 5% EDTA was performed prior to Epon embedding (Renault *et al.*, 1994*b*). Observations were performed using a JEOL JEM 1200EX transmission electron microscope operating at 60 kV.

■ **Nucleic acid extraction.** Purified virus pellets were suspended in 0·5 ml extraction buffer (100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA, 0·5% SDS, pH 8) using a Potter homogenizer. Proteinase K was added to a concentration of 0·2 mg/ml and the suspension was incubated at 50–56 °C for 3 h. Proteinase K was added again to a final concentration of 0·4 mg/ml and the incubation proceeded at 50–56 °C for 15 h. Nucleic acid was extracted twice with phenol–chloroform–isoamyl alcohol (25 : 24 : 1) and twice with chloroform–isoamyl alcohol (24 : 1). A 10 µl aliquot of 10 mg/ml glycogen was then added to the aqueous phase in order to improve nucleic acid recovery, followed by half a volume of 7·5 M ammonium acetate and two volumes of absolute ethanol. The nucleic acid was allowed to precipitate for 1 h at −80 °C, pelleted, rinsed with 70% ethanol, dried and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). DNA was directly extracted from larval *C. gigas* as described above but an additional RNase treatment was carried out (Sambrook *et al.*, 1982). DNA from adult and juvenile *C. gigas* was extracted by powdering them in liquid nitrogen, treating with an electric mincer, and extracting DNA as described above for larvae.

■ **Agarose gel electrophoresis.** DNA extracted from purified virus was digested with *Eco*RI or *Bgl*II. Electrophoretic separation of restriction fragments was performed in 0·7% agarose. Plasmid digests were run in 1% agarose gels. Ethidium bromide incorporated into the buffer (0·5 µg/ml) was used to stain DNA bands. Phage lambda *Eco*RI and *Hin*dIII DNA double digests were used as mass markers (Eurogentec). Patterns of DNA bands were analysed using Electro 2.00 (Alcatel) image analysis software.

■ **Partial cloning and screening.** Viral DNA was digested with *Eco*RI and ligated into the *Eco*RI site of pBluescript KS II−. Transformation was carried out using competent *Escherichia coli* XL-1 Blue cells. The boiling method was used to obtain minipreparations for screening transformed plasmids, and large-scale preparations made by alkaline lysis and CsCl purification were used for plasmids of interest (Sambrook *et al.*, 1982).

■ **DNA labelling, dot blot preparation and hybridization.** After denaturation, DNA was spotted onto nylon Hybond-N+ membranes (Amersham) following established procedures (Costanzi & Gillespie, 1987). Dot blots were prepared with positive controls of DNA extracted from purified virus particles, and recombinant plasmids corresponding to the cloned fragments were used as probes. The negative control was

DNA extracted from healthy adult *C. gigas*. Other blots were performed using DNA extracted from virus-infected juveniles (one batch) and larval *C. gigas* (four different batches). For each sample and control DNA, quantification was determined by spectrophotometer at 260 nm. Then spots were added to the membrane in tenfold serial dilutions containing 1 µg to 0·1 pg DNA. Probes were prepared from cloned fragments purified after agarose gel electrophoresis of *Eco*RI-digested plasmids. Cloned DNA fragments were recovered from excised agarose bands (Sambrook *et al.*, 1982) and the ECL kit (Amersham) was used according to the manufacturer's instructions.

■ **DNA sequencing.** The OmniBase DNA sequencing kit (Promega) was used for sequencing according to the manufacturer's instructions. Sequences were obtained after dried polyacrylamide gels were exposed to X-ray films.

■ **Sequence alignment.** Alignment of nucleotide sequences was performed using the BLAST algorithm described by Altschul *et al.* (1990) and obtained through web site http://www.ncbi.nlm.nih.gov/. Both Blastn and Blastx programs were used.

Results and Discussion

Purification

Before carrying out the purification steps, it was necessary to grind infected material efficiently in order to release virus particles, as oyster larval tissues are surrounded by a shell. As no reference procedure was available, several approaches were taken, including decalcification in 5% EDTA and different mechanical treatments. Efficient dissociation of tissues was achieved when decalcification in 5% EDTA for 2 h was carried out followed by treatment using an Ultra-Turrax tissue homogenizer. However, this method did not lead to purification of virus particles, perhaps because of disassembly of particles during the decalcification step. An alternative method was successful. This consisted of adding sand to the larval samples before treatment using a Ultra-Turrax tissue homogenizer. This mechanical treatment efficiently ground the shells and dissociated tissues and cells.

Different buffers were also assessed for virus purification, taking into account that the larvae were obtained from a marine environment of approximately 1000 mOsm. First attempts using a standard Tris–NaCl–EDTA (pH 8) buffer (Hayashi *et al.*, 1987; Seal & St Jeor, 1988) adjusted to 1000 mOsm with NaCl failed to yield purified virus. The best results were obtained using sea water. This empirical 'buffer' was used for further purification attempts. Also, as material used for purification consisted of diseased and dead animal tissues, it was supplemented with a protease inhibitor in order to protect virus particles from degradative enzymes.

Sucrose gradients were used for purifying virus particles as it was likely that the ground oyster tissues contained virus particles in relatively small concentrations, perhaps partially degraded in comparison to other herpesviruses usually obtained from infected cell cultures. Preformed sucrose gradients are frequently used for purification of herpesvirus particles (Hayashi *et al.*, 1987, 1989; Seal & St Jeor, 1988; Kanto *et al.*, 1994), and enable particles to be separated according to size and density. Other methods of separation, such as isopycnic CsCl gradients, were considered less promising since they are more likely to result in damage to virus particles.

Analysis of sucrose gradient fractions was performed by TEM. TEM after negative staining with PTA did not lead to clear identification of virions (Fig. 1*a*). This was due in part to the presence of a virus envelope and to the presence of cellular debris in most of the purified fractions. Therefore, it was necessary to use an alternative method based on the observation of ultrathin sections of Epon-embedded pellets. This method was time consuming, but facilitated clear identification of the different types of virus particles (capsids and enveloped virions). These particles were found mainly at the 40–50% interface of sucrose gradients. A virus suspension composed of capsids and enveloped particles was recovered from this fraction (Fig. 1*b*). Spherical or polygonal particles, 70–80 nm in diameter, were observed. Some particles appear empty and others contain an electron-dense core. Enveloped particles larger in diameter (110–130 nm) and with a reduced tegument were also detected. A few particles were also found at the 30–40% interface, but were contaminated by abundant cellular debris. Only the 40–50% pure fractions were retained for further studies of the viral genome. The purification method used was reproducible and yielded sufficient quantities of herpes-like virus purified particles to permit subsequent studies of the genome.

Indeed, herpesviruses provide a more difficult challenge than most viruses with regard to the preparation of pure virus particles. Techniques which work well with one virus strain in a particular cell line do not work for other strains of virus in the same cells or for the same strain in different cells. Using 5–45% sucrose gradients, herpes simplex viruses can be clearly observed as a fluffy white band at the centre of the gradient. This band contains the single peak of infectious virus (Killington & Powell, 1985). For gallid herpesvirus 3 (Marek's disease herpesvirus type 2), enveloped particles are detected after centrifugation in sucrose gradients at 48–50% sucrose (Cauchy, 1985).

Nucleic acid extraction and characterization

For this study, a batch of 17·5 g virus-infected *C. gigas* larvae was used. DNA was extracted from approximately half of the final pellet of purified virions. A total of 500 µg of genomic viral DNA was obtained. Analysis on a 0·7% agarose gel revealed a single band of high molecular mass DNA (Fig. 2, lane c). The lack of degraded DNA showed that both the virus purification and DNA extraction methods adequately preserved the integrity of viral DNA.

Digestion with *Eco*RI or *Bgl*II yielded different patterns composed of more than 24 different bands each (Fig. 2, lanes a and b). The sizes of the *Eco*RI and *Bgl*II restriction fragments

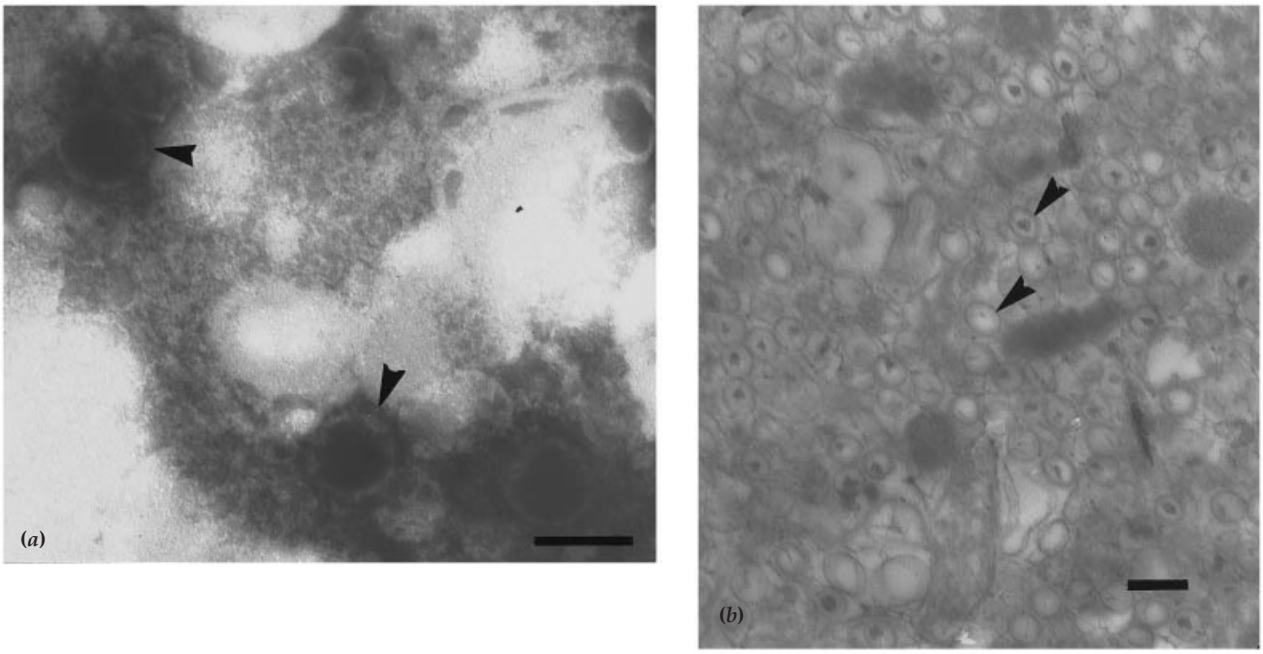

Fig. 1. TEM examination of purified virus particles from infected oyster larvae. (*a*) Negative staining of virus suspensions with PTA. Bar, 100 nm. (*b*) Virus pellets were fixed and embedded in Epon resin, and stained ultrathin sections were then contrasted with uranyl acetate and lead citrate. Bar, 200 nm. Arrowheads indicate virus particles.

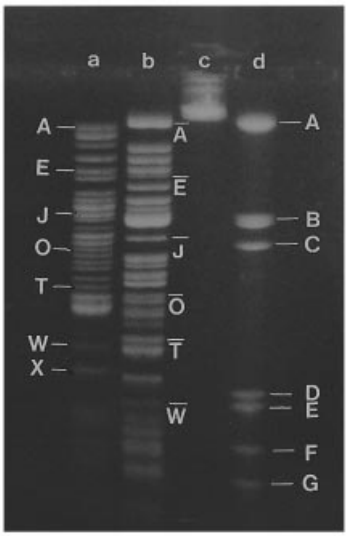

Fig. 2. Ethidium bromide-stained agarose gel showing restriction endonuclease patterns of oyster herpes-like virus DNA digested with *Bgl*II (lane a) or *Eco*RI (lane b). Fragment nomenclature is indicated. Undigested DNA is shown in lane c, and molecular mass markers in lane d (A, 21·23 kbp; B, 5·15–4·98–4·25 kbp; C, 3·52 kbp; D, 2·02 kbp; E, 1·91 kbp; F, 1·58 kbp; and G, 1·38 kbp).

were calculated using the Electro 2.00 software (Table 1). The size of the viral genome was estimated by addition of the 23 largest *Eco*RI restriction fragments (A to W) and, considering that the intense bands A and I each consist of two fragments, an estimated genome size of 177·63 kbp was obtained. The addition of the 24 largest *Bgl*II restriction fragments (A to X) gave a value of 177·19 kbp. Considering the presence of several smaller bands of undefined sizes, the genome size was estimated as approximately 180 kbp. Although this value remains to be defined more precisely using an appropriate method, such as pulsed field gel electrophoresis, it does lie within the range of genome sizes characteristic of the *Herpesviridae* (125–250 kbp).

Partial cloning of the virus genome and specificity of cloned fragments

Our objective was to clone several restriction fragments and check their specificity in order to prepare specific probes for further investigations. Preparation of a complete clone library for this virus remains to be accomplished. To date, 300 transformants have been screened by plasmid isolation. Sizes of cloned fragments ranged from approximately 0·5 to 3·5 kbp.

Four cloned *Eco*RI fragments were chosen arbitrarily for further investigations. From their sizes, these fragments were thought to be the *Eco*RI fragments K (3·4 kbp), T (2·57 kbp), V (2·18 kbp) and Z (1 kbp). The results for fragment V are shown in Fig. 3. Hybridization to dot blots specifically identified positive control DNA (Fig. 3 *a*) and also DNA extracted from virus-infected spat and larvae (Fig. 3 *b*). No hybridization was detected to DNA extracted from healthy oysters (1 µg to 0·1 ng) (Fig. 3). The other three fragments also showed specific hybridization (data not shown).

The best sensitivity was obtained with peroxidase-labelled probes K and V, which enabled detection of positive hybridization to as little as 1 ng (8×10^{-6} pmole) of genomic

Table 1. Restriction fragments and sizes

After digestion of virus genomic DNA by *Eco*RI or *Bgl*II, restriction patterns exhibit more than 24 fragments of different sizes, summarized below. From the restriction patterns, an approximate size for the virus genome was calculated.

*Eco*RI restriction pattern		*Bgl*II restriction pattern	
Fragment	Size (kbp)	Fragment	Size (kbp)
A	> 21·23	A	20·84
B	16·87	B	19·29
C	14·98	C	17·74
D	13·47	D	15·61
E	12·90	E	13·28
F	10·44	F	12·12
G	8·17	G	9·60
H	6·85	H	8·25
I	5·52	I	6·89
J	3·82	J	5·53
K	3·40	K	5·15
L	3·34	L	4·38
M	3·22	M	3·97
N	3·14	N	3·72
O	3·03	O	3·56
P	2·97	P	3·45
Q	2·85	Q	3·37
R	2·81	R	3·34
S	2·71	S	3·20
T	2·57	T	3·10
U	2·45	U	3·01
V	2·18	V	2·88
W	1·96	W	2·64
X and other smaller bands	< 1·95	X	2·27
		Other smaller bands	< 2·25
Total A to W (A and I repeated twice)	**177·63**	**Total A to X**	**177·19**

virus DNA, and also to 100 ng (38×10^{-6} pmole) of K plasmid or 10 ng ($2{\cdot}9 \times 10^{-6}$ pmole) of V plasmid. For DNA extracted from virus-infected animals, the sensitivity of hybridization depended on the sample. The detection limit was 100 ng for DNA extracted from infected spat and 1–10 ng for DNA extracted from different batches of infected larval *C. gigas*.

Partial sequencing and sequence alignment

Partial sequencing of one cloned fragment was performed. The accession number of the 266 bp sequence obtained is HJ223507. No significant similarities with any other available DNA or imputed protein sequence was obtained, even with sequences from other *Herpesviridae* or other viruses.

Conclusions

This study reports the first successful purification of a virus pathogen for *C. gigas*, a bivalve mollusc. It was prompted by reports of substantial mortalities in cultured larval and spat Japanese oysters since 1991, in France and in other countries (Hine *et al.*, 1992; Nicolas *et al.*, 1992; Renault *et al.*, 1994*a*, *b*). Selected cloned fragments proved to be specific for hybridization to blotted virus DNA or DNA from infected animals. These cloned fragments may now form the basis for establishment of new methods, particularly those utilizing PCR, for diagnosis of this virus infection in oysters. The availability of a procedure enabling purification of virus particles from infected animals may also permit production of specific antibodies by immunization of laboratory animals, and establishment of diagnostic methods based on immunological detection. Development of methods for detecting this virus in oysters is likely to aid understanding of the infection at different stages of oyster development and epidemiological surveys of oyster stocks.

This paper also reports the first description of the genome of a herpes-like virus with an invertebrate host. The DNA sequence data obtained in this study are too limited to enable

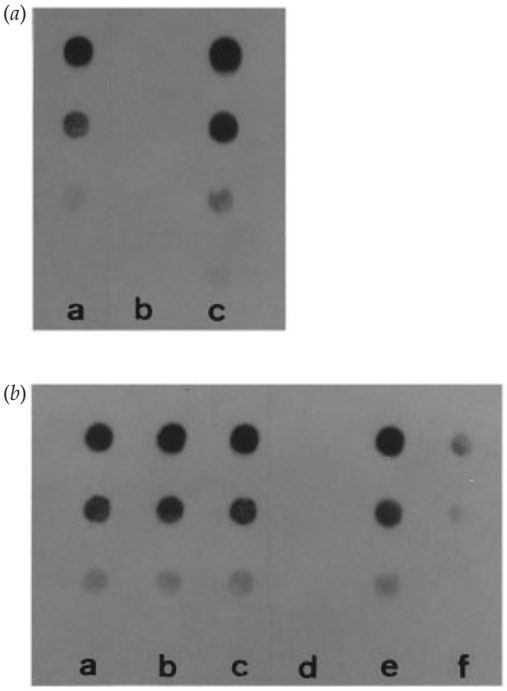

Fig. 3. (*a*) Dot blot showing hybridization of peroxidase-labelled *Eco*RI fragment V to DNA from V plasmid (a), healthy oysters (b) and purified virus (c). Samples of 100 ng to 0·1 ng of DNA were used. (*b*) Dot blot showing hybridization of peroxidase-labelled *Eco*RI fragment V to DNA from virus-infected spat (a–c), healthy oysters (d) and virus-infected larvae (e, f). The samples contained tenfold dilutions of DNA from 0 µg to 10 ng (a–c) or 1 µg to 1 ng (d–f).

conclusions to be drawn regarding relationships between the oyster virus and herpesviruses of vertebrates. However, derivation of more extensive DNA sequence information will facilitate detailed phylogenetic studies in future.

This study could not have been completed without the valuable help and confidence of some private French shellfish farmers. This research was supported in part by the Conseil Général de la Charente-Maritime and by a grant of the Ministère de l'Aménagement du Territoire, de l'Equipement et des Transports (Fonds d'Aide à la Recherche et à l'Innovation, no. 95.07.01). We also thank Dr A. Davison (MRC Virology Unit, Glasgow) for critical comments on the manuscript.

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3. 2. 2. Un bon en avant : le séquençage complet du génome (Davison A. J., Trus B. L., Cheng N, A. C. Steven, Watson M. S., Cunnigham C, Le Deuff R.-M. & T. Renault, 2005, Journal of General Virology, 86: 41-53)

La purification du virus à partir de larves d'huître creuse, *C. gigas*, infectées et l'extraction de l'ADN viral à partir de particules a permis de réaliser le séquençage complet du génome et d'analyser sa structure (dans le cadre d'un projet de recherche financé par l'Union Européenne, VINO).

La taille du génome viral est aujourd'hui estimée autour de 207 kpb. Ce matériel génétique est organisé en deux segments uniques de tailles différentes, U_L (Unique Long, 167843 pb) et U_S, (Unique Short, 3370 pb), flanqués de régions répétées inversées, TR_L (Terminal Repeat Long et IR_L (Internal Repeat Short) (7584 pb) de part et d'autre de U_L, TR_S et IR_S (9774 pb) de part et d'autre de U_S (**Davison *et al.*, 2005**). Enfin, une séquence de 1510 pb, nommée X, est située entre IR_L et IR_S. Ce type d'organisation du génome est observé chez certains herpès virus de vertébrés (herpes simplex virus et cytomégalovirus humain). Par ailleurs, des expériences de southern blot réalisées à l'aide de sondes générées par PCR et spécifiques des extrémités des segments U_L et U_S ont permis de montrer que ces deux régions uniques étaient présentes au sein du génome viral avec deux orientations inverses. Quatre isomères sont ainsi représentés. Cette caractéristique est retrouvée chez les herpès virus infectant les vertébrés. Enfin, une proportion (20-25%) des génomes viraux contient une région de 4.8 kpb dans le segment U_L possédant une orientation inversée. Le génome peut donc présenter des formes différentes. Ce résultat n'est pas surprenant dans la mesure où l'ADN ayant fait l'objet du séquençage a été extrait de particules purifiées à partir de larves naturellement infectées. Le matériel viral utilisé ne correspondait donc pas à un matériel clonal.

Une analyse détaillée des séquences a permis d'identifier 136 cadres de lecture et 124 gènes codant pour une protéine (**Davison *et al.*, 2005**). Trente huit d'entre eux peuvent être regroupés en douze familles de gènes apparentés (protéines sécrétées, protéines associées aux membranes, hélicases, inhibiteurs d'apoptose, déoxyuridine triphosphatase et RING-finger protéines). Sept gènes codent pour des enzymes (DNA polymérase, déoxyuridine triphosphatase, sous-unités d'une ribonucléotide réductase, hélicase, primase et sous-unité ATPase d'une terminase). Sept protéines montrent des homologies avec des protéines inhibitrices de l'apoptose d'origine virale ou cellulaire. Ce type de protéine est en particulier synthétisé par les baculovirus et les entomopoxvirus, virus infectant les insectes. Ce résultat souligne l'importance du phénomène d'apoptose chez les invertébrés pour lutter contre les maladies virales. Dix cadres de lecture codent pour des protéines de membrane de classe 1 et 17 autres protéines contiennent un domaine hydrophobe indiquant une possible assocation avec les membranes.

Si la structure du génome viral s'apparente à celle d'HSV-1, l'analyse de sa séquence laisse apparaître très peu d'homologie avec les autres membres de la famille des *Herpesviridae* (**Le Deuff & Renault, 1999** ; **Davison *et al.*, 2005**). Les comparaisons de séquence n'ont pas permis d'identifier de protéines virales présentant des homologies avec les protéines de capsides d'autres herpès virus. De plus, il n'a pas été possible d'identifier des gènes du virus n'ayant des homologues que chez d'autres herpès virus, à l'exception d'un gène codant pour une sous-unité ATPase de la terminase. Cette enzyme est impliquée dans l'empaquetage de l'ADN viral dans la capside. Des gènes homologues sont présents chez tous les herpès virus et les seuls homologues non herpès virus sont codés par le phage T4 et des bactériophages apparentés. Les gènes du phage T4 et du virus infectant les larves d'huître ne présentant pas

d'intron, alors que leurs homologues chez les herpès virus de mammifères et d'oiseaux en possèdent un et que deux introns sont observés chez les herpès virus de poissons et d'amphibiens.

De plus, la cadre de lecture ORF30 code pour une protéine présentant des homologies avec le domaine N terminal riche en cystéines d'une protéine de fonction inconnue retrouvée uniquement dans deux sous-familles d'herpès virus de vertébrés (*Beta-* et *Gammaherpesvirinae*) (UL92 pour le virus HCMV et ORF31 pour le virus HHV8, human herpes virus 8).

Des analyses en cryo-microscopie et une reconstitution en trois dimensions de la structure des capsides virales isolées à partir de larves d'huîtres infectées ont montré que ces structures possédaient toutes les caractéristiques de celles des herpès virus (**Davison *et al.*, 2005**). Elles apparaissent comme un assemblage de 162 capsomères, dont 12 pentamériques et 150 hexamériques, et présentent une symétrie icosaédrique.

Les **caractéristiques morphologiques des virions**, les **sites de réplication** du virus, la nature de l'acide nucléique viral (**ADN double brin de grande taille**), **l'organisation du génome**, la présence de **certains gènes (terminase)** et la **structure fine des capsides** sont autant d'éléments qui permettent de conclure qu'il s'agit bien d'un **herpès virus**. Ainsi, les données disponibles laissent supposer que les herpès virus de mammifères et d'oiseaux, ceux infectant les poissons et les amphibiens et ceux présents chez les invertébrés formeraient trois lignées, le virus décrit chez l'huître creuse, *C. gigas*, étant le premier membre identifié du troisième et dernier groupe. Ce virus a été nommé Ostreid Herpes virus 1 (OsHV-1). D'après ce modèle, OsHV-1 aurait divergé il y un milliard d'années et les virus de poissons il y a 400 millions d'années. Dans le cas des virus humains, une divergence de 1% en séquence nucléotides semble indique une séparation de l'ordre de 100 000 années.

Les travaux de **caractérisation moléculaire du virus infectant les bivalves** ont permis de vérifier que ce virus n'était **pas d'origine anthropique**, en d'autres termes qu'il ne correspondait pas à une accumulation d'un agent infectieux d'origine humaine ou animale liée aux capacités de filtration des coquillages considérés. De plus, la spécificité d'hôte des herpès virus et la distance phylogénétique entre les virus affectant l'homme et ceux affectant les mollusques font que la probabilité d'une possible transmission d'OsHV-1 à l'homme est nulle. Bien que faisant preuve d'un large spectre d'hôtes parmi différentes espèces de mollusques bivalves, le virus OsHV-1 n'a encore jamais été détecté dans une autre classe, un autre embranchement ou un autre phylum.

Le travail de séquençage réalisé a ouvert de nombreuses perspectives comme

(i) le développement d'outils de diagnostic moléculaire
(ii) l'étude de la diversité génétique des herpès virus infectant les mollusques
(iii) et les interactions entre hôte, agent infectieux et environnement.

Les **informations de séquence** obtenues ont en particulier permis de **développer différents outils de diagnostic** basés sur les techniques de **PCR et d'hybridation *in situ***. Ces outils de diagnostic moléculaire, en particulier la PCR, sont aujourd'hui couramment utilisés pour réaliser des analyses lors d'épisodes de mortalités inexpliquées chez les bivalves marins.

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A novel class of herpesvirus with bivalve hosts

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Ostreid herpesvirus 1 (OsHV-1) is the only member of the *Herpesviridae* that has an invertebrate host and is associated with sporadic mortality in the Pacific oyster (*Crassostrea gigas*) and other bivalve species. Cryo-electron microscopy of purified capsids revealed the distinctive $T=16$ icosahedral structure characteristic of herpesviruses, although the preparations examined lacked pentons. The gross genome organization of OsHV-1 was similar to that of certain mammalian herpesviruses (including herpes simplex virus and human cytomegalovirus), consisting of two invertible unique regions (U_L, 167·8 kbp; U_S, 3·4 kbp) each flanked by inverted repeats (TR_L/IR_L, 7·6 kbp; TR_S/IR_S, 9·8 kbp), with an additional unique sequence (X, 1·5 kbp) between IR_L and IR_S. Of the 124 unique genes predicted from the 207 439 bp genome sequence, 38 were members of 12 families of related genes and encoded products related to helicases, inhibitors of apoptosis, deoxyuridine triphosphatase and RING-finger proteins, in addition to membrane-associated proteins. Eight genes in three of the families appeared to be fragmented. Other genes that did not belong to the families were predicted to encode DNA polymerase, the two subunits of ribonucleotide reductase, a helicase, a primase, the ATPase subunit of terminase, a RecB-like protein, additional RING-like proteins, an ion channel and several other membrane-associated proteins. Sequence comparisons showed that OsHV-1 is at best tenuously related to the two classes of vertebrate herpesviruses (those associated with mammals, birds and reptiles, and those associated with bony fish and amphibians). OsHV-1 thus represents a third major class of the herpesviruses.

INTRODUCTION

Viruses are assigned to the family *Herpesviridae* on the basis of morphological criteria and have been identified in a wide range of vertebrates and one invertebrate, the Pacific oyster, *Crassostrea gigas* (Minson *et al.*, 2000). The vertebrate herpesviruses fall into two major phylogenetic groups. Those in the first group have mammalian or avian hosts and are classified into three subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*) that share extensive genetic relationships (McGeoch *et al.*, 2000). Reptilian herpesviruses probably also belong among the *Alphaherpesvirinae* (Nigro *et al.*, 2004; Quackenbush *et al.*, 1998; Une *et al.*, 2000; Yu *et al.*, 2001). Viruses in the second group infect amphibians or bony fish and again are interrelated (Bernard & Mercier, 1993; Davison, 1998; Davison *et al.*, 1999). Genetic evidence for a common evolutionary origin for the two groups is tenuous, however, since not a single herpesvirus-specific gene is detectably conserved in both. The only completely sequenced lower vertebrate herpesvirus, channel catfish virus (CCV), does share a few genes with the higher vertebrate group, but these have counterparts in other organisms (Davison, 1992). The conserved gene that comes closest to being herpesvirus specific encodes the putative ATPase subunit of the terminase, an enzyme complex involved in packaging viral DNA into preformed capsids. However, the presence of a distantly related gene in bacteriophage T4 leaves open the possibility of convergent evolution, with the two herpesvirus lineages having acquired this function independently. Nonetheless, similarities between the two vertebrate

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SDS-PAGE analysis of the OsHV-1 capsid proteins is available as supplementary material in JGV Online.

herpesvirus groups in capsid structure (Booy *et al.*, 1996) and mechanisms of capsid maturation (Davison & Davison, 1995) tip the balance of evidence in favour of a common origin.

Particles morphologically similar to herpesviruses were first reported in an invertebrate (the Eastern oyster, *Crassostrea virginica*) by Farley *et al.* (1972) from the USA. Infections were detected contemporaneously in the UK in another oyster species, *Ostrea edulis* (D. J. Alderman, personal communication). Herpes-like viruses have since been identified in various marine bivalve species throughout the world, including the Pacific oyster, *C. gigas* (Hine *et al.*, 1992; Nicolas *et al.*, 1992; Renault *et al.*, 1994b), European flat oyster, *O. edulis* (Comps & Cochennec, 1993; Renault *et al.*, 2000a), Antipodean flat oyster, *Ostrea angasi* (Hine & Thorne, 1997), Chilean oyster, *Tiostrea chilensis* (Hine, 1997; Hine *et al.*, 1998), carpet shell clam, *Ruditapes decussatus* (Renault & Arzul, 2001), Manila clam, *Ruditapes philippinarum* (Renault *et al.*, 2001) and great scallop, *Pecten maximus* (Arzul *et al.*, 2001a). Infections are often associated with sporadic episodes of high mortality among larvae and juveniles (Renault, 1998; Renault *et al.*, 1994a, 1995). PCR-based diagnostic methods (Arzul *et al.*, 2002; Barbosa-Solomieu *et al.*, 2004; Lipart & Renault, 2002; Renault & Arzul, 2001; Renault *et al.*, 2000a, b) have facilitated epidemiological investigations, for example showing that healthy adult animals can harbour the viral genome. Transmission experiments have demonstrated the virulence of the virus (Le Deuff *et al.*, 1994) and indicated that a single species is probably responsible for all the infections observed (Arzul *et al.*, 2001b, c). The ability of the virus to cause disease in a wide range of bivalve species may indicate that it has emerged as a consequence of selection under intensive farming conditions (Arzul *et al.*, 2001c). The earliest genetic evidence for its presence in bivalves was obtained by *in situ* hybridization analysis of samples of *O. edulis* collected in 1976 in the UK (R.-M. Le Deuff, unpublished data).

The virus isolated from infected *C. gigas* larvae has been classified as a member of the *Herpesviridae* under the name ostreid herpesvirus 1 (OsHV-1) (Minson *et al.*, 2000). Initial characterization yielded an estimated genome size of 180 kbp, and a small (266 bp) fragment demonstrated no sequence similarity to other herpesviruses (Le Deuff & Renault, 1999). The purpose of the present work was to analyse the capsid morphology and genome sequence of OsHV-1 in order to assess its relationship to vertebrate herpesviruses.

METHODS

Purification of OsHV-1 capsids. Frozen infected *C. gigas* larvae (0·5–1·0 ml packed volume) that were PCR-positive for OsHV-1 were ground with an equal volume of sand in 9 ml NTE (0·5 M NaCl, 20 mM Tris/HCl, pH 7·5, 2 mM EDTA) by using a pestle and mortar maintained on ice. Triton X-100 [10 % (v/v) in NTE] was added to a final concentration of 1 % (v/v) and the mixture was probe sonicated on ice for 1 min. Capsids were prepared essentially as described by Davison & Davison (1995), with all centrifugation steps carried out in a Sorvall TST-41 rotor at 4 °C. The sonicated material was centrifuged for 10 min at 10 000 r.p.m. (maximum 17 000 ***g***). The supernatant was placed in a fresh tube and underlayed with 1 ml 40 % (w/v) sucrose in NTE and centrifuged for 1 h at 20 000 r.p.m. (maximum 68 000 ***g***). The pellet was resuspended in 0·5 ml NTE and centrifuged on a linear 10–40 % (w/v) sucrose gradient in NTE for 1 h at 20 000 r.p.m. The capsid band was visualized using illumination from a light pipe and isolated by side puncture of the tube, diluted in 10 ml PBS and pelleted for 1 h at 20 000 r.p.m. The capsids were resuspended in approximately 10 µl PBS and frozen and transported on dry ice. A second batch was prepared similarly, except that the concentration of NaCl in NTE was reduced to 0·05 M in order to reduce ionic disruption and the capsids were transported on wet ice.

Cryo-electron microscopy of OsHV-1 capsids. Cryo-electron microscopy was performed as described by Newcomb *et al.* (2000) using an FEI CM-200 FEG electron microscope operating at 120 kV and low-electron-dose methods. Seven micrographs, recorded at a magnification of × 38 000 and digitized on a Perkin-Elmer 1010MG microdensitometer at 25 µm per pixel, yielded a total of 248 particle images at 6·6 Å (0·66 nm) per pixel. These data were analysed and a reconstruction calculated using the polar Fourier transform (PFT) model-based procedure to determine orientations and origins (Baker & Cheng, 1996). A previously calculated map of herpes simplex virus type 1 (HSV-1) A-capsids (Cheng *et al.*, 2002) was used as a starting model. After four cycles of PFT refinement, a stable three-dimensional reconstruction was calculated from 161 images to a resolution of 25 Å (0·25 nm).

Isolation of OsHV-1 virion DNA. Cell-culture methods for growth of OsHV-1 do not exist. Consequently, OsHV-1 DNA was extracted from virions purified from infected *C. gigas* larvae obtained from a commercial hatchery (Le Deuff & Renault, 1999).

Cloning of OsHV-1 DNA fragments. Cosmid libraries were prepared by inserting OsHV-1 DNA partially digested with *Bam*HI or *Sau*3AI into the *Bam*HI site of a derivative of Supercos 1 (Stratagene) (Cunningham & Davison, 1993) and packaging the products into bacteriophage λ particles using a Stratagene III XL kit. A total of 320 cosmids was analysed by *Bam*HI digestion.

A primary bacteriophage λ library of OsHV-1 *Bam*HI fragments (5×10^4 recombinants) was produced using the Lambda ZAP II vector (Stratagene) and a Stratagene III Gold packaging kit. The amplified library was subjected to the manufacturer's mass excision protocol to derive a plasmid library. A total of 84 plasmids was assessed by *Bam*HI digestion.

A plasmid library of OsHV-1 *Bam*HI fragments inserted directly into the *Bam*HI site of the pUC18 vector was generated. A total of 288 plasmids was characterized by *Bam*HI digestion. Attempts were made to clone several fragments absent from this and the excised λ libraries by purifying OsHV-1 *Bam*HI fragments from an agarose gel and inserting them into pUC18. The resulting clones were analysed by digestion with appropriate restriction endonucleases, using genome sequence data to predict cleavage patterns. The identities of inserts in the final plasmid library were confirmed by DNA sequencing.

Analysis of OsHV-1 genome structure. Restriction endonuclease and Southern blot hybridization analyses (the latter employing ^{32}P-labelled DNA probes) were carried out using standard procedures.

Sequencing of the OsHV-1 genome. An M13mp19 library of random OsHV-1 DNA fragments was sequenced using an ABI PRISM 377 instrument. The sequence database was compiled from electropherograms using Pregap4 and Gap4 (Staden *et al.*, 2000) and Phred (Ewing & Green, 1998; Ewing *et al.*, 1998). Regions of

ambiguity were resolved by sequencing PCR products directly or as plasmid clones. The sequence was determined to a mean redundancy of 10·8, and 96·1 % was obtained on both strands. The purity of the OsHV-1 DNA preparation, as judged from the number of M13 clones whose sequence did not match the database, was 99 %.

The genome sequence was reconstructed from the database after locating the genome termini, which were predicted approximately from restriction endonuclease profiles and mapped experimentally as described by Davison *et al.* (2003). This involved ligating a partially double-stranded adaptor to flush-ended OsHV-1 DNA and carrying out PCR using an adaptor-specific primer plus a primer specific for the left or right terminal region of the genome (5′-GAGTGCC-CAGGTGCATTATG-3′ or 5′-CACGGCCGTTGTATAGGAGG-3′, respectively). The junction between forms of the terminal sequences present internally in the genome was amplified using the two OsHV-1-specific primers. Products were cloned as plasmids and sequenced.

The genome sequence was analysed using locally implemented forms of the GCG (Accelrys), CLUSTAL W (Thompson *et al.*, 1994) and Ptrans programs (Taylor, 1986) and online versions of the BLAST (Altschul *et al.*, 1997), FastA (Pearson & Lipman, 1988), SignalP v2.0 (Nielsen *et al.*, 1997) and PRED-TMR2 programs (Pasquier & Hamodrakas, 1999).

RESULTS AND DISCUSSION

Capsid structure of OsHV-1

Despite husbanding OsHV-1 capsids in small volumes, the low yields rendered sparse fields of particles when prepared for cryo-electron microscopic observation over holey carbon films. Nevertheless, by searching for relatively well-populated areas and combining data from multiple micrographs, sufficient images were accumulated to yield a reconstruction at a resolution of approximately 25 Å (0·25 nm).

OsHV-1 capsids were similar in overall appearance to those of other herpesviruses that have been studied to date (Booy *et al.*, 1996; Cheng *et al.*, 2002; Trus *et al.*, 1999, 2001). The diameter estimated from cryo-electron microscopic images (approx. 116 nm) was slightly less than that of HSV-1 capsids (125 nm). This is somewhat surprising in view of the larger genome size (207 kbp compared with 152 kbp), but the DNA could be accommodated if packaged to the same density as in human cytomegalovirus (HCMV) (Bhella *et al.*, 2000). Most particles were roughly hexagonal in outline, corresponding to views close to three- or twofold axes of symmetry (Fig. 1). The striking feature was the double layer of density around the peripheries. In this respect, they differed from other herpesvirus capsids, which tend to have serrated peripheries generated by external protrusions seen in side-view. The reconstruction shown in Fig. 2(a–c) provides an explanation for this difference, which arose because the OsHV-1 triplexes extended radially almost as far outwards as the hexon protrusions. The double layer of peripheral density was occasionally interrupted by low-density patches (Fig. 1).

Reconstruction of the OsHV-1 capsid (Fig. 2a–c) revealed an icosahedral structure with a triangulation number of

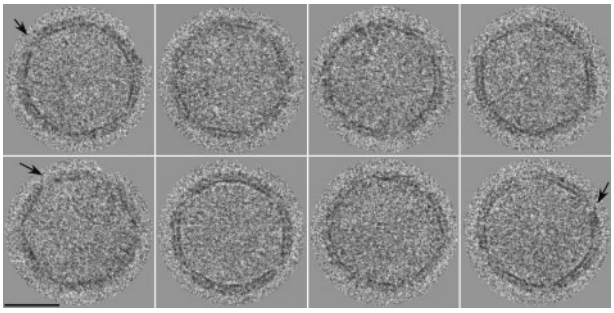


Fig. 1. Cryo-electron micrographs of individual OsHV-1 capsids. The arrowheads denote patches of low peripheral density due to the absence of pentons. Bar, 50 nm.

$T=16$. This confirmed that OsHV-1 was a herpesvirus, since this surface lattice geometry has been observed only with members of this family. A most striking feature was the large holes at the vertices, marking the absence of pentons from these sites. These holes explained the low-density patches seen on the original images (Fig. 1). Observations of HSV-1 have shown that pentons are more susceptible to proteolytic degradation than hexons (Trus *et al.*, 1996) and pentons may be extracted selectively by treatment with denaturants at concentrations that leave the rest of the surface lattice intact (Fig. 2d and e) (Newcomb *et al.*, 1993). There is other evidence that HSV-1 pentons are conformationally different from hexons (Cheng *et al.*, 2002). Unlike

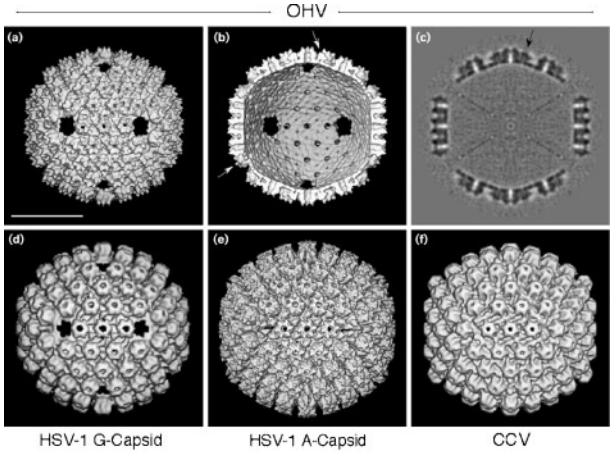


Fig. 2. Cryo-electron microscopic reconstruction of the OsHV-1 capsid viewed along a twofold axis of symmetry: (a) outside surface, (b) inside surface and (c) central section. The arrows indicate density corresponding to the triplexes. For comparison, HSV-1 capsids are shown: (d) lacking pentons owing to treatment with guanidine hydrochloride (G-capsid; reproduced from Newcomb *et al.*, 1993, by permission of Elsevier) and (e) an untreated reference (A-capsid). For further comparison, (f) shows the capsid of CCV (reproduced from Booy *et al.*, 1996, by permission of Elsevier). The OsHV-1 capsid is slightly smaller (by approx. 7 %) than that of HSV-1. Bar, 50 nm.

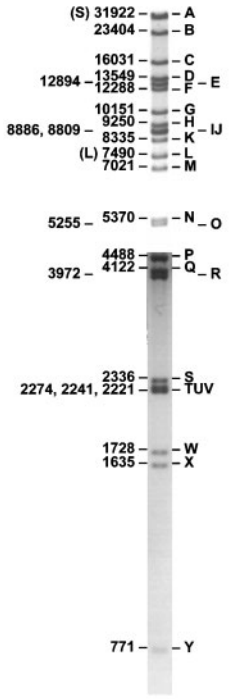
hexons, they do not bind the small capsid protein VP26 (Booy *et al.*, 1994), and certain monoclonal antibodies differentiate between the penton and hexon conformations of the 150 kDa major capsid protein VP5 (Trus *et al.*, 1992). Another example of pentons representing the weakest points in an icosahedral shell is provided by their heat-induced loss from mature bacteriophage P22 capsids (Teschke *et al.*, 2003). The OsHV-1 capsids were not exposed to denaturants or heat, and the holes were observed in both batches studied. By analogy with known properties of other herpesviruses, we supposed that 11 of the OsHV-1 vertices were originally occupied by pentons of the major capsid protein and the twelfth by a portal protein complex, but that OsHV-1 vertex proteins are more selectively labile than those of HSV-1 and were degraded or dislodged at some point during the isolation procedure. The reconstructed OsHV-1 capsid was similar to that of HSV-1 with its pentons extracted (Fig. 2d) and, taking into account the absence of pentons, to the intact HSV-1 and CCV capsids (Fig. 2e and f).

Other features of the OsHV-1 capsid were also distinctively herpesvirus-like. These included prominent external protrusions at the hexon sites, rising above the continuous floor region, and the relatively flat and featureless appearance of the inner surface. Because the facets were rather flat, not round, it was likely that the particles analysed were derived from mature capsids rather than precursor procapsids. Masses corresponding to triplexes were present at all sites of local threefold symmetry (best seen at a glancing angle and in cross-section; upper arrows in Fig. 2b and c). In other previously characterized herpesvirus capsids, triplexes are heterotrimers consisting of two copies of a protein of about 35 kDa and one copy of another protein of approximately 50 or 35 kDa, depending on the virus (Trus *et al.*, 2001). The triplexes initially play a morphogenic role in the assembly of the procapsid and then, in the mature capsid, switch to a structural role as clamps that stabilize the joints between groups of three neighbouring capsomeres (Steven & Spear, 1997).

Many higher vertebrate herpesviruses have a small capsid protein (10–15 kDa; VP26 in HSV-1) that caps the hexons but not the pentons and whose presence may be inferred from comparing hexon and penton structures (Wingfield *et al.*, 1997). Such a comparison was not possible with the preparations of OsHV-1 capsids and thus the question of whether OsHV-1 has a corresponding protein remains unanswered. However, we noted the presence of a protein of appropriate size among those generated by SDS-PAGE of OsHV-1 capsids (see supplementary Fig. 1, available at JGV Online). These proteins included species of 150 kDa (presumably the major capsid protein) and 14 kDa, plus less-abundant species of 40, 32 and 27 kDa.

Genome structure of OsHV-1

The *Bam*HI profile of OsHV-1 DNA is shown in Fig. 3, alongside a list indicating the order of fragments along the genome and the identities of fragments that were cloned as cosmids or plasmids. Two of the five fragments that were not cloned as plasmids were terminal (A, L) and would not be



Fragment	Size (bp)		Cosmid	Plasmid
	Estimated	Actual		
L	7600	7490		
J	9000	8809	■	■
N	5300	5370	■	■
c	100	123	■	■
M	7100	7021	■	■
Y	760	771	■	■
Z	400	403	■	■
Q	4100	4122	■	■
a	150	241	■	■
W	1750	1728	■	■
K	8500	8335	■	■
D	13500	13549	■	■
X	1650	1635	■	■
C	16000	16031	■	■
F	12300	12288	■	■
B	23000	23404	■	■
T	2250	2274	■	■
R	3900	3972		
U	2200	2241		■
H	9400	9250		■
V	2200	2221		■
I	9000	8886		
S	2300	2336		■
P	4500	4488		■
E	13000	12894		
G	10500	10151	■	■
O	5200	5255	■	■
b	150	229	■	■
A	30000	31922		
Total	**205810**	**207439**		

Fig. 3. Sizes of OsHV-1 *Bam*HI fragments and bacterial clones obtained. The *Bam*HI profile of OsHV-1 DNA is shown to the left, with each fragment indicated by a letter and size (bp) derived from the genome sequence. The upper and lower portions were obtained from 0·6 and 1·5 % (w/v) agarose gels, respectively, stained with ethidium bromide. Fragments containing the left (L) and right (S) genome termini are indicated. Four small fragments (Z, a, b and c) detected in other experiments are not visible in these gels. To the right, the *Bam*HI fragments are listed as arranged along the genome from left to right, with estimated sizes (derived by agarose gel electrophoresis) and actual sizes (calculated from the genome sequence). Dark-grey shading shows fragments cloned successfully into cosmids (*Bam*HI and *Sau*3AI libraries with inserts of 35–45 kbp containing several contiguous *Bam*HI fragments) or plasmids (containing individual *Bam*HI fragments). Light-grey shading shows fragments from which portions were present in cosmids (at the ends of inserts in the *Sau*3AI library).

expected to be represented. The locations of the other three uncloned fragments (R, I and E), plus the minimum size requirement for a cosmid insert (35 kbp), had the effect of excluding from the cosmid library five intervening fragments (U, H, V, S and P) that were obtained as plasmids. Two regions of the genome, in fragments R and E, were also not represented in the M13 library, necessitating direct sequencing of PCR products. The sequence features presumably responsible for failure to clone certain regions of the genome were not readily apparent.

The structure of the sequence database implied that the genome contained two unique regions (U_L and U_S; 167 843 and 3370 bp, respectively), each flanked by an inverted repeat (TR_L/IR_L and TR_S/IR_S; 7584 and 9774 bp, respectively), with the internal copies of the repeats separated by a third unique region (X; 1510 bp). Thus, the genome may be represented as TR_L–U_L–IR_L–X–IR_S–U_S–TR_S. The total genome size was 207 439 bp, close to the sum of the estimated sizes of *Bam*HI fragments (Fig. 3) and larger than the value published initially (180 kbp; Le Deuff & Renault, 1999). The nucleotide composition was 38·7 mol% G+C.

The 5′ ends of the genome were not unique, but a predominant form was apparent for each (Fig. 4). PCR of the region between IR_L and IR_S yielded two products (data not shown), the larger containing X, as expected, and the smaller lacking it. The latter would correspond to a ligated form of the termini if the 3′ end of each terminus possessed unpaired nucleotides complementary to those at the other (Fig. 4), as is the case for vertebrate herpesviruses (Mocarski & Roizman, 1982). In experiments in which probes from X and flanking regions of IR_L and IR_S were hybridized to *Hin*dIII fragments of OsHV-1 DNA (Fig. 5a), each probe detected the 7645 bp fragment containing X and adjacent portions of IR_L and IR_S. The IR_L probe also detected the 1245 bp terminal fragment from TR_L (electrophoresed from the gel in Fig. 5a, but confirmed in other experiments) and the IR_S probe detected the 4890 bp terminal fragment from TR_S. In addition, the X and IR_L probes hybridized to a 2755 bp fragment of low abundance, which represented the terminal fragment from TR_L joined to a copy of X. However, a fragment corresponding to the fused form of the genome termini with X lacking (6135 bp) was not detected. These results indicated that the major genome form is as described above, that a small proportion of molecules contains a copy of X at the left genome end and that an even smaller proportion may lack the internal copy of X.

Vertebrate herpesvirus genomes containing two unique regions flanked by large inverted repeats, such as those of HSV-1 and HCMV, exist in virions as four isomers differing in the relative orientations of the unique regions (Hayward *et al.*, 1975; Weststrate *et al.*, 1980). As a consequence, certain restriction endonucleases produce 'half-molar' or 'quarter-molar' fragments from the IR_L–IR_S junction and half-molar fragments from one or both genome termini,

Left terminus

```
2          ~TGGGGGGGCTGT
4          ~TGGGGGG
```

Right terminus

```
4                              TCCCCAA~
1                               CCCCAA~
```

IR_L-IR_S junction

```
1          ~TGGGGGGGCTGTTACTCCCCAA~
1          ~TGGGGGGGCTGT..CTCCCCAA~
1          ~TGGGGGGGCTGT...TCCCCAA~
1          ~TGGGGGGGCTGC...TCCCCAA~
1          ~TGGGGGGGCTG....TCCCCAA~
5          ~TGGGGGGGC......TCCCCAA~
```

Fig. 4. Sequences of the OsHV-1 genome termini in comparison with the junction in a minority of molecules that corresponds to a fusion of the termini (i.e. the major junction lacking X). The sequences of the termini have been inverted and complemented so that they align with the junction. All sequences are shown as a single 5′→3′ strand. A tilde (~) indicates the location of contiguous sequence, which is not shown, and the terminal nucleotides are underlined. The number of clones obtained for each sequence is shown to the left. The possibility that the junction was an artefact generated by priming at one terminus by the other, utilizing unpaired nucleotides, rather than having originated from genomes lacking X internally, is rendered unlikely by the small number of unpaired nucleotides potentially present (0–9 at each terminus, with a mode of 2).

depending on whether they cleave the inverted repeats. The sizes of fragments produced by digestion of OsHV-1 DNA with a variety of restriction endonucleases showed that genome isomerization was a property of OsHV-1, and their abundance indicated that virion DNA contained approximately equimolar amounts of the four isomers. For example, *Cla*I, which cleaves U_L and U_S but neither repeat, produced four half-molar fragments of 10 256–12 421 bp and four quarter-molar fragments of 22 262–25 319 bp (Fig. 5b). Hybridization experiments using probes from the ends of U_L and U_S confirmed the identities of these fragments, each probe detecting terminal and junction fragments of appropriate sizes (Fig. 5c). Thus, probes from the left and right ends of U_L and the left and right ends of U_S hybridized to terminal fragments of 11 388, 10 256, 10 496 and 12 421 bp, respectively, and also to pairs of junction fragments (the components largely indistinguishable from each other in Fig. 5c) of 25 319/

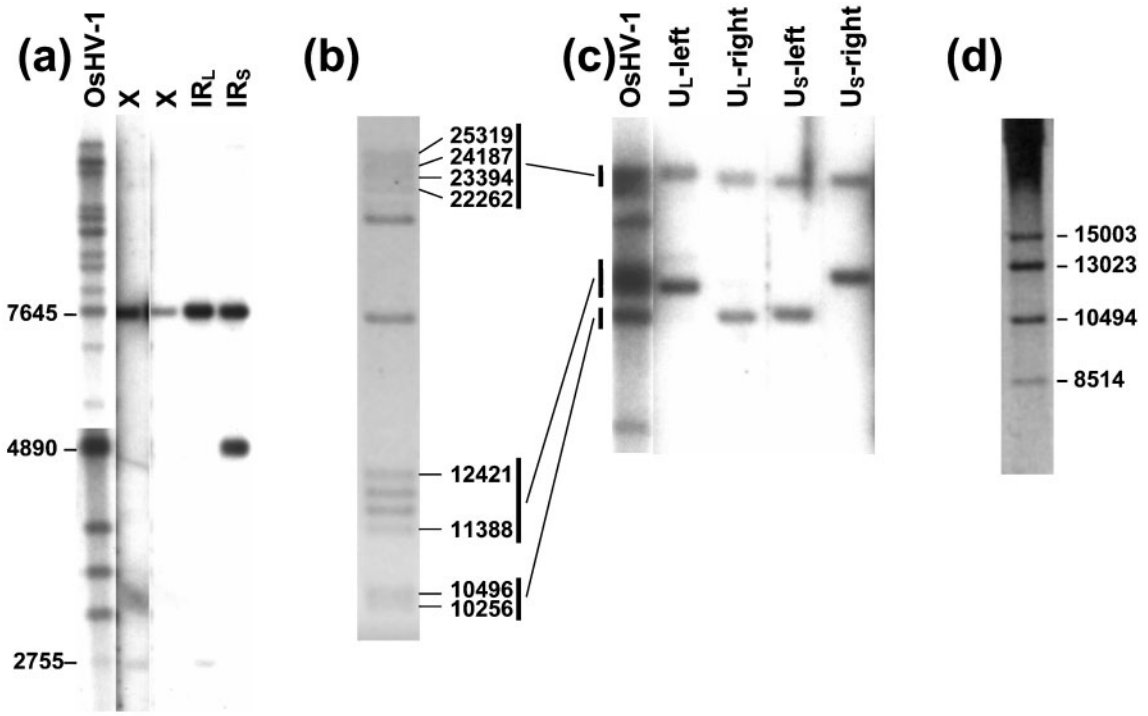


Fig. 5. Data confirming the OsHV-1 genome structure. Standard size markers were included in all gels, but are not shown. All fragment sizes (bp) were derived from the genome sequence. (a) Autoradiograph showing the results of hybridizing radiolabelled probes to a *Hin*dIII digest of OsHV-1 DNA transferred from a 0·6 % (w/v) agarose gel. The probes were OsHV-1 DNA and regions from X (nt 184202–184416, as a PCR product excised from a plasmid) and IR_L and IR_S immediately adjacent to X (nt 182634–183011 and 184522–184878, respectively, as PCR products). A longer exposure of the lower half of the first lane has been superimposed and a longer exposure of the result obtained using the X probe (third lane) is provided in the second lane. (b) Ethidium bromide-stained 0·6 % (w/v) agarose gel showing larger *Cla*I fragments of OsHV-1 DNA, with sizes of submolar bands from the genome termini and the IR_L–IR_S junction indicated. (c) Autoradiograph showing the results of hybridizing radiolabelled probes to a *Cla*I digest of OsHV-1 DNA transferred from a 0·6 % (w/v) agarose gel. The probes were OsHV-1 DNA and cloned PCR products from the left and right ends of U_L (nt 7586–7785 and 175227–175426, respectively) and the left and right ends of U_S (nt 194324–194539 and 197461–197662, respectively). The correspondence with fragments shown in (b) is indicated. (d) Ethidium bromide-stained 0·7 % (w/v) agarose gel showing fragments in the *Kpn*I profile of OsHV-1 DNA. Major and minor fragments diagnostic of inversion of a 4·8 kbp region in U_L are indicated.

23 394, 24 187/22 262, 23 394/22 262 and 25 319/24 187 bp, respectively.

A further complexity in genome structure was revealed by several of the M13 clones used to construct the sequence database. These implied that a minor population of genomes existed with an inversion of a 4·8 kbp region in the centre of U_L (green bar in Fig. 6). This was supported by the detection of minor restriction endonuclease fragments whose sizes were consistent with them containing the two junctions between the inverting region and flanking sequences in the minor population. For example, both minor (8514 and 15 003 bp) and major fragments (10 494 and 13 023 bp) were detected in the *Kpn*I profile (Fig. 5d). The proportions indicated that approximately 25 % of the DNA population contained the 4·8 kbp region in inverse orientation.

The preparations of OsHV-1 DNA examined therefore contained a mixture of genome forms. Some were an inherent property of the genome structure and others probably reflect the fact that the DNA originated from a virus that had not been clonally purified. The major structure, TR_L–U_L–IR_L–X–IR_S–U_S–TR_S, was present by virtue of inversion of U_L and U_S as four isomers in approximately equimolar amounts. Some molecules contained an additional copy of X at the left terminus and some might have lacked X internally. This could represent X–TR_L–U_L–IR_L–X–IR_S–U_S–TR_S, perhaps with X–TR_L–U_L–IR_L–IR_S–U_S–TR_S and TR_L–U_L–IR_L–IR_S–U_S–TR_S, each again as four isomers. Since herpesvirus genomes are considered to be packaged into capsids from head-to-tail concatemers, some of these minor forms might result from rare cleavage of concatemers at the X–TR_S, rather than IR_L–IR_S, junction. Some genomes also contained a 4·8 kbp

region within U_L that was inverted. Presumably, this population also consisted of the various permutations described above.

Genetic content of OsHV-1

In order to identify potential protein-coding regions in the genome (Fig. 6), an initial set of open reading frames (ORFs) was defined, in which each ORF consisted of at least 100 codons and was flanked by termination codons. Smaller ORFs overlapping larger ones by more than 60 % of their length were then discounted, except for ORF17 and ORF18, which overlapped slightly larger ORFs on the opposing strand. These were retained because each encoded a putative membrane protein and ORF17 was followed by a potential polyadenylation signal. One ORF of 117 codons was discounted because it spanned the X–R_S junction, had an unusually high G+C content and lacked a potential polyadenylation signal. Three additional ORFs (ORF16, ORF36 and ORF37) smaller than 100 codons were then added to the set. ORF16 and ORF36 did not overlap other ORFs and encoded putative membrane proteins, and ORF37 overlapped ORF38 to a small extent. In order to represent protein-coding regions, the 5′ end of each ORF was trimmed to the first ATG codon, except for ORF120, where use of the second ATG gave rise to a strong prediction for a signal sequence at the N terminus of the encoded protein. The analysis indicated the presence of 124 unique protein-coding genes (Fig. 6). Owing to the presence of the inverted repeats, 12 ORFs were duplicated, resulting in a total of 136 genes in the genome. These numbers included several fragmented genes (see below), each of which was counted as a single ORF. All ORFs were followed by a potential polyadenylation signal or were a member of a potential 3′-coterminal set. No indications of splicing emerged from sequence analysis.

One striking property of the gene complement was the presence of 38 genes in 12 families of related genes (Fig. 6). These included two families whose products were predicted to be secreted (ORF13 with two members and ORF50 with four), three families predicted to encode membrane-associated proteins (ORF41 with three members, ORF54 with two and ORF88 with four), one family whose products contained motifs V and VI of SF2 helicases (ORF7 with three members; motifs defined by Gorbalenya & Koonin, 1993), one family whose products were related to inhibitors of apoptosis (BIR with four members), one family derived from a deoxyuridine triphosphatase gene [(dut) with three members, of which probably only ORF75 encoded an active enzyme; active site motifs defined by McGeoch, 1990], two families of RING-finger genes (RING-1 with four members and RING-2 with two) and two other families (ORF4 with five members and ORF35 with two). The ancient nature of the duplication events that have resulted in these families was illustrated by the fact that family members were distantly related and generally widely distributed in the genome. Gene families are also prevalent in vertebrate herpesviruses and numerous in the larger genomes (Chee *et al.*, 1990).

The BIR family had four members (ORF42, ORF87, ORF99 and ORF106) that encoded products belonging to a family of viral and cellular proteins known as 'baculovirus inhibitor of apoptosis repeat proteins' (BIRPs), several of which have been shown to have anti-apoptotic activity (reviewed by Miller, 1999). The ORF42 and ORF106 proteins also contained a RING finger. BIRP-regulated pathways are evidently important mediators of defence against viral infection in invertebrates, since BIR genes are found not only in OsHV-1 but also in other large DNA viruses with invertebrate (insect) hosts, including members of the *Baculoviridae* (Crook *et al.*, 1993), *Ascoviridae* (Stasiak *et al.*, 2000), *Poxviridae* (Afonso *et al.*, 1999), *Iridoviridae* (Jakob *et al.*, 2001) and *Asfarviridae* (Yáñez *et al.*, 1995). In contrast, large DNA viruses of vertebrates do not encode BIRPs and subvert host apoptotic defences by other means.

Another characteristic of the OsHV-1 genome was the presence of disrupted genes in certain of the gene families (ORF5 in the ORF41 family; ORF32, ORF63 and ORF65 in the ORF32 family; and ORF50, ORF62, ORF73 and ORF105 in the ORF50 family). Although the disrupted genes contained more than one ORF, they were counted as single entities in calculating the OsHV-1 gene complement and Fig. 6 shows the locations of the intact coding regions that presumably existed before fragmentation occurred. The genes involved were detected from amino acid sequence alignments as between two and five tandem ORFs arranged in different reading frames or separated by termination codons, but similar to successive regions in relatives that were fragmented differently or not at all. Most or all are presumably non-functional and it was notable that no intact member of the ORF50 family and only one intact member of the ORF32 family remained. The degree of divergence between sequences generally precluded identifying precisely the locations and, in some cases, the number of fragments. As an example, Fig. 7 shows details of the proposed disintegration of ORF5 in relation to its intact relatives. There are four fragments, separated by two frameshifts and one termination codon. It was notable that the ORF5 protein also lacked a C-terminal region that was present in the related proteins and contained a putative transmembrane domain. This raised the possibility that ORF5 has been affected by deletion of this region. Moreover, ORF5 was more extensively deleted in an OsHV-1 variant (orange bar in Fig. 6; Arzul *et al.*, 2001c; the end nearer U_L was not mapped precisely). The ability to align sequences within gene families was essential for detecting disrupted genes and it cannot be ruled out that genes not belonging to families may also be disrupted. Gene fragmentation has not been observed in vertebrate herpesviruses, but is a characteristic of many members of the *Poxviridae* (reviewed by Gubser *et al.*, 2004). It is not possible to discern at present whether apparent loss of gene function in OsHV-1 reflects the selection of a pathogenic variant from a genetically intact

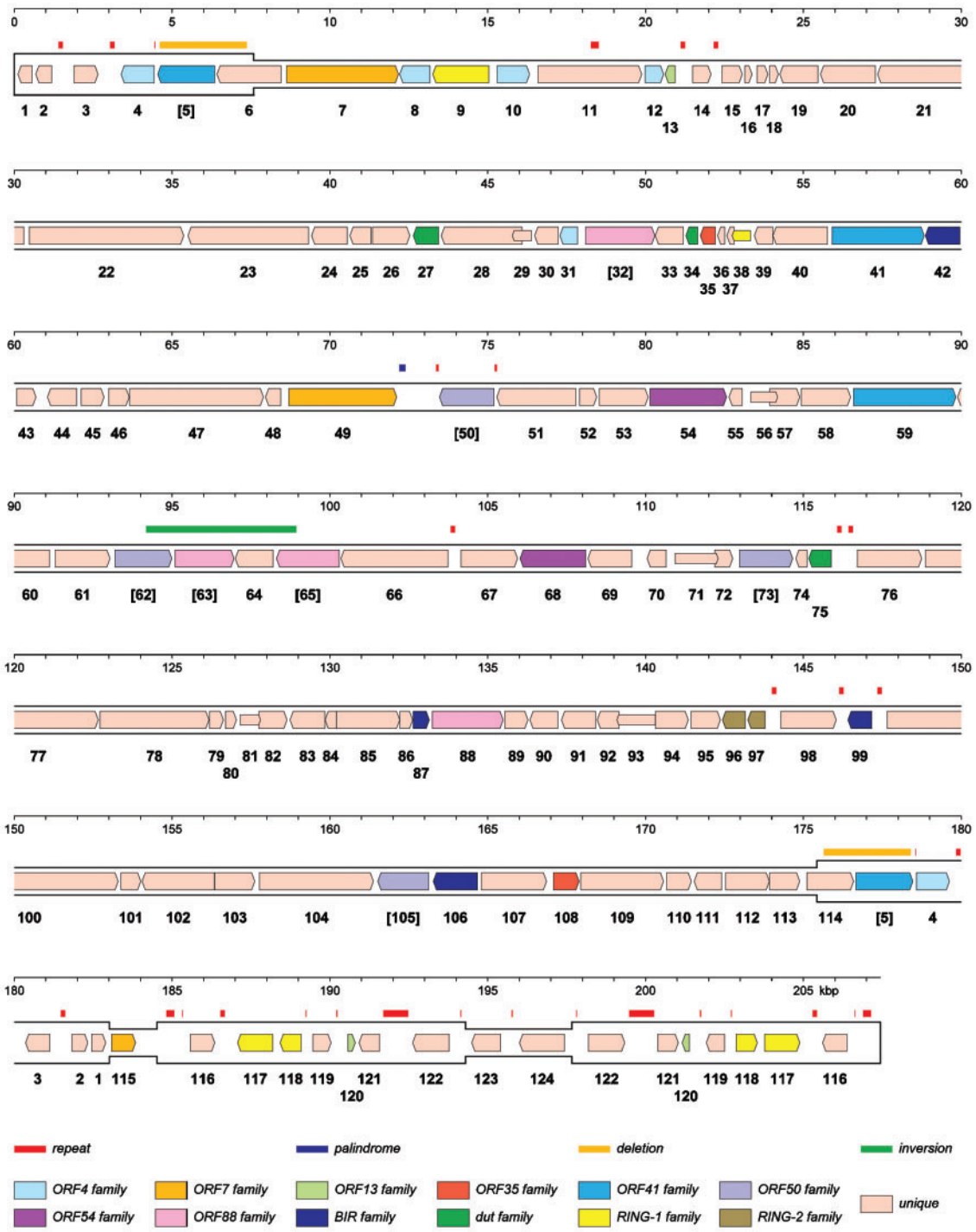


Fig. 6. Layout of genes in the OsHV-1 genome. The scale is in kbp and the 'ORF' prefix is omitted throughout. The inverted repeats TR_L/IR_L (ORF1–ORF6) and TR_S/IR_S (ORF116–ORF122) are shown in a thicker format than the unique regions U_L (ORF6–ORF114), U_S (ORF123–ORF124) and X (ORF115). By reference to the key, coloured bars above the genome denote features in the DNA sequence described in the text and coloured arrows in the genome indicate protein-coding regions grouped according to the key. Square brackets apply to genes whose fragmented protein-coding regions are depicted as intact.

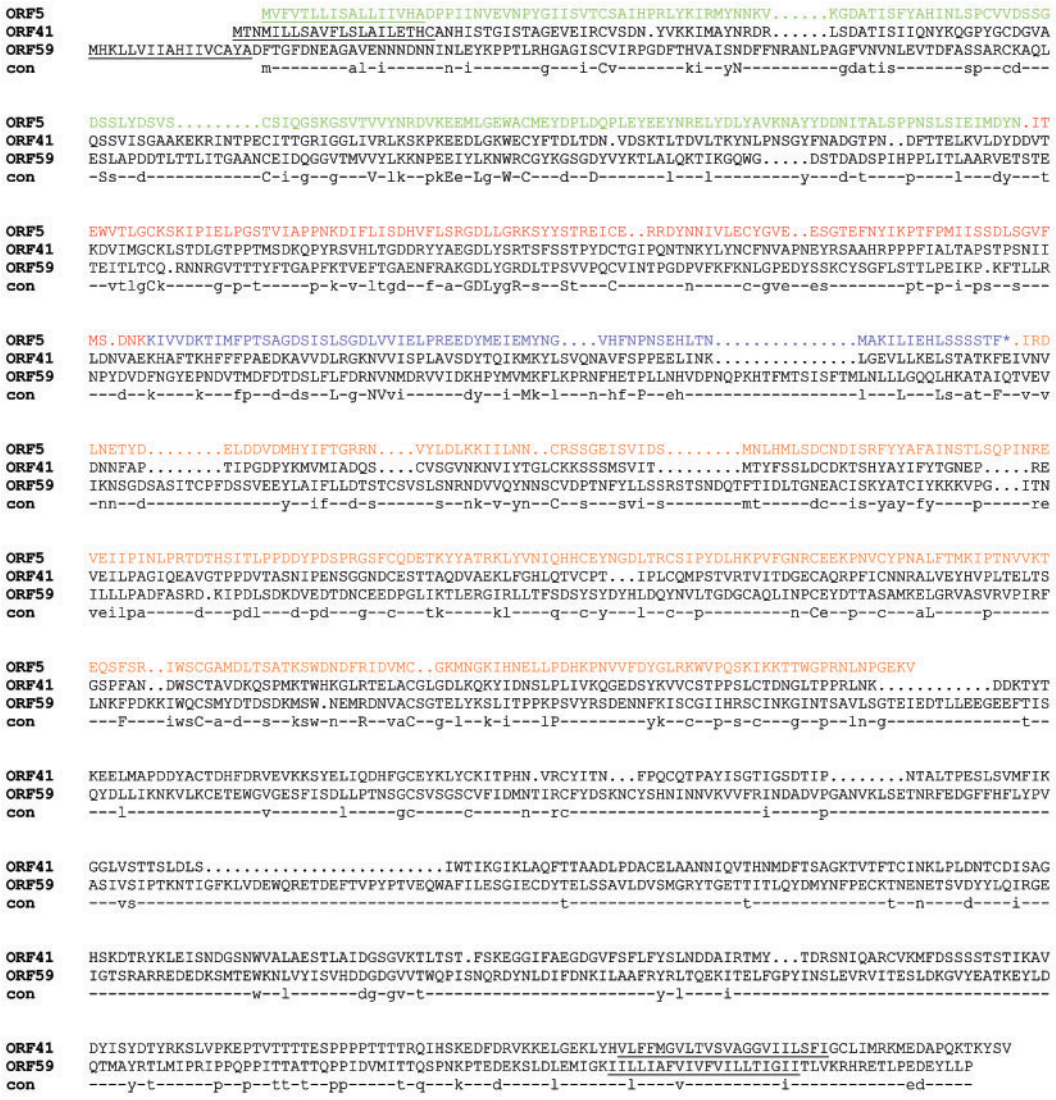


Fig. 7. Amino acid sequence alignment of ORF41 and ORF59 with sequences that make up ORF5, an apparently disrupted member of the gene family. The four separate coding regions that comprise ORF5 are shown in different colours. The first three are in different reading frames and the third and fourth are in the same reading frame but separated by a termination codon (asterisk). The ends of these regions are identified with a degree of uncertainty, but are contiguous with respect to the DNA sequence (i.e. there are no gaps). Predicted signal and transmembrane sequences are underlined near the N and C termini of the proteins, respectively. The consensus (con) is shown in upper case for residues that are identical in all three proteins and in lower case for residues that are identical in two.

ancestor, which may have been more species restricted and benign, as a result of modern large-scale shellfish farming activities.

The gene layout in the 4·8 kbp region within U_L that is inverted in some genomes (green bar in Fig. 6) was difficult to assess, since it contained all or part of three disrupted genes. The interpretation supported from alignments was that the minor form (utilized in Fig. 6) was the parent of the major form. In the latter, ORF62 and ORF65, both of which were disrupted, are present as fragmented genes representing hybrids between the ORF50 and ORF88 families. This complex rearrangement was presumably arrived at by a series of recombination and mutation events whose order and effect are obscure.

Database searches provided functional information on 25 OsHV-1 genes that were not members of families. These included genes encoding a tentative primase (ORF24; based on a short motif defined by Klinedinst & Challberg, 1994), two subunits of ribonucleotide reductase (ORF20 and ORF51), a helicase (ORF67), the catalytic subunit of a DNA

polymerase δ (ORF100), the ATPase subunit of terminase (ORF109; Davison, 2002) and two RING-like proteins (ORF53 and ORF124). The ORF95 protein was related to a family of conserved eukaryotic proteins (e.g. the FLJ21144 protein in humans), which are also more distantly related to the nuclease domain in bacterial RecB proteins (Aravind *et al.*, 1999; Chang & Julin, 2001). The ORF30 protein was related in an N-terminal cysteine-rich domain to a protein of unknown function in two subfamilies (*Beta-* and *Gammaherpesvirinae*) of the higher vertebrate herpesviruses (e.g. UL92 in HCMV and ORF31 in human herpesvirus 8). The consensus of this domain is $CX_{20–21}CX_2CX_3HXCX_5CX_{6–10}CX_3G$. A total of 15 genes encoded proteins that had predicted signal or transmembrane sequences and therefore may be associated with membranes, one (ORF57) specifying a protein that was related to a cellular chloride ion channel.

The OsHV-1 sequence contained several families of imperfect repeats, ranging in size from 3 to 146 bp (red bars in Fig. 6). Many were present as a single copy at each locus, and all but one were intergenic. Also, a large palindrome (Fig. 8) was located between ORF49 and ORF50 (blue bar in Fig. 6), most of which was deleted upon cloning into plasmids. By analogy with higher vertebrate herpesviruses in the subfamily *Alphaherpesvirinae* (Stow & McMonagle, 1983; Weller *et al.*, 1985) and the *Roseolovirus* genus of the subfamily *Betaherpesvirinae* (Inoue *et al.*, 1994), the palindrome is a candidate for an origin of DNA replication.

Evolution and taxonomy of OsHV-1

Despite the similarities in capsid structure between OsHV-1 and the two vertebrate herpesvirus groups, sequence comparisons did not reveal any OsHV-1 proteins with convincing similarity to known capsid proteins of either group. Moreover, global comparisons failed to identify OsHV-1 genes that had homologues only in other herpesviruses, with the exception of ORF30, which had a domain found to date elsewhere only in a subset of mammalian herpesviruses but not in CCV. ORF109, which encoded the putative ATPase subunit of the OsHV-1 terminase, had relatives in all vertebrate herpesviruses and in T4-related bacteriophages. The OsHV-1 and T4 genes are not spliced, whereas those in higher and lower vertebrate herpesviruses contain one and two introns, respectively. In principle, phylogenetic analysis of ORF109 and its counterparts should reveal the branching order, but it was not possible to draw a conclusion beyond that of the three groups being highly diverged. Thus, although a scheme may be proposed in which OsHV-1 and the two vertebrate herpesvirus groups descended from a common ancestor with divergence so extensive that sequence similarity is not detectable, the evidence must be regarded as scant. Nonetheless, it is clear that herpesviruses fall into three major classes, one associated with mammals, birds and reptiles, one with amphibians and bony fish, and one with bivalves. The logical means of establishing this in formal taxonomy would be to establish three virus families incorporating the three groups as listed above, under the umbrella of an order (*Herpesvirales*).

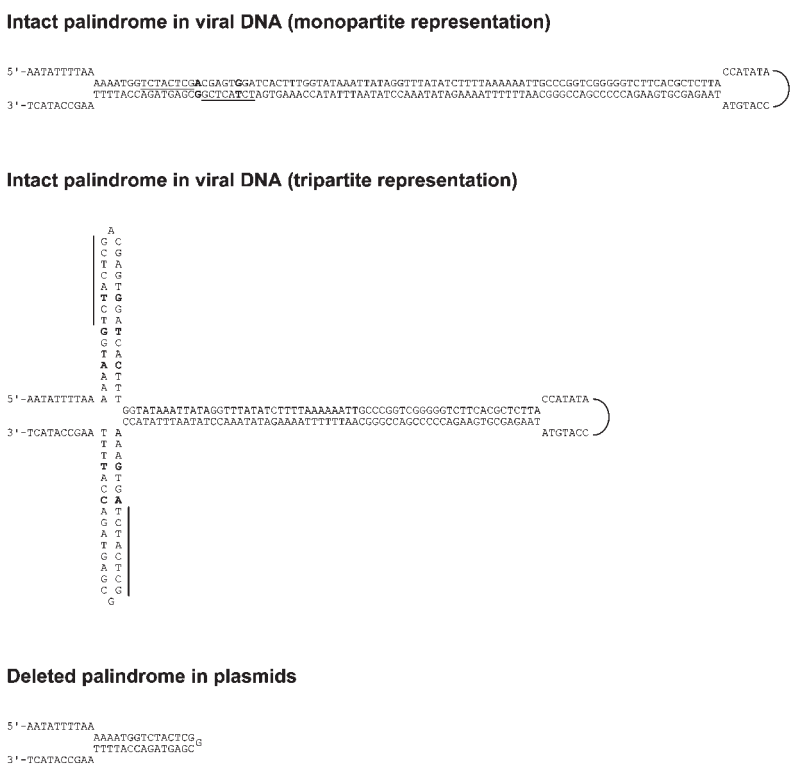


Fig. 8. Structure of the intact OsHV-1 palindrome in viral DNA and its deleted form in plasmids. Base pairing within one of the two DNA strands is shown. Non-Watson–Crick base paired nucleotides within the paired regions are shown in bold. The regions between which the deletion occurred in plasmids are indicated by horizontal or vertical lines on the viral sequence.

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3. 3. A la chasse aux virus: un besoin d'outils de diagnostic performants

Les descriptions multiples de virus de type herpès apparaissent comme un élément poussant à les étudier au mieux. Cependant, la **technique la plus utilisée pour rechercher des agents infectieux chez les mollusques** a été et demeure aujourd'hui encore **l'histologie**. Cette méthode est assez inadaptée pour le diagnostic des infections virales. Elle a été souvent complétée par des analyses en microscopie électronique à transmission. Cependant, ces deux techniques sont longues à mettre en œuvre et ne sont pas les meilleurs outils pour réaliser des suivis de terrain. Par ailleurs, **l'absence de lignées cellulaires de mollusques marins** complique la tâche du pathologiste. En effet, pour analyser des échantillons suspects, ce dernier ne peut pas cibler la recherche d'effets cyto-pathogènes *in vitro*, technique de choix pour le diagnostic virologique. Enfin, le diagnostic passe chez les coquillages par une mise en évidence des agents infectieux eux-mêmes. Les mollusques ne possédant **pas de réponse immunitaire adaptative et de mémoire immunitaire** (absence de lymphocytes et d'anticorps), il n'est pas possible de rechercher les traces laissées par le passage d'un agent pathogène (diagnostic indirect comme la sérologie). **Seules peuvent être mises en œuvre des techniques de diagnostic direct**.

Concernant le diagnostic des infections à virus de type herpès chez les coquillages, une avancée a été faite avec la purification du virus infectant les larves d'huître creuse, C. *gigas,* (**Le Deuff & Renault, 1999**) et le séquençage complet de son génome (**Davison *et al.*, 2005**).

3. 3. 1. Développement d'outils moléculaires spécifiques d'OsHV-1 (Renault T., Le Deuff R.-M., Lipart C. & C. Delsert, Journal of Virological Methods, 2000, 88: 41-50) et (Batista F. M., Taris N., Boudry P. & T. Renault, 2007, Journal of Virological Methods, 139(1): 1-11)

A l'aide des données initiales de séquence obtenues pour le virus infectant les larves d'huître creuse (**Le Deuff & Renault, 1999**), un protocole de PCR nichée (**Renault *et al.*, 2000a**) a été développé au Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France). La PCR offre de nombreux avantages pour le diagnostic des maladies infectieuses (Henson & French, 1993 ; Jones & Bej, 1994 ; Martin, 1994). Concernant le virus infectant l'huître creuse, les avantages recherchés étaient une grande sensibilité et une grande spécificité, un traitement simple des échantillons et la disponibilité des réactifs nécessaires. Deux couples d'amorces ont été dessinés (A3/A4 et A5/A6, région A du génome viral codant pour une protéine de fonction inconnue). La spécificité de l'amplification a été testée en utilisant de l'ADN viral extrait de particules purifiées, de l'ADN d'huître creuse et des ADN provenant de différents herpès virus de vertébrés (poissons, amphibiens et homme). Des produits de PCR ont été obtenus uniquement avec l'ADN extrait du virus de l'huître. Des échantillons infectées et non infectés (larves contrôlées en microscopie électronique) ont ensuite fait l'objet d'une analyse. Le protocole choisi pour préparer les échantillons était un protocole simple (broyage en eau distillée et traitement par la chaleur). Ce protocole comparé à des protocoles plus classiques d'extraction de l'ADN (phénol/chloroforme) a montré son efficacité et sa robustesse. Un des objectif fixés dans ce travail était de transférer rapidement la technique d'analyse par PCR à d'autres laboratoires Ifremer, non spécialisés en biologie moléculaire. Il y avait donc besoin d'un protocole ne nécessitant pas de connaissances spécifiques en matière d'extraction d'ADN. Une **corrélation entre observation de mortalités anormales, détection de particules virales en microscopie électronique et obtention de produits de PCR** a été mise en évidence, l'avantage de cette dernière technique étant de pourvoir disposer d'un **résultat en moins de 24 heures**.

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Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France

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Abstract

A PCR-based procedure for detecting a herpes-like virus that infects the Japanese oyster, *Crassostrea gigas*, in France was developed. Two primers were designed to provide specific amplification products ranging in size from 917 to 1001 bp when carried out on oyster herpes-like virus DNA. No amplification was observed of oyster genomic DNA nor of the DNA from vertebrate herpesviruses. Crude samples were prepared and submitted to nested PCR, allowing amplification of DNA fragments of the expected size when carried out on infected larval and spat samples. The procedure used to prepare the sample for PCR was found to be critical because of the presence of unidentified substances in oyster tissues that inhibit the PCR reaction. A rapid and convenient sample preparation using ground tissues allowed a sensitive detection of the herpes-like virus infected oysters. The ability of the defined PCR protocol to diagnose herpes-like virus infections in oysters was compared to the transmission electron microscopy technique using 15 *C. gigas* larval batches with or without mortalities. PCR amplification is as sensitive a diagnostic assay for herpes-like virus as transmission electron microscopy. However, the nested PCR protocol is more convenient and less time consuming. The relationship between reported mortalities among *C. gigas* oyster spat and herpes-like virus DNA detection by PCR was also investigated. Statistical analysis showed that virus detection and mortalities are correlated. This observation highlights the importance of studying the causative role of herpes-like virus in oyster spat mortalities. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Herpes-like virus; Japanese oyster; *Crassostrea gigas*; Detection; Polymerase chain reaction

1. Introduction

The Pacific oyster, *Crassostrea gigas*, is the most important species of bivalve mollusc reared in the world (FAO, 1989) and is of particular importance for French mariculture. Cultivation may be endangered by the occurrence of epizootics, especially virus diseases, which are considered one of the major risks to production. Indeed, mortalities have been described among different species of ostreids associated with the presence of viruses belonging to various families. The first description of a virus was reported in adult Eastern oysters, *Crassostrea virginica*, with

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the detection of particles indicating membership of the *Herpesviridae* (Farley et al., 1972). More remarkably, mass mortalities of adult Portuguese oyster, *Crassostrea angulata*, among French livestocks (between 1967 and 1973) were associated with iridovirus infections (Comps et al., 1976; Comps and Bonami, 1977; Comps and Duthoit, 1979). Other viruses described in ostreids are members of the *Iridoviridae*, *Papovaviridae*, *Togaviridae*, *Retroviridae* and *Reoviridae* (Farley, 1976, 1978; Elston, 1979; Meyers, 1979; Elston and Wilkinson, 1985).

More recently, in 1991, viruses interpreted as belonging to the *Herpesviridae* were associated with high mortality rates of hatchery-reared larval *C. gigas* in France (Nicolas et al., 1992) and in New Zealand (Hine et al., 1992). Since 1992 sporadic high mortalities of larval *C. gigas* are observed regularly in some private French hatcheries, occurring each year during the summer period in association with a herpes-like virus (Renault et al., 1994b). The pathogenicity of the virus was demonstrated by experimental transmission of the infection to axenic *C. gigas* larvae (Le Deuff et al., 1994). Additionally, some mortalities reported among *Ostrea edulis* and *C. gigas* spat in France since 1992 have also been associated with detection of a herpes-like virus (Comps and Cochennec, 1993; Renault et al., 1994a). More recently, replication of herpes-like viruses was observed in *Ostrea angasi* adults in Australia (Hine and Thorne, 1997) and in larval *Tiostrea chilensis* in New Zealand (Hine, 1997; Hine et al., 1998).

Herpes-like virus infections in oysters belonging to the genera *Ostrea* and *Crassostrea* seem to be ubiquitous and are associated with substantial mortalities. These observations highlight the importance of developing a range of diagnostic methods in order to assess the causative role of the viruses in oyster mortalities and to limit the possible economic impact of the infections by studying virus epidemiology and preventing the potential spread of the infections. To diagnose herpes-like virus infections, the basic method for examination of suspect samples is still light microscopy. This method appears poorly adapted to virus diseases and needs to be improved upon by other techniques such as transmission electron microscopy. Both techniques are time consuming and inadequate for epidemiological surveys. In addition, research into virus cytopathogenic effects in cell cultures is impossible because of the lack of bivalve cell lines. Serological methods are also not available due to the absence of immunoglobulin production in molluscs, and no molecular method for diagnosing oyster herpes-like virus infections has been published. A breakthrough was achieved recently in the development of a protocol, based on sucrose gradient centrifugation, for purifying oyster herpes-like virus particles from fresh infected larval *C. gigas* (Le Deuff and Renault, 1999). This advance has served as an appropriate platform for generating molecular biological reagents to diagnose virus infections in oysters.

The aim of the present study was to develop a procedure to detect herpes-like virus in French oysters using the polymerase chain reaction (PCR) (Saiki et al., 1985; Mullis et al., 1986). PCR offers many advantages for disease diagnosis (Henson and French, 1993; Jones and Bej, 1994; Martin, 1994). With regard to herpes-like viruses from oysters, important advantages include its high sensitivity, pathogen specificity, ease of sample processing, and availability of reagents. A nested PCR protocol would be useful for disease diagnosis and also for studying the basic biology of these viruses and their causative role in bivalve mortality.

2. Material and methods

2.1. Clinical samples

C. gigas oyster larvae and spat were investigated by PCR. Samples were collected from 1992 until 1997 from broods, some of which presented high mortalities, and were kept frozen at −20°C after sampling. Some samples were examined by transmission electron microscopy.

2.2. Electron microscopy

For transmission electron microscopy, larvae were fixed for 1 h in cold 2.5% glutaraldehyde in

0.2 M cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide in the same buffer (1 h, 4°C). After dehydration by means of an ascending ethanol series, specimens were embedded in Epon resin. Ultrathin sections were cut with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM 1200 EX transmission electron microscope at 80 kV.

2.3. Virus and oyster DNAs

Herpes-like virus DNA was extracted from purified virus pellets. The protocol used was described previously (Le Deuff, 1995; Le Deuff and Renault, 1999). Viral DNA was resuspended in TE buffer (10 mM Tris; 1 mM EDTA; pH 8) and DNA concentration was determined by spectrophotometry at 260 nm. It was adjusted to 100 ng per μl in TE buffer. Tenfold serial dilutions from 10 ng/μl to 1 fg/μl were prepared in the same buffer.

Herpes simplex virus 1 (HHV1), herpes simplex virus 2 (HHV2), Epstein-Barr virus (HHV4), human cytomegalovirus (HHV5), human herpesvirus 6 (HHV6), ictalurid herpesvirus 1 (CCV), salmonid herpesvirus 2 (*Oncorhynchus masou* virus, OMV) and frog herpesvirus (FV4) DNAs were extracted from infected cell cultures.

Oyster DNA was derived from healthy adult *C. gigas* (La Tremblade, Charente Maritime) and prepared as described previously (Le Deuff, 1995).

2.4. Sequencing

Viral DNA was digested using *Eco*RI and ligated into the *Eco*RI-linearized pBluescript KS II-plasmid (Stratagene, USA). DNA was transformed into competent *Escherichia coli* XL1 Blue cells (Stratagene, USA) and clones were submitted to restriction enzyme analysis. Screening of recombinant clones was done as described previously (Le Deuff and Renault, 1999). A positive clone was sequenced partially as double-stranded DNA, according to the manufacturer's (Pharmacia, Sweden) recommendations. The GenBank accession numbers are AF 173837 and AF 173838.

2.5. Design of primers

PCR primers were derived from the above sequence. Forwards primers (A3 and A5) and reverse primers (A4 and A6) were designed which provided two primer-pair combinations (A3/A4 and A5/A6), with expected PCR products that ranged, respectively, in size from 1001 to 917 bp. Primer sequences used were:

A3: 5′GCCAACCGTTGGAACCATAACAAGCG3′
A4: 5′GGGAATGAGGTGAACGAAACTATAGACC3′
A5: 5′CGCCCCAACCACGATTTTTCACTGACCC3′
A6:
5′CCCGTCAGATATAGGATGAGATTTG3′

Primers were supplied by Eurogentec (Belgium). A3/A4 were used as external primers and A5/A6 as internal primers in the nested PCR protocol.

2.6. Sample preparation for PCR

A total of 50 mg of frozen larvae were weighed out in an Eppendorf microtube and ground in 50 μl of distilled water using a disposable tissue homogeniser. For spat, 30 individuals (six pools of five animals) were analyzed for each batch. Animals were removed from the shell and dried on paper. The technique used to grind spat samples depended on the animal size. For spat up to 12 mm in shell size, groups of five animals were weighed out in 1.5-ml Eppendorf microtubes and double distilled water was added at 1 ml/g of tissue. Animals were ground using a disposable tissue homogeniser. For spat larger than 12 mm in shell size, five individuals were ground with a rubber mallet in plastic bags without adding double distilled water. After cutting a corner of the bag, 0.5 g of ground tissue was recovered into a 1.5-ml Eppendorf tube and 0.5 ml of double distilled water was added. Ground larval and spat samples were vortexed and denatured in a boiling water bath for 10 min. Samples were mixed again

and centrifuged at 10 000 rpm for 5 min. Supernatants were recovered and immediately diluted tenfold in double distilled water and frozen at − 20°C.

2.7. PCR conditions

Thermal cycling was carried out using a Crocodile III thermal cycler (Appligene Oncor, France). The 50-μl PCR reactions were undertaken using the Goldstar Polymerase (Eurogentec, Belgium), each containing the appropriate reaction buffer (Eurogentec, Belgium), 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM $MgCl_2$, and 2.5 U of DNA polymerase. Various amounts of template DNA were added under a volume of 1 μl. After heating the samples for 2 min at 94°C, 35 cycles were carried out followed by a final elongation step of 5 min at 72°C. Each of the 35 cycles consisted of a DNA melting step at 94°C for 1 min, a primer annealing step for 1 min at 50°C and a primer elongation step at 72°C for 1 min. The reaction conditions in the second reaction were the same as for the first reaction, except for template DNA volume (0.5 μl of first PCR reaction products). Then 10 μl of each PCR products was size-selected on 1% agarose gels, stained with ethidium bromide (0.5 μg/ml) and visualized using a 302-nm UV transilluminator. The size of the DNA products was determined relative to those of size markers (Eurogentec, marker 1 or marker 2). Relative amounts of amplification products within the same gel were made by comparing bands intensities on photographs.

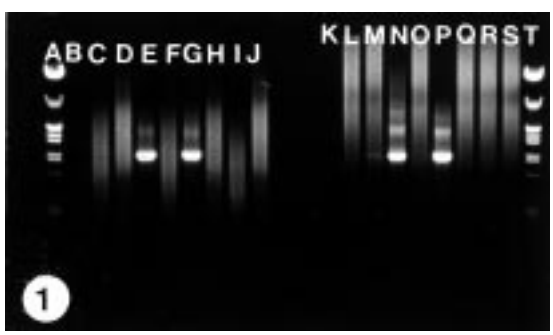

Fig. 1. Amplification products using virus DNA, plasmid DNA and oyster DNA as template. Lanes B–J: first PCR reaction products (primer pair A3/A4); lanes K–S: second PCR reaction products (primer pair A5/A6). Lanes A and T show the migration of molecular weight markers (Eurogentec M2). Lanes B and K: control reactions without DNA polymerase; lanes C, F, H, J, L, O, Q and S: control reactions without virus DNA as template; lanes D and M: virus DNA (1 pg); lanes E and N: virus DNA (10 ng); lanes G and P: plasmid DNA (100 pg); lanes I and R: oyster DNA (0.8 μg).

3. Results

3.1. Optimization of the herpes-like virus specific amplification

When the nested PCR protocol was carried out with the A3/A4 and A5/A6 primers on corresponding cloned virus DNA and viral DNA isolated from purified virus particles, it allowed the amplification of DNA fragments of the expected sizes (Fig. 1, lanes E, G, N and P). Meanwhile no amplification was observed when genomic DNA of *C. gigas* was used as template (Fig. 1, lanes I and R) or when virus DNA or DNA polymerase was omitted (Fig. 1, lanes B, K, C, F, H, J, L, O, Q and S) indicating that the amplicons produced were the results of template-dependent DNA amplification and that PCR reagents were not contaminated with viral DNA. PCR reactions carried out on the DNA from various herpesviruses infecting human or lower vertebrates did not amplify DNA fragments (data not shown) indicating that the nested PCR reaction is specific to the herpes-like virus infecting *C. gigas*. Magnesium chloride concentrations appeared to be critical, depending on the amount of viral DNA used in the PCR reaction. A 2.5-mM concentration of $MgCl_2$ allowed production of visible amounts of amplicons when 1 pg of virus DNA was used. Primer concentrations also appeared to be critical, depending on the amount of viral DNA used in PCR reactions. PCR2 reactions provided visible amplicons on agarose gel for 50, 100, 200 and 300 ng of primers when 1 pg of virus DNA was used as template, but the reaction failed to produce visible amplicons for 400 and 500 ng of primers (data not shown).

Fig. 2. Effect of DNA polymerase concentration on virus DNA detection by nested PCR using A3/A4 and A5/A6 primer pairs. Lane A: molecular weight markers (Eurogentec M2); lanes C–G: second PCR reaction products for 0.2 pg of virus DNA as template; lanes I–M: second PCR reaction products for 0.5 pg of virus DNA as template. Lanes C and I: 0.1 U of Taq polymerase/μl; lanes D and J: 0.006 U/μl; lanes E and K: 0.01 U/μl; lanes F and L: 0.03 U/μl; lanes G and M: 0.05 U/μl. Lanes B and H show control reactions without template.

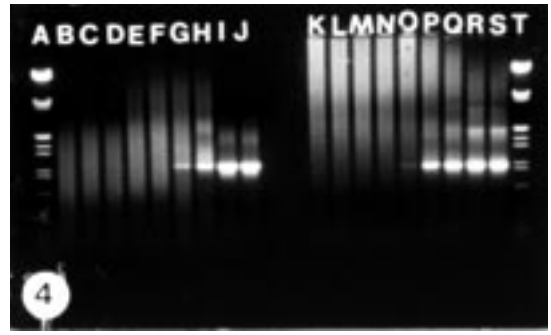

Fig. 4. Sensitivity of viral DNA detection by nested PCR using the A3/A4 and A5/A6 primer pairs. Results were obtained on a serial dilution (1:10) of viral DNA from 1 fg to 10 ng. Lanes B–J: PCR1; lanes K–S: PCR2. Lanes A and T: molecular marker M2 (Eurogentec, Belgium). Lanes B and K: control reactions without template. Lanes C and L: 1 fg of viral DNA. Lanes J and S: 10 ng of viral DNA.

Although thermal cycling was carried out routinely using 50°C as annealing temperature in PCR1 and PCR2 reactions, 45 and 55°C also yielded specific products. Amplicon yield increased as the DNA polymerase concentration increased from 0.01 to 0.05 U/μl (Figs. 2 and 3). Increasing the DNA polymerase concentration above 0.05 U/μl resulted in inhibitory effects (Fig. 2). Using DNA extracted from purified virus particles, the minimum quantity of template needed to produce sufficient amounts of DNA for visualization was 0.1–1 pg for the second PCR reaction (Fig. 4). The yield of amplicons increased when increasing the amount of template and reached a maximum when 100 pg of virus DNA was used (Fig. 4). When more than 1 ng of template virus

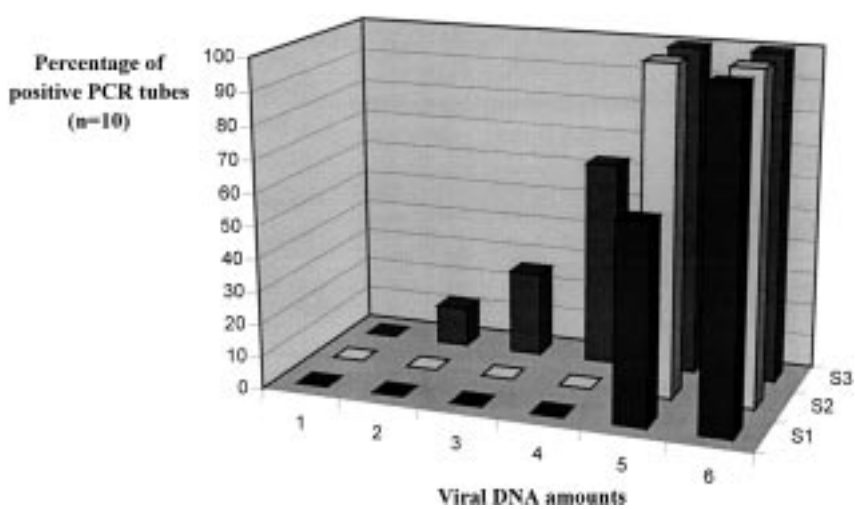

Fig. 3. The percentage of positive tubes ($n = 10$) after PCR2 is indicated with different concentrations of DNA polymerase (S1: 0.01 U/μl, S2: 0.03 U/μl and S3: 0.05 U/μl). Ten PCR reactions were performed for each DNA polymerase concentration and different amounts of virus DNA (1: 1 fg, 2: 10 fg, 3: 100 fg, 4: 200 fg, 5: 500 fg and 6: 1 pg).

DNA was used, visible non-specific amplification products were detected (Fig. 4). Cycle number did not appear to be critical. All combinations (30, 35 and 40 cycles for PCR1 and PCR2) produced visible amounts of PCR products (data not shown).

3.2. PCR with oyster specimens

The effect of inhibitors contained in animal tissues on the PCR reaction was investigated in mixing experiments (Table 1). The first PCR reactions (PCR1) were carried out using different amounts of virus DNA extracted from purified particles (1 ng to 10 fg) as template, mixed with increasing amounts of non-infected spat tissues (0.01, 0.05, 0.1, 0.5, 1, 2 and 6 mg per tube). Spat tissues were prepared by grinding oysters controlled as negative by TEM for virus detection (6 g of tissues in 1 ml of double distilled water) in an Eppendorf tube. Second PCR reactions (PCR2) were performed using 0.5 μl of first PCR reaction product. Viral DNA yielded a detectable amplification product of the expected size after PCR2 when PCR1 was carried out on 1 ng to 100 fg (Table 1) in absence of oyster tissues. Increasing the amount of non-infected spat tissues gradually inhibited the first and second PCR reactions; this inhibitory effect depended on the amount of virus DNA introduced in PCR1 (Table 1). The specific reaction product was observed for PCR2 when PCR1 was performed in the presence of 6 or 2 mg of non-infected spat sample on 1 ng of virus DNA but not on 100 pg of viral DNA (Table 1). Thus, the inhibitory effect could be overcome partially by increasing the amount of virus DNA introduced in PCR1. Non-infected spat tissues (6, 2, 1, 0.5, 0.1, 0.05 and 0.01 mg), in the absence of viral DNA, failed to produce amplicons (Table 1).

In order to optimize our reaction conditions, we use routinely tenfold diluted the oyster tissues (1 g/ml in distilled water) immediately after grinding, so that the amount of tissue added to each PCR reaction was 0.1 mg. This dilution procedure gave more positive results (66.7 versus 40%, $n = 15$) than weighing a lesser amount (0.1 g) of tissue without further dilution.

The possible inhibitory effect of the reagents contained in the PCR1 reaction on the PCR2 reaction was tested. PCR1 was carried out using 100 fg, 200 fg and 10 pg of DNA extracted from purified particles as template and different amounts of PCR1 reaction (1, 0.8, 0.5, 0.3, 0.2 and 0.1 μl) were then used for PCR2 reaction. PCR2 reaction product was observed for each amount of PCR1 reaction, although the signal was less intense when using 1 μl of PCR1 reaction than for lower amounts (data not shown).

The procedure used to prepare each sample prior to PCR is known to be critical for the success of the amplification reaction. Alternative techniques were examined such as the use of

Table 1
Effect of different amounts of non-infected oyster tissues on the detection of amplicons in the PCR2 reaction[a]

Non-infected oyster tissue dilutions	Virus DNA						
	0	10 fg	100 fg	1 pg	10 pg	100 pg	1 ng
Pure	1−	1−	1−	2−	2−	2−	1+/1−
1:3	1−	1−	1−	2−	2−	2−	3+
1:6	1−	1−	1−	2−	1+/1−	1+/1−	3+
1:12	1−	6−	6−	2+/5−	1+	1+	2+
1:60	1−	6−	1+/5−	5+/2−	2+	2+	3+
1:120	1−	6−	6−	4+/3−	1+/1−	2+	3+
1:600	1−	1−	1+	2+	1+/1−	2+	2+
None	1−	1−	1+	1+	2+	2+	3+

[a] For each amount of non-infected oyster tissue and virus DNA, the number of positive and negative PCR reaction is given. A total of 0.5 U of DNA polymerase was added to each reaction. Non-infected oyster tissue dilutions were obtained from a ground tissue preparation (6 g per 1 ml) in distilled water. ND, not done.

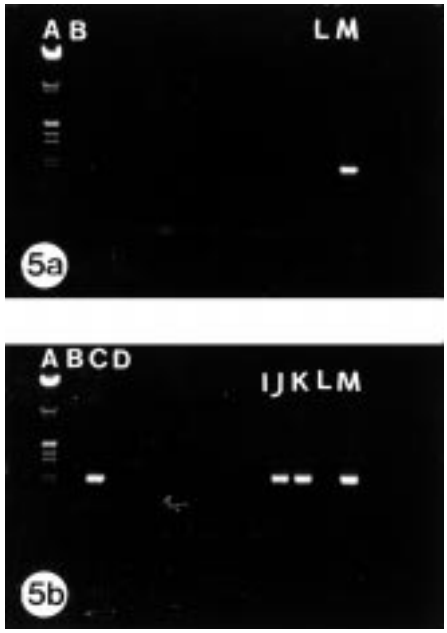

Fig. 5. Effect of sample preparation on virus DNA detection by nested PCR using the A3/A4 and A5/A6 primer pairs. (A) Samples treated by Chelex-100 (PCR2 products). (B) Samples treated by grinding and heating in distilled water (PCR2 products). Lanes B–M: oyster samples. Lane A: M2 molecular marker (Eurogentec, Belgium).

Chelex-100 (Walsh et al., 1991) and the combination of proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. Both techniques yielded false negative results (Fig. 5A and B). The protocol that provided constantly the best detection, for larvae and as well as for spat, was to grind animals in double distilled water (1 g/ml), and to denature the samples in a boiling water bath for 10 min. Samples were then centrifuged at 10 000 rpm for 5 min and the supernatant was recovered and immediately diluted tenfold in double distilled water and kept frozen at -20°C. This protocol allowed detection of amplification from different clinical samples with high mortality rates (Fig. 6).

3.3. Diagnosis comparison and correlation with mortalities

As a validation of the assay, 15 oyster larval batches either with or without mortalities were analysed by both transmission electron microscopy (TEM) and the nested PCR reaction as described above. Our data showed that the ten samples with high mortality rates at the time of sampling were positive for both TEM and nested PCR while the five healthy samples were negative for both techniques.

A total of 660 oyster spat corresponding to 20 different batches were submitted for PCR analysis. Of these 335 were animals originated from 12 batches with mortalities and 325 were animals from eight batches without mortality. Pools of five animals were analysed. A total of 27 oyster pools of five animals were diagnosed as positive and corresponded to animal batches with mortalities (Table 2), while no positive pool was detected on oyster batches without mortality (Table 2). A χ^2 test indicated that reported mortalities for PCR analysed spat samples had an influence on the viral DNA detection ($\chi^2 = 33.9$, $\alpha = 0.001$).

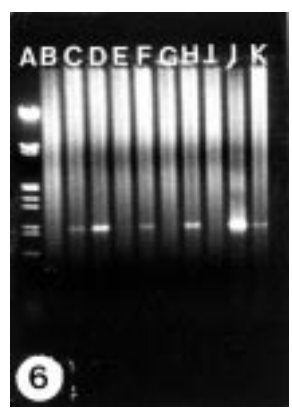

Fig. 6. Analysis of *C. gigas* larvae and spat samples by the nested PCR using the A3/A4 and A5/A6 primer pairs revealed on ethidium bromide-stained agarose gel of the PCR2 product. Lanes B and I: control reactions with no template DNA; lanes J and K: positive control corresponding to 10 ng and 0.5 pg of virus DNA, respectively. Lanes C–E: *C. gigas* larval samples; lanes F–H: *C. gigas* spat samples. Lanes C, D, F and H: samples controlled as infected by TEM examination; lanes E and G samples controlled as non-infected by TEM examination.

Table 2
Relationship between mortalities and herpes-like virus DNA detection[a]

	Individuals	Number of batches	PCR (+)	PCR (−)	Total number of PCR analysis
Batch with mortality	335	12	27 (40%)	40 (60%)	67
Batch without mortality	325	8	0 (0%)	65 (100%)	65
Total	660	20	27 (19.7%)	105 (80.3%)	132

[a] Pools of five individuals were analysed using the nested PCR reaction as above. PCR diagnosis was recorded either as (+) or (−) when the PCR2 amplification product was detected or not after analysis on ethidium bromide-stained agarose gel.

4. Discussion

The recent appearance of herpes-like viruses infecting different oyster species highlights the need for diagnostic methods. The detection techniques currently available, histology and TEM, are less than completely satisfactory with regards to sensitivity, time and cost efficiency. PCR-based detection systems for vertebrate herpesvirus infections are well documented (Boyle and Blackwell, 1991; Casas et al., 1996; Cassinotti et al., 1996; de Gee et al., 1996; Torigoae et al., 1996). Progress has also been made recently in developing methods for detection of human shellfish-transmitted viruses (Lees et al., 1994; Le Guyader et al., 1994; Atmar et al., 1995). However, to date no molecular methods for detecting viruses infecting bivalves, particularly oyster herpes-like virus, has been published. This PCR-based detection procedure is the first developed for a herpes-like virus infecting the Pacific oyster, *C. gigas*, in France.

Two primer pairs (A3/A4 and A5/A6) were designed using the partial sequence of a cloned virus DNA fragment (Le Deuff, 1995; Le Deuff and Renault, 1999) and were shown to be specific for the oyster herpes-like virus, providing PCR products of expected sizes. In our method 2.5 mM of $MgCl_2$ and 0.05 U/μl of DNA polymerase were the optimal conditions. Other parameters such as annealing temperatures and primers or template concentrations (Innis and Gelfand, 1990) were tested. The detection procedure was found to be specific, not leading to false positives, thus indicating that the primer sequences are not detected in the oyster genome under these conditions. The nested PCR assay detected routinely as little as 500 fg of virus DNA extracted from purified particles which corresponds approximately to 2500 genome copies, assuming a genome size of 180 kbp (Le Deuff, 1995; Le Deuff and Renault, 1999). The oyster herpes-like virus primers failed to produce DNA fragments of the expected size when human, fish and frog herpesvirus DNAs were used as template. This observation supports the conclusion that the primers are specific for the oyster herpes-like virus.

Concerning the different procedures of sample preparation from oyster specimens, boiling of ground tissues was the preferred method, because it was simple. Extensive purification procedures must be weighed against the increased risk of sample contamination at each step of manipulation. A possible explanation for false-negative results observed when methods such as Chelex-100 DNA preparation was used is that this method involves some sample dilution, therefore allowing only the use of a fraction of the original sample. However the presence of compounds in oysters that inhibit the PCR reaction might be a potential problem in using ground tissues, leading to false negative results. In order to minimize the inhibitory effects due to these compounds, a tenfold dilution was used for PCR amplification and 0.1 mg of oyster tissues was found to be sufficient for a sensitive detection of viral DNA.

Diagnosis by PCR amplification on ten batches of larval oysters with a high mortality rate was confirmed by TEM examination, thus providing a validation test. The sensitivity of the amplification reaction to the presence of less than 500 fg of virus DNA (2500 copies of viral genome) suggests the PCR technique can detect a small number of virions in an isolate corresponding to several thousand larvae.

Statistical analysis of data on a larger sample of spat displaying either or not mortalities, shows that the viral DNA detection by PCR depends on the mortality status of the analysed batches. Indeed, a χ^2 test indicates that reported mortalities have an influence on the viral DNA detection ($\chi^2 = 33.9$, $\alpha = 0.001$). This observation highlights the importance in studying the causative role of herpes-like virus in oyster spat mortalities.

These results indicate that the nested PCR protocol represents a fast, sensitive and reliable method for detecting the herpes-like virus in oysters. This procedure will be useful in the future not only for disease diagnosis but also for studies on the basic biology and pathogenicity of the emerging bivalve herpes-like viruses.

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Par la suite, sur la base de l'obtention de la **séquence complète du génome d'OsHV-1** (**Davison *et al.*, 2005**), de **nombreux autres couples d'amorces de PCR,** ciblant différents gènes ont été dessinés. Le très petit nombre d'homologies observées avec les autres herpès virus en terme de séquences et le fait que le virus OsHV-1 soit le premier membre décrit d'un nouveau groupe d'herpès virus n'a pas facilité le choix des gènes cibles. Il est à noter que le gène codant pour l'ADN polymérase n'a pas été sélectionné. En effet, cette enzyme chez OsHV-1 ne présente pas plus d'homologie avec les autres herpès virus qu'avec d'autres organismes.

Le choix a porté en particulier sur deux gènes codant respectivement pour une **protéine inhibitrice de l'apoptose (IAP, région B)** et une **glycoprotéine membranaire putative (GP)**, et une région du génome viral **(région C)** codant pour deux protéines de fonction inconnue. Les deux premiers gènes ont été sélectionnés sur la base des fonctions des protéines qu'ils codent (intérêt en terme de compréhension des interactions entre le virus et son hôte, en plus d'un intérêt diagnostique). Par ailleurs, ces deux gènes ne présentent que très peu de variabilité (Arul, 2001). Pour la région C, au contraire, une variabilité importante a pu être notée avec la détection d'un variant présentant une délétion de plus de 2 kpb. L'intérêt de cibler cette région en plus de sa variabilité est qu'elle est présente dans le génome en deux copies (TR_L et IR_L). Dans ces conditions, il était possible d'imaginer une plus grande sensibilité de la détection par PCR ce qui a pu être démontré par la suite.

Une technique de **PCR compétitive** (**Arzul *et al.*, 2000a** ; **Renault *et al.*, 2004**) a aussi été développée en utilisant des amorces déjà disponibles et en construisant un standard interne qui diffère de l'ADN viral cible par une délétion de 76 paires de base. Ce standard a été obtenu par PCR et cloné. Il est ajouté à une concentration définie dans les échantillons à analyser. Il est ensuite co-amplifié en présence de l'ADN viral recherché (si ce dernier est présent). Les produits de PCR obtenus à partir des deux types de matrice (standard interne et ADN viral) peuvent être différenciés en gel d'agarose sur la base de leurs tailles respectives. La méthode se montre sensible permettant la détection d'1 fg d'ADN viral pour 0,5 mg de tissus. Elle a été utilisée pour rechercher des inhibiteurs de la PCR dans différents types d'échantillon. Aucun effet inhibiteur n'a été ainsi mis en évidence dans les tissus de naissain broyés et traités par la chaleur (validant ainsi le protocole préalablement établi, **Renault *et al.*, 2000**). Ce n'est pas le cas lorsque ce même protocole de préparation est utilisé pour des échantillons d'huîtres adultes. Cette approche a permis de monter que pour les animaux adultes, il était nécessaire d'utiliser un protocole autre (extraction phénol/chloroforme). Sur cette base, il a été possible de **détecter de l'ADN viral chez des individus adultes ne présentant aucun symptôme** et des **quantifications** ont pu être réalisées (**Renault *et al.*, 2005**) :

- entre 1,5 pg et 325 pg d'ADN viral ont été détectés dans 0,5 mg de tissus d'huîtres adultes asymptomatiques,
- entre 750 pg to 35 ng d'ADN viral dans 0,5 mg de tissus de naissain infecté présentant des mortalités (de 2 à 100 fois plus par rapport à la quantité maximale détectée chez des adultes asymptomatiques).

Ces résultats montrent l'intérêt des outils moléculaires non pas seulement pour le diagnostic, mais aussi pour mieux comprendre la maladie et les interactions qui peuvent exister entre l'agent infectieux et son hôte. Chez **les huîtres adultes**, **le virus** peut donc être **présent sans induire de symptômes**. Cette observation tend à montrer que comme pour les autres herpès virus, OsHV-1 est capable de persister chez son hôte toute la vie durant de ce dernier.

Des amorces ont aussi été dessinées dans l'objectif de réaliser des **amplifications à partir de matériel extrait de blocs histologiques**. En effet, il est apparu nécessaire de disposer d'outils permettant de rechercher le virus sur du matériel biologique archivé. Dans ce cas, la taille des produits de PCR ne doit pas excéder 200 pb. En effet, la fixation et le traitement des échantillons pour l'histologie s'accompagnent d'une dégradation (fragmentation) des ADN. Le protocole d'extraction développé ainsi que les amorces dessinées ont été utilisés pour démontrer la **présence d'ADN viral dans des échantillons d'huîtres conservés en blocs histologiques depuis plus de dix ans** (**Barbosa Solomieu *et al.*, 2004**). Très récemment, ce type d'approche a également permis d'obtenir au laboratoire (Ifremer, La Tremblade, France) des informations de séquence à partir d'un bloc histologique correspondant à une huître plate prélevée et fixée en 1979 (matériel conservé depuis 28 ans). Cette approche présente un intérêt évident pour réaliser des travaux d'identification et de taxonomie plusieurs années, voir plusieurs décennies après la collecte d'animaux et la survenue d'un épisode de mortalité.

Le développement de nouveaux outils de diagnostic doit comprendre une étape de validation. Celle-ci intègre en particulier des **essais de comparaison entre laboratoires** afin de vérifier la reproductibilité et la robustesse de la technique mise au point. Pour les outils de PCR spécifiques du virus OsHV-1, une telle approche a été mise en œuvre dans le cadre d'un projet financé par l'Union Européenne (projet VINO). En effet, un essai inter-laboratoire a été réalisé. Il impliquait trois laboratoires européens (Espagne, Irlande et Royaume Uni) et a été effectué sur la base d'un matériel biologique commun (ADN viral et échantillons de larves et de naissain infectés et non infectés) fournis par le Laboratoire de Génétique et Pathologie de La Tremblade (Ifremer, France, Participant A).

Des essais préliminaires ont été réalisés par les différents participants en utilisant des dilutions sériées d'ADN viral. Ils ont permis de montrer que 10 à 100 fg pouvaient être détectés en routine par les différents laboratoires en utilisant le couple d'amorces C2/C6. Le couple d'amorces C5/C13 permet de détecter de manière systématique 1 fg d'ADN viral. Ce dernier résultat (grande sensibilité) peut être expliqué par le fait que le produit d'amplification est de petite taille (environ 200 pb) et que de ce fait le rendement d'amplification est élevé. De plus, ce couple d'amorces cible une zone du génome répétée (région C, cf. page 81).

Trente échantillons de référence (larves et naissain) ont été analysés en PCR avec les deux couples d'amorces (C2/C6 et C5/C13). Onze de ces échantillons ont été fournis comme étant négatifs et les 19 autres comme positifs par le Laboratoire de Génétique et Pathologie (Tableau II, colonnes Participant A). Les résultats obtenus par les différents laboratoires sont rapportés dans le Tableau II. Globalement, il est possible d'observer une concordance pour les échantillons infectés. En effet, 16 des 19 échantillons définis comme positifs ont donné une amplification en PCR pour les trois laboratoires B, C et D quel que soit le couple d'amorces utilisé (Tableau II). Seuls trois échantillons (12, 27 et 28) n'ont pas été systématiquement trouvés positifs (Tableau II). Concernant les échantillons négatifs, pour les laboratoires B et D, il y a congruence avec le statut prédéfini pour les onze échantillons. Les résultats obtenus par le laboratoire C sont positifs pour de nombreux échantillons (Tableau II). Ces résultats pourraient s'expliquer par un problème de contamination.

Ces premiers résultats sont très encourageants. Et si ce type d'essai nécessite bien sûr d'être répété, il est cependant important de noter que c'est la **première démarche de validation inter-laboratoire entreprise pour un outil de diagnostic moléculaire développé pour la recherche d'un agent infectant les mollusques bivalves**.

Tableau II - Essai inter-laboratoire pour la valadition de la détection d'OsHV-1 par PCR (projet européen VINO)

N° échantillon	Résultats de PCR							
	C2/C6				C5/13			
	A	B	C	D	A	B	C	D
1	-	-	+	-	-	-	+	-
2	-	-	-	-	-	-	+	-
3	-	-	+	-	-	-	-	-
4	-	-	-	-	-	-	+	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	+	-
7	-	-	+?	-	-	-	+	-
8	-	-	+	-	-	-	+	-
9	-	-	+	-	-	-	+	-
10	-	-	-	-	-	-	+	-
11	-	-	-?	-	-	-	+	-
12	+	+	+	+	+	+	-	-
13	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	-	+
18	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+
27	+	-	+	+	+	+	+	+
28	+	-	-	-	+	-	-	-
29	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+

A: laboratoire ayant fourni le matériel biologique de référence ; B, C et D: laboratoires ayant réalisé l'essai inter-laboratoire. Les résultats concordants sont représentés en vert et en rose ceux non concordants.

Plusieurs protocoles de PCR et couples d'amorces sont aujourd'hui disponibles. **Une revue a été récemment publiée** sur le sujet par **Batista *et al.* (2007)** dans laquelle il a été tenté d'une part de faire de manière la plus exhaustive possible le point sur l'existant et d'autre part de réaliser une analyse critique. Il faut noter qu'un certain nombre d'amorces utilisées au Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France) n'a pas été publié à ce jour. En particulier, des amorces ciblant le gène codant pour la sous-unité ATPase de la terminase ont été récemment dessinées et utilisées.

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Review

Detection of ostreid herpesvirus 1 DNA by PCR in bivalve molluscs: A critical review

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Abstract

Herpes-like viral infections have been reported in different bivalve mollusc species throughout the world. High mortalities among hatchery-reared larvae and juveniles of different bivalve species have been associated often with such infections. The diagnosis of herpes-like viruses in bivalve molluscs has been performed traditionally by light and transmission electron microscopy. The genome sequencing of one of these viruses, oyster herpesvirus 1 (OsHV-1), allowed the development of DNA-based diagnostic techniques. The polymerase chain reaction (PCR) has been used for the detection of OsHV-1 DNA in bivalve molluscs at different development stages. In addition, the PCR used for detection of OsHV-1 has also allowed the amplification of DNA from an OsHV-1 variant. The literature on DNA extraction methods, primers, PCR strategies, and confirmatory procedures used for the detection and identification of herpesviruses that infect bivalve molluscs are reviewed.
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Keywords: Herpesvirus; OsHV-1; Detection; PCR; Bivalve molluscs; Oyster

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1. Introduction

Herpes-like viral infections have been identified in various marine bivalve mollusc species throughout the world, including the USA (Farley et al., 1972; Friedman et al., 2005), New Zealand (Hine et al., 1992), France (Nicolas et al., 1992; Arzul et al., 2001b; Renault and Arzul, 2001; Renault et al., 2000b, 2001b), Australia (Hine and Thorne, 1997), and Mexico (Vásquez-Yeomans et al., 2004). The first description of a virus morphologically similar to members of the *Herpesviridae* family in a bivalve mollusc was made by Farley et al. (1972) in the eastern oyster, *Crassostrea virginica*. Since then, a wide host range has been reported for herpes and herpes-like viruses infecting bivalve species, including the Pacific oyster *C. gigas* (Hine et al., 1992), the European oyster *Ostrea edulis* (Nicolas et al., 1992), the Antipodean flat oyster *O. angasi* (Hine and Thorne, 1997), the Chilean oyster *Tiostrea chilensis* (Hine et al., 1998), the Manila clam *Ruditapes philippinarum* (Renault et al., 2001a), the carpet shell clam *R. decussatus* (Renault and Arzul, 2001), the Portuguese oyster *C. angulata* (Arzul et al., 2001a), the Suminoe oyster *C. rivularis* (Arzul et al., 2001a) and the French scallop *Pecten maximus* (Arzul et al., 2001b). High mortalities among hatchery-reared larvae (Hine et al., 1992; Nicolas et al., 1992; Renault et al., 1994; Renault and Arzul, 2001) and juveniles (Comps and Cochennec, 1993; Renault et al., 1994, 2000b; Friedman et al., 2005) of different bivalve species have often been associated with herpes and herpes-like virus infections. Observations by transmission electron microscopy (TEM) revealed that larvae exhibit generalized infections, whereas focal infections were generally observed in spat (Renault et al., 1994). Viral infections were also observed in adult bivalves (Hine and Thorne, 1997; Arzul et al., 2001b, 2002; Vásquez-Yeomans et al., 2004), but adults are apparently less sensitive to such infections as compared to younger stages (Arzul et al., 2002; Renault and Novoa, 2004). The pathogenicity of herpes-like viruses was demonstrated by experimental transmission assays either for larval stages of *C. gigas* (Le Deuff et al., 1994) and other bivalve species (Arzul et al., 2001a,b). Similar experiments were conducted with juveniles and adults of *C. gigas*, but the results were inconclusive (Renault and Novoa, 2004). It is noteworthy that a highly pathogenic herpes-like virus was observed recently by TEM in the gastropod mollusc *Haliotis diversicolor supertexta* in Taiwan associated with high mortality rates (Chang et al., 2005).

The development of a method for purifying herpes-like virus particles from infected *C. gigas* larvae facilitated the extraction of viral DNA and a partial genome characterization (Le Deuff and Renault, 1999). The genome was subsequently completely sequenced (GenBank accession no. AY509253) revealing a tenuous relationship with other herpesviruses. This oyster virus was classified as a member of the *Herpesviridae* family under the name oyster herpesvirus 1 (OsHV-1) (Minson et al., 2000) and was considered the only member of a new major class of herpesvirus (Davison, 2002; Davison et al., 2005). A variant of OsHV-1 (OsHV-1var) was also described in larvae of different bivalve species (Arzul et al., 2001b,c) and OsHV-1 and OsHV-1var are considered representatives of a single viral species. In contrast with vertebrate herpesviruses, which are generally confined to a single host, OsHV-1 has been identified in several bivalve species and interspecies viral transmission was demonstrated (Arzul et al., 2001a; Friedman et al., unpublished data).

Detection of viruses in mollusc bivalves cannot be done by classic serological methods because molluscs do not produce antibodies. In addition, viral replication in cell culture to facilitate the diagnosis is not possible due to the absence of mollusc cell lines. The diagnosis of herpes-like virus infections has traditionally been performed by light microscopy, as a first approach in order to detect cytological abnormalities, followed by transmission electron microscopy to complete the diagnosis (Hine and Thorne, 1997; Renault et al., 2000b). These procedures are time consuming, impractical for epidemiological surveys, and some viruses can be difficult to detect and identify when present in low amounts. As a result of these limitations, other diagnostic methods have been developed such as immunochemistry and nucleic acid-based techniques. A protocol using polyclonal antibodies produced in BalbC mice immunized with viral particles purified from infected *C. gigas* larvae has been used to detect OsHV-1 proteins (Le Deuff, 1995; Arzul et al., 2002). Another technique that has also been developed is *in situ* hybridization, which allows the detection of viral DNA (Renault and Lipart, 1998; Lipart and Renault, 2002; Barbosa-Solomieu et al., 2004). Both techniques have high sensitivity and specificity, and allow the visualization of viral proteins or DNA, respectively. Polymerase chain reaction (PCR) diagnostic methods have also been developed for detection of OsHV-1 DNA (Renault et al., 2000a; Renault and Arzul, 2001; Arzul et al., 2001a,b,c). PCR is considered a suitable tool for the diagnosis of OsHV-1 infections owing its specificity, high sensitivity relative to other methods (e.g., TEM), ease of sample processing, availability of reagents, and time and cost efficiency. However, as this test only detects viral nucleic acid, other tests such as histology, ISH or antigen-based tests must confirm PCR tests, particularly in a new species or new geographic location. In areas where the pathogen is endemic, PCR alone may be sufficient for diagnosis of its presence. A competitive PCR method was developed that can be used to demonstrate the presence of PCR inhibitors or to quantify OsHV-1 DNA (Renault et al., 2004). Different methods of DNA extraction (Renault et al., 2000a; Arzul et al., 2002; Batista et al., 2005; Friedman et al., 2005) as well as various primer pairs (Renault et al., 2000a; Arzul et al., 2001a,b,c; Renault and Arzul, 2001; Barbosa-Solomieu et al., 2004, 2005) have been designed and used to detect viral DNA using one-round or nested PCR. In addition, the PCR conditions used for detection of OsHV-1 also allow amplification of DNA from other closely related herpesviruses in different bivalve species, as was the case of OsHV-1var (Arzul et al., 2001b,c). In order to confirm the authenticity of PCR products, different techniques have been used, including digestion of PCR products with restriction enzymes (PCR-RFLP) (Arzul et al., 2001c; Renault et al., 2004; Barbosa-Solomieu et al., 2004, 2005) and DNA sequencing. The objectives of the present work are: (1) to provide a review of the different DNA extraction methods, PCR protocols, primers and confirmatory procedures that have been used in the detection of OsHV-1 DNA by PCR; (2) to discuss the adequacy of the

Table 1
Techniques used to extract nucleic acids from bivalve molluscs samples for detection of herpesvirus DNA by PCR

Development stage	Sampling conditions	Method	Reference
Larvae and spat	Frozen at −20 °C	Grinding, boiling	Renault et al. (2000a)
Larvae	Frozen at −80 °C	Washing, grinding, digestion with proteinase K, boiling	Batista et al. (2005)
Spat	Fresh material and samples fixed in 95% ethanol	Commercial kit (lysis, digestion with proteinase K, DNA binding to silica gel)	Friedman et al. (2005) and Burge et al. (2006)
Adults	Frozen at −80 °C	Grinding, digestion with proteinase K, phenol–chloroform purification	Le Deuff (1995)
Adults and spat	Fixed in Davidson's and Carson's solutions, paraffin-embedded	Deparaffination, digestion with proteinase K, heating	Barbosa-Solomieu et al. (2004)
Adults	Fixed in Davidson's solution	Digestion with proteinase K, heating	Barbosa-Solomieu et al. (2005)
Adults	Fixed in Davidson's solution	Digestion with proteinase K, phenol–chloroform purification	Unpublished data
Adults	95% ethanol (gill)	Digestion with proteinase K, phenol–chloroform purification	Unpublished data

different methods based on published and unpublished data; (3) to address different aspects that should be considered in order to avoid false PCR results.

2. Nucleic acid extraction techniques

The exquisite sensitivity of the PCR reaction can allow the amplification of DNA even when a very small number of target molecules are present. Nevertheless, the type of samples analysed as well as the procedure used for nucleic acid extraction can greatly affect the success of the amplification reaction. If samples cannot be processed immediately after harvesting they should be conserved and stored under conditions that preserve the integrity of nucleic acids. Extraction of nucleic acids from fresh frozen samples (e.g., larval or adult tissues), stored at −20 or −80 °C, generally yields good quality DNA for OsHV-1 detection by PCR (Renault et al., 2000a; Arzul et al., 2002; Batista et al., 2005). Samples fixed and stored using non-denatured ethanol have also been used for amplification of OsHV-1 DNA (Friedman et al., 2005; Burge et al., 2006; Renault et al., unpublished data). Other trials using formalin fixed samples (e.g., Davidson's and Carson's solutions) have also allowed amplification of herpesvirus DNA from bivalve mollusc samples (Barbosa-Solomieu et al., 2005). However, nucleic acid extraction from formalin-fixed tissues usually yields DNA that is degraded and can affect subsequent nucleic acid-based techniques (Jackson et al., 1990; An and Fleming, 1991), especially those involving larger amplified fragments such as that produced by the nested A primer test of OsHV-1 at 917 bp (Renault et al., 2000a).

Several techniques have been used for the extraction of nucleic acids from different types of bivalve mollusc samples in order to detect herpesvirus DNA by PCR (Table 1). Renault et al. (2000a) used a simple method of nucleic acid extraction from frozen oyster specimens by grinding and boiling the tissues. This method allowed the extraction of nucleic acids and subsequently amplification of viral DNA from larvae and spat of *C. gigas* infected with a herpes-like virus as confirmed by TEM. However, this technique does not appear suitable to amplify OsHV-1 DNA from adults. Other techniques of DNA extraction were also examined by Renault et al. (2000a) namely, Chelex-100 and a combination of proteinase K digestion with phenol/chloroform purification, but both techniques yielded false-negative PCR results. It was also noticed that extraction by boiling ground oyster tissues in some cases produced compounds that inhibited the amplification reaction (Renault et al., 2000a). A method of DNA extraction from oyster larvae was reported by Batista et al. (2005) that combines the use of proteinase K, Tween-20 and boiling. This method allowed the amplification of OsHV-1 DNA by PCR from a small amount of larvae (3 mg). Commercial DNA extraction kits (e.g. Qiagen's QIAamp DNA mini kit using the tissue protocol) have also been used to extract amplifiable herpesvirus DNA from *C. gigas* spat (Friedman et al., 2005; Burge et al., 2006). These authors obtained good quality DNA but variable PCR results depending on the amount of template DNA used. Herpesvirus DNA has also been extracted from *C. gigas* adults by grinding, digestion with proteinase K and phenol/chloroform purification from fresh frozen tissues (Le Deuff, 1995).

For decades, specimens of bivalve molluscs have been routinely fixed in formalin and embedded in paraffin for histological analyses. Although extraction of sufficient quantity of amplifiable DNA from these tissues can be difficult (Jackson et al., 1990; An and Fleming, 1991), these samples constitute invaluable resources for pathological analysis using DNA-based techniques. Barbosa-Solomieu et al. (2004) could extract nucleic acids from fixed paraffin-embedded archival samples by deparaffination, digestion with proteinase K and heating. The method allowed the amplification of small fragments (196 and 207 bp) of OsHV-1 DNA, and the absence of PCR inhibitors was demonstrated by amplification of an internal standard. A similar protocol was successfully used by Barbosa-Solomieu et al. (2005), who extracted nucleic acids from oyster tissues preserved in Davidson's solution, which allowed the detection of OsHV-1 by PCR. A study in which the previous technique and a variation of this procedure, using phenol/chloroform purification and ethanol precipitation instead of the heating step, revealed PCR inhibition in 29 and 5% of the cases, respectively

(Batista, personal communication). The samples used in that study had been fixed in Davidson's solution and later transferred to 70% ethanol. The greater inhibition observed with the boiling protocol could be due to the presence of cell components and DNA degrading enzymes that may have been removed with the phenol/chloroform purification step. Indeed, the removal of inhibitory effects by phenol/chloroform purification has been reported (An and Fleming, 1991). The effect of inhibitors may also be reduced during the PCR reaction with the use of bovine serum albumin (BSA) as outlined below (Friedman et al., 2005). However, methods of nucleic acid extraction with fewer steps should be used preferentially in order to reduce the risk of sample contamination.

3. PCR analysis

Different PCR strategies can be used to detect viral DNA. Some methods utilize one-round PCR (or single PCR) in which the DNA sequence is amplified using one primer pair. Other methods use nested PCR in which first round amplification products are subjected to a second round of amplification and hence two primer pairs are used.

3.1. PCR primers

Based on OsHV-1 sequence data several oligonucleotide primers have been designed for the detection of herpesvirus DNA in bivalve molluscs (Table 2). Different primer pair combinations (Table 3) have been used to amplify DNA by PCR from regions A, B, C and Gp of the virus genome (Fig. 1). The A region encodes a protein of unknown function; the B region encodes a putative inhibitor of apoptosis belonging to the IAP family; the C region encodes parts of two proteins of unknown functions and is present twice in the genome (being located in the inverted repeats TR_L and IR_L) (Davison et al., 2005); and the Gp region encodes a putative glycoprotein (Arzul et al., 2001b,c).

3.2. Diagnostic specificity and sensitivity

Specificity is defined as the ability of an assay to amplify DNA only from the target agent. Sensitivity (or limit of detection) is defined as the smallest quantity of viral DNA that can be systematically detected.

Two pairs of primers (A3/A4 and A5/A6) corresponding to the A region of the OsHV-1 genome have been used to detect

Table 2
Oligonucleotide primers used for OsHV-1 detection by PCR

Name	Primer sequence (5′–3′)	Forward/reverse	Reference
Region A			
A3	GCCAACCGTTGGAACCATAACAAGCG	Forward	Renault et al. (2000a)
A3	GCCAACCGTTGGAACCATAACA**G**GCG	Forward	*
A4	GGGAATGAGGTGAACGAAACTATAGACC	Reverse	Renault et al. (2000a)
A5	CGCCCCAACCACGATTTTTCACTGACCC	Forward	Renault et al. (2000a)
A6	CCCGCTAGATATAGGATGAGATTTG	Reverse	Renault et al. (2000a)
A6	CCCG**T**CTAGATATAGGATGAGATTTG	Reverse	*
Region B			
B1	ATGTAATGGGTGGTGGTGCT	Forward	Arzul et al. (2001c)
B2	CAACAGCTTTGGAGGTTGGT	Reverse	Arzul et al. (2001c)
B3	GTGGAGGTGGCTGTTGAAAT	Forward	Arzul et al. (2001b)
B4	ACTGGGATCCGACTGACAAC	Reverse	Arzul et al. (2001b)
Region C			
C1	TTCCCCTCGAGGTAGCTTTT	Forward/reverse	Arzul et al. (2001c)
C2	CTCTTTACCATGAAGATACCCACC	Forward/reverse	Arzul et al. (2001c)
C4	GCAGTTGTGGTATACTCGAGATTG	Forward/reverse	Arzul et al. (2001c)
C5	CCGTGACTTCTATGGGTATGTCAG	Forward/reverse	Arzul et al. (2001c)
C6	GTGCACGGCTTACCATTTTT	Forward/reverse	Arzul et al. (2001c)
C9	GAGGGAAATTTGCGAGAGAA	Forward/reverse	Barbosa-Solomieu et al. (2004)
C10	ATCACCGGCAGACGTAGG	Forward/reverse	Barbosa-Solomieu et al. (2004)
C11	GAGGGAAATTTGCGAGAGAG	Forward/reverse	Barbosa-Solomieu et al. (2005)
C13	CCTCGAGGTAGCTTTTGTCAAG	Forward/reverse	Renault et al. (2004)
C14	CCGTGACTTCTATGGGTATG	Forward/reverse	Renault et al. (2004)
C15	GATTACCCAGATTCCCCTC	Forward/reverse	Renault et al. (2004)
Region Gp			
Gp3	GGTTGTGGGTTTGGAAATGT	Forward	Arzul et al. (2001b)
Gp4	GGCGTCCAAACTCGATTAAA	Reverse	Arzul et al. (2001b)
Gp7	TTACACCTTTGCCGGTGAAT	Forward	Unpublished data
Gp8	TCACATCACTTGGTGGCAAT	Reverse	Unpublished data
Gp10	AAGCAAATGACACGACACCA	Reverse	Unpublished data
Gp17	AACCACCACACAAGCTCCTC	Forward	Unpublished data
Gp18	ACATCTGGTGGTGGGATAGG	Reverse	Unpublished data

* The sequence of primer A3 and A6 based on OsHV-1 genome is presented in the table with a G instead of A for primer A3 in the underline position and with the insertion of a T in the underline position for primer A6.

Table 3
Primer pairs, product size and restriction enzymes analysis of PCR products used for the detection of OsHV-1 (OsHV-1var) DNA

Primer pair	Product size (bp)	Restriction enzyme analysis		
		Enzyme	Fragments size (bp)	Reference
Region A				
A3/A4	1001 (ca. 1001)	*Bxt*I	765/236	Renault et al. (2001b)
A5/A6	917 (UPS)			
Region B				
B1/B2	464 (ca. 464)			
B3/B2	332 (ca. 332)			
B3/B4	207 (ca. 207)	*Tru*9I	121/85	Barbosa-Solomieu et al. (2004)
Region C				
C1/C4	538 (NA)			
C1/C6	896 (NA)	*Mbo*I	747/94/55	Renault et al. (2004)
C2/C4	352 (180)			
C2/C6	709 (ca. 529)	*Xho*I	375/335 (375/155)	Arzul et al. (2001c)
C13/C5	765 (UPS)			
C9/C10	197 (UPS)	*Sau*3AI	118/78	Barbosa-Solomieu et al. (2004)
C11/C10	197 (UPS)			
C15/C14	780 (UPS)			
Region Gp				
Gp3/Gp4	698 (698)			
Gp3/Gp10	1735 (UPS)			
Gp7/Gp8	699 (UPS)			
Gp17/Gp18	306 (UPS)			

UPS: unknown product size; NA: no amplification.

herpesvirus DNA in larvae and spat via nested PCR (Renault et al., 2000a). The specificity of these primer pairs was evaluated using DNA from *C. gigas* as well as DNA from vertebrate herpesviruses. The detection procedure was found to be specific, only amplifying OsHV-1 DNA and not leading to false-positives. The sensitivity of nested PCR using on A3/A4 and A5/A6 primer pairs was also tested. As little as 500 fg of virus DNA extracted from purified particles were routinely detected. The amplification sensitivity decreased with increasing amounts of non-infected oyster spat tissues (Renault et al., 2000a). The one-round PCR assay with the A3/A4 primer pair not only allowed amplification of OsHV-1 DNA but also the detection of a variant of this virus (OsHV-1var) in *C. gigas* and *R. philippinarum* larvae (Arzul et al., 2001c).

The combination of primer pairs A3/A4 and A5/A6 allowed less PCR amplification than C2/C6 (21.4% *versus* 32.4%) when the same larval samples were analysed (Renault and Arzul, 2001). The differences observed in herpesvirus DNA detection suggested that the one-round PCR with C2/C6 was more useful for epidemiological surveys than the nested PCR using A3/A4 and A5/A6 (Renault and Arzul, 2001). Moreover, Renault et al. (2004) observed that the C2/C6 primer pair systematically

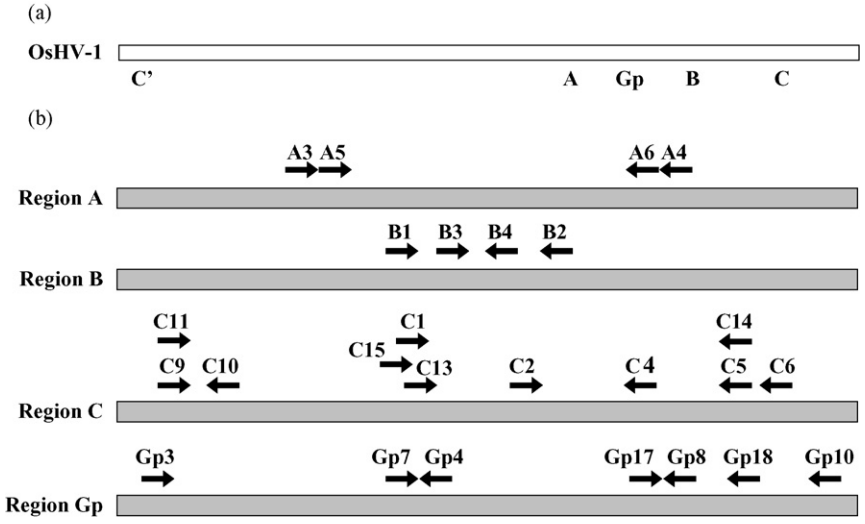

Fig. 1. General scheme of OsHV-1 genome. (a) Position of the regions A, B, C and Gp. C′ represent the position of the inverted repeat of the C region. (b) Diagrams with primers (arrows) annealing sites on the four regions.

Table 4
Sensitivity and adequacy of some of the primer pairs that have been mostly used to detect herpesvirus DNA from bivalve mollusc samples by PCR

Primer pair	Sensitivity	Positive points	Limitations
One-round PCR			
A3/A4	ND	Also allows the amplification of OsHV-1var and the OsHV detected in USA[a]	
C1/C6	1 fg[b]	Target fragment present twice	Failed to amplify OsHV-1var[c]
C2/C6	1 fg[b]	Target fragment present twice; allows the differentiation of OsHV-1 from OsHV-1var[c]	Failed to amplify OsHV detected in USA[a]
C13/C5	10 fg[d]	Target fragment present twice	Extraneous bands observed
Gp3/Gp4	10 fg[d]	Target region apparently more conserved; also allows the amplification of OsHV-1var[c]	
Nested PCR			
A3/A4–A5/A6	500 fg[e]	PCR conditions optimized; also allows the amplification of OsHV detected in USA[a]	Low sensitivity
C13/C5–C2/C4	1 fg[f]	Target fragment present twice; allows the differentiation of OsHV-1 from OsHV-1var[c]	Extraneous bands observed

ND: not determined.
[a] Friedman et al. (2005).
[b] Renault et al. (2004).
[c] Arzul et al. (2001b,c).
[d] Vigneron et al. (2004).
[e] Renault et al. (2000a).
[f] Unpublished data.

allowed the detection of 1 fg of purified viral DNA, and was more sensitive than the nested PCR with primer pairs A3/A4 and A5/A6 (detection limit of ca. 500 fg of purified viral DNA). The higher sensitivity of the one-round PCR with the primer pair C2/C6 can be partially explained by the fact that the targeted fragment is present twice in the OsHV-1 genome (Arzul et al., 2001b,c). However, other primer pairs designed in the C region (C13/C5 and C15/C14) failed to produce detectable amplicons with amounts of purified viral DNA detected with the C2/C6 primer pair (Renault et al., 2004). Vigneron et al. (2004) observed a detection limit of 10 fg of purified viral DNA for both primer pairs C13/C5 and Gp3/Gp4 (Table 4), the later target region being only present in one copy in the OsHV-1 genome. On the other hand, Friedman et al. (2005) detected herpesvirus DNA in *C. gigas* juveniles with A3/A4 and A5/A6 whereas no PCR products were obtained with primer pair C2/C6. In addition, partial sequencing of the amplified A region revealed a single nucleotide difference compared to the reference sequence of OsHV-1 (Arzul et al., 2001c). The herpesvirus described by Friedman et al. (2005) is very closely related to OsHV-1 based on the sequence of the A region. Non-amplification with C2/C6 primer pair could be due to nucleotide substitutions and/or insertions/deletions in the C region. The variant of OsHV-1 described by Arzul et al. (2001b,c) presented a major deletion of 2.8 kbp in the C region as well as other nucleotide deletions, substitutions and insertions. No amplification of OsHV-1var DNA was observed when primer pairs C1/C4 and C1/C6 were used. Whereas amplicons were produced with primer pairs C2/C4 and C2/C6; however, these amplicons were smaller than those obtained with reference viral DNA (Arzul et al., 2001c). In contrast, the PCR products obtained from samples infected with OsHV-1 and OsHV-1var using pair primers A3/A4, B1/B2, B3/B4 and Gp3/Gp4 were the same size (Arzul et al., 2001b,c). Nevertheless, some nucleotide differences (substitutions) in the sequence of the Gp3/Gp4 fragment were observed between the two types of OsHV-1 (Arzul et al., 2001c). It is noteworthy that primers used to detect bivalve herpesviruses where designed based on specific OsHV-1 sequences. Hence, putative polymorphisms in primer-binding sites can result in non-amplification or lower sensitivity of the PCR analysis to detect other related herpesviruses such as OsHV-1var.

OsHV-1 was also detected by PCR in asymptomatic *C. gigas* adults using the primer pairs B3/B2 and C2/C6 (Arzul et al., 2002). Both primer pairs gave concordant PCR results. Although other techniques, *in situ* hybridization and immunohistochemistry, were also used by Arzul et al. (2002) to detect herpesvirus DNA and proteins, respectively PCR was the most sensitive method. Mantle and gills appear as organs of choice for OsHV-1 detection in adult oysters (Arzul et al., 2002).

Different PCR strategies and primer pairs were used to analyse hatchery-reared oyster larvae (Fig. 2) (Batista, personal communication). The larvae were collected at different stages of development and immediately frozen at −80 °C. Samples for PCR analysis were prepared according to the method used by Renault et al. (2000a). No differences were observed in the number of virus DNA positive samples with the different primer pairs. Amplification artifacts were observed with the C9/C10 primer pair yielding PCR products of various sizes. However, no positive PCR results were obtained for the samples showing extraneous bands when other primer pairs were used. Amplicons approximately 200 bp smaller than the expected size were obtained with the C13/C5 primer pair. Based on their size, these PCR products could be interpreted as the amplification of OsHV-1var DNA. However, when PCR analyses were performed with others primer pairs (i.e., A3/A4, B3/B4 and Gp3/Gp4), that have been used to amplify DNA of OsHV-1var in previous studies (Arzul et al., 2001b,c), no PCR products were obtained (data not shown). These results suggest that the PCR condi-

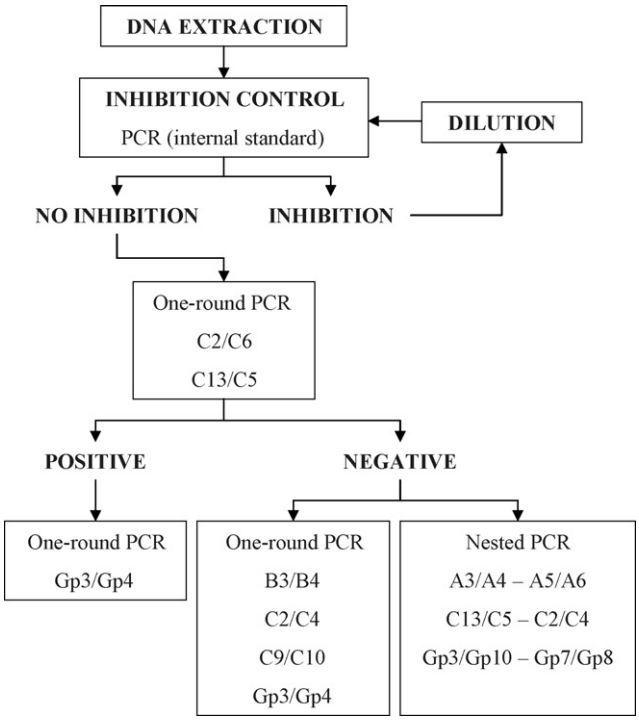

Fig. 2. Experimental procedure scheme used for the detection of OsHV-1 DNA.

tions used with the primer pairs C9/C10 and C13/C5 should be optimized in order to eliminate the non-specific amplification observed.

3.3. Amplification from formalin fixed samples

Samples for histopathological examination are usually formalin fixed using different types of fixatives (e.g., 10% formalin, Davidson's solution, Carson's solution). The quality and quantity of the DNA extracted from these types of samples is generally low due to degradation and leaching of nucleic acids during the fixation procedure, particularly under prolonged fixation durations (more than 24–48 h). Primer pairs that amplify small fragments can be used to increase the efficiency of the amplification reaction, in particular, when the template DNA is highly fragmented (Coates et al., 1991). Barbosa-Solomieu et al. (2004) used primers pairs C9/C10 and B3/B4 successfully to amplify OsHV-1 fragments of 196 and 207 bp, respectively, from formalin-fixed paraffin-embedded archival samples. Despite the similar size of the amplified fragments, a higher number of PCR positive results were obtained with primer pair C9/C10 compared to B3/B4 (Barbosa-Solomieu et al., 2004). In contrast, no detectable PCR products were obtained in previous assays using primers that generate larger fragments (600–1000 bp) when the same type of samples were analysed (Renault, personal communication).

3.4. Competitive PCR

A competitive PCR method was developed in order to detect and quantify herpesvirus DNA (Renault et al., 2004). The method was based on co-amplification of OsHV-1 DNA and a mimic molecule, which differs from the target DNA by a deletion of 76 bp, using the same set of primers (C1/C6 or C2/C6). Production of the mimic molecule was done by PCR using the C1 primer and a modified primer (C116). The size differences of amplicons obtained from viral DNA and the mimic molecule can easily be observed by electrophoresis on agarose gel. This method has been successfully used to quantify herpesvirus DNA (Arzul et al., 2002) and to detect the presence or absence of PCR inhibitory factors (Barbosa-Solomieu et al., 2004; Renault et al., 2004; Batista et al., 2005).

4. Analysis of amplification products

Assessing the specificity of a PCR only on the basis of amplicon size determined by agarose gel electrophoresis can result in false-positive results, especially in PCR assays, that have not been properly validated. Different techniques have been used to verify the specificity of PCR products from bivalve herpesviruses, including Southern blot analysis, restriction digestion and sequencing. Arzul et al. (2001c) used a digoxigenin-labelled probe in order to verify the specificity of amplification products by Southern blot hybridization. Digestion of PCR products with restriction enzymes has been the technique most frequently used to confirm the specificity of the amplification of herpesvirus DNA (Table 3). The method is simple and the results can be obtained in a few hours. Nevertheless, insufficient or absent digestion caused by the presence of certain inhibitory compounds or low enzyme activity due to sub-optimal storage conditions can lead to misinterpretations of the results. Consequently, restriction digestion of PCR products obtained from reference OsHV-1 DNA (e.g., the positive control) should be used in every assay as an external control of the digestion reaction. Sequencing of PCR products is the most accurate method for determining the authenticity of the amplicons. Despite the accuracy of sequencing, this technique it is not very practical for amplicon confirmation and the cost is high specially when analysing a large number of samples. Nevertheless, it can provide unique information about the virus detected and hence allow the identification of new herpesviruses or variants of OsHV-1. If the previous confirmatory methods are not available, different primer pairs targeting different areas of the viral genome can also be used to confirm the presence of the target viral DNA.

During an outbreak of OsHV-1 in hatchery-reared larvae, PCR products obtained with the primer pairs C2/C4 and Gp17/Gp18 were cloned and sequenced (Batista, personal communication). Although no differences were observed in the sequence of the majority of clones when compared with the reference virus (OsHV-1) some clones presented single nucleotide polymorphism (Fig. 3). The polymorphism observed could be due to polymerase errors and not because of the presence of more than one type of viruses. In order to eliminate these uncertainties, high-fidelity polymerases (e.g., Goldstar Polymerase, Eurogentec, Belgium) should be used. Sequencing can also be performed directly from the PCR

```
Reference  ctctttaccatgaagatacccaccAATGTGGTAAAGACGGAACAATCTTTTTCTAGGATA
Variant-A  ..........................................................

Reference  TGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATAATGATTTTAGA
Variant-A  ..........................................................

Reference  ATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTATTGCCCGACCAC
Variant-A  ..........................................................

Reference  AAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAA
Variant-A  ..........................................................

Reference  AAAACCACATGGGGGCCAAGGAATTTAAACCCCGGGGAAAAAGTATAAATAGGCGCGATT
Variant-A  ..........................................................

Reference  TGTCAGTTTAGAATCATACCCACACACTcaatctcgagtataccacaactgc
Variant-A  ..C.....................................................
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(a)

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Reference  aaccaccacacaagctcctcCTCCACCTATCGTTATTAATACCACAGCAGCACCTTTGGC
Variant-B  ..........................................................

Reference  GCCCATTACCAATGCCACATTGCCACCAAGTGATGTGATCACACCGGAGGCTGTGAATTT
Variant-B  ..........................................................

Reference  AACAGATGATACCCCTGTTGTAAATGAACCGGTAAATTCTACATTTATCAATGATACGGA
Variant-B  ..........................................................

Reference  TGTATTAGATGATTCTCCCACCACCTCTGCTCCACAAGCGCCTGGTATAGTTGGTATAAT
Variant-B  ..........................................A...............

Reference  TGTAAATAAGATTACAACAACACCTGCACCATCCATCGGTAGGGCGcctatcccaccacc
Variant-B  ..........................................................

Reference  agatgt
Variant-B  ......
```

(b)

Fig. 3. Comparison of the nucleotide sequence of (a) the C2–C4 fragment (variant-A) and (b) the Gp17–Gp18 fragment (variant-B) from oyster larvae with reference OsHV-1 DNA. Primer sequences are in lower cases.

products since putative polymerase errors will be underrepresented.

5. PCR results and infection levels

Amplification of nucleic acid by PCR has revolutionized the diagnosis of viral infections, in particular when it is difficult or impossible to cultivate the infectious agent due to a lack of continuous cell lines. However, caution should be applied when assigning a virus to a disease based only on the detection of its genomic material. The presence of virus nucleic acid could be due to latent, low-grade persistent or active infections (Kleinschmidt-DeMasters et al., 2001), or to the presence of virions in the gastrointestinal tract and not due to actual infection. In the case of latent infections, even if highly sensitive PCR methods are used in association with well-established nucleic acid extraction procedures, viral DNA may not be detected (e.g., Khadijah et al., 2003). Consequently, negative PCR results always have to be interpreted with some caution.

Detection of herpesvirus DNA by PCR has been reported in larvae of bivalve species in which intracellular viral particles were observed by TEM (Arzul et al., 2001a; Renault et al., 2001b). Moreover, positive PCR results have been associated with high mortality rates during larval rearing (Renault et al., 2000a; Arzul et al., 2001a,b,c; Renault and Arzul, 2001). The failure to detect viral DNA by PCR does not necessarily mean that the animals are not infected, as low levels of viral DNA in tissues may be below the threshold of the detection method. It has been observed that the number of positive PCR results in moribund *C. gigas* juveniles is higher than in apparently healthy individuals (C. Garcia, IFREMER LGP LaTremblade, personal communication; Burge et al., 2006). OsHV-1 has been detected by PCR in apparently healthy *C. gigas* adults, which suggest that the virus is able to persist in adult oysters without inducing disease and mortality (Arzul et al., 2002).

6. Avoiding false PCR results

6.1. False-positive results

The high sensitivity that makes PCR a very powerful technique for the amplification of DNA can also be seen as a negative aspect because of inadvertent contamination by nucleic acids. Even very low levels of contamination can result in false-positive results. There are basically three major sources of contamination: (1) target DNA previously extracted (e.g., in reagents used for the PCR reaction); (2) PCR products obtained from previous assays; and (3) cloned target molecules in plasmids (Rys and Persing, 1993). Contamination of PCR reaction by previously amplified sequences is the most likely form of contamination and occurs especially in environments where the same amplification is run repeatedly (e.g., diagnostic laboratories).

In order to avoid contamination, separate work areas should be used for: (1) preparation and storage of reagents; (2) sample preparation; (3) preparation of amplification mixture components; (4) PCR amplification; and (5) analysis of amplicons. Equipment (e.g., pipetting devices) and reagents should not be exchanged between the separate work areas. Preparation of amplification mixture components is best carried out in a hood equipped with UV lights (Fox et al., 1991). These lights should be turned on before and after preparation of the PCR mixture. To limit contamination, all pipetting procedures should be performed using filter tips, and disposable gloves should always be worn and changed frequently. Cross-contamination can also be minimized throughout the elimination of contaminants from surfaces and equipments with sodium hypochlorite (e.g., decontamination of laboratory bench with 10% bleach). For each PCR analysis, at least one negative control (containing distilled water instead of template DNA) per five diagnostic samples should be used. Due to the high sensitivity of PCR (i.e., less than 1 fg of purified viral DNA can be detected), the nature of samples, the environment (e.g., seawater, hatchery) and the time of sampling (e.g., mortality outbreaks), particular care should be taken during sample collection and preparation. Although, this type of contamination is most frequently encountered, plasmid contamination may also be problematic due to the ease of plasmid aerosol formation. To avoid plasmid contamination, in addition to the precautions listed above, a dedicated room where plasmids are handled is recommended.

6.2. False-negative results

There are several factors that can cause false-negative PCR results such as: (1) pipetting errors; (2) inadequate sample preparation; (3) nucleic acid degradation and capture; and (4) polymerase inhibition. Inadequate sample preparation can yield insufficient amounts of target molecules as well as poor quality DNA (e.g., poor lysis efficiency). The method of nucleic acid extraction may also yield compounds that can inhibit the PCR reaction by degradation and sequestration of target DNA and/or inhibiting polymerase (Wilson, 1997). The presence of inhibitor factors can also depend on the development stage of the animal. For example, severe PCR inhibition has been observed in oyster larvae 1 day after fertilization, but not in older larvae from the same larval batch (Batista, personal communication). PCR inhibition was also observed in adult oyster samples using ground tissues, but not in spat samples (Renault and Novoa, 2004). Therefore, it is advisable to employ standard molecules as indicators of the efficacy of the reaction (Ballagi-Pordány and Belák, 1996), such as the internal standard developed by Renault et al. (2004). To assure that the extraction method used yields good quality DNA and also to evaluate the presence of inhibitory factors, universal primers that amplify DNA of the hosts can be used (Le Roux et al., 1999; Batista et al., 2005). The universal primers developed by Le Roux et al. (1999) have been successfully used to amplify DNA from several species including the oysters *C. gigas* and *O. edulis* (Audemard et al., 2002; Friedman et al., 2005). The dilution of inhibited samples provides a rapid and simple way to overcome this problem (Renault et al., 2000a). However, sample dilution can reduce the number of target molecules below the limit of the detection method. Commercial DNA purification kits procedures can also be used to remove inhibitors and at the same time concentrate the total DNA extracted (An and Fleming, 1991; Wiedbrauk et al., 1995). The addition of BSA can effectively reduce PCR inhibition (Friedman et al., 2005).

7. Final considerations

PCR has been used successfully to detect the DNA of herpesvirus infecting several species of bivalve molluscs. Although PCR specificity has been assessed for some of the primer pairs used to detect herpesvirus DNA (i.e., A3/A4, A5/A6, C1/C6,

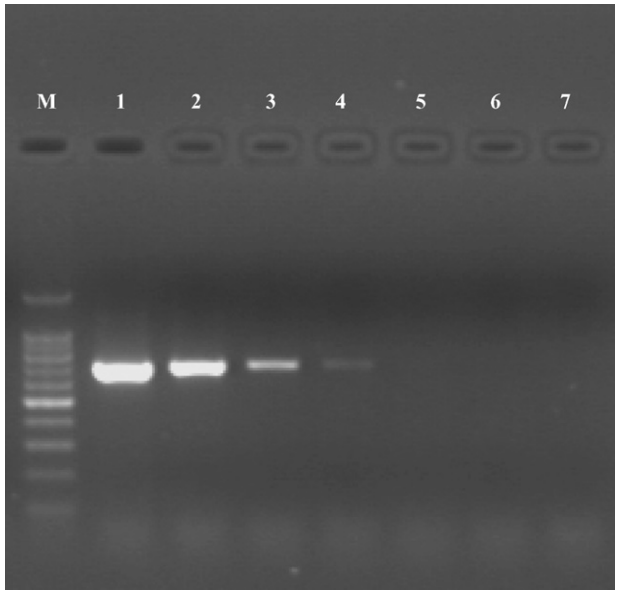

Fig. 4. Sensitivity of OsHV-1 DNA detection by PCR using the C2/C6 primer pair when 500 ng of non-infected *C. gigas* DNA was added. Results were obtained on a serial dilution (1:10) of purified viral DNA. Lane 1: 10 pg of purified viral DNA; lane 2: 1 pg of purified viral DNA; lane 3: 100 fg of purified viral DNA; lane 4: 10 fg of purified viral DNA; lane 5: 1 fg of purified viral DNA; lane 6: 100 ag of purified viral DNA; lane 7: negative control; lane M: size markers (100 bp DNA ladder, Promega).

Table 5
Recommendations for the detection of herpesvirus DNA by PCR in bivalve mollusc samples

Steps	Recommendations
Sampling	Samples should be preferably fresh frozen (−20 °C, −80 °C or liquid nitrogen), if not possible they can be fixed in 70% ethanol and stored at 4 °C If samples are formalin-fixed (e.g., Davidson's and Carson's solutions), over-fixation should be avoided (over 24–48 h) and subsequently transferred to 70% ethanol and stored at 4 °C Among the different formalin-based fixatives Davison's solution should be preferentially used in order to better preserve nucleic acids integrity
DNA extraction	Sample preparation should be done in a specific area and protocols used should be adequate to the type of material Extraction by boiling ground tissues can be used for larval samples or as below DNA extraction from spat and adult animals can be performed by proteinase K digestion followed by phenol/chloroform purification and ethanol precipitation or Qiagen extraction kits The presence of PCR inhibitors should always be checked (e.g., internal control) The quality and quantity of the nucleic acids extracted as well as the presence of inhibitory factors can be assessed throughout the use of universal primers To eliminate PCR inhibitors, dilution of the DNA preparation can be done. However, this procedure will also reduce the putative number of target molecules
PCR and primers	Positive (i.e., viral DNA) and negative (e.g., distilled water) controls should be always used. At least one negative control per five diagnostic samples should be used BSA should be added to the PCR master mix to reduce inhibition The selected primers should detect both OsVH-1 and OsHV-1var DNA The use of more than one primer pair is advantageous, such as C2/C6 and Gp3/Gp4 (several primer pairs targeting different areas of the viral genome) Since no gain in sensitivity has been documented by nested PCR procedures, its use should be avoided in order to reduce the risk of contamination
Analysis of amplicons	Confirmation of positive PCR results can be done by restriction enzyme analysis or Southern blot hybridization In each restriction enzyme analysis, amplicons obtained from reference OsHV-1 DNA should also be digested (positive control) Sequencing of PCR products can also be used to confirm the authenticity of amplicons and will provide additional information about the identity of the fragments amplified

C2/C6, C13/C5 and C14/C15), this has not done been done for all primer pairs used routinely. Moreover, the amplification conditions that have been used in PCR assays using different primer pairs were based on the conditions optimized for A3/A4 and A5/A6 (Renault et al., 2000a). The sensitivity (between 1 and 10 fg of purified viral DNA) observed for the majority of the primer pairs (e.g., C2/C6, Gp3/Gp4) suggests that the PCR conditions did not have a significant negative influence on the detection limit. However, it should be noted that non-specific amplification has been reported, which may be due to sub-optimal PCR conditions (e.g., annealing temperature and Mg^{2+} concentration). For example, detection of bands with unexpected sizes was observed in PCR assays with the C2/C6 primer pair. The template DNA used for these assays was extracted from the gills (preserved in 95% ethanol) of adult oysters by digestion with proteinase K followed by phenol/chloroform purification and ethanol precipitation. However, when using an annealing temperature of 60 °C (an increase of 10 °C) these extraneous bands were not observed (unpublished data). Under these conditions, the minimum template quantity needed systematically to detect amplicons was 10 fg of purified viral DNA extracted from purified particles. The same sensitivity was also observed when 500 ng of DNA from non-infected *C. gigas* was added (Fig. 4). Consequently, validation and standardization of the PCR conditions for the primer pairs that are apparently more useful for epidemiological surveys should be performed. The adequacy of some of the primer pairs often used is presented in Table 4, and practical recommendations for the detection of herpesvirus DNA by PCR in bivalve mollusc samples are given in Table 5. Development of a quantitative real-time PCR test is needed for simultaneous identification and quantification of virus copies (viral load) in hosts or environmental samples as the conventional end-point PCR tested developed to date provide qualitative data (presence or absence).

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Au cours de l'année 2006, deux nouvelles techniques de diagnostic ont été dévéloppées : **PCR en temps réel** et **mini-array**.

Un protocole de PCR en temps réel a été mis point au sein du LGP pour la détection et la quantification du virus OsHV-1 en utilisant un appareil Stratagène Mx3000P et une méthodologie basée sur l'utilisation du SyberGreen. La sensibilité du diagnostic est 10 à 100 fois supérieure à celle observées pour les techniques de PCR classique (simple PCR ou PCR nichée). Cette méthode ouvre la voie à de nouvelles approches, notamment au travers de l'étude de la **corrélation entre « charge » virale et mortalité chez l'huître creuse, *C. gigas***. Des données sur les « charges » virales associées à des épisodes de mortalité ont ainsi été obtenues très récemment, renforçant les connaissances sur les interactions hôte/virus. En 2006, plus de 600 échantillons de *C. gigas* ont été analysés par cette technique. Cet outil a permis également de préciser l'efficacité de divers protocoles d'extraction d'ADN. Cette nouvelle technique a fait l'objet de la préparation d'un manuscrit soumis pour publication à Journal of Virological Methods en 2007 (**Pepin J.-F., Riou A. & T. Renault, Rapid and sensitive detection of Ostreid Herpesvirus 1 (OsHV-1) in oyster samples by Real-Time PCR**).

En partenariat avec la société SkuldTech (Montpellier, France), un pilote pour le diagnostic d'OsHV-1 par mini-array a également été développé. L'objectif final visé est un **kit de diagnostic «tout en un» transférable** à terme aux **laboratoires de contrôle et aux professionnels écloseurs/nurseurs pour de l'autocontrôle**. Fin 2006, des prototypes du kit ont été obtenus et répondaient aux attentes en terme de spécificité et sensibilité. Ce nouvel outil a été présenté aux écloseurs professionnels français au cours d'une réunion organisée par l'Ifremer en février 2007 et a suscité un vif intérêt.

La technologie mini-array est une version des outils de haute technologie que constituent les puces à ADN. Elle repose sur l'amplification enzymatique de fragments d'ADN ciblés par PCR suivie de la visualisation des produits amplifiés par hybridation moléculaire sur des membranes prêtes à l'emploi (Figure 5). Contrairement au dot blot, avec la méthode mini-array, il s'agit de l'hybridation d'un produit de PCR sur un second produit de PCR. La cible (produit de PCR spécifique) est hybridée sur une sonde préalablement déposée sur un support poreux (membrane nylon). La méthode de détection possède une sensibilité et une spécificité accrues, en comparaison avec des méthodes courantes d'analyse de matériel génétique (ADN) amplifié par PCR.

La mise en place du mini-array a impliqué plusieurs étapes de développement et d'optimisation : choix des séquences virales cibles et des amorces, optimisation de la PCR et de la PCR multiplexe (préparation des cibles), préparation des sondes et des membranes, optimisation du protocole d'hybridation, des lavages et du protocole de révélation, estimation de la sensibilité et de la spécificité analytiques et fabrication et essais de deux prototypes (versions « plasmide » et « 28S »).

A l'issue des différentes étapes de développement, deux versions du kit de détection d'OsHV-1 par mini-array ont été mises au point. Le choix des amorces utilisées permet aux deux versions du kit de détecter le virus OsHV-1 ainsi que son variant (OsHV-1 var) (**Arzul *et al.*, 2001**). La différence entre les deux prototypes consiste au fait que des gènes de contrôle différents ont été intégrés dans chacune des versions. Ces gènes permettent de s'assurer de l'efficacité du test, puisque leur amplification indique l'absence d'inhibition.

La première version (version 28S) contient un gène interne obtenu à partir de la séquence du gène 28S de l'huître creuse. Le gène interne de la deuxième version (version plasmide) est un plasmide dont la séquence n'a aucun rapport avec le génome de l'huître, ni avec celui du virus.

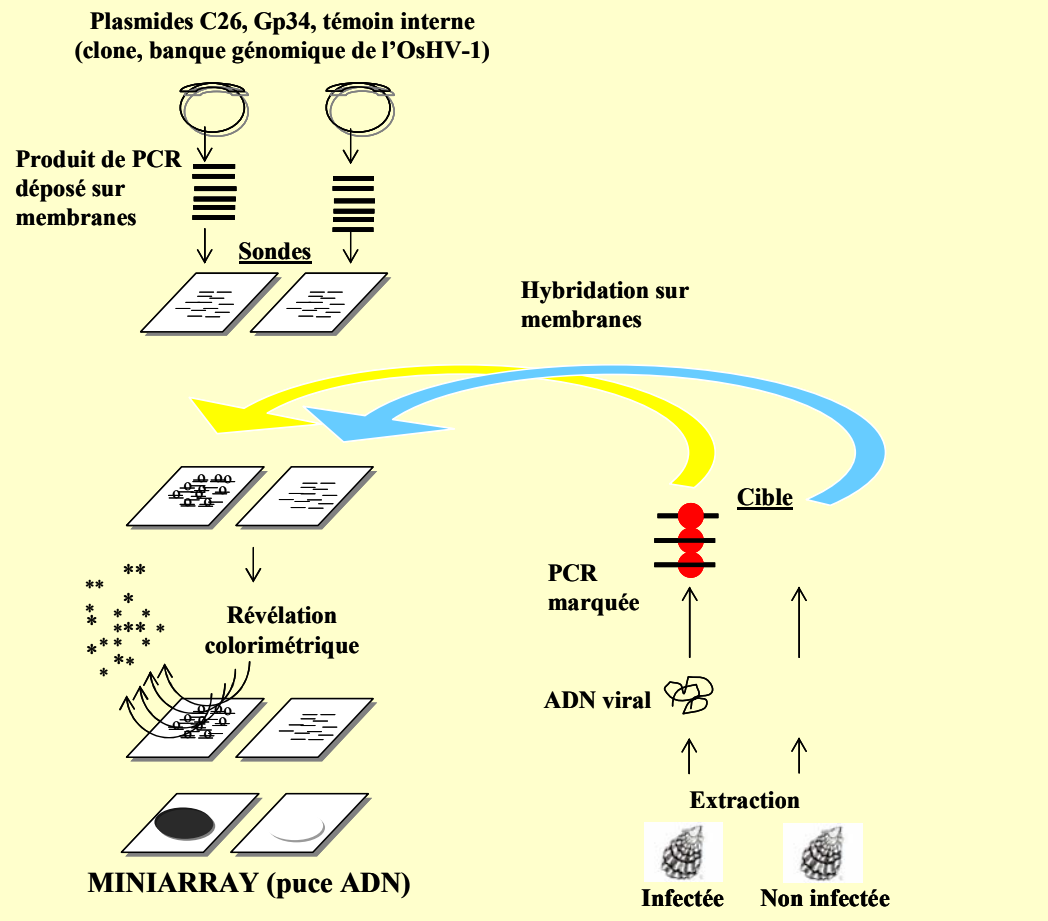
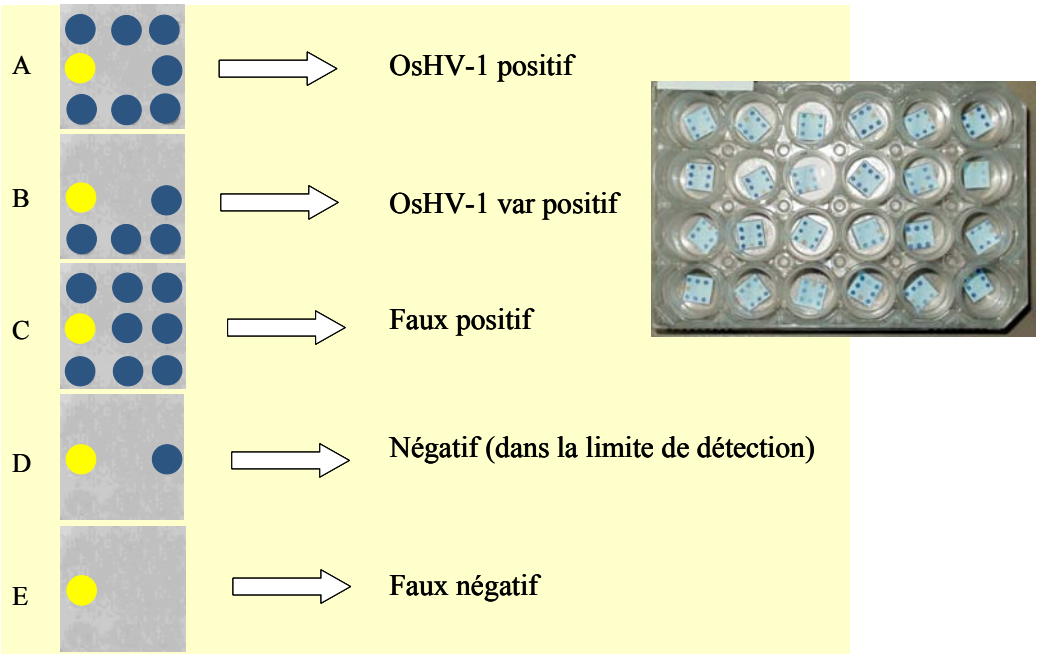


Figure 5. *Principe de fonctionnement du mini-array (puce ADN).*

La sensibilité et la spécificité analytiques de la PCR multiplexe («premier étage» du mini-array) ont été évaluées par analyse des produits de PCR sur gel. Elles ont été comparées avec celles de la PCR conventionnelle. La sensibilité et la spécificité analytiques du mini-array ont été également évaluées par PCR quantitative en temps réel (Light Cycler, Roche) (cf. page 97). Le mini-array développé permet de détecter une quantité d'ADN viral faible (20 copies d'ADN viral par µL d'échantillon) et sa spécificité a été validée par l'analyse des courbes de fusion de la PCR en temps réel.

L'interprétation des résultats du test de détection du kit se fait à l'œil nu. La Figure 6 regroupe les différents types de résultat qui peuvent être observés. Ainsi, le test permet la détection du virus OsHV-1 (Figure 6, A) et du variant OsHV-1 var (Figure 6, B). Un dispositif de mise en évidence de marquage non spécifique (mauvaise manipulation, quantité de cible excessive…) est intégré sur la membrane (Figure 6, C). Comme mentionné précédemment, un contrôle interne est inclus afin de minimiser les risques de qualifier à tort des échantillons comme négatifs (Figure 6, D : échantillon négatif, E : faux négatif).



Figures 6. *Mode d'emploi du kit de détection de l'OsHV-1 et de l'OsHV-1 var (mini-array)*

Les résultats du projet devraient être valorisés aussi bien sur le plan scientifique, par leur publication dans des revues spécialisées, que sur le plan commercial, par le dépôt d'un brevet d'application et la mise sur le marché du kit (Figure 7) (à l'issue du processus de validation). Au terme de sa validation, cet outil pourra devenir un solide **test de criblage terrain** et contribuer au développement de pratiques aquacoles et à l'établissement de **procédures fiables de bio sécurisation de la filière conchylicole**.

Figure 7. *Kit de détection de l'OsHV-1 et ses principaux composants et réactifs*

Des techniques d'hybridation *in situ* (HIS) ont également été développées (**Renault & Lipart, 1998** ; **Lipart & Renault, 2002** ; **Arzul *et al.*, 2002**). Ces méthodes permettent en particulier de détecter le virus OsHV-1 de manière spécifique chez des individus présentant des niveaux d'infection faibles. Elles sont relativement longues à mettre en œuvre. Elles nécessitent en effet une étape initiale comprenant la fixation des échantillons et la préparation de coupes histologiques. Cependant, en virologie, chez les mollusques, ces méthodes restent d'une aide précieuse. En effet, elles permettent de **confirmer une suspicion d'infection virale posée par le biais de l'histologie**.

Dans le cadre du projet européen VINO, un essai inter-laboratoire de validation de la détection du virus OsHV-1 par HIS a été aussi réalisé. Il impliquait trois laboratoires européens (Espagne, Irlande et Royaume Uni) et a été effectué sur la base d'un matériel biologique commun.

Trente échantillons de référence (coupes histologiques) ont été fournis par le Laboratoire de Génétique et Pathologie de La Tremblade (Ifremer, France, Participant A) : dix échantillons négatifs et 20 positifs (Tableau III, colonne Participant A) correspondant à des juvéniles d'huître creuse, *C. gigas*, collectés en 1994 et 1995. Ces échantillons ont été analysés en histologie au moment du prélèvement, mais ils ont également fait l'objet d'une analyse en PCR *a posteriori* (ADN extrait de coupes histologiques) afin de confirmer leur statut (infecté ou non infecté) sur la base d'un outil de diagnostic moléculaire, l'histologie ne permettant que de poser un diagnostic de suspicion d'infection sur la base de l'observation d'anomalies cellulaires et nucléaires.

Les résultats obtenus par les différents laboratoires (B, C et D) sont rapportés dans le Tableau III. Sur la base du statut des échantillons de référence défini par le participant A, les laboratoires B, C et D ont trouvé de faux positifs et de faux négatifs (Tableau III). Pour la laboratoire B, sur les dix échantillons qualifiés comme négatifs, sept ont été détectés positifs. Ce résultat montre la difficulté d'interprétation de lames en hybridation *in situ* et la nécessité d'une validation intégrant des sessions de formation à la lecture. Pour le laboratoire D les résultats sont globalement concordants. En effet, seuls deux faux négatifs ont été observés.

Au vu des résultats obtenus, ce type d'essai nécessite bien sûr d'être répété. Cependant, **ce test** était le **premier** du genre pour une **technique d'HIS permettant le diagnostic d'un agent infectant les mollusques bivalves**.

Tableau III - Essai inter-laboratoire pour la valadition de la détection d'OsHV-1 par HIS (projet européen VINO)

N° échantilllon	ISH C1/C6 A	B	C	D
1	-	?	-	-
2	-	+	-	-
3	-	+	-	-
4	-	+	-	-
5	-	+	-	-
6	-	+	+	-
7	-	ND	ND	-
8	-	-	+	-
9	-	+	+	-
10	-	+	?	-
11	+	+	+	-
12	+	+	+	-
13	+	+	+	+
14	+	+	+	+
15	+	+	?	+
16	+	+	-	+
17	+	+	+	+
18	+	+	+	+
19	+	+	+	+
20	+	+	-	+
21	+	+	-	+
22	+	+	+	ND
23	+	+	?	ND
24	+	+	+	ND
25	+	+	-	ND
26	+	+	+	ND
27	+	-	+	ND
28	+	-	-	ND
29	+	+	+	ND
30	+	-	+	ND

A: laboratoire ayant fourni le matériel biologique de référence ; B, C et D: laboratoires ayant réalisé l'essai inter-laboratoire. ND: non déterminé (lames de référence expédiées brisées à l'arrivée) , ?: interprétation difficile. Les résultats concordants sont représentés en vert et en rose ceux non concordants.

*3. 3. 2. La PCR : une expérience de dix ans en terme de diagnostic (*Renault T. & I. Arzul, 2001, Journal of Fish Diseases, 24 : 161-167) et (Burge C. A., Judah L. R., Conquest L. L., Griffin F. J., Cheney D. P, Suhrbier A., Vadopalas B., Olin P. G., Renault T. & C. S. Friedman 2007, Journal of Shellfish Research, 26 (1): 163–172).

L'élevage des coquillages en écloserie est un processus biologique complexe dont le succès n'est pas toujours assuré. Des mortalités sont ainsi observées de manière régulière dans les écloseries de coquillages en Europe. Des changements dans les facteurs physico-chimiques de l'environnement (température, présence d'algues ou de polluants) ou des maladies infectieuses peuvent influencer la réussite de ces élevages. C'est dans ce contexte que les pratiques d'élevage en écloserie prennent aujourd'hui en compte les données de la recherche en terme de nutrition et de prophylaxie vis à vis des infections impliquant des bactéries du genre *Vibrio*.

Cependant, depuis 1991, des épisodes de mortalité ont été observés et associés à la détection de virus de type herpès dans différentes écloseries (Nicolas *et al.*, 1992 ; Hine *et al.*, 1992). Cette association entre mortalité et infection virale nous a incité à déterminer l'implication exacte du virus OsHV-1 en tant qu'agent causal.

Ainsi, dans le cadre d'un projet européen (ROMEO) dont l'objectif principal était d'analyser les causes de mortalité en écloseries de coquillages (qualité de l'eau et de la nourriture, malades infectieuses), la technique de PCR a été utilisée pour rechercher la présence du virus OsHV-1 chez des échantillons de larves de différentes espèces de coquillages collectés dans plusieurs écloseries européennes (**Renault & Arzul., 2001**).

L'étude a porté sur 81 échantillons prélevés dans cinq écloseries (France, Espagne, Royaume Uni). Sur la base d'une recherche d'ADN du virus OsHV-1 par PCR, 21% (17 lots) des 81 échantillons prélevés entre 1998 et 2000 sont apparus positifs. Ces résultats ont confirmé des données précédemment obtenues montrant que le virus OsHV-1 était **détecté dans des écloseries commerciales en France**. De plus, des **résultats positifs** ont été obtenus pour des **échantillons provenant d'Espagne et du Royaume Uni**.

L'**ADN viral** a pu être détecté chez **quatre espèces de bivalve différentes : *C. gigas*, *O. edulis*, *R. philippinarum* et *R. decussatus***. Pour cette dernière espèce, c'est la première fois que la détection du virus était rapportée.

Par ailleurs, 76,5% des échantillons positifs en PCR correspondaient à des lots de larves présentant des mortalités anormales. Ainsi, à la différence des autres facteurs pris en compte et suivis au cours du projet ROMEO (qualité de l'eau et présence de polluants, qualité de la nourriture et présence de bactéries), seule la détection du virus a montré une forte corrélation avec les épisodes de mortalité anormale**. L'infection virale a pu ainsi être identifiée comme le principal facteur explicatif au cours de l'étude**. Ce résultat est à mettre en perspective avec l'étude menée par **Le Deuff *et al.* (1994)** ayant permis de démontrer le pouvoir infectieux du virus de type herpès infectant les larves d'huître creuse, *C. gigas*.

Journal of Fish Diseases 2001, **24,** 161–167

Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: specific viral DNA detection by PCR

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Abstract

Periodic losses in oyster hatcheries are regularly reported in Europe. Herpes-like virus infections seem to play a key role. Polymerase chain reaction (PCR) was used to investigate the presence of herpes-like virus DNA in larval samples belonging to different bivalve species from different geographical origins. Seventeen samples of the 81 analysed appeared positive for the herpes-like virus DNA by PCR. These results confirm previous data indicating that herpes-like virus infections occur in commercial French hatcheries. Polymerase chain reaction positive results were also obtained for bivalve larval samples originating from Spain and the UK. The number of virus DNA positive samples depended on the primer pair used. The primer pair C2/C6 appears well adapted for herpes-like virus DNA detection because of processing ease and great sensitivity. Positive samples were observed in four bivalve species: *Crassostrea gigas*, *Ostrea edulis*, *Ruditapes decussatus* and *Ruditapes philippinarum.* Herpes-like virus DNA detection is reported in larval *R. decussatus* for the first time. Many samples in which viral DNA was detected by PCR correspond to larval batches presenting mortalities. Herpes-like viruses may be one of the causative agents of mortalities observed in bivalve hatcheries.

Keywords: shellfish hatchery, mortality, herpes-like virus, viral infection, *Crassostrea gigas*, *Ostrea edulis*, *Ruditapes philippinarum*, *Ruditapes decussatus*, Europe.

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Introduction

Crassostrea gigas is increasingly being cultured in a great number of countries from animals originally introduced from Japan and is one of the most exploited bivalve species in the countries of the European Union (EU). Although no serious pathogen has been detected previously in Japanese oysters in Europe, mortalities have been observed since the introduction of this bivalve species into EU countries. Mortalities have been described among different species of ostreids and are associated with the presence of viruses belonging to various families. The first description of a virus was reported in adult eastern oysters, *C. virginica,* with the detection of particles indicating a member of the *Herpesviridae* (Farley, Banfield, Kasnic & Foster 1972). Mass mortalities of adult Portuguese oysters, *C. angulata,* in France between 1967 and 1973 were associated with iridovirus infections (Comps, Bonami, Vago & Campillo 1976; Comps & Bonami 1977; Comps & Duthoit 1979). Other viruses described in ostreids include members of the *Iridoviridae*, *Papovaviridae*, *Togaviridae*, *Retroviridae* and *Reoviridae* (Farley 1976, 1978; Elston 1979; Meyers 1979; Elston & Wilkinson 1985).

In 1991, viruses interpreted as belonging to the *Herpesviridae* were associated with high mortality rates of hatchery-reared larval *C. gigas* in France (Nicolas, Comps & Cochennec 1992) and in New Zealand (Hine, Wesney & Hay 1992). Since 1992 sporadic high mortalities of larval *C. gigas* have been regularly observed in some commercial French hatcheries, occurring each year during the summer in association with a herpes-like virus (Renault, Cochennec, Le Deuff & Chollet 1994a). The pathogenicity of the virus was demonstrated by experimental transmission of the infection to

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healthy *C. gigas* larvae (Le Deuff, Nicolas, Renault & Cochennec 1994). Since 1993, sporadic high mortalities have occurred also in some batches of Pacific oyster spat cultured in different French locations (Renault, Le Deuff, Cochennec & Maffart 1994b). In addition, herpesvirus infections were reported in spat and larvae of the European flat oyster, *Ostrea edulis,* in France (Comps & Cochennec 1993; Renault, Le Deuff, Chollet, Cochennec & Gerard 2000a). Concomitant mortalities were observed among larvae and spat of *C. gigas* and *O. edulis* in 1994 and 1995, with the detection of herpes-like virus particles by transmission electron microscopy (Renault *et al.* 2000a). Replication of herpes-like viruses was also described in *O. angasi* adults in Australia (Hine & Thorne 1997), in larval *Tiostrea chilensis* in New Zealand (Hine 1997; Hine, Wesney, Besant 1998) and in larval *Ruditapes philippinarum* in France (Renault 1998). Unexplained mortalities were observed in recent years among *C. gigas* larvae in the UK and Spain, although samples were not examined. High losses were reported among Japanese oyster spat in Ireland in 1994 and 1995. No obvious cause of mortalities was determined (Culloty & Mulcahy, unpublished data). Screening using conventional light microscopy yielded little apart from some cell damage, most noticeably enlarged cell nuclei and marginated chromatin. Results may indicate that herpes-like virus is present in at least one site on the south coast of Ireland (Culloty & Mulcahy, unpublished data).

Herpes-like virus infections in bivalves are often associated with substantial mortalities. These observations highlight the importance of testing a range of efficient diagnostic methods in order to assess the causative role of herpes-like viruses in bivalve mortalities. To diagnose herpes-like virus infections, the basic method for examination of suspect samples is still light microscopy. This method appears poorly adapted to viral diseases and needs to be improved upon by other techniques such as transmission electron microscopy. Both techniques are time consuming and inadequate for epidemiological surveys. In addition, research into virus cytopathogenic effects in cell cultures is impossible because of the lack of bivalve cell lines. Serological methods are also not available because of the absence of immunoglobulin production in molluscs. A breakthrough was achieved recently in the development of a protocol, based on sucrose gradient centrifugation, for purifying oyster herpes-like virus particles from fresh infected larval *C. gigas* (Le Deuff & Renault 1999). This advance permitted the generation of molecular biological reagents to diagnose virus infections (Renault & Lipart 1998; Renault, Le Deuff, Lipart & Delsert 2000b). A procedure to detect herpes-like virus in French oysters using the polymerase chain reaction (PCR) (Saiki, Scharf, Falcoma, Mullis, Horn, Erlich & Arnheim 1985; Mullis, Falcoma, Scharf, Snikl, Horn & Erlich 1986) was developed (Renault *et al.* 2000b). Polymerase chain reaction offers many advantages for disease diagnosis (Jones & Bej 1994; Martin 1994) including its extreme sensitivity, pathogen specificity, ease of sample processing and availability of reagents.

The observed association between oyster mortality and herpes-like virus infections make it imperative to determine the extent to which the virus is involved as a causative agent of massive mortalities in different European countries and to survey infections epidemiologically in different European shellfish hatcheries. The aim of this study was to use molecular reagents (PCR) specific for oyster herpes-like viruses in order to analyse larval samples belonging to different bivalve species from different geographical origins.

Materials and methods

Clinical samples

Eighty one larval bivalve batches were obtained from five different hatcheries located in France, Spain and the UK: 21 samples (26%) from Hatchery 1 (UK), seven samples (9%) from Hatchery 2 (UK), 34 samples (42%) from Hatchery 3 (France), 10 samples (12%) from Hatchery 4 (France) and nine samples (11%) from Hatchery 5 (Spain). Animals were 8–13 days-old and of four bivalve species: *C. gigas* (77%), *O. edulis* (4%), *R. philippinarum* (4%) and *Ruditapes decussatus* (15%). The percentage values of samples analysed for each bivalve species depended on the hatchery involved (Fig. 1). Samples were collected from broods, some of which presented high mortalities from November 1997 to June 2000, and were frozen at −20 °C as soon as possible after sampling. Frozen samples were sent to the IFREMER laboratory (La Tremblade, Charente Maritime, France) on dry ice.

Virus DNA

Herpes-like virus DNA was extracted from purified virus pellets. The protocol used was as previously

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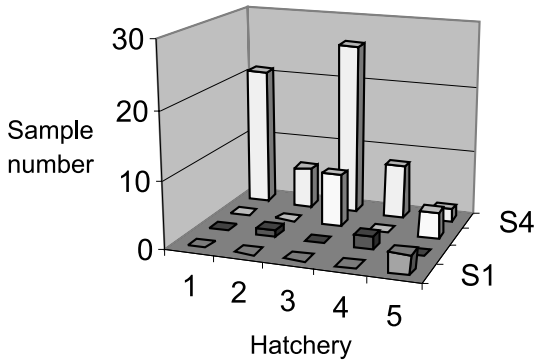

Figure 1 Distribution of larval samples in relation to hatchery of origin and bivalve species. Numbers denote hatcheries. S1: *R. philippinarum*, S2: *O. edulis*, S3: *R. decussatus*, S4: *C. gigas*.

described (Le Deuff 1995; Le Deuff & Renault 1999). Briefly, purified virus particles were suspended in extraction buffer (100 mM NaCl; 10 mM Tris; 25 mM EDTA; 0.5% SDS; pH 8), then proteinase K (0.2 mg mL^{-1}) was added. DNA was extracted using phenol/chloroform/isoamyl alcohol (24/23/1) and precipitated. It was resuspended in TE buffer (10 mM Tris; 1 mM EDTA; pH 8). The DNA concentration was determined using a Perkin–Elmer spectrophotometer at 260 nm. A virus DNA suspension was prepared at 100 ng μL^{-1} in TE buffer and used as a positive control for PCR analysis.

Sample preparation for PCR

Frozen larvae of 50 mg were weighed out in an Eppendorf microtube and ground in 50 μL of distilled water as previously described (Renault *et al.* 2000b). Larval samples were denatured in a boiling water bath for 10 min. Samples were centrifuged at 1000 g r.p.m. for 5 min. Supernatants were recovered, immediately 10-fold diluted in double distilled water and frozen at −20 °C.

PCR primers

Seven PCR primers were derived from herpes-like viral DNA sequences (Renault *et al.* 2000b; Davison, unpublished data). Three forward primers (C6, A3 and A5) and four reverse primers (C1, C2, A4 and A6) were designed (Table 1) which provided four primer-pair combinations (C1/C6, C2/C6, A3/A4 and A5/A6) with expected PCR products that ranged, respectively, in size 896 pb, 709 pb, 1001 pb and 917 bp. For the nested PCR protocol, A3/A4 were used as external primers and A5/A6 as internal primers. The primer pairs C1/C6 and C2/C6 were used in simple PCR protocols.

Table 1 Specific oligonucleotides used in the present study

Name	Sequence
A3	5′ GCCAACCGTTGGAACCATAACAAGC 3′
A4	5′ GGGAATGAGGTGAACGAAACTATAGACC 3′
A5	5′ CGCCCCAACCACGATTTTTCACTGACCC 3′
A6	5′ CCCGTCAGATATAGGATGAGATTTG 3′
C1	5′ TTCCCCTCGAGGTAGCTTTT 3′
C2	5′ CTCTTTACCATGAAGATACCCACC 3′
C6	5′ GTGCACGGCTTACCATTTTT 3′

PCR conditions

Fifty microlitre PCR reactions were performed, each containing the appropriate reaction buffer, 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM $MgCl_2$, 2.5 units of DNA polymerase Goldstar (Eurogentec, Belgium) and 1 μL of template DNA. After heating samples for 2 min at 94 °C, 35 cycles were performed followed by a final elongation step of 5 min at 72 °C. Each of the 35 cycles consisted of a DNA melting step at 94 °C for 1 min, a primer annealing step for 1 min at 50 °C for the C1/C6, C2/C6, A3/A4 and A5/A6 pairs and a primer elongation step at 72 °C for 1 min. For the nested PCR using the A3/A4 and A5/A6 primer pairs, reaction conditions were the same for the second reaction, except for template DNA volume. For the second PCR reaction 0.5 μL of first PCR reaction products was used.

PCR products were separated by size by electrophoresis on 1% agarose gels. Gels were stained with ethidium bromide (0.5 μg mL^{-1}). Ten microlitre of each PCR product was loaded per lane. The DNA was visualized using a 302-nm UV transilluminator and the gels photographed. Sizes of the DNA products were determined relative to those of size markers (Eurogentec, Belgium).

As a result of the high sensitivity of PCR, special precautions were adopted to avoid contamination. All steps of this study, including sample preparation, preparation of amplification mixture components and PCR amplification were carried out in separate rooms. To limit contamination, all pipetting procedures were performed using filter tips.

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Results

Viral DNA detection by PCR

Amplification products were observed by PCR analysis with expected sizes on agarose gels from different clinical samples (Fig. 2, Table 1). The PCR analyses were performed using different primer pairs: 65 (38%) using the primer pair C1/C6, 66 (39%) using the primer pair C2/C6 and 39 (23%) using the nested PCR protocol with A3/A4 and A5/A6 (Renault *et al.* 2000b). Twenty-eight samples were analysed using the three sets of primers and other samples were analysed using only one or two primer pairs. Positive results shown in Table 2 are for any primer pair used.

Seventeen samples of the 81 analysed (21%) appeared positive for herpes-like virus DNA by PCR (Table 2). However, for two samples (Hatchery 1), the band of expected size was faint on agarose gels. Positive samples were observed in the four bivalve species: 11 *C. gigas* samples (64.7%), two *O. edulis* samples (11.8%), three *R. decussatus* samples (17.6%) and one *R. philippinarum* sample (5.9%) (Table 2). Positive samples were observed in four hatcheries: Hatchery 1, Hatchery 3, Hatchery 4 and Hatchery 5 (Fig. 3, Table 2). All samples originating from Hatchery 2 were negative for the detection of herpes-like virus DNA by PCR.

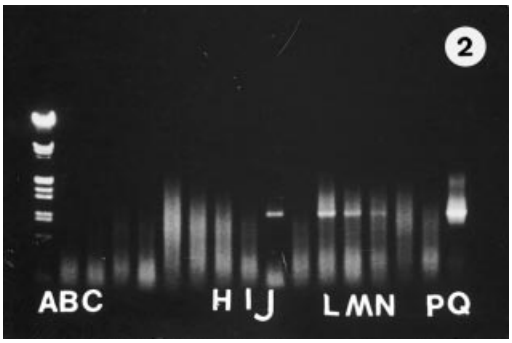

Figure 2 Analysis of bivalve larval samples by nested PCR using the A3/A4 and A5/A6 primer pairs. Agarose gel electrophoresis of the first PCR reaction products. Lane A shows the migration of molecular weight marker (Eurogentec MT2). Lanes B, I and P: control reactions without template DNA; lane Q: positive control corresponding to 0.5 ng of herpes-like viral DNA. Lanes B to H: *C. gigas* larval samples (Hatchery 1). Lanes J to O: *R. decussatus* larval samples (Hatchery 3). Positive larval samples: lanes J, L, M and N.

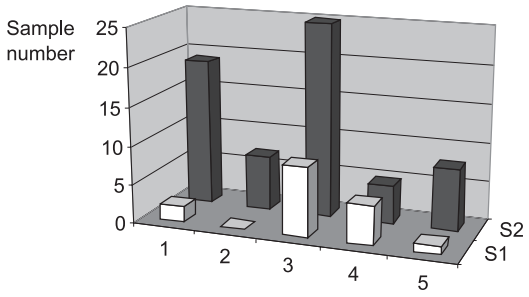

Figure 3 Detection of herpes-like virus DNA by PCR. 1: Hatchery 1, 2: Hatchery 2, 3: Hatchery 3, 4: Hatchery 4 and 5: Hatchery 5. S1: PCR positive samples and S2: PCR negative samples.

PCR results and primer pairs used

The number of virus DNA positive samples depended on the primer pair used for PCR. Twenty eight larval samples were analysed using the three sets of primers (C1/C6, C2/C6 and A3/A4 – A5/A6) corresponding to 84 PCR analyses (28 × 3). Two samples appeared positive (2/28: 7.1%) when the primer pair C1/C6 was used. Nine samples (9/28: 32.4%) allowed the detection of a band of expected size on agarose gels when the PCR reactions were

Table 2 Herpes-like virus DNA detection by PCR in bivalve larval samples

	Crassostrea gigas		*Ostrea edulis*		*Ruditapes decussatus*		*R. philippinarum*	
	+	−	+	−	+	−	+	−
Hatchery 1	2	19	0	0	0	0	0	0
Hatchery 2	0	6	0	1	0	0	0	0
Hatchery 3	6	20	0	0	3	5	0	0
Hatchery 4	3	5	2	0	0	0	0	0
Hatchery 5	0	2	0	0	0	4	1	2
Sub-total	11	52	2	1	3	9	1	2
Total	63		3		12		3	

+: Detection of bands of expected sizes on agarose gels using primer pairs C1/C6, C2/C6 or A3/A4 and A5/A6; –: No band of expected size detected on agarose gels.

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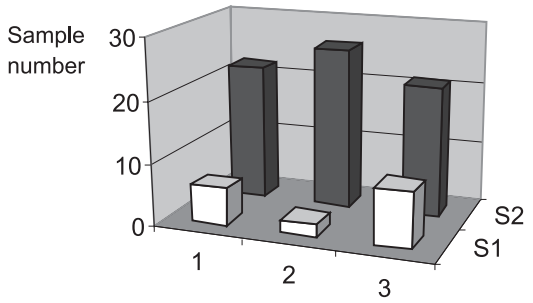

Figure 4 Detection of herpes-like virus DNA by PCR. 1: A3/A4 and A5/A6, 2: C1/C6 and 3: C2/C6. S1: PCR positive tubes. S2: PCR negative tubes.

carried out with the primer pair C2/C6. A band of expected size was observed in six samples (6/28: 21.4%) when the nested PCR protocol (A3/A4 and A5/A6) was used. The primer pair C1/C6 allowed less PCR amplification than C2/C6 (7.1% vs. 32.4%) or A3/A4 – A5/A6 (7.1% vs. 21.4%) (Fig. 4).

Mortality and positive PCR results

Thirteen PCR positive batches (76.5%) corresponded to animals presenting mortalities. No information was available for one PCR positive sample (Hatchery 5). Two PCR positive samples corresponded to *O. edulis* larvae not experiencing mortalities at the time of sampling (Hatchery 4). One sample giving a PCR positive result corresponded to *C. gigas* larvae originating from Hatchery 1 which presented slow growth without mortality.

Discussion

Currently available detection techniques for herpes-like viral infections in bivalves, i.e. histology and transmission electron microscopy, are less than totally satisfactory with regard to sensitivity, time and cost efficiency. Progress has been recently made in developing molecular methods including PCR for detection of a herpes-like virus infecting *C. gigas* larvae (Renault & Lipart 1998; Renault *et al.* 2000b) and PCR was used in this study to investigate the presence of herpes-like virus DNA in several bivalve species from different geographical origins.

Positive samples were observed in four hatcheries from three European countries (France, Spain and the UK). For the French hatcheries, nine and five samples, respectively, showed positive PCR results. These results confirm previous data indicating that herpes-like viral infections occur in commercial hatcheries in France (Nicolas *et al.* 1992; Renault *et al.* 1994b, 2000a). Positive PCR results have also been obtained for a bivalve larval sample originating from Spain and in two samples from the UK. However, further analysis is needed to confirm these results. Some samples originating from Spain and the UK were thawed when they were received at the laboratory for PCR analysis. Negative PCR results or detection of bands of unexpected sizes on agarose gels may correspond to damaged viral DNA and thus the number of positive PCR results may be underestimated.

The PCR procedure used was found to be specific, not leading to false positive results (Renault *et al.* 2000b; Arzul, Renault, Lipart, Davison, in press). The oyster herpes-like virus primers used here failed to produce amplicons when human, fish and frog herpesviruses DNA or oyster DNA were used as templates (Renault *et al.* 2000b; Arzul *et al.*, in press). Moreover, diagnosis of herpes-like virus infections by the PCR protocol in 15 batches of larval oysters was previously confirmed by transmission electron microscopy examination, providing an initial validation test (Renault *et al.* 2000a).

The detection of herpes-like virus DNA is reported here for the first time in larval *R. decussatus*. Thus, in Europe, herpes-like viruses may be found in four bivalve species at the larval stage (Nicolas *et al.* 1992; Renault *et al.* 1994b, 2000a; Renault 1998). The specific detection of viral DNA in several bivalves species highlights the need to study potential interspecific transmission of herpes-like viruses. It is not known whether these agents represent different viruses, as might be anticipated from the fact that vertebrate herpesviruses are invariably associated with individual host species.

The detection of viral DNA was associated in 13 samples (76.5%) with high mortality rates. Herpes-like viruses may be interpreted as one of the causative agents of the reported mortalities. It is well established in the literature that herpes-like viruses infect larval cells (Hine *et al.* 1992, 1998; Nicolas *et al.* 1992; Renault *et al.* 1994b; 2000a) and induce mortality (Le Deuff *et al.* 1994; Le Deuff, Renault & Gerard 1996). Transmission electron microscopy data show that viral particles are present inside the cells of the different larval bivalve species and are not merely an external contaminant. The successful experimental transmission

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of a herpes-like viral disease has been reported in axenic *C. gigas* larvae (Le Deuff *et al.* 1994, 1996). After inoculation of the viral suspension, healthy larvae died quickly and cumulative mortalities reached 100% on the sixth day post-infection. Transmission electron microscopy examination revealed the presence of viral particles in inoculated larvae, whilst in control animals neither lesions, nor virus, was detected.

The differences observed in herpes-like virus DNA detection depending on the PCR primer pairs used indicates that some primer pairs are more adapted for epidemiological surveys than others. Thus, the primer pair C2/C6 seems well adapted for herpes-like virus DNA detection because of processing ease (simple PCR reactions) and great sensitivity. The PCR protocol using the primer pair C2/C6 limits reagent use and reduces contamination in comparison with the nested PCR protocol using the A4/A5 and A5/A6 primer pairs (Renault *et al.* 2000b). A rapid response can be obtained for herpes-like virus detection and the availability of a sensitive and reliable PCR technique to detect herpes-like virus DNA in less than 24 h may be useful to manage larval rearing in commercial hatcheries. The detection of viral DNA by PCR in larval batches presenting mortalities may help to avoid the spread of viral infections among broodstocks.

Acknowledgments

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En France, les mortalités associées au virus OsHV-1 chez les coquillages concernent essentiellement les larves et les juvéniles. Dans le cadre du Réseau de Pathologie des Mollusques (REPAMO, réseau Ifremer), des analyses de coquillages lors d'épisodes de mortalité anormale ont permis de détecter ce virus chez différentes espèces (huître creuse, huître plate, palourdes, coquille Saint Jacques) dans différentes zones conchylicoles françaises ainsi que dans des écloseries et des nurseries.

L'Ifremer assure pour le compte de la Direction des Pêches Marines et de l'Aquaculture (DPM), par le biais du réseau REPAMO et de la Cellule Analytique du LGP La Tremblade, une **surveillance des mortalités anormales de coquillages au niveau national en terme de pathologie**. Cette surveillance a pour objectif de maintenir un état de veille vis-à-vis de l'émergence de nouveaux agents pathogènes et de nouvelles souches d'agents déjà présents sur le territoire, et vis-à-vis de l'introduction d'agents pathogènes exotiques. Il répond plus particulièrement à l'exigence réglementaire de la Directive 95/70/CE, transposée en droit national par l'article R.236-14 du Code Rural. Il s'agit en particulier d'écarter ou de confirmer l'implication d'un agent infectieux lors d'un épisode de mortalité anormale.

La notion de **mortalité anormale** est définie de manière réglementaire au niveau communautaire (Directive Européenne 95/70/EC) : c'est une **mortalité soudaine affectant plus de 15% d'un stock et survenant dans une période de 15 jours**. Pour chaque cas de mortalité anormale déclarée (par un conchyliculteur), une enquête épidémiologique est réalisée auprès du professionnel concerné sur la base d'un questionnaire type. Ce document comprend quatre parties intégrant des informations sur le lot (espèce, origine, âge, histoire), sur l'épisode de mortalité anormale, sur les techniques d'élevage utilisées (densité d'animaux, transferts, manipulations) et sur les conditions environnementales (température, salinité, …). L'ensemble de ces informations est intégré dans une base de données confidentielle (base REPAMO). L'objectif est de collecter des informations pour tenter de trouver une explication au phénomène de mortalité observé.

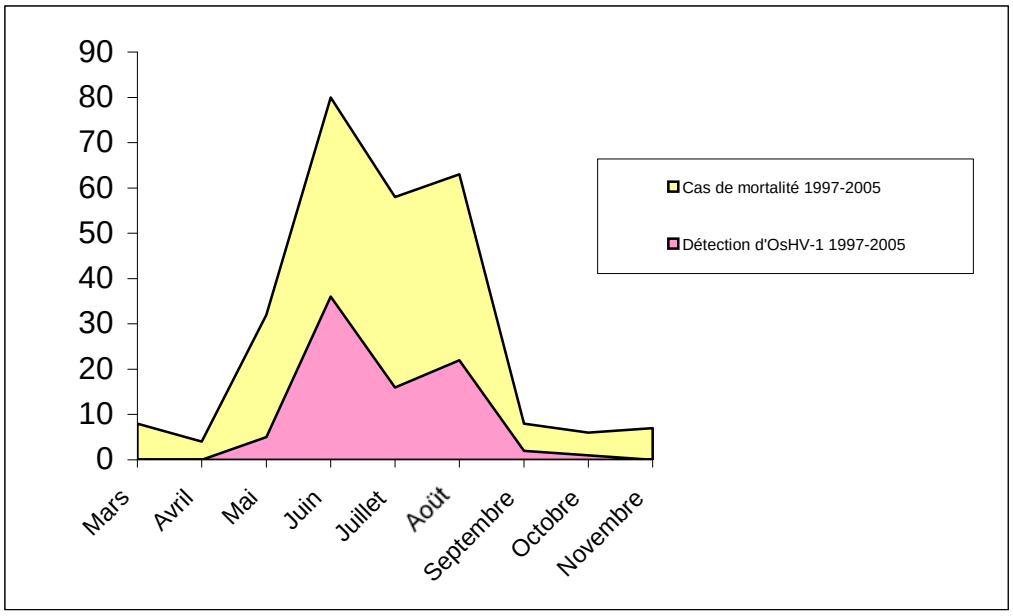
Des échantillons peuvent également être prélevés pour réaliser des analyses (intégration de la recherche du virus OsHV-1 par PCR) afin de confirmer ou d'infirmer une hypothèse infectieuse. Cette approche repose sur une **surveillance passive** : elle est basée sur les déclarations faites par les professionnels eux-mêmes. Cela peut conduire à une sous-estimation des mortalités et à des informations inexactes concernant la période de mortalité ainsi que son niveau (Moyse, 1993 ; Toma *et al.*, 2000).

Par le biais de cette approche de surveillance, il a été possible de **détecter le virus OsHV-1 au cours d'épisodes de mortalité anormale** aussi bien **sur l'estran que dans des écloseries nurseries** plusieurs années de suite (Table IV, Figure 8).

Tableau IV - Echantillons d'huître creuse, *C. gigas,* collectés durant des épisodes de mortalité sur l'estran et dans des écloseries/nurseries, en France, entre 1997 et 2005 et détection du virus OsHV-1 par PCR

Année	Estran			Ecloseries/Nurseries		
	Nombre d'échantillons	Nombre d'échantillons négatifs pour la détection d'OsHV-1	Nombre d'échantillons positifs pour la détection d'OsHV-1	Nombre d'échantillons	Nombre d'échantillons négatifs pour la détection d'OsHV-1	Nombre d'échantillons positifs pour la détection d'OsHV-1
1997	46	36	10	5	2	3
1998	25	24	1	2	1	1
1999	18	13	5	1	1	0
2000	30	23	7	6	4	2
2001	28	23	5	2	0	2
2002	10	8	2	7	4	3
2003	16	9	7	8	4	4
2004	22	13	9	8	6	2
2005	11	8	3	6	4	2
Total	206	152	54	45	26	19

Figure 8. *Cas de mortalité anormale rapportés chez l'huître creuse, C. gigas, entre 1997 et 2005 et détection du virus OsHV-1 par PCR*



Les analyses réalisées ont permis de montrer une **typologie particulière des mortalités associées à la détection du virus OsHV-1 chez les juvéniles d'huître creuse élevés sur estran**. Elles apparaissent en **tâches**. Cette observation peut être liée à la nature du virus. En effet, les herpès virus sont des virus enveloppés qui présentent une faible capacité de persister dans le milieu extérieur et pour lesquels la transmission passe le plus généralement par un contact direct entre individus infectés et individus sains (Van Regenmortel *et al.*, 2000). La persistance du virus OsHV-1 dans le milieu extérieur, en particulier dans l'eau de mer, est mal connue. Cependant, il a été démontré que la température était un facteur important influant la détection du virus dans l'eau (**Vigneron *et al.*, 2004**). De plus, l'eau de mer peut contenir

diverses substances (nucléases ou molécules à activité virucide) (Bettarel *et al.*, 2000) qui peuvent être à l'origine de la destruction du virus. Dans ce contexte, le virus OsHV-1 lorsqu'il est excrété par les animaux malades doit principalement infecter des huîtres saines dans un proche voisinage. La **dissémination probablement réduite du virus** doit jouer un rôle dans la typologie des mortalités (en tâches). Le point d'origine de l'infection virale reste aujourd'hui incertaine : soit le virus est introduit avec les animaux, soit il existe un réservoir dans le milieu naturel. La première hypothèse peut être étayée par le fait que les herpès virus possèdent la capacité de persister chez l'hôte tout au long de sa vie et que l'expression virale est associée à des conditions stressantes (Van Regenmortel *et al.*, 2006). Si la latence n'a pas été démontrée de manière non équivoque à ce jour pour le virus OsHV-1, la détection d'ADN et de protéines virales a été rapportée chez des huîtres adultes asymptomatiques (**Arzul *et al.*, 2002**). Par ailleurs, dans les **écloseries/nurseries**, les **mortalités associées aux infections à OsHV-1** sont **massives et uniformes**. Cette observation peut traduire une dissémination plus aisée du virus, liée à de fortes densités et aux flux hydrauliques.

Les mortalités associées à la détection du virus OsHV-1 surviennent en période estivale (Figure 9). Cette observation suggère une **influence de la température de l'eau**. Il a été démontré qu'au stade larvaire chez l'huître creuse, *C. gigas*, ce facteur influait sur l'expression du virus (**Le Deuff *et al.*, 1996**). Chez le naissain, l'effet de la température est aussi fortement suspecté (**Renault *et al.*,1994b** ; Friedman *et al.*, 2005 ; Burge *et al.*, 2006). Les données REPAMO montrent que le virus est principalement détecté au printemps et en été et qu'il apparaît d'abord en Méditerranée où les températures de l'eau augmentent le plus précocement (Figure 10). Ensuite, la détection d'OsHV-1 se fait le long du littoral français de manière séquentiel du sud vers le nord (Figure 10). Le virus est généralement observé après une augmentation soudaine de la température et il ne l'est plus lorsque la température de l'eau reste élevée sur une longue période. Le même type de constat a pu être fait aux USA en Californie : des mortalités de juvéniles d'huître creuse sont observées juste après une élévation brutale de la température (Burge *et al.*, 2006).

Figure 9. *Détection du virus OsHV-1 par PCR au cours de l'année (pour l'ensemble des analyses réalisées entre 1997 et 2005)*

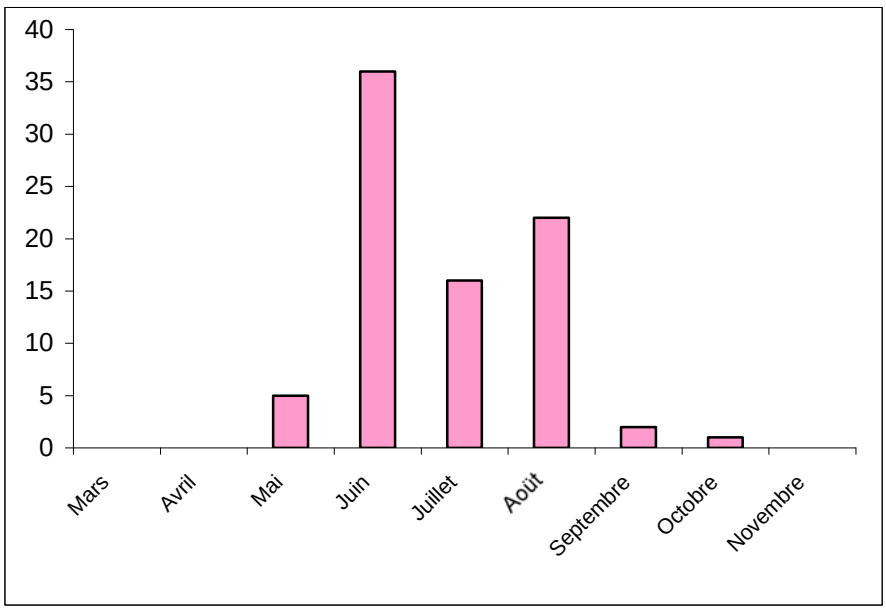
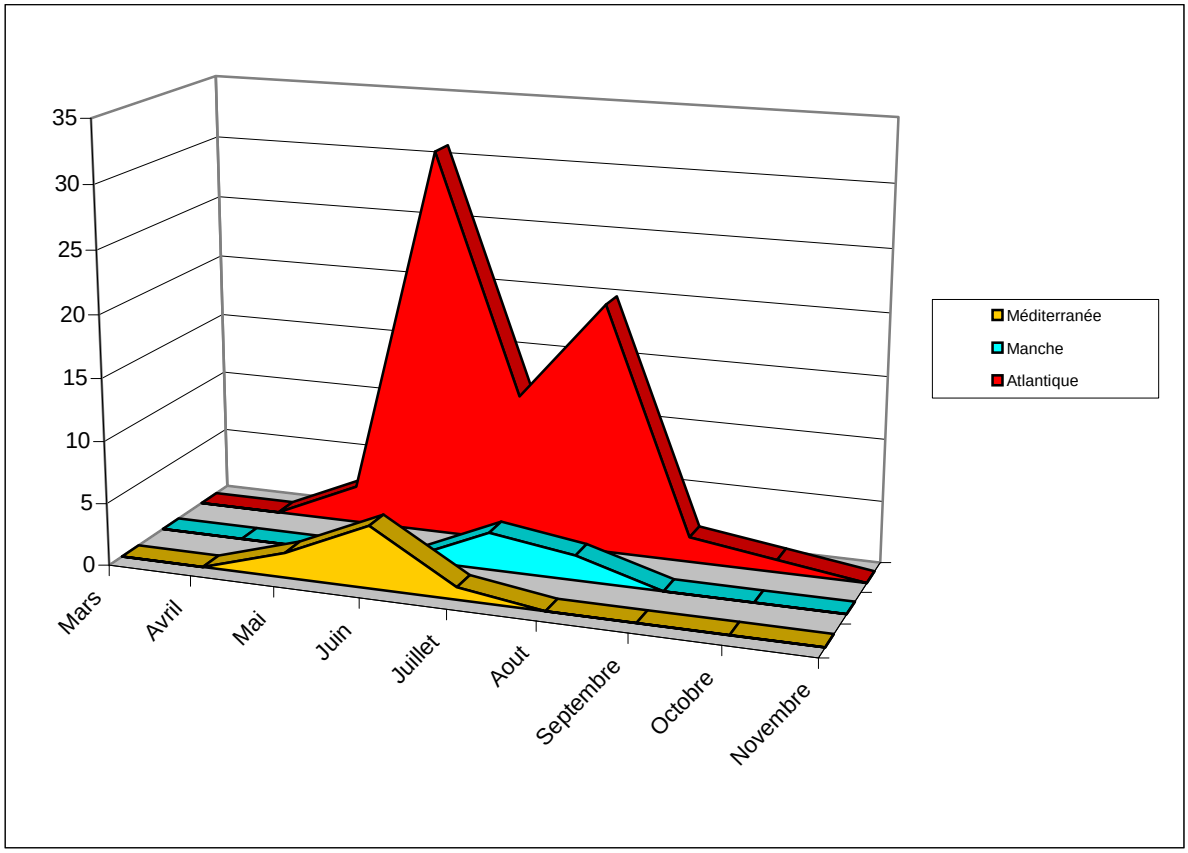
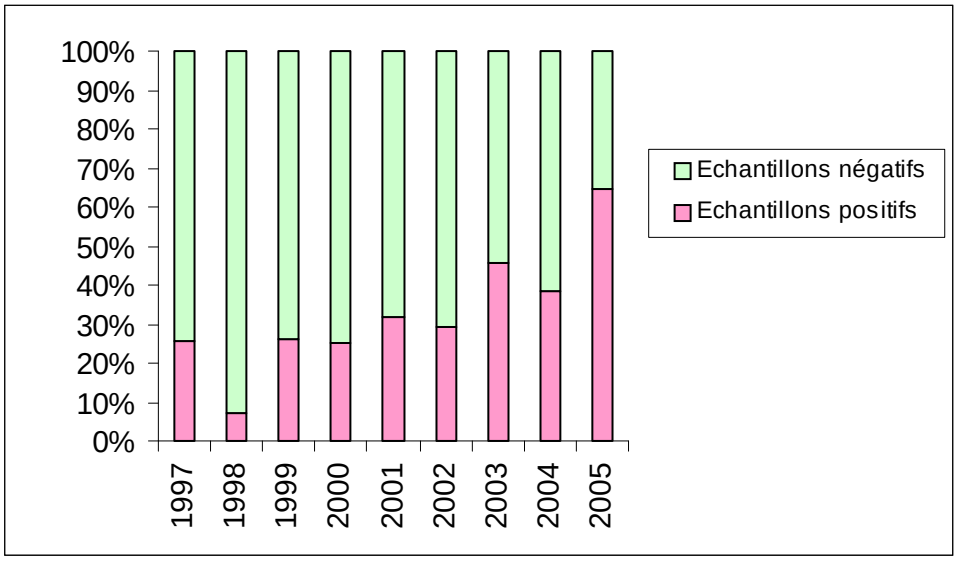


Figure 10. *Origine géographique des échantillons et détection du virus OsHV-1 par PCR (pour l'ensemble des analyses réalisées entre 1997 et 2005)*



La fréquence de détection du virus OsHV-1 par PCR a augmenté à partir de 2003 (Figure 11). Ce résultat peut être expliqué par des modifications en terme de collecte des échantillons et de procédure d'analyse.

Figure 11. *Fréquence de détection du virus OsHV-1 par PCR en fonction des années*



Un effort d'information a été réalisé auprès des professionnels conchyliculteurs afin de favoriser les déclarations des épisodes de mortalité et de réduire le temps entre observation de la mortalité et prélèvement d'un échantillon pour analyse.

En effet, il a été montré que la probabilité de **détecter le virus** était **augmentée** pour des **animaux prélevés rapidement après le début d'un épisode de mortalité** et si **l'échantillon contenait des huîtres moribondes** (huîtres « baillantes », restant ouvertes à l'émersion) (Tableau V). La capacité de détecter le virus est fortement liée à la qualité du prélèvement. Pour les coquillages, l'observation des mortalités et la collecte d'échantillons sont totalement dépendantes de l'accès aux animaux qui très souvent ne peut se faire qu'une semaine sur deux (cycle des marées). Enfin, à partir de 2003, ce sont de nouvelles amorces qui ont été utilisées pour le diagnostic du virus par PCR, amorces qui permettent d'augmenter la sensibilité de la technique.

Tableau V - Détection du virus OsHV-1 par PCR chez l'huître creuse*, C. gigas*, en fonction du type d'échantillon (présence/absence d'huîtres moribondes) et de la date de prélèvement par rapport à la date de la mortalité

Résultats de PCR : détection d'OsHV-1	Type d'échantillon		Date de collecte versus date de la mortalité		
	Présence d'huîtres moribondes	Absence d'huîtres moribondes	< 1 semaine	> 1 semaine	Pas d'information
Positifs	45	28	59	7	7
Négatifs	23	155	78	57	43
Total	68	183	137	64	50

Deux mesures peuvent être préconisées afin d'améliorer la détection du virus lors d'épisodes de mortalité :

- collecter les échantillons le plus rapidement possible après l'observation d'une mortalité (moins d'une semaine) ;
- collecter des animaux vivants et des animaux moribonds, la détection du virus étant plus fréquente sur ce dernier type d'animaux.

Bien que ces recommandations apparaissent évidentes pour le pathologiste, leur mise en application n'est pas simple pour les mollusques. En effet, en particulier pour les huîtres, l'élevage se fait principalement sur l'estran. Les animaux ne sont donc accessibles que de manière sporadique au moment de la marée basse et uniquement lorsque le coefficient de marée le permet. Ceci ne facilite pas les observations et les prélèvements. L'accès aux parcs d'élevage demande également des moyens logistiques lourds (nécessité de disposer d'un bateau en général). En d'autres termes, il est matériellement très difficile, voir **impossible**, pour les ostréiculteurs de réaliser une **surveillance quotidienne des animaux**.

Malgré ces difficultés, des suivis ciblés (collaboration avec des professionnels conchyliculteurs) pourraient apporter des informations complémentaires importantes en terme d'implication du virus dans les mortalités observées sur estran. En effet, la collecte de prélèvements réalisés de manière régulière avec un pas de temps serré (avant, pendant et après un épisode de mortalité) apparaît adaptée pour répondre à cette question. De plus, la PCR quantitative en temps réel offre aujourd'hui un outil de choix afin de mettre en relation une « charge » virale et l'observation de mortalités.

Afin de mieux comprendre l'implication du virus dans les phénomènes de mortalités anormales observés chez l'huître creuse, *C. gigas*, une **analyse de causalité** a été menée sur la base des données obtenues dans le cadre du REPAMO **entre 1997 et 2005**.

La démonstration d'un lien de causalité entre un agent pathogène et des mortalités observées sur le terrain n'est pas seulement basée sur la possibilité de reproduire la maladie expérimentalement, mais doit aussi prendre en compte l'idée que la cause de la mortalité peut être une combinaison de facteurs. Le développement de principes afin d'aider à l'évaluation des théories sur la cause des maladies a été initié par Koch (1882) et Henlé et complété par Hill (1965) et Evans (1976). Hill et Evans ont formulé une liste de postulats qui sont aujourd'hui largement acceptés comme guides pour évaluer un lien de causalité en épidémiologie. L'établissement d'une relation causale est utile à la fois dans un but cognitif et afin de proposer des politiques de prévention en santé animale. Il s'agit cependant d'une tâche extrêmement complexe. Les critères de causalité de Hill constituent dans ce contexte une aide dans l'évaluation de la nature causale d'une association malgré leurs nombreuses limites et l'impossibilité d'aboutir à une conclusion formelle.

L'analyse du lien de causalité entre la détection du virus OsHV-1 et les mortalités anormales de naissain d'huîtres creuses a été basée sur une approche de classement et de pondération semi-quantitative des critères définis par Hill et Evans (Evans, 1965 ; Hill, 1976 ; Cameron *et al.*, 2006). L'ensemble des critères, au nombre de 10, a été positionné sur une échelle arbitraire de 0 (pas d'évidence supportée par des données) à 3 (forte évidence). Ensuite, chaque critère a été pondéré (1 ou 2, 2 pour les critères interprétés comme les plus importants). Cette approche en prenant en compte l'ensemble des critères a permis d'établir un score.

Une **relation de causalité modérée entre mortalité et détection du virus OsHV-1 en PCR chez le naissain dans le milieu** a pu être ainsi définie (Tableaux VI et VII). Il est important de noter que les analyses pour la recherche du virus OsHV-1 ont été réalisées en PCR ce qui fait l'originalité de ce suivi épidémiologique. Le Laboratoire de Génétique et Pathologie de La Tremblade (Ifremer, France) peut se prévaloir d'une expérience unique en terme de **mise en œuvre d'un outil de diagnostic moléculaire chez les mollusques**, avec une **utilisation continue de la PCR sur une période de dix ans**. C'est grâce à cette expérience qu'il a été possible d'entreprendre une étude de causalité dans la mesure où des informations étaient disponibles pour une longue période.

S'il a été possible d'identifier une relation de causalité entre mortalités d'huîtres creuses et détection du virus par PCR grâce à un suivi épidémiologique sur plusieurs années, des informations complémentaires sont encore nécessaires. La nécessité de disposer de ces informations complémentaires peut expliquer le fait que la relation trouvée soit modérée.

Tableau VII - Interprétation des résultats en fonction des scores obtenus selon Cameron *et al.* (2006)

Score	Conclusion
0	Absence d'évidence
> 0 et ≤ 10	Très faible évidence
> 10 et ≤ 19	Faible évidence
> 19 et ≤ 29	Evidence modérée
> 29 et ≤ 38	Evidence élevée
> 38 et ≤ 48	Evidence très élevée

L'ensemble des résultats obtenus dans le cadre du REPAMO et concernant la recherche du virus OsHV-1 fait l'objet de la préparation d'un manuscrit (**Garcia C., Miossec L., Arzul I., Thébault A., Robert M., Chollet B., Francois C., Joly J.-P. & T. Renault, Oyster Herpes virus-1 and its relationship with *Crassostrea gigas* spat mortality in France: synthesis of a 9 year study**).

Tableau VI - Mortalité et détection de virus OsHV-1 par PCR chez l'huître creuse, *C. gigas*, lors de mortalités anormales. Niveaux théoriques de lien de causalité sur la base de 10 critères. Les critères considérés comme les plus importants sont multipliés par 2.

Niveau d'évidence		**Absent**	**Faible**	**Modéré**	**Forte**	**Niveau d'incertitude**				**Sources**
	Scale of relevant criteria	0	1	2	3	Pas de données	1 (faible)	2 (modéré)	3 (forte)	
Force de l'association	x 2					X				
Reproductibilité des résultats	x 1				3		X			Présente étude ; Burge et al. 2006a ; Burge *et al.* 2006b ; Renault & Novoa 2004 , Renault *et al.* 1995
Spécificité	x 1			2			X			Burge *et al.* 2006a ; Renault & Novoa 2004 ; Arzul 2001 ; Renault *et al.* 1994a
Temporalité	x 2				6			X		Burge *et al.* 2006a ; Arzul 2001
Gradient biologique	x 2		2						X	Présente étude; Burge *et al.* 2006a
Plausibilité biologique	x 1				3		X			Burge *et al.* 2006a ; Barbosa *et al.* 2005 ; Renault & Novoa 2004 ; Arzul 2001 Le Deuff 1995
Cohérence biologique	x 1				3		X			Burge *et al.* 2006a ; Friedman *et al.* 2005 ; Renault & Novoa 2004 ; Arzul 2001
Données expérimentales	x 2		2						X	Renault & Novoa 2004
Analogie	x 1				3		X			Renault & Novoa 2004 ; Kimura *et al*. 1981 ; Poulos *et al.* 2006
Intervention	x 2								X	Présente étude
Sous total			**4**	**6**	**14**					
Total	**24**									

Aux USA, des mortalités de juvéniles d'huître creuse, *C. gigas*, sont aussi observées régulièrement en période estivale depuis 1993 (Friedman *et al.*, 1997 ; Cherr & Friedman, 1998). Dans la Baie de Tomales (Californie), ces épisodes sont généralement de courte durée (une à deux semaines) et sont associés à des températures élevées durant l'été et à la prolifération d'algues (Cherr & Friedman 1998). Les pertes cumulées peuvent être très importantes et atteindre 90% des effectifs. L'impact économique que peut avoir ce phénomène explique les nombreux travaux scientifiques entrepris afin de tenter d'en définir les causes et de proposer des solutions.

Initialement, les sites les plus à l'intérieur de la Baie de Tomales (les plus estuariens) présentaient des pertes plus faibles ou décalées dans le temps par rapport aux sites plus à l'extérieur de la baie (conditions plus océaniques) (Cole, 1989 ; Judah 2002). Les animaux nouvellement implantés sur les sites d'élevage et les huîtres les plus jeunes (de moins d'un an) étaient les individus les plus touchés par les mortalités. Les huîtres de deux ans ne présentaient pas ou peu de mortalité (Cherr & Friedman, 1998).

Dans le travail réalisé par **Burge *et al.* (2007)**, différents facteurs (température de l'eau, présence d'agents infectieux et prolifération d'algues) ont été étudiés en utilisant trois stocks différents d'huîtres, mis en élevage sur trois sites (Inner Tomales Bay, Outer Tomales Bay et Bodega Harbor) à l'automne 2000 et au printemps 2001, en Baie de Tomales (Californie, USA). La croissance, la mortalité ainsi que le statut infectieux des animaux ont ensuite été suivis. Une expérimentation de même type a également été réalisée sur un autre site (Totten Inlet) dans l'Etat de Washington (USA) en utilisant les mêmes cohortes d'huîtres que celles placées dans le Baie de Tomales. Les plus fortes mortalités ont été observées sur le site le moins océanique de la baie (Inner Bay) et sont apparues corrélées aux températures les plus élevées relevées dans cette zone (25°C). Ce sont les huîtres présentant les croissances les plus importantes qui étaient préférentiellement affectées. Des différences significatives en terme de mortalité cumulée ont également été observées entre les différents stocks. De plus, les analyses histologiques ont permis de mettre en évidence chez les animaux au cours des épisodes de mortalité des anomalies dans les tissus conjonctifs et la glande digestive, similaires à celles précédemment décrites chez les huîtres creuses infectées par un herpès virus.

En conclusion, il a été possible de démonter grâce à cette étude (**Burge *et al.*, 2007**) et d'autres travaux (Friedman *et al.*, 2005; Burge *et al.*, 2006) une corrélation entre températures élevées et apparition de mortalité entre 2001 et 2003 en Baie de Tomales (Californie, USA). Par ailleurs, pour cette période, une infection à herpès virus a pu être suspectée sur la base d'analyses histologiques (**Burge *et al.*, 2007**) et confirmée par des analyses moléculaires (PCR utilisant des amorces spécifiques pour OsHV-1) (Friedman *et al.*, 2005; Burge *et al.*, 2006). Le récent développement d'outils de diagnostic spécifiques du virus OsHV-1 permettait en effet d'investiguer l'hypothèse infectieuse dans le cadre de suivis de terrain. Il est aujourd'hui suspecté que **le virus OsHV-1 ait été impliqué dans les phénomènes de mortalité en Baie de Tomales dès 1993**. Une **analyse rétrospective de matériel archivé** devrait permettre de confirmer ou d'infirmer cette hypothèse.

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SUMMER SEED MORTALITY OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* THUNBERG GROWN IN TOMALES BAY, CALIFORNIA, USA: THE INFLUENCE OF OYSTER STOCK, PLANTING TIME, PATHOGENS, AND ENVIRONMENTAL STRESSORS

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ABSTRACT Summer seed mortality (SSM) has occurred yearly in Tomales Bay, California since 1993. SSM has resulted in up to 90% cumulative losses, and has been associated with extreme temperature, phytoplankton blooms, and an oyster herpesvirus. In this study, three stocks of Pacific oysters were planted at three sites in California (Inner Tomales Bay, Outer Tomales Bay, and Bodega Harbor) in October of 2000 (Fall) and April of 2001 (Spring) and monitored for mortality, growth, and health status. In April of 2001, a similar study was conducted in Totten Inlet, WA state using cohorts of oysters planted in California; animals were monitored for mortality and growth. Temperature data were collected at all sites; phytoplankton abundance data were collected at the California sites. Mortality occurred only at the Inner Tomales Bay site where losses were correlated with maximum temperatures ($r = 0.949$) and preferentially affected faster growing oysters ($r = 0.916$). Significant differences in cumulative mortality were identified among oysters stocks and two of the three oysters stocks planted in the fall outperformed their cohorts planted in the spring ($P < 0.0001$). Microscopic changes in connective tissue and digestive tubules are consistent with previous observations of herpesvirus infections in oysters including: diffuse to multifocal pertibular hemocyte infiltration, diapedesis, dilation of the digestive tubules, nuclear hypertrophy, and chromatin margination. Nuclear hypertrophy and chromatin margination, in particular, are suggestive of herpesvirus infections; these histological changes were rare indicating the need to use multiple diagnostic methods when oyster herpesviruses are suspected to cause SSM. Temperature maxima (~25°C) experienced at the Inner Tomales Bay site are not considered extreme for Pacific oyster survival; the association between oyster herpesviruses and temperature in Tomales Bay, California is discussed.

KEY WORDS: Pacific oysters, *Crassostrea gigas*, mortality, temperature, oyster herpesvirus, Tomales Bay, California

INTRODUCTION

Significant recurrent summer losses of the Pacific oyster, *Crassostrea gigas* Thunberg, have occurred globally for over five decades and concern over its impact on oyster aquaculture has heightened in recent years (Imai et al. 1965, Glude 1974, Perdue et al. 1981, Cheney et al. 2000, Soletchnik et al. 2005). These losses, termed "summer mortality" are typically prolonged and affect older, reproductively mature animals during summer months (Koganezawa 1974, Glude 1974, Cheney et al. 2000). Summer mortality has been associated with multiple stressors including phytoplankton blooms (Cho 1979), warm water temperatures, changes in salinity, dissolved oxygen (Perdue et al. 1981, Perdue 1983), and pathogens (Friedman et al. 1991). More recently, losses have been reported in seed oysters during summer months and have been attributed to "summer mortality;" however, recent research suggests differences between "summer mortality" of adult oysters and seed mortalities. Seed mortalities typically affect the smallest animals, are short in duration, and have been associated with warm water temperatures and an oyster herpesvirus (Renault et al. 1994a, 1994b, Renault et al. 2000, Friedman et al. 2005, Burge et al. 2006). To differentiate between adult summer mortality, losses of seed oyster during summer months are referred to as "summer seed mortality" or SSM.

SSM has been observed among Pacific oyster seed, *Crassostrea gigas* (Thunberg) in Tomales Bay, California (Friedman et al. 1997, Cherr & Friedman 1998) and France (see review by Renault et al. 1994a, 1994b.) since 1993. Prior to this time average losses from plant to harvest (~18 mo) ranged from 15% to 35% (J. Finger, Hog Island Oyster Co., pers. comm.). In Tomales Bay, SSM events are typically short in duration (1–2 wk) and have been associated with elevated summer water temperatures and phytoplankton blooms (Cherr & Friedman 1998). Initially, culture facilities located near the inner reaches of Tomales Bay, experienced delayed and/or lower losses than those in outer Tomales Bay locations where more productive phytoplankton communities and oceanic conditions have been documented (Cole 1989, Judah 2002). Growers reported that new seed and younger animals seemed to be more susceptible to these mortality events than older individuals. Survivors from 1993 plantings experienced lower losses than those planted oysters in 1994 (Cherr & Friedman 1998). Only Pacific oysters in Tomales Bay appear to be affected by SSM; clams (*Venerupis*

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philippinarum), mussels (*Mytilus galloprovincialis*) and eastern (*C. virginica*), Olympia (*Ostrea conchapilia* = *O. lurida*), Kumamoto (*C. sikamea*), and flat oysters (*Ostrea edulis*) appear to be unaffected (J. Finger, pers. comm.).

In France, SSM occurs among *C. gigas* and *O. edulis* in the nursery and early grow-out in the field and has been associated with a herpesvirus infection (ostreid herpesvirus 1: OsHV-1) (Comps & Cochennec 1993, Renault et al. 1994a, 1994b, Renault et al. 2000). OsHV induced losses of larvae also occur in several bivalve species in France: Pacific oysters, *C. gigas* (Nicolas et al. 1992), Manila clams, *Venerupis* (=*Ruditapes*) *phillippinarum* (Renault & Arzul 2001, Renault et al. 2001), carpet clams, *Ruditapes decussatus* (Renault & Arzul 2001, Arzul et al. 2001b), and French scallops, *Pecten maximus* (Arzul et al. 2001a); and in New Zealand: Pacific oysters, *C. gigas,* (Hine et al. 1992) and flat oysters *Tiostrea chilensis* (Hine et al. 1998). A similar virus was recently reported from Tomales Bay (Friedman et al. 2005) and is associated with SSM in Pacific oysters (Burge et al. 2006).

Multiple stressors such as elevated water temperature, pathogens (i.e., oyster herpesvirus), and/or phytoplankton blooms have been suggested as potential causes of SSM in Tomales Bay, CA. We examined the role of these stressors in SSM and hypothesize that SSM may be mitigated by the selection of mortality resistant stocks and outplant time. Therefore, the objective of this sentinel field study is to test the association of SSM with water temperature, phytoplankton blooms, and pathogens in Tomales Bay using three oyster stocks outplanted in the fall and the spring.

MATERIALS AND METHODS

Sentinel Monitoring Surveys: 2000–2001

Oyster Stocks and Study Sites

Monitoring surveys were conducted in California, USA at 3 sites: Inner Tomales Bay, Outer Tomales Bay, and Bodega Harbor; and at a single site in Washington state: Totten Inlet (Fig. 1). Oyster stocks were obtained from 2 suppliers: (1) Strait of Georgia, Washington state (WA-1) and (2) Yaquina Bay, Oregon (OR-1, predicted to be a high performing line based on previous survival and growth and OR-2, predicted to be a poor performing line) (C. S. Friedman unpubl. data), donated by the Molluscan Broodstock Program (C. Langdon, OR State University). Oyster condition was assessed in all stocks ($n = 20$ animals from each stock on each date) on October 10, 2000 and March 30, 2001 and no abnormalities were observed.

Pacific oyster stocks were planted in the fall of 2000 (October 27, 2000) in Tomales Bay (Inner and Outer Bay sites) and Bodega Harbor (California, USA). Cohorts of the animals planted in fall of 2000 were over-wintered at the source hatcheries and planted in the spring of 2001 (April 11, 2001) in Tomales Bay (Inner and Outer Bay sites), Bodega Harbor, and Totten Inlet. Three bags of oysters per stock were planted in 1 cm individual mesh Nytex bags (61 × 122 cm) tied to metal racks approximately 30.5 cm above the sediment at the +30.5 cm MLLW. One thousand oysters were planted per bag at all California sites; in Washington state 200 oysters were planted per bag. Variation in culture methods between California and Washington sites reflect differences in established culture methods in each area.

Mortality, Growth, and Condition

Seed mortality in replicate bags of oysters was monitored monthly after the outplant of the Spring cohort by counting the number of dead oysters (empty shells or gaping oysters) from a random sample of 100 oysters per bag. Monthly growth was monitored by measuring oyster height (length) to the nearest mm using vernier calipers except at Totten Inlet, where oysters were measured only at the beginning and end of the experiment. Animal condition was assessed using routine paraffin histology to detect the presence of morphological abnormalities and or pathogens. Oysters stocks planted in the Fall of 2000 were examined on April 11, 2001 ($n = 90$: 10 animals collected from each stock from Bodega Harbor, Outer Tomales Bay, and Inner Tomales Bay on April 11). A subset of oysters from all stocks were examined during mortality episodes in Tomales Bay ($n =$ 27 survivors collected on June 5 and July 23). A standard cross section that included gills, mantle, gonad, and digestive gland was excised from each oyster and placed in Invertebrate Davidson solution (Shaw & Battle 1957) for 24 h; a subset of samples collected were processed for routine paraffin histology. Deparaffinized, 5-μm tissue sections were stained with hematoxylin and eosin (Luna 1968) and viewed by light microscopy to assess the presence of parasites and or morphological changes.

Environmental Parameters

Temperature and phytoplankton abundance were monitored over the course of this survey. Temperature data were recorded at each site with continuous data loggers (YSI Inc.) and were binned into 1-h intervals for analysis. Phytoplankton samples were collected from Inner and Outer Tomales Bay sites and Bodega Harbor (May 1 to August 20, 2001). Weekly, near-surface samples were collected from each site throughout the study period by lowering a bucket just below the surface. In addition twice-daily samples were collected in 2001 at Inner and Outer Tomales Bay sites using automated water samplers (Sigma 900 AS). At the Inner Bay site the sampler was located on a floating dock in water 0.6–30.5-m deep depending on the tidal height) with the intake tube held at 15.2 cm below the surface. At the Outer Bay, a floating dock was not available so the sampler was located on a fixed platform with the intake set at 0.5 m above the mud surface and adjacent to oyster culture racks. A shortcoming of the fixed platform was that the intake was at times exposed to air at very low tides and thus some samples were missed. In addition, samples drawn when tides were lower than 0.6 m were often filled with organic detritus, making it difficult or impossible to identify and enumerate the phytoplankton cells. Comparison of cell counts from AS and bucket grabs at the same location and time revealed that the AS samples were similar in species composition and cell density ($P = 0.36$, T-test; Sokal & Rohlf 1995). Thus data collected using both approaches are reported herein.

Phytoplankton samples were immediately preserved with 2% Lugol iodine solution and stored in a cool dark room at the Bodega Marine Laboratory until they were analyzed using the Utermohl technique (Utermohl 1958). Typically 50-mL subsamples were examined by quantifying phytoplankton to the species level or higher taxonomic affiliation (e.g., genus) according to Thomas (1997). Ten fields (or more for up to 200 cells) of view at 200× magnification were enumerated for each sample. Only cells >7-μm diameter were counted.

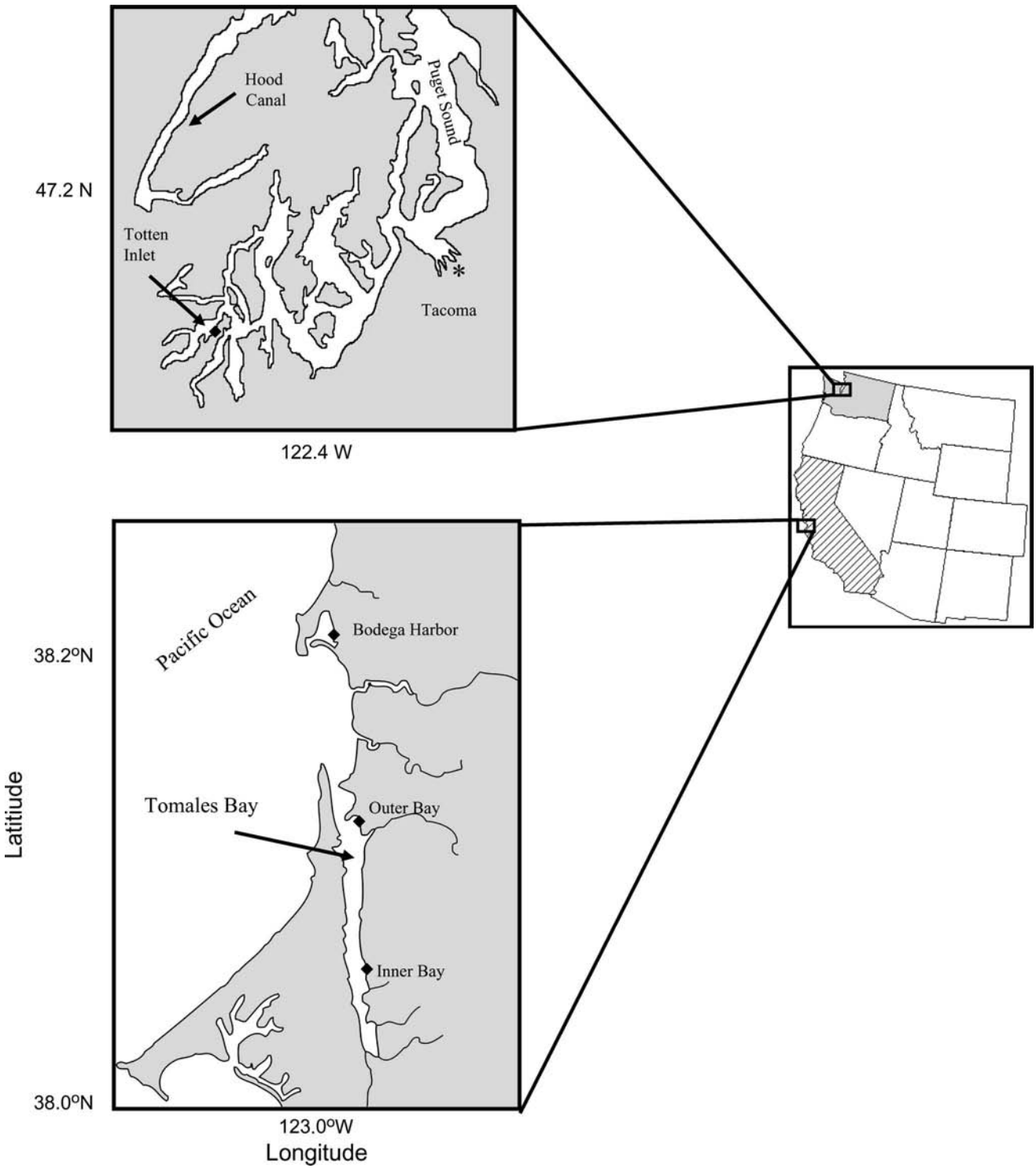

Figure 1. Map of field planting locations in California: Tomales Bay (Inner and Outer Bay sites), Bodega Harbor and Washington: Totten Inlet. Bodega Harbor and Totten Inlet are control sites where seed oyster mortality has not been documented.

Data Analysis

Differences among cumulative mortality (all sites) and final shell height (Bodega Harbor and Tomales Bay sites in 2001) were analyzed among stocks by a one-way analysis of variance and differences identified using a Tukey Test (Zar 1999). Finite mortality rates were calculated as the number of oysters that died from one mortality survey to the next and as total inferred mortalities divided by the number of oysters originally outplanted. Mortality data were arcsine transformed, which establishes the variance for percentage data (Zar 1999). Pearson moment correlation (r) was used to test for a linear relationship between growth rate and mortality (Sigma Stat 2.02).

Potential stressors, including daily mean temperature, temperature exposure, binned temperature (mean, maximum, and minimum), and phytoplankton assemblages were compared among sites or to mortality as appropriate. Daily mean temperatures at each site were found to be serially auto-correlated, with the same first-order serial correlation. To compare mean temperature at each site (April 25 to August 21, 2001) six Z-tests were calculated to compare the four means using an adjustment factor for first-order serial correlation (Ramsey & Schafer 2002) corrected with a Bonferroni P value. Hours of temperature over 16°C, 18°C, 20°C, 22°C, and 24°C for each two-week period were quantified from January to August in 2001, and data collected between April 25, 2001 to August 21, 2001 were compared between sites using a one-way ANOVA. Temperature mean, maxima, and minima were binned between mortality surveys, and the Spearman Rank Correlation (SRC) was used to test if a linear relationship existed between binned temperature and mortality (SPSS 11.5). Similarly, the SRC was used to test for a linear relationship between mortality and binned phytoplankton groupings defined as total mean cells and mean densities of dinoflagellates (including the potentially toxic *Akashiwo sanguinea*), diatoms and flagellates at Inner and Outer Tomales Bay sites and Bodega Harbor.

RESULTS

Sentinel Field Surveys: 2000 and 2001

Mortality, Growth, and Condition

Cumulative mortality was significantly different among sites ($P < 0.0001$, Table 1) and stocks ($P < 0.0001$, Fig. 2). In two of the three stocks examined (WA-1 and OR-2), mortality was significantly lower in the Fall outplants than the Spring outplants ($P < 0.0001$). Mortality was higher at the Inner Bay site (40% to 100%) than at the Outer Bay site (0–25%) or Bodega Harbor and Totten Inlet sites where losses were minimal (0% to 2%). A stock by site interaction was detected because of the site-specific mortality affecting stocks grown at the Inner Bay site. At the Inner Bay site, mortality was highest between May 23 and June 8; for the reminder of the experiment, mortality was low for all groups except for the Fall outplant of OR-2. At the Outer Bay site, instantaneous mortality was less than 6% per

TABLE 1.

Instantaneous[1] and cumulative (cum.)[2] mortalities of three stocks[3] of Pacific oysters in 2000–2001.

		Percent Mortality (%)					
Site[4]	Date	WA-1(F)[5]	WA-1(S)[5]	OR-1(F)[5]	OR-1(S)[5]	OR-2(F)[5]	OR-2(S)[5]
IB	5/23	0	0	0	0	0	0
	6/8[6]	34.6 ± 10.2	66.81 ± 11.64	36.80 ± 13.46	60.69 ± 8.00	32.84 ± 28.55	100
	6/26	6.67 ± 5.01	23.33 ± 11.84	13.00 ± 2.00	25.67 ± 15.14	18.00 ± 11.27	0
	7/24	1.50 ± 0.62	2.50 ± 4.42	2.00 ± 3.33	4.67 ± 4.73	39.98 ± 40.55	0
	8/21	0.20 ± 0.20	0.67 ± 1.21	3.00 ± 1.00	0	0	0
	9/17	0.33 ± 0.21	1.17 ± 0.98	0	6.67 ± 11.55	0.33 ± 0.33	0
	Cum.	40.03 ± 11.30	76.66 ± 6.42	47.45 ± 10.87	74.85 ± 1.75	56.34 ± 15.45	100
OB	5/24	0	0	0	0	0	0
	6/25	0	0	0	0	0	0
	7/23	0.50 ± 1.22	0.80 ± 2.00	0	0	0.01 ± 0.01	0
	8/6	0	5.30 ± 3.35	0	0	0.01 ± 0.01	12.50 ± 17.7
	8/22	0	5.50 ± 6.97	0	0	0	2.50 ± 3.50
	9/18	0	0.10 ± 0.40	0	0	0	9.65 ± 12.23
	Cum.	0.50 ± 1.22	11.34 ± 10.50	0	0	0.01 ± 0.01	24.88 ± 6.47
BH	5/29	0	0	0	0	0	0
	6/28	0	0	0	0	0	0.01 ± 0.01
	7/25	0	0	0	0	0	0
	9/18	0	0	0	0	0	0
	Cum.	0	0	0	0	0	0.01 ± 0.01
TI	7/31	NA[7]	NA[7]	NA[7]	0	NA[7]	0
	8/15	NA[7]	NA[7]	NA[7]	0.30 ± 0.45	NA[7]	0.50 ± 0.58
	8/30	NA[7]	NA[7]	NA[7]	0.20 ± 0.45	NA[7]	0.34 ± 0.58
	9/13	NA[7]	NA[7]	NA[7]	0	NA[7]	0.17 ± 0.29
	10.20	NA[7]	NA[7]	NA[7]	0	NA[7]	1.18 ± 1.27
	Cum.	NA[7]	NA[7]	NA[7]	0.50 ± 0.71	NA[7]	2.33 ± 2.02

[1] At each sample date, mortality is listed as instantaneous (+/– standard deviation) or the proportion of animals dead during this sample period.
[2] Cumulative mortality is the number of oysters that died through the whole experiment divided by the # of oysters per bag.
[3] Oysters stocks are as follows: WA-1, a hatchery stock from Georgia Strait, WA State; OR-1, a high performing family line from Yaquina Bay; Oregon; OR-2, a low performing family line from Yaquina Bay, OR.
[4] Sites are abbreviated as follows: Inner Tomales Bay, IB; Outer Tomales Bay, OB; Bodega Harbor, BH, and Totten Inlet, TI.
[5] F denotes fall and S denotes spring outplants.
[6] IB was monitored on both 6/5/2001 and 6/8/2001 and the column labeled 6/8/2001 is the average mortality for the two days.
[7] Only OR-1 and OR-2 were outplanted in the Spring in Totten Inlet.

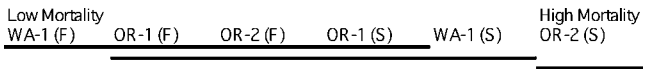

Figure 2. Statistical differences ($P < 0.0001$) in cumulative mortality (arcsine transformed) among the three stocks of Pacific oysters grown in Tomales Bay, CA in 2000–2001.

sampling except for the Spring outplant of OR-2, which experienced losses ranging from 9.7% to 12.5%.

Oyster shell heights are summarized in Table 2. Oysters planted in the Spring were significantly larger than those planted in the Fall at planting time ($P < 0.0001$). However, oysters that were over-wintered in the bay (Fall outplants) were significantly larger than their Spring cohorts when the first morphometric measurement was taken in late May ($P < 0.0001$). Oysters grown at the Inner Bay site grew significantly larger than oysters grown in the Outer Bay or Bodega Harbor sites ($P < 0.0001$). Outer Bay oysters were also larger than those grown in Bodega Harbor ($P < 0.0001$). Final shell heights differed among stocks and between outplant time at all sites ($P < 0.0001$, Fig. 3). Growth rate and cumulative mortality were significantly correlated at the Inner Bay site where losses were highest ($P < 0.05$, $r = 0.916$). Growth rate and cumulative mortality were not significantly correlated at the Outer Bay ($P > 0.05$) or Bodega Harbor ($P > 0.05$) sites.

Animals were examined using light microscopic examination of standard hemotoxylin and eosin tissues. Normal tissue architecture was noted in oysters examined before outplant, pre-mortality, and oysters grown at the Outer Bay site during and after mortality outbreaks (July 23). Oysters collected during the 5 of June 2001 mortality event ($n = 27$ survivors) at the Inner Bay site revealed morphological changes in the digestive gland and connective tissues. Microscopic changes in tissue architecture were observed in 13 of 27 surviving oysters and included diffuse to multifocal peritubular hemocyte infiltration, diapedesis and dilation of digestive tubules some of which contained cellular debris (Fig. 4). Nuclear changes, including hypertrophy, chromatin margination, and diffuse central eosinophilia were seen in several cells within connective tissue adjacent to digestive tubules (Fig. 4D).

Environmental Parameters

Differences in mean daily temperature exposure were detected among sites in 2001 (Fig. 5, $P < 0.05$). Mean temperature exposure was the same among sites in Tomales Bay ($P < 0.05$) and was similar between the Outer Bay site and Totten Inlet ($P > 0.05$) sites. Inner Bay oysters were exposed to higher mean temperatures than oysters reared at Totten Inlet ($P < 0.05$). Oysters grown at Bodega Harbor experienced lower mean temperatures than oysters grown at both Tomales Bay sites (Inner Bay: $P < 0.0001$, Outer Bay: $P < 0.01$) and Totten Inlet ($P = 0.00957$ uncorrected, $P = 0.0574$ Bonferroni corrected). The marginal P value for the comparison of mean temperatures between Bodega Harbor and Totten Inlet may be explained by the conservative nature of the Bonferroni method, and the highly significant nature of the original P value of 0.00957, indicating that oysters grown in Bodega Harbor experienced lower mean water temperatures than those grown at Totten Inlet.

Significant trends were observed when oyster losses were examined in relation to temperatures. Mortality surveys

TABLE 2.

Shell height (mean ± SD) of three stocks[1] of Pacific oysters in 2000–2001.

		Oyster Height					
Site[2]	**Date**	**WA-1(F)[3]**	**WA-1(S)[3]**	**OR-1(F)[3]**	**OR-1(S)[3]**	**OR-2(F)[3]**	**OR-2(S)[3]**
IB	Pre-plant[4]	9.5 ± 1.9	10.4 ± 1.8	4.7 ± 1.7	6.0 ± 2.6	6.4 ± 0.7	9.5 ± 1.2
	5/24	58.0 ± 14.2	18.8 ± 5.4	45.6 ± 8.6	15.7 ± 4.3	45.5 ± 20.4	19.8 ± 6.3
	7/24	59.2 ± 15.8	45.8 ± 10.4	66.6 ± 12.6	41.7 ± 7.8	64.3 ± 13.1	NA[5]
	9/17	75.0 ± 15.4	64.4 ±	12.7	56.7 ± 10.6	76.3 ± 12.9	NA[5]
	Total change[6]	65.6	54	70.6	50.7	69.9	NA[5]
OB	Pre-plant[4]	9.5 ± 1.9	10.4 ± 1.8	4.7 ± 1.7	6.0 ± 2.6	6.4 ± 0.7	14.4 ± 8.0
	5/25	41.1 ± 14.5	15.1 ± 4.4	41.4 ± 9.9	11.3 ± 3.9	37.8 ± 18.9	14.4 ± 8.0
	7/23	64.8 ± 13.7	41.3 ± 11.9	53.6 ± 12.1	29.6 ± 13.6	58.5 ± 12.5	36.9 ± 9.4
	9/18	80.7 ± 17.1	54.4 ± 11.3	67.7 ± 14.7	50.4 ± 14.6	70.8 ± 14.5	52.2 ± 14.2
	Total change[6]	71.3	43.9	63	44.4	64.4	42.6
BH	Pre-plant[4]	9.5 ± 1.9	10.4 ± 1.8	4.7 ± 1.7	6.0 ± 2.6	6.4 ± 0.7	9.5 ± 1.2
	5/29	40.7 ± 10.6	15 ± 2.7	22.1 ± 8.1	8.3 ± 3.7	29.6 ± 8.6	14.1 ± 5.0
	9/17	54.3 ± 16.6	36.2 ± 12.2	56.9 ± 16.0	26.5 ± 10.9	54.7 ± 11.3	38.2 ± 14.7
	Total change[6]	44.9	25.7	52.2	20.6	48.3	28.6
TI	Pre-plant[4]	NA	NA	NA	7.3 ± 2.0	NA	11.5 ± 4.1
	10/20	NA	NA	NA	60.8 ± 7.8	NA	47.2 ± 8.3
	Total change[6]	NA	NA	NA	53.6	NA	35.7

[1] Oysters stocks are as follows: WA-1, a hatchery stock from Georgia Strait, WA State; OR-1, a high performing family line from Yaquina Bay, OR; OR-2, a low performing family line from Yaquina Bay, OR.
[2] Sites are abbreviated as follows: Inner Tomales Bay, IB; Outer Tomales Bay, OB; Bodega Harbor, BH; and Totten Inlet, TI.
[3] F denotes fall and S denotes spring outplants.
[4] Oysters planted in fall were measured on 11/27/2000 and oysters planted in the spring were measured on 4/11/2001.
[5] 100% mortality was observed in the OR-2 on 6/8/2001.
[6] Total height change is expressed as the average final shell height minus the average initial shell height.

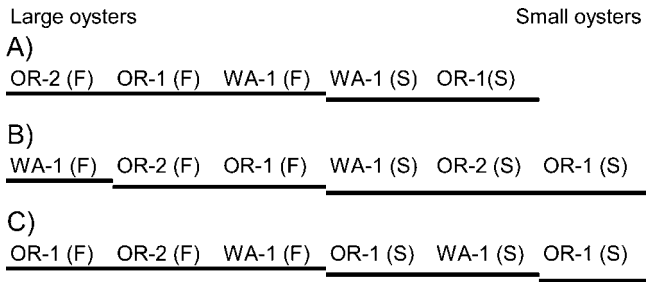

Figure 3. Statistical differences ($P < 0.0001$) among final shell height in the three stocks of Pacific oysters grown at (a) Inner Tomales Bay, (b) Outer Tomales Bay, and (c) Bodega Harbor in 2000–2001.

conducted on June 5 and 8 revealed high losses in all groups, particularly in Spring outplants at the Inner Bay site. In fact, maximum temperatures significantly correlated with mortality at the Inner Bay site only ($r = 0.949$, $P < 0.05$). The first temperature maximum over 24°C occurred 15 days before losses of up to 50% at the Inner Bay site (Fig. 6). Immediately prior to oyster losses, during the period of May 19 to June 8, all sites had a temperature maximum exceeding 24°C (Fig. 5), however oysters grown at the Inner Bay site experienced several temperature maximums of >24°C prior to losses (Fig. 6). No correlation was detected between high temperatures and mortality at the Outer Bay, Bodega Harbor, or Totten Inlet sites ($P > 0.05$). Mortality events were not significantly correlated with the average ($P > 0.05$), minimum ($P > 0.05$), or temperature ranges ($P > 0.05$) at any of the three sites. On a monthly basis, the Inner Bay experienced a greater number of hours of exposure than all other sites to temperatures in excess of 16°C, 18°C, 20°C, and 22°C, and Totten Inlet experienced the most hours greater than 24°C (Fig. 7). When comparing duration of temperatures in excess of 16°C, 18°C, 20°C, 22°C, and 24°C between sites, only temperatures in excess of 18°C ($P < 0.05$), 20°C ($P < 0.05$), and 22°C ($P < 0.05$) presented differences among sites (Fig. 8).

Diatoms dominated phytoplankton assemblages at the Tomales Bay sites and at the Bodega Harbor site in July (Fig. 9). In May, June, and August dinoflagellates and other flagellates dominated the composition of samples at the Bodega Harbor site. No correlation between phytoplankton groupings (total cells, mean dinoflagellates (including the potentially toxic *Akashiwo sanguinea*), mean diatoms, and mean flagellates) and mortality ($P > 0.05$ for all listed groupings) was found at either Tomales Bay site, or Bodega Harbor.

DISCUSSION

In our 2000–2001 field study, a commercially available oyster stock, WA-1, performed equally or better than other stocks including those from specific family lines used in this study (OR-1 and OR-2) indicating that selection of oyster stocks (or family lines) may help mitigate losses of Pacific oyster seed caused by SSM. Differences in oyster genetics (oyster stocks or family lines) have been shown to influence seed oyster survival (Degremont et al. 2005), and survival can have a strong genetic basis in areas of seed mortality (Degremont et al. 2007).

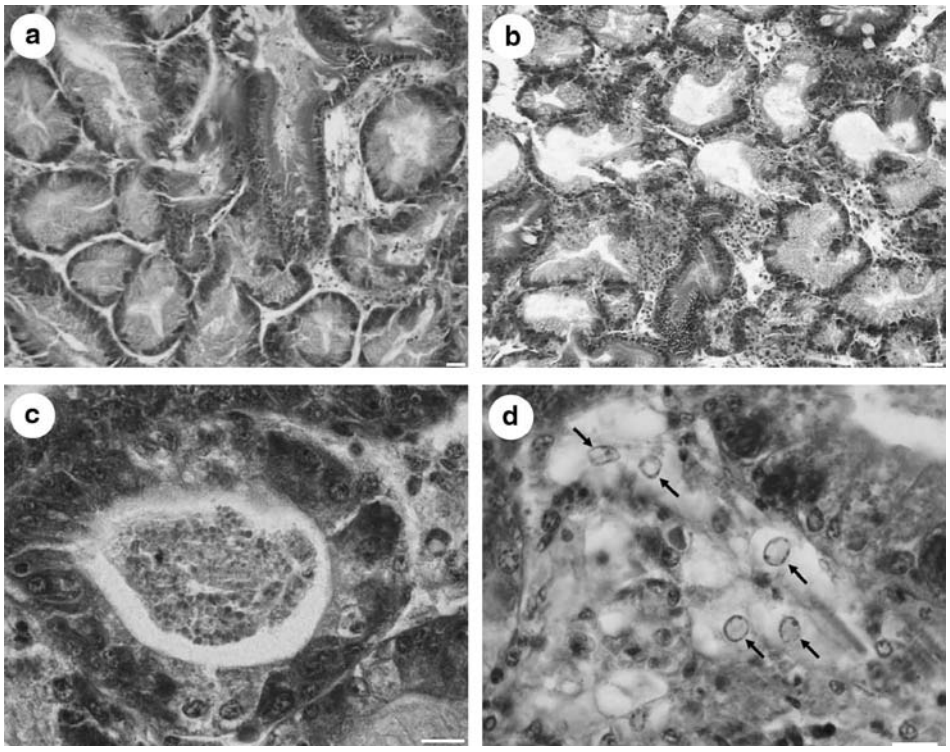

Figure 4. Normal and abnormal tissue and cellular architecture of oysters collected at the Inner Bay site during the June 5, 2001 mortality event (a) Normal digestive gland architecture, Scale bar = 20 μm, (b) Hemocyte infiltration (inflammation) surrounding dilated digestive tubules, Scale bar = 20 μm, (c) Dilated digestive tubule containing cellular debris, scale bar = 10 μm, and (d) Enlarged cell nuclei with chromatin margination suggestive of herpesvirus infection (arrows), scale bar = 10 μm. Hematoxylin and eosin.

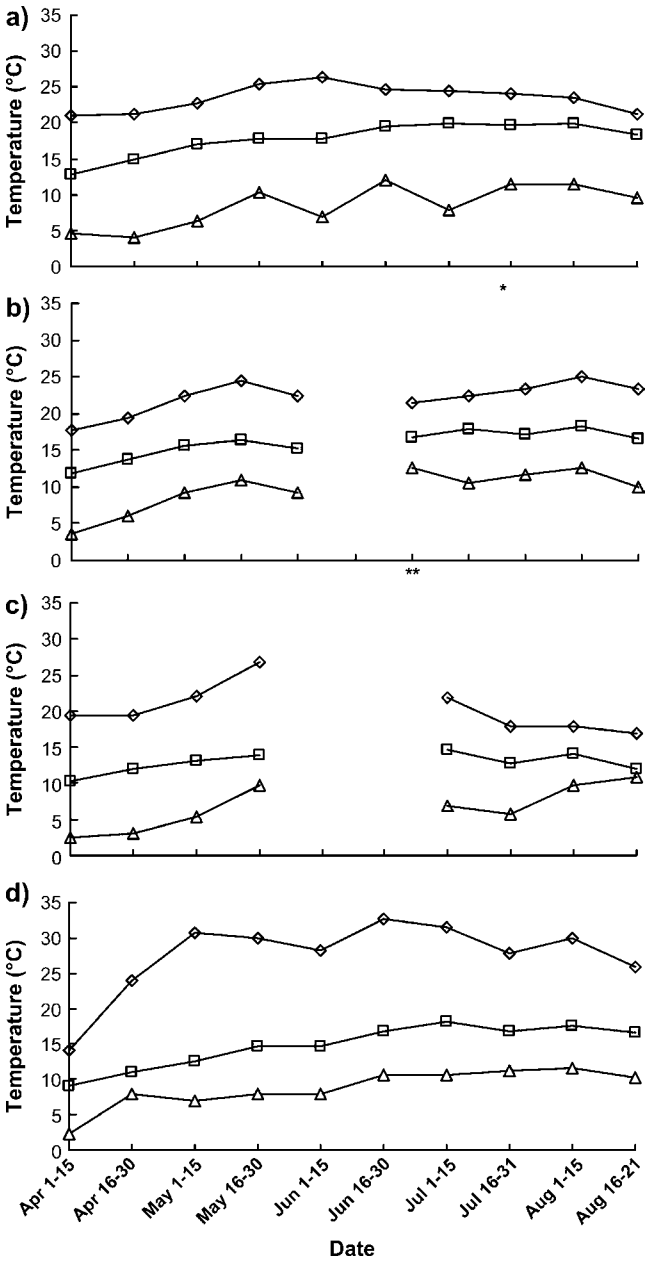

Figure 5. Bimonthly temperature maxima (◇), means (□), and minima (Δ) at (a) Inner Tomales Bay, (b) Outer Tomales Bay, (c) Bodega Harbor, and (d) Totten Inlet from April 1 to August 21, 2001. *July 24–31. **June 25–30.

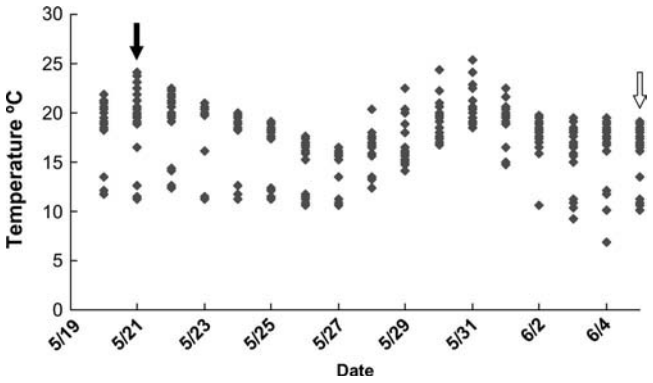

Figure 6. Daily Inner Tomales Bay temperatures between May 20, 2001 and June 5, 2001. On May 21, 2001 temperatures exceeded 24°C for the first time (dark arrow). On June 5, 2001, the first mortality of the summer was observed (light arrow).

Furthermore, the use-selected lines may enhance disease resistance in seed oysters (Calvo et al. 2003). Outplant time helped mitigate losses from SSM; our study demonstrated for the first time that oyster cohorts planted in the Fall outperformed cohorts planted the following Spring, the standard industry plant time. Thus losses may be mitigated or dampened by careful selection of planting time. Fall cohorts may have outperformed those planted in the Spring because (1) Fall cohorts had a longer time to acclimate to Tomales Bay conditions or (2) Fall cohorts were of a much larger size than Spring cohorts when mortality occurred. In support of the second lines of reasoning, five stocks of oysters were outplanted in Tomales Bay in Spring only, and higher survival was documented in stocks with larger individuals (Burge et al. 2006).

No correlation between phytoplankton blooms and mortality was observed in the 2000–2001 experiment reported herein. Phytoplankton blooms have been associated with SSM in Tomales Bay (Cherr & Friedman 1998) and were also related to mortalities in adult oysters from a 1998 field study of Cheney et al. (2000). These data suggest that phytoplankton blooms are not consistently related to oyster losses in Tomales Bay and that other stressors such as elevated water temperature or a pathogen (an oyster herpesvirus) are involved.

Temperature exposure and maxima (~25°C) have been repeatedly associated with SSM in Tomales Bay (Cherr & Friedman 1998, Burge et al. 2006) and were associated with losses in this study. Burge et al. (2006) examined the relationship between temperature and mortalities at the Inner Tomales Bay site considering three years (2001, 2002, and 2003), and the only temperature consistently related to mortalities were total exposure and degree hours per day in excess of 24°C and 25°C. These yearly maximum temperatures are not considered to be extreme for Pacific oyster survival (Cheney et al. 2000). In fact, oysters can exhibit phenotypic plasticity where chronic exposure to sublethal temperature stress can protect oysters even at otherwise lethal temperatures of 41°C to 46°C (Clegg et al. 1998, Hamdoun et al. 2003) far in excess of those observed in this study. In addition, immune function (hemocyte mortality, phagocytosis, esterase, and aminopeptidase activities) in adult Pacific oysters appears to be unaffected at 25°C based on *in vivo* and *in vitro* exposure of hemocytes for 2 and 4 h, respectively (Gagnaire et al. 2006), suggesting that seed oyster immunity should not be compromised at 25°C. Therefore, temperature is unlikely to be the lone stressor involved in SSM in Tomales Bay, CA.

Histological examination of oysters sampled during the Inner Bay mortality event suggests herpesvirus infection based on nuclear changes, including cells with hypertrophy and chromatin margination. Histological micrographs similar to those provided herein have been documented in herpesvirus infected seed oysters in France (Renault et al. 1995) and Tomales Bay (Burge et al. 2006). In the present study, extreme losses were documented when temperatures exceeded 24°C to 25°C levels, which are believed to lead to viral expression and mortality in OsHV-1 infected larvae (Le Deuff et al. 1996) and

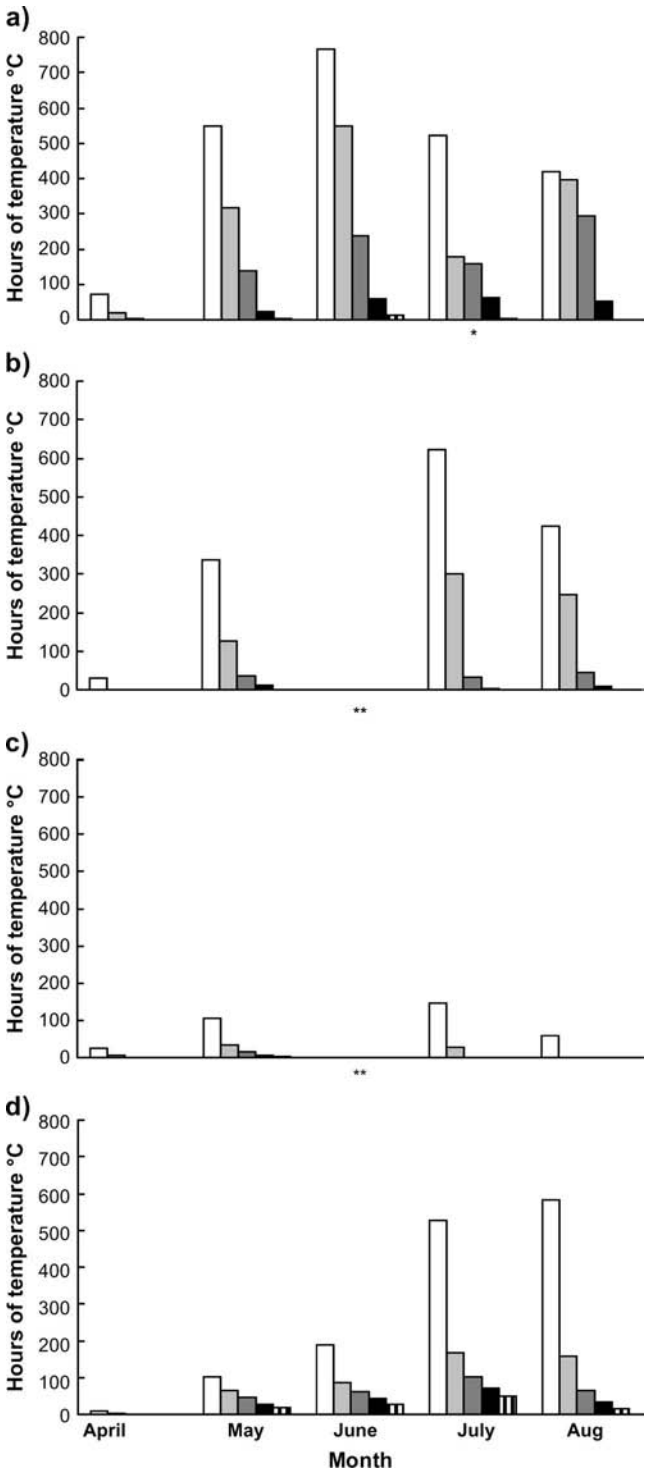

Figure 7. Hours exposed to temperatures in excess of 16°C (white bar), 18°C (light gray bar), 20°C (gray bar), 22°C (black bar), and 24°C (striped bar) at the (a) Inner Tomales Bay, (b) Outer Tomales Bay, (c) Bodega Harbor, and (d) Totten Inlet from April 1 to August 21, 2001. *Data from July 10 through 27 are not available for the Inner Bay site. **June data are not available for Bodega Harbor and the Outer Bay sites.

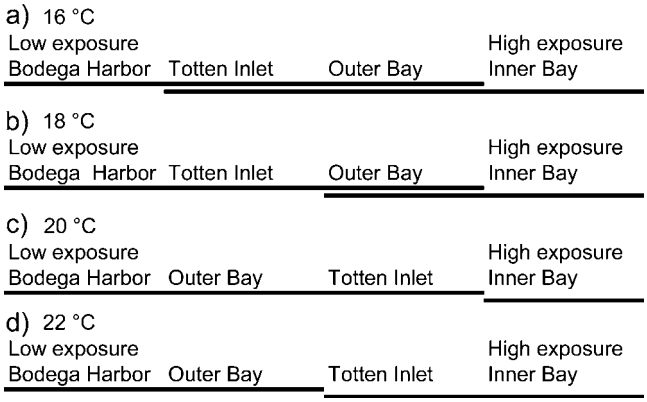

Figure 8. Statistical differences ($P < 0.05$) in temperature exposure at temperatures greater than (a) 16°C, (b) 18°C, (c) 20°C, and (d) 22°C experienced by Pacific oysters grown at Bodega Harbor, Totten Inlet, Outer Tomales Bay, and Inner Tomales Bay in 2001.

seed (Burge et al. 2006). Therefore, correlation of high temperature with SSM is likely caused by expression of OsHV infections (Burge et al. 2006).

Not all oysters collected during the Inner Bay mortality event exhibited histological changes, and nuclear changes suggestive of herpesvirus infection were rare. At the Inner Bay site, 13 of 27 oysters collected during mortality events exhibited typical changes in tissue and cellular architecture including: dilated digestive gland tubules, pertibular to multifocal hemocyte infiltration, and cells undergoing diapedesis across digestive gland tubules. Although nuclear changes characteristic of herpesvirus infection have been reliably documented in larval oysters (Hine et al. 1992, Renault et al. 1994a); observations of nuclear changes in seed infected by oyster herpesviruses are rare (Friedman et al. 2005, Burge et al. 2006). Non-moribun oysters collected postmortality may be survivors of OsHV infections,

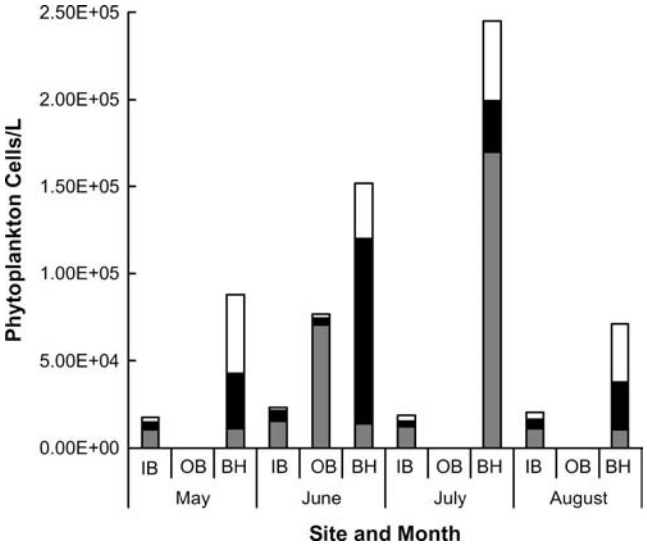

Figure 9. Tomales Bay phytoplankton seasonal trends including diatoms (gray bars), dinoflagellates (black bars), and other flagellates (white bars). Note that although losses began at the Inner Bay site in early June 2001, no phytoplankton bloom was recorded.

and may not have active, productive herpesvirus infections; instead they may have low level productive yet asymptomatic, or latent herpesvirus infections where visible damage to host cells may not occur (Barbosa-Solomieu et al. 2004) as observed in many oyster samples collected after the early June mortality event in the present study. Thus oysters with inactive infections (survivors) may not exhibit histological changes suggestive of herpesviruses. Therefore, the use of multiple diagnostic tests (i.e., PCR, *in situ* hybridization, and histology) are important in diagnosing ''unknown'' oyster seed mortalities, particularly if OsHV is a possible cause (Lipart & Renault 2002). Additionally, OsHV-1 detection using the PCR is significantly improved if oyster samples contain moribund oysters or are collected less than one week from the beginning of the mortality (Celine Garcia, IFREMER, pers. comm.).

In conclusion, mortalities and elevated temperatures have been associated with oyster losses since 1993 and this association was clearly demonstrated between 2001 to 2003 in Tomales Bay. In the later three years, oyster herpesviral infection has been suspected as playing a role in SSM of oysters grown in Tomales Bay based on histology (2001, the present study) and molecular data from 2002 (Friedman et al. 2005) and 2003 (Burge et al. 2006). Given that SSM has been associated with OsHV in France beginning in 1993 (Renault et al. 1994a, 1994b), it is hypothesized that OsHV was involved in SSM in Tomales Bay as early as 1993 when losses were first observed (Cherr & Friedman 1998). A retrospective analysis of archived tissues for evidence of OsHV infection would test this hypothesis. Our observation of an association between presence of an oyster herpesvirus and mortality (Burge et al. 2006) suggests that further studies on the influence of OsHV on Pacific oyster seed survival are needed.

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Par ailleurs, des examens menés sur des huîtres creuses, *C. gigas*, adultes en France dans le cadre de travaux de recherche ont permis de détecter la présence d'ADN et de protéines du virus OsHV-1 chez environ 80% des animaux analysés en absence de signes cliniques et de mortalité (**Arzul *et al.*, 2002**). L'ensemble de ces données suggère que le virus OsHV-1 est présent avec une forte prévalence dans les populations d'huîtres creuses en France. Comme les autres herpès virus, le virus infectant l'huître creuse, *C. gigas,* semble capable de persister chez son hôte. Les huîtres adultes pourraient jouer un rôle de porteurs sains et de réservoirs favorisant la transmission du virus (**Arzul *et al.*, 2002**).

Sur le base de l'utilisation d'outils spécifiques de détection moléculaire (PCR et HIS), il a été montré chez l'huître creuse que le statut infectieux des géniteurs pouvait avoir une influence sur la survie de la descendance au stade larvaire (**Barbosa Solomieu *et al.*, 2004**). En particulier, les croisements impliquant un mâle porteur du virus et une femelle indemne donnent une descendance présentant des taux de survie larvaire faibles (**Barbosa Solomieu *et al.*, 2004**). Les résultats obtenus laissent suspecter que les huîtres femelles pourraient transmettre à leur descendance une sorte de protection vis à vis de l'infection virale.

Le virus OsHV-1 a été détecté en France lors d'épisodes de mortalité anormale chez différentes espèces de bivalves. Des essais en laboratoire de transmission interspécifique de l'infection virale ainsi que des travaux basés sur l'identification moléculaire des agents viraux responsables de mortalités ont permis de montrer avec certitude qu'un même et seul virus, le virus OsHV-1, était capable d'infecter l'huître creuse, la palourde japonaise et la coquille Saint Jacques (**Arzul *et al.*, 2001a** ; **Renault *et al.*, 2001**). Le virus OsHV-1 montre ainsi un large spectre d'hôtes, ce qui n'est pas une caractéristique commune aux virus de la famille des *Herpesviridae*. Cependant, les espèces affectées par OsHV-1 sont exclusivement des mollusques bivalves.

Les outils de diagnostic spécifiques du virus OsHV-1 ont été utilisés pour démontrer la présence de ce virus chez l'huître creuse, *C. gigas*, aux USA et d'associer sa détection à des phénomènes de mortalités anormales. Les outils de diagnostic moléculaires sont aujourd'hui d'une aide précieuse pour traquer les virus infectant les coquillages. Ils sont également d'une grande utilité pour réaliser des analyses rétrospectives sur du matériel biologique archivé.

Cependant, la **difficulté de disposer d'échantillons adaptés** persiste en pathologie des coquillages. En effet, les coquillages sont le plus souvent élevés sur l'estran et ne sont accessibles qu'à certaines périodes. Dans ces conditions, un suivi journalier des animaux est impossible et **disposer de coquillages en cours de mortalité peut s'avérer une gageure**.

3. 3. 3. Virus infectant les ormeaux et outils de diagnostic spécifiques d'OsHV-1

Les outils développés pour le diagnostic moléculaire du virus OsHV-1 (PCR et HIS) ont été testés sur des échantillons d'ormeaux infectés par un virus de type herpès à Taiwan (Chang *et al.*, 2005). Par ailleurs, dans le cadre de l'accueil du Dr Chang (Department of Veterinary Medicine, College of Bio-Resources and Agriculture, Université de Taipei, Taiwan) au sein du laboratoire LGP (Ifremer, La Tremblade, France) en 2006, d'autres essais ont été réalisés avec 15 couples d'amorces différents ciblant cinq régions du génome du virus OsHV-1. L'ensemble des tests n'a pas permis d'obtenir d'amplification spécifique. Des amplicons ont été observés pour certaines amorces, mais le clonage et le séquençage de ces produits de PCR ont permis de monter qu'il s'agissait d'amplifications non spécifiques. Des résultats comparables ont été obtenus pour des échantillons d'ormeaux infectés australiens (Dr S. Corbeil, Australian Animal Health Laboratory, CSIRO, Geelong, Victoria, Australie, communication personnelle).

Pour les analyses en HIS dans de cadre de l'accueil du Dr Chang au sein du laboratoire (LGP, Ifremer, La Tremblade, France) en 2006, les résultats sont beaucoup plus prometteurs. En effet, **des marquages** ont été observés dans différents tissus, **ganglions nerveux** (Figure 12) et **tissus conjonctifs de la glande digestive** (Figure 13) avec une sonde spécifique du virus OsHV-1. Des analyses en HIS ont également été réalisées sur coupes d'ormeaux infectés provenant d'Australie et ont permis d'observer des marquages similaires dans les tissus nerveux et les tissus conjonctifs, autour de la zone buccale en particulier.

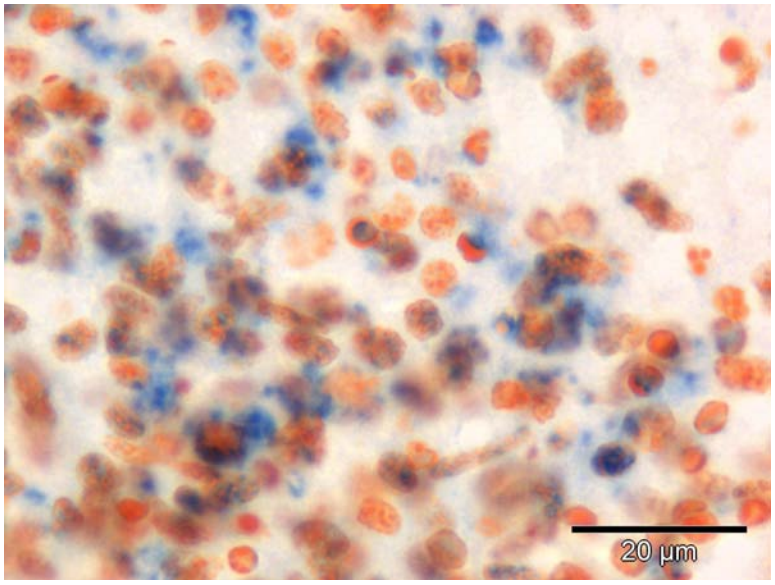

Figure 12. *HIS réalisée avec une sonde spécifique du virus OsHV-1. Marquage (coloration bleue) obtenu sur coupe d'ormeau infecté par un virus de type herpès provenant de Taiwan (ganglion nerveux)*

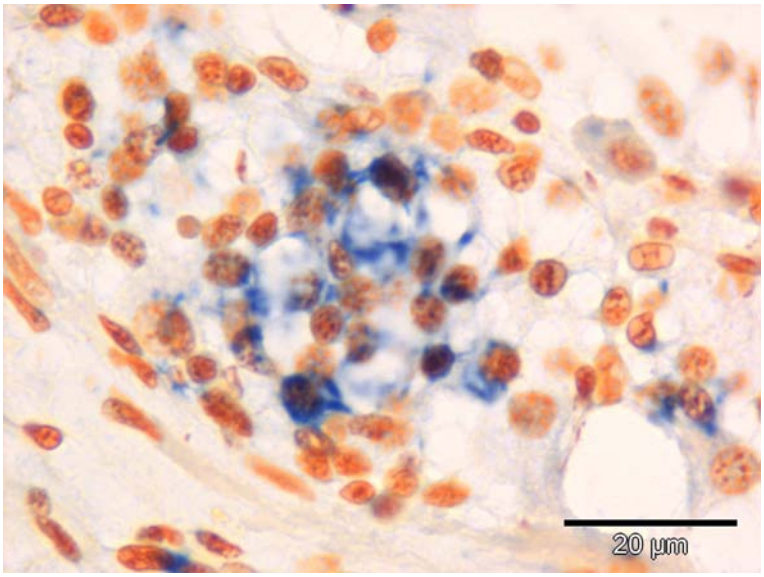

Figure 13. *HIS réalisée avec une sonde spécifique du virus OsHV-1. Marquage (coloration bleue) obtenu sur coupe d'ormeau infecté par un virus de type herpès provenant de Taiwan (tissu conjonctif de la glande digestive)*

Ces résultats tendent à montrer que le ou les virus infectant les ormeaux à Taiwan et en Australie n'est ou ne sont pas le virus OsHV-1, mais qu'il(s) partage(nt) cependant des séquences avec le virus infectant les bivalves (OsHV-1). L'utilisation en HIS d'une sonde marquée de plus de 700 pb peut expliquer les résultats obtenus. Cette sonde peut en effet s'hybrider de manière incomplète, mais suffisante pour induire un signal sur coupes histologiques. Il apparaît ainsi que dans l'attente du développement d'outils spécifiques du/des virus infectant les ormeaux dans la zone Pacifique, la technique d'HIS basée sur l'utilisation de sondes dirigées contre OsHV-1 peut être une approche de choix pour confirmer une suspicion posée en histologie chez ces gastéropodes marins. Par ailleurs, les résultats obtenus en PCR sur les échantillons d'ormeaux confirment la spécificité des amorces dessinées pour diagnostiquer OsHV-1. C'est une confirmation *a posteriori* des choix faits en terme de sélection des régions pour le dessin des amorces spécifique d'OsHV-1.

Le virus OsHV-1 n'apparaît donc pas comme l'unique représentant des herpès virus infectant les invertébrés. Cela n'est pas inattendu dans la mesure où cette famille de virus est parmi celle qui compte le plus de membres.

Il apparaît aussi d'intérêt pour le LGP (Ifremer, La Tremblade, France) de développer un modèle de maladie expérimentale utilisant l'espèce d'ormeau présente en France, *Haliotis tuberculata*. Au vu des récents résultats obtenus à Taiwan et en Australie, les ormeaux apparaissent comme particulièrement sensibles aux infections à virus de type herpès : très fortes mortalités et rapidité d'évolution de la maladie. Il n'a pas été possible à ce jour de développer au LGP un protocole reproductible d'infection expérimentale avec le virus OsHV-1 chez les huîtres juvéniles ou adultes. L'infection peut par contre être reproduite au stade larvaire sans difficulté (**Le Deuff *et al.*, 1994**).

Des mortalités ont été observées ces dernières années en France chez l'ormeau *H. tuberculata* et n'ont pas été systématiquement expliquées. Dans ce contexte, une recherche de virus de type herpès semble nécessaire à mettre en œuvre.

3. 4. Un sosie chez un protiste : une énigme à résoudre

Des observations en microscopie électronique à transmission, réalisées au Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France) (**Renault *et al.*, 2003**), ont permis de détecter de manière fortuite des **particules virales de type herpès au sein d'un organisme unicellulaire** (Figures 14, 15 et 16), présent dans des élevages larvaires d'huître creuse, *C. gigas*. Cet organisme a été interprété comme étant un **champignon** sur la base de ses caractéristiques structurales. La détection préalable du virus OsHV-1 par PCR dans ces échantillons de larves d'huîtres s'était révélée positive. Cependant, aucune caractérisation moléculaire de l'organisme unicellulaire infecté n'a pu être réalisée à ce jour.

Des capsides et des nucléocapsides sont ainsi observées dans le noyau de cet organisme unicellulaire (Figure 14). Elles présentent une symétrie icosaédrique et un diamètre d'environ 70 nm. Des particules enveloppées sont retrouvées dans le cytoplasme (Figure 15) et semblent également s'accumuler dans l'espace présent entre la membrane cytoplasmique et une structure lamellaire plus externe (paroi) (Figures 15 et 16).

Figure 14. *Organisme unicellulaire (champignon) observé dans un élevage de larves d'huître creuse, C. gigas, et montrant des capsides et des nucléocapsides dans le noyau ; barre=100 nm*

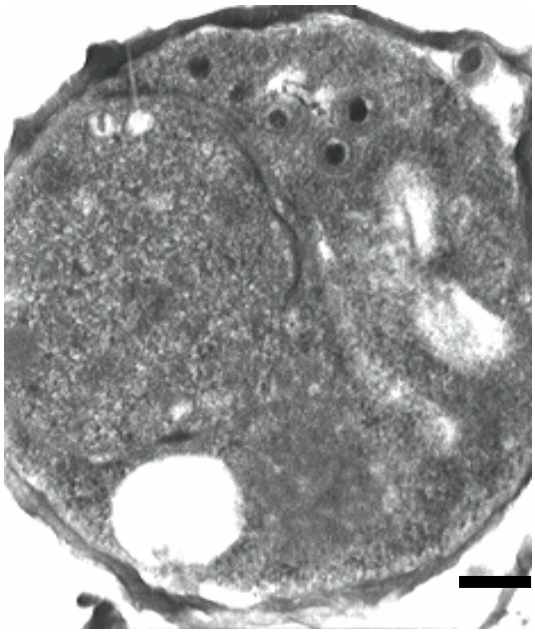

Figure 15. *Organisme unicellulaire (champignon) observé dans un élevage de larves d'huître creuse, C. gigas, et montrant des capsides et des nucléocapsides dans le noyau ainsi que des particules enveloppées dans le cytoplsame ; barre=200 nm*

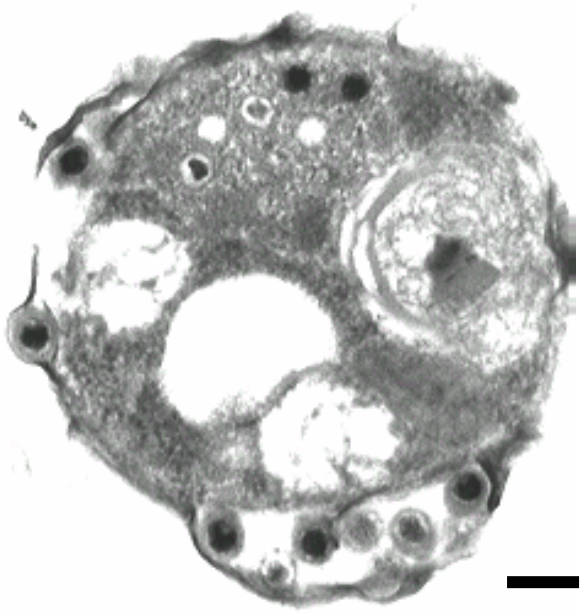

Figure 16. *Organisme unicellulaire (champignon) observé dans un élevage de larves d'huître creuse, C. gigas, et montrant des capsides et des nucléocapsides dans le noyau ainsi que des particules enveloppées dans l'espace entre la membrane cytoplsamique et la paroi ; barre=200 nm*

Par ailleurs, il est important de noter que cet **organisme unicellulaire** a été détecté soit sous **forme libre**, soit **dans les larves d'huîtres en position intra-cellulaire dans des vacuoles** (Figure 17) Dans ce cas, des particule virales sont observées à l'extérieur du champignon, dans la lumirère des vacuoles (Figure 18). Il semble qu'elles aient été libérées après dégradation de la paroi des organismes unicellulaires. Il est possible du fait de ces observations de suspecter un phénomène de phagocytose qui aboutirait à la dégradation de la paroi du champignon et à la libération de particules virales piégées entre la membrane cytoplasmique et la paroi de cet organisme unicellulaire.

Les observations en microscopie électronique à transmission réalisées sur ces échantillons d'huîtres n'ont pas permis de détecter de particules virales de type herpès à l'intérieur des noyaux des cellules larvaires alors que les analyses en PCR avec des amorces spécifiques d'OsHV-1 étaient positives. Ce type d'observation aurait été un élément intéressant pour étayer l'hypothèse d'une possible transmission du virus aux animaux via un organisme unicellulaire. La question demeure sans réponse à ce jour.

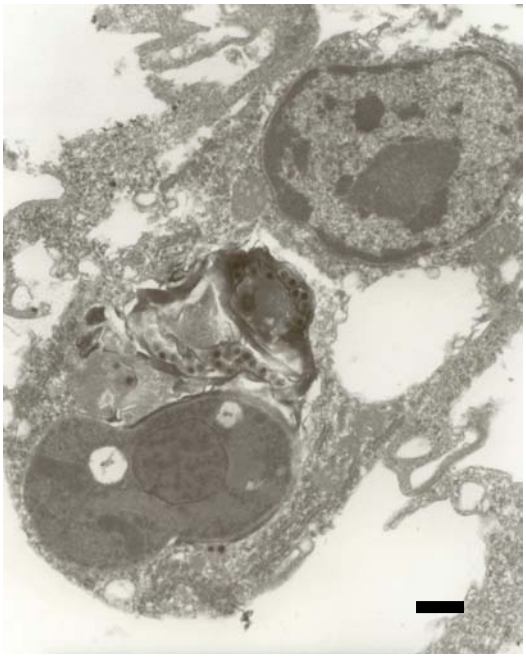

Figure 17. *Organisme unicellulaire (champignon) observé dans le cytoplasme d'une cellule de larve d'huître creuse, C. gigas ; barre=500 nm*

Ces résultats, confrontés à la littérature scientifique soulèvent de nombreuses interrogations :

- OsHV-1 est-il spécifique des mollusques?
- Existe-t-il d'autres virus de type herpès non encore décrits qui peuvent infecter les organismes marins autres que les mollusques?
- OsHV-1 est-il capable d'infecter des champignons dans le milieu marin?

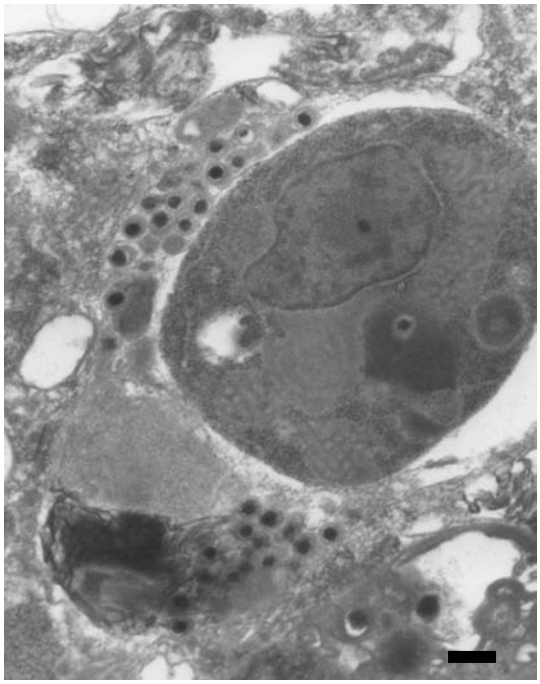

Figure 18. *Présence de particules virales dans le cytoplasme d'une cellule de larve d'huître creuse, Crassostrea gigas, au sein d'une vacuole; barre=200 nm*

Des particules virales de type herpès ont été précédemment rapportées chez différentes espèces de champignon (Kazama & Schornstein, 1972 ; 1973 ; Styer & Corbett, 1978). Des particules ont été observées chez le **champignon, *Phytophtora parasitica var. parasitica***, parasite d'une plante (Styer & Corbett, 1978).

En 1972, une culture d'un **champignon isolé de l'estuaire York River (Virginie, Etats-Unis) et identifié comme *Thraustochytrium sp***., a été décrite infectée par un **virus enveloppé** sur la base d'observations réalisées en microscopie électronique à transmission (Kazama & Schornstein, 1972 ; 1973). Les nucléocapsides détectées présentent un diamètre de 110 nm et les particules virales extracellulaires mesurent 280 nm. La présence d'une enveloppe, la symétrie icosaédrique et la taille des capsides, le matériel génétique de type ADN et la localisation des virions (présence de capsides dans le noyau et de particules enveloppées dans le cytoplasme et en position extracellulaire) ont permis de **rapprocher le virus de la famille des *Herpesviridae*** (Kazama & Schornstein, 1972). Jusqu'à alors, aucune description de virus à ADN enveloppé n'avait été rapportée chez un champignon. Les cellules fungiques infectées par le virus semblent ne contenir que peu d'organites par rapport aux cellules non infectées (Kazama & Schornstein, 1973). Seules les champignons mis en culture depuis plus de cinq heures en milieu pauvre contiennent des particules virales (Kazama & Schornstein, 1972). Le virus n'est par ailleurs détecté que dans une proportion des cellules (16 %). Aucune particule virale n'a été observée dans les champignon récemment mis en culture ni dans le champignon à un stade de développement plus avancé (Kazama & Schornstein, 1972). Des conditions favorables au développement du champignon ne permettent pas au virus de s'exprimer, alors que des conditions stressantes favorisent l'expression de ce dernier. Ainsi, un milieu de culture riche en nutriments ne permet pas d'observer de particules virales au sein de la culture (Kazama, 1973). Un milieu pauvre telle que de l'eau de mer auto clavée permet d'obtenir 60% à 70 % de cellules infectées (Kazama, 1973).

Il a été possible de prendre contact avec le Dr Kazama, bien qu'il soit aujourd'hui à la retraite. L'objectif était de pouvoir disposer de cultures de *Thraustochytrium sp.* infectées par le virus de type herpès afin de tenter de le purifier et de le caractériser d'un point de vue moléculaire. Malheureusement, aucun isolat et aucune culture n'ayant été conservé, cette approche n'a pas pu être menée à bien.

Des **particules virales de type herpès** ont également été observées par Perkins, au sein d'une autre espèce de **champignon marin, *Schizochytrium aggregatum*** (Kazama, 1972). *Thraustochytrium sp.* et *S. aggregatum* appartiennent à la même famille des *Traustochytriaceae* de l'ordre des Saprolegniales (Kazama, 1972 ; Kazama, 1973 ; Perkins, 1974). Les thraustochytrides présentent des similitudes importantes avec les labyrinthulides, mais font partie de deux familles distinctes, respectivement la famille *Thraustochytriaceae* et la famille *Labyrinthulaceae* (Honda *et al.*, 1999). Ce sont des organismes d'apparence fongique non-chlorophylliens. Tous appartiennent au phylum des Eumycètes, plus précisément des Labyrinthulomycota et à la classe des Oomycètes (Porter, 1990). Ils sont retrouvés dans **les eaux marines et les estuaires**, où ils vivent sur les débris organiques, les algues et les plantes marines.

En 1987, Bower a décrit une infection due à un parasite chez des ormeaux appartenant à l'espèce *Haliotis kamtschatkana,* au stade juvénile (Bower, 1987). En 1989, au Canada, un parasite nommé QPX pour Quahaug Parasite X a été associé à d'importantes mortalités chez le clam, *Mercenaria mercenaria,* aux stades juvénile et adulte (Whyte *et al*., 1994). Les symptômes de la maladie ainsi que la morphologie du parasite ont permis de rapprocher l'agent QPX des thraustochytrides et des labyrinthulides (Whyte *et al*., 1994). D'autres infections dues à un parasite semblable à QPX, engendrant des mortalités chez des clams originaires de diverses écloseries et à différents stades de développement, ont été rapportées dans plusieurs sites aux Etats-Unis (Smolowitz *et al*., 1998). Il semble que ce soit un seul et même parasite qui soit responsable de ces différents épisodes de mortalité ou des espèces proches (Stokes *et al*., 2002). Suite à des analyses moléculaires, ce ou ces **parasites** ont été intégrés au phylum des **Labyrinthulomycota** (Stokes *et al*., 2002). Cependant, l'espèce n'a pu être identifiée avec certitude du fait du manque de données de séquence (Stokes *et al*., 2002). Le fait que ce parasite soit intégré au phylum des Labyrinthulomycota et soit un agent pathogène pour les mollusques apporte matière à questionnement. Les champignons unicellulaires peuvent faire partie de l'alimentation des mollusques. Est-il possible que **les virus de type herpès infectant des champignons aient pu évoluer de telle manière à franchir la barrière d'espèce au contact des mollusques bivalves**? S'agit-il de virus distincts appartenant simplement à une même famille?

D'autre part, des **particules virales d'allure herpétique** ont également été observées dans des cultures de l'**algue unicellulaire *Ostreococcus tauri*** (Moreau, Laboratoire Arago, Observatoire Océanologique, Banyuls sur Mer, France, communication personnelle). Certaines cultures analysées se sont avérées être infectées à un faible pourcentage. Cette algue fait également partie de l'alimentation de l'huître creuse, *C. gigas* (Dupuy *et al*., 2000). Son abondance parmi l'ensemble du phytoplancton est maximale en été (Dupuy *et al*., 2000). Est-il possible qu'il y ait un **lien avec les épisodes de mortalité dus à OsHV-1** puisqu'ils ont principalement lieu en période estivale?

L'existence de virus de type herpès infectant des organismes unicellulaires marins tels que des champignons ou des algues constitue aujourd'hui une question d'intérêt. La recherche de ces virus et de leur mode de transmission pourrait contribuer à la compréhension de l'adaptation des virus à leurs hôtes et éventuellement du franchissement des barrières d'espèce.

Récemment, un birnavirus (marine birnavirus, MABV) infectant les coquillages et les poissons a été détecté dans le zooplancton (Kitamura *et al*., 2002 ; Kitamura *et al*., 2003). C'est la première fois que le génome viral d'un birnavirus marin est détecté dans diverses espèces identifiées de zooplancton. Toutefois, cette détection n'a pu être associée à un phénomène d'infection en particulier ou à une accumulation du MABV dans le zooplancton (Kitamura *et al*., 2003). La détection du matériel génétique de MABV est positive aussi bien à partir d'échantillons d'eau (filtrés à 0,22 µm) sur toute la colonne d'eau (2-45 m) (Kitamura *et al*., 2002) qu'à partir d'échantillons de zooplancton (Kitamura *et al.*, 2003). Le **zooplancton** serait ainsi un **vecteur du MABV** de par son **statut de proie pour les juvéniles de poissons et de coquillages** de la zone étudiée (Kitamura *et al*., 2003). Ainsi, l'hypothèse qu'un organisme unicellulaire marin puisse constituer un vecteur d'un virus n'est pas nouvelle. Cependant, aucun vecteur ou hôte réservoir n'a jamais été rapporté au sein de la famille des *Herpesviridae*.

Aucune lignée cellulaire de bivalve n'est actuellement disponible afin de permettre la réplication du virus OsHV-1. Les herpès virus infectant les coquillages ne se répliquent pas sur cellules d'insectes, de poissons et de mammifères, ni en primo cultures de cellules d'huître (**Le Deuff *et al.*, 1994b** ; **Renault *et al.*, 1995**). Le fait de détecter des **virus de type herpès en réplication au sein d'organismes marins unicellulaires** laisse entrevoir des possibilités de disposer d'un **système de production de particules virales**. Bien qu'il soit possible que les virus de type herpès infectant les champignons soient différents d'OsHV-1, est-il envisageable que ce dernier puisse infecter ces organismes unicellulaires et s'y répliquer ? Des **essais d'infection expérimentale de champignons marins unicellulaires par OsHV-1** pourraient être envisagés.

Les herpès virus infectant les coquillages apparaissent comme des virus « anciens » ayant évolués avec leurs hôtes. Cependant, les nombreuses et récentes descriptions de ce type de virus pourraient bien être liées au développement de la conchyliculture et de nouvelles techniques (développement des écloseries/nurseries dans lesquelles les densités sont élévées, les espèces peuvent être mélangées et les conditions d'élévage peuvent être très différentes de celles rencontrées dans le milieu extérieur) ainsi qu'à des modifications de l'environnement (pollution d'origine anthropique, réchauffement climatique).

4. Facteurs de risque d'apparition ou d'émergence de maladies infectieuses: Relations hôtes, agent pathogènes et environnement

4. 1. Immunité innée ou comment se défendre vis à vis des bio-agresseurs quand on est un coquillage

La compréhension des mécanismes de défense développés par les mollusques est une approche fondamentale pour la pérennité de la conchyliculture. La disparition de l'huître portugaise, *Crassostrea angulata*, du littoral français a bouleversé l'ostréiculture à la fin des années 60. Cette dernière a du et a pu s'adapter en substituant *C. angulata* par l'huître creuse japonaise, *C. gigas*, espèce peu ou pas sensible aux infections impliquant des virus de type iridovirus. Une nouvelle épizootie de ce type aurait de graves conséquences économiques et sociales pour le monde ostréicole. Il apparaît dans ce contexte important de mieux comprendre les mécanismes de défense développés par les coquillages.

Les mécanismes de défense développés par les bivalves reposent sur une réponse innée non adaptative sans phénomène de mémoire immunitaire (absence de lymphocytes et pas de production d'anticorps). Les effecteurs de l'immunité chez les mollusques sont principalement les hémocytes présents dans l'hémolymphe et divers effecteurs humoraux. Les hémocytes sont capables de phagocytose et de diapédèse. Ils synthétisent également des molécules immunoactives telles que les lectines ou le lysozyme.

4. 1. 1. Les hémocytes (Xue Q. & T. Renault**,** 2001, Developmental and Comparative Immunology, 25: 187-194.)

Les mollusques bivalves possèdent un système circulatoire de type semi-ouvert, comprenant un cœur et des vaisseaux, où circule l'hémolymphe (Cheng, 1981). L'hémolymphe assure la distribution des hémocytes à travers tout l'organisme. Ces cellules sont présentes dans la circulation (vaisseaux et sinus hemolymphatiques), mais aussi dans les tissus et organes.

Divers types hémocytaires ont pu être observés et différentes classifications ont été proposées. Chez l'huître creuse, *C. gigas*, de deux à quatre types cellulaires ont pu être distingués (Tableau VIII). Cheng (1981) a proposé un schéma intégrant des granulocytes, des hyalinocytes et des cellules séreuses comme les trois types cellulaires principaux. Tous les sous-types hémocytaires observés et pouvant être assignés à une de ces trois catégories correspondraient à différents stades de développement d'une même lignée.

L'origine des hémocytes reste inconnue et leur capacité à se multiplier dans l'hémolymphe n'a jamais été démontrée. L'existence d'un ou plusieurs sites d'hématopoïèse a été suspectée. Certains auteurs attribuent cette fonction à un organe en particulier alors que d'autres considèrent que le tractus digestif ou les tissus conjonctifs pourraient intervenir comme centre d'hématopoïèse (Lorteau *et al.*, 1995). Aucune de ces hypothèses n'a été vérifiée à ce jour.

Le rôle des cellules séreuses reste inconnu, mais les hyalinocytes et les granulocytes sont des cellules capables de phagocytose, le principal mécanisme de défense chez les mollusques (Feng, 1988).

Tableau VIII : Différents types cellulaires décrits chez l'huître creuse, *C. gigas*

Références	Types cellulaires	Critères*	Techniques utilisées
Bachère *et al.*, 1988a ; 1988b	- Granulocytes - Petits hyalinocytes - Hyalinocytes intermédiaires - Grands hyalinocytes	- Morphologie - Densité en gradient de Percoll	- Microscopie photonique - Microscopie électronique - Séparation en gradient de Percoll
Chagot, 1989	- Granulocytes (sous type A, B et C) - Grands hyalinocytes - Petits hyalinocytes	- Morphologie - Activités enzymatiques	- Microscopie photonique - Microscopie électronique - Cytochimie
Auffret, 1989 ; 1995	- Hémocytes granuleux (basophiles et acidophiles - Hyalinocytes (hémocytes agranuleux et ameobocytes agranuleux)	- Morphologie - Activités enzymatiques	- Microscopie photonique - Microscopie électronique
Chagot *et al.*, 1992 Mourton *et al.*, 1992 La Peyre *et al.*, 1995	- Granulocytes - Grands hyalinocytes - Petits hyalinocytes	- Morphologie - Activités enzymatiques	- Microscopie photonique - Microscopie électronique - Cytochimie

*Critères morphologiques: formes, rapport nucléocytoplasmique, granulations cytoplasmiques et affinité tinctoriales.

Si différentes techniques incluant la microscopie photonique, la microscopie électronique à transmission et des analyses biochimiques ont été mises en oeuvre pour caractériser les hémocytes chez les mollusques, la classification sur la base de la présence de marqueurs membranaires n'a été à ce jour que peu développée. Les anticorps sont largement utilisés afin d'identifier les cellules de l'immunité chez les vertébrés et apparaissent comme des outils de choix pour le phénotypage cellulaire. C'est dans ce contexte, qu'un travail de recherche a été développé au sein du Laboratoire de Génétique et Pathologie (Ifremer, La Temblade, France) avec pour objectif principal d'obtenir des anticorps monoclonaux spécifiques des hémocytes de l'huître plate, *O. edulis,* (**Xue & Renault, 2001**).

Ce sont des populations hémocytaires séparées (granulocytes et hyalinocytes) qui ont servi pour les immunisations des souris et la production des anticorps monoclonaux. Sur l'ensemble des hybridomes produits, un seul possédait une **spécificité marquée pour les granulocytes** sans montrer de réactivité vis à vis des hyalinocytes. L'épitope reconnu par cet anticorps est présent dans les granules. Par contre, aucun anticorps spécifique des hyalinocytes n'a été obtenu. Ce résultat pourrait s'expliquer par le fait que les granulocytes dériveraient des hyalinocytes et que de ce fait ils possèderaient la majorité des épitopes portés par les cellules hyalines.

L'anticorps monoclonal spécifique des granulocytes a été utilisé pour étudier la distribution de ce type cellulaire chez l'huître plate, *O. edulis*, à différents stades de développement. Les cellules marquées (granulocytes) ont été détectées dans le tissu conjonctif de différents organes (branchies, manteau, glande digestive, …). Il est à noter qu'aucun marquage n'a été observé chez les **larves** (âgées de 8-10 jours) analysées laissant suspecter l'existence d'un **système de défense immature** à ce stade de développement.

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Monoclonal antibodies to European flat oyster *Ostrea edulis* hemocytes: characterization and tissue distribution of granulocytes in adult and developing animals

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Abstract

Monoclonal antibodies specific for hemocyte sub-populations of the European flat oyster, *Ostrea edulis*, were prepared using separated granulocytes and hyalinocytes as antigen sources. Six monoclonal antibodies specific for hemocytes were selected. Five of them showed a specificity for more than one hemocyte type and one hybridoma produced a monoclonal antibody reacting specifically with granulocytes. At the ultrastructural level, this monoclonal antibody demonstrated epitopes principally in dense cytoplasmic granules of granulocytes. Western blotting analysis indicated that a peptide of 50 kDa was recognized by this antibody. It was therefore used to investigate granulocyte distribution and ontogenesis in European flat oysters using immunohistochemistry. Granulocytes were mostly observed in connective tissues in different organs. Their distribution pattern in digestive gland, mantle, gills and gonad may indicate different functional status. Morover, the absence of granulocytes in early larval stages can partly correspond to an immature immune system in oyster larvae. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: European flat oyster; *Ostrea edulis*; Monoclonal antibody; Hemocyte; Granulocytes; Tissue distribution; Ontogenesis

1. Introduction

Bivalve hemocytes are not homogenous in views of both morphology and function [1–11]. For European flat oyster, *Ostrea edulis*, hemocytes have been classified into granulocytes, large and small hyalinocytes on morphological basis [4,12,13]. However, the knowledge concerning hemocyte sub-populations and their involvment in host defense mechanisms is limited. Certain investigators used enzymes and lectins in defining mollusc hemocyte types [14–18]. The application of monoclonal antibodies in studying immune cells has greatly promoted immunological researches in vertebrates including fish [19–28]. However, the application of monoclonal antibodies in molluscan hemocyte studies is still limited to a few species [29–34]. This paper describes the preparation of monoclonal antibodies against European flat oyster, *Ostrea edulis*, hemocytes using separated cell sub-populations as antigen sources. The main objective of the study was to obtain specific antibodies to be able to define hemocyte sub-populations present in hemolymph. A monoclonal antibody specific for granulocytes was obtained and it was

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therefore used to investigate tissue distribution and ontogenesis of this hemocyte type in European flat oysters using immunohistochemistry.

2. Material and methods

2.1. Oysters

Adult European flat oysters, *Ostrea edulis*, 7–9 cm in shell length were purchased from shellfish farmers in Marenne Oléron Bay (Charente Maritime, France). Animals were processed immediately after arriving in laboratory. Larval and juvenile individuals originated from a private hatchery in Bourgneuf Bay (Vendée, France).

2.2. Antigen preparation

European flat oyster hemolymphs were collected in the pericardial cavity of healthy animals. Hemocytes were then separated into sub-populations by centrifugation in Percoll and Ficoll gradients as previously described [13]. Cell fractions G, F1 and F2 were used as antigen sources for mouse immunization and represented granulocytes, large and small hyalinocytes, respectively.

2.3. Immunization

Hemocyte pellets (2.5×10^6 cells) were suspended in 20 μl of filtrated 0.9% (w/v) NaCl solution and 180 μl of complete Freund's adjuvant were then added. Eight-week-old Balb/c mice were immunised via intraperitoneal injections of hemocyte preparations (100 μl per mouse). Two mice were injected at the same time with one hemocyte sub-population preparation (G, F1 or F2). Three concurrent booster injections were given at 2 week intervals. Four days before cell fusion, ascitic fluids and sera was collected to estimate antibody titers by indirect immunofluorescence and mice received the last intravenous booster.

2.4. Hybridoma production

Four days following the final injection, spleen cells were fused with SP2O myeloma cells in a ratio of 2:1 using polyethylene glycol (PEG 1500, 50%). Cell fusions using −80°C stored splenocytes were carried out with a similar procedure. Culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) on fixed hemocytes (0.8% glutaraldehyde, 10 min) in 96 well plates. Fixed hemocytes were treated with 3% Triton × 100 in PBS for 10 min. Hemocyte endogenous peroxidase was eliminated by 20 min incubation with 0.3% hydrogen peroxide in methanol. After 30 min incubation in 6% skim dried milk (Régilait), culture supernatants were incubated for 60 min. Peroxidase labeled sheep anti-mouse IgG and IgM (diluted 1:250 in PBS, Sanofi Diagnostics Pasteur) were then incubated for 60 min. After each antibody incubation, five washes in PBS (5 min) were carried out. The immunoreactivity of culture supernatants was monitored by adding *O*-phenylenediamine (0.4 mg/l, Sigma) and hydrogen peroxide (0.02% v/v) for 30 min. Plates were analysed with a Titerteck Multiskan (MCC/340) plate reader at 450 nm. Positive hybridomas were further tested by immunocytochemical techniques using centrifuged hemocytes. Monoclonal antibodies were then isotyped with a haemagglutination test kit (Serotec). Negative controls included hemocytes and PBS, hemocytes minus primary antibodies and hemocytes plus preimmune mouse sera. Positive controls corresponded to immune mouse sera and ascitic fluids obtained four days before cell fusion.

Culture supernatants with high antibody titers were collected and applied directly. Antibodies were also produced by injecting hybridomas into peritoneal cavity of 10-week-old Balb/c mice. After hybridoma cell proliferation, ascitic fluids with high antibody titers were obtained in 4–5 weeks.

2.5. Immunocytochemistry

European flat oyster hemocyte monolayers were prepared by cytocentrifugation (Hettich, 50 g, 1 min, 4°C) on histological slides (1.5×10^6 hemocytes per slide). Cells were fixed for 10 min in methanol or acetone at 4°C.

For indirect immunofluorescence labeling, centrifuged hemocyte slides were saturated with 6% (w/v) skim dried milk (Régilait). Then, two incubations were successively performed with hemocyte specific antibodies and fluoresceine conjugates (sheep anti-mouse IgG (H + L), diluted 1:250 in PBS, Sanofi-Diagnostics Pasteur), 60 min each. The secondary antibody was incubated in the presence of Evan's

blue (0.5% v/v, Sanofi-Diagnostics Pasteur). After antibody incubations, five washes in PBS (5 min) were carried out. The controls were as for ELISA but also included hemocytes plus a monoclonal antibody against CD45 (NCL-LCA, Novo Castra Laboratories).

Indirect immunoperoxidase staining on cytocentrifuged hemocytes was conducted following a similar procedure with a treatment with 0.3% (v/v) H_2O_2 in methanol for 40 min before saturation with skim dried milk and using peroxidase labeled secondary antibodies (sheep anti-mouse IgG (H + L), diluted 1: 250 in PBS, Sanofi-Diagnostics Pasteur). In addition, slides were treated in 0.06% (w/v) 3, 3′ diaminobenzidine in PBS supplemented with H_2O_2 (0.3%, v/v) and counter stained with Unna's blue.

Adult oyster tissues were fixed in Davidson's solution. Larvae and juveniles were fixed in Bouin's solution. Before preparing paraffin blocks, larvae and juveniles were decalicified in 5% EDTA in PBS for 20–24 h at 4°C. Tissue sections of 7 μm thick were treated with the indirect immunoperoxidase staining procedure previously described.

2.6. Western blotting

Oyster hemocyte pellets (3×10^6 cells) were resuspended in 100 μl lysis buffer (Tris 1.2 g/l; sodium chloride 8.77 g/l; EDTA 0.29 g/l; NP40 5 ml/l; pH 7.4). After 5 min incubation at room temperature, protein concentrations of lysates were estimated using a commercial kit (Micro BCA Protein Assay Reagent Kit, Pierce) and then adjusted to 2 mg/ml with distilled water. After dilution (1:1) in denaturing buffer (2-mercaptoethanol 1 ml/l; glycerol 98% 2 ml/l; sodium dodecyl sulfate 0.4 g/l; bromophenol blue 0.1% in distilled water 0.2 ml/l), samples were boiled for 5 min. Proteins were then separated by electrophoresis on 9–17.5% acrylamide/bis-acrylamide gradient gels by running at 25 mA for 40 min in a Bio-Rad Mini-Protean II cell. Total proteins (20 μg) were loaded on each well. Separated proteins were transferred at 100 V for 60 min to a nitrocellulose membrane in a Bio-Rad Trans-blot apparatus. The specific peptides were detected by immunoenzymatic staining in a similar procedure to indirect immunoperoxidase for centrifuged hemocytes and histological sections.

2.7. Ultrastructural localization

To prevent granulocyte degranulation, European flat oyster hemocytes were collected in cold 0.25% glutaradehyde–3% paraformaldehyde in cacodylate buffer. They were immediatly centrifuged (640 g, 4°C, 15 min.) and fixed again in the same fixative solution overnight at 4°C. Following dehydration in ethanol series, cells were infiltrated and embedded in LR White resin (Sigma). Sections collected on nickel grids were, after rehydration, saturated with 5% skim dried milk (Régilait) in PBS (30 min). They were then incubated with primary antibody diluted in PBS (1:50 for ascitic fluids, 1:2 for cell culture supernatants), for 2 h. After washes in PBS, an incubation with colloidal gold labeled secondary antibody (goat anti-mouse IgM + IgG, 10 nm, TEBU) diluted to 1:10 in PBS was carried out for 60 min. Grids were stained in uranyl acetate and observed with a transmission electron microscope (JEOL JEM 1200EX) at 80 kV.

2.8. Data analysis

Mean values and p values in comparing data by Student's *t*-test were obtained using Excel software. $P < 0.05$ was regarded as significant.

3. Results

3.1. Monoclonal antibody preparation

Seven fusions were performed, two of which were conducted with splenocytes conserved at −80°C, and up to 536 hybridomas were obtained. The mean fusing rates were 52% and 30% for the fresh splenocytes and −80°C conserved cells, respectively. No significant difference in fusing rate was observed. Among the 79 hybridomas producing antibodies positive for *Ostrea edulis* hemocytes, six were selected on the basis of their characteristic staining pattern, and cloned for further tests. The six hybridomas were designated GP_2E_{11}, $F_1P_1D_4$, $F_1P_3F_{11}$, $F_1P_2F_6$, $F_1P_3G_2$ and $F_2P_3G_8$ and were isotyped as IgG1, IgG2a, IgG1, IgG1, IgG2b and IgG2a, respectively.

3.2. Characterization of monoclonal antibodies

The monoclonal antibody GP_2E_{11} reacted with the

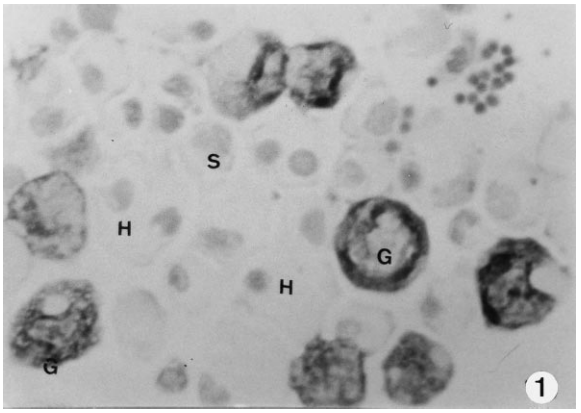


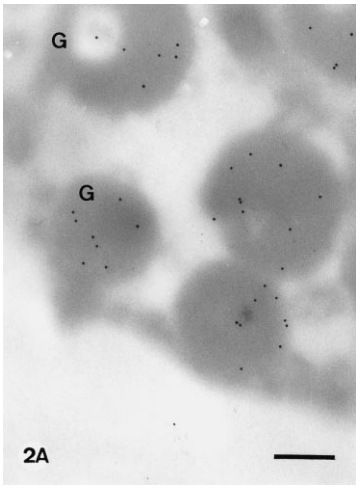
Fig. 1. Antibody specificity identified by indirect immunoperoxidase on centrifuged hemocytes for the monoclonal antibody GP_2E_{11} (G: granulocytes, H: large hyalinocytes and S: small hyalinocytes).

granule-containing hemocytes when indirect immunofluorescence staining was used (data not shown). In hemocyte preparations labeled by indirect immunoperoxidase technique, all cells possessing morphological characteristics of granulocytes were specifically labeled (Fig. 1). The agranulocytes (large and small hyalinocytes) showed no specific reactivity (Fig. 1).

The other antibodies ($F_1P_1D_4$, $F_1P_3F_{11}$, $F_1P_3G_2$, $F_2P_3G_8$ and $F_1P_2F_6$) reacted also with granulocytes (data not shown). However, their epitope location was different from each other. The antibody $F_1P_1D_4$ showed a specific reactivity to granulocytes and small hyalinocytes. $F_2P_3G_8$ evenly labeled the granulocyte and small hyalinocyte cytoplasm. The antibody $F_1P_2F_6$ recognised big sized hemocytes including large hyalinocytes and granulocytes. $F_1P_3G_2$ reacted with the hemocyte surface of several cell types. According to the indirect immunoperoxidase staining of centrifuged hemocytes, $F_1P_3F_{11}$ bound to some cytoplasmic granules in granulocytes and small hyalinocytes.

3.3. Ultrastructural localization

Monoclonal antibodies GP_2E_{11} and $F_1P_1D_4$ were tested for epitope ultrastructural localization because of their strong reaction on centrifuged hemocytes. The monoclonal antibody GP_2E_{11} reacted with the matrix of dense granules present in granulocyte cytoplasm (Fig. 2(A)). For the monoclonal antibody $F_1P_1D_4$



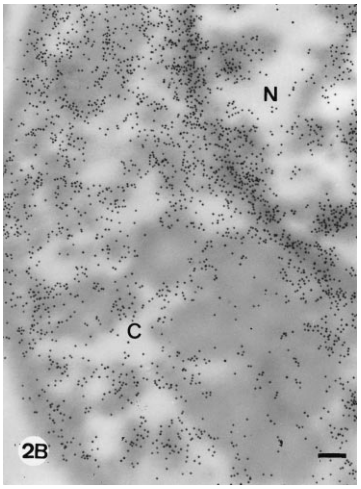


Fig. 2. (A) and (B): Antibody specificity identified by immunogold labeling. (A) The monoclonal antibody GP_2E_{11} (G: granules, bar: 100 nm). (B) The monoclonal antibody $F_1P_1D_4$ (N: nucleus, C: cytoplasm, bar: 200 nm).

specific labelling was observed in the cytoplasm and the nucleus of granulocytes and small hyalinocytes (Fig. 2(B)).

3.4. Western blotting

A single peptide was identified by the antibody GP_2E_{11} in granulocyte lysates prepared from the granulocyte sub-population [13] with a molecular mass of 50 kDa (Fig. 3).

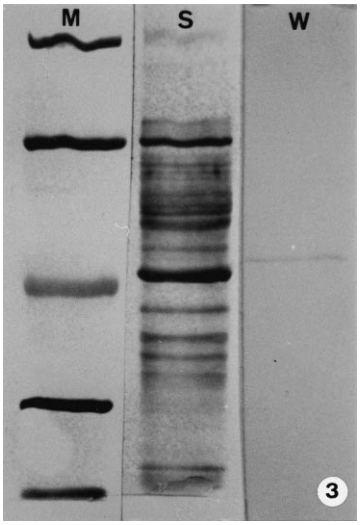


Fig. 3. The specificity of the monoclonal antibody GP_2E_{11} analyzed by Western blotting. M: molecular weight markers, from top to bottom correspond to 94.0, 67.0, 43.0, 30.0 and 20.1 kDa; S: silver nitrate staining; W: Western blotting.

3.5. Granulocyte counting using indirect immunoperoxidase technique

The percentages of cells labeled by the monoclonal antibody GP_2E_{11} on centrifuged hemocytes with immuno-enzymatic staining were compared with granulocyte percentages determined according to Hemacolor kit (Merck) staining for 20 oysters. Statistical analysis (Student's *t*-test) indicated a significant positive correlation between the two techniques ($P < 0.01$, Fig. 4).

3.6. Granulocyte tissue distribution

The monoclonal antibody GP_2E_{11} was applied for studying granulocyte tissue distribution in European flat oysters. For healthy oysters, granulocytes were abundant in mantle, digestive gland and gill connective tissues (data not shown). In connective tissues underneath mantle epithelia, granulocytes were often present as cell clusters. They were also observed between epithelial cells in digestive tubules and intestine, mantle and gills. In gonads, granulocytes distributed in connective tissues but not within gametic folliculi. The morphology of granulocytes detected in tissues was similar to those present in the hemolymph. In digestive gland and mantle epithelia, the basal membrane between connective tissue and epithelium appeared intact even though granulocytes existed in both sides. Granulocytes were also observed in the connective tissues of kidney and adductor muscle as well as in heart tissue. However, the cell density was much lower than in the organs described above.

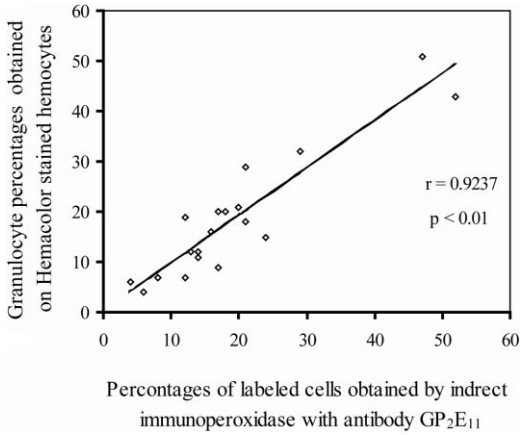


Fig. 4. Correlation between percentages of labeled cells determined by indirect immunoperoxidase using the monoclonal antibody GP_2E_{11} and those obtained for Hemacolor stained granulocytes.

A primary analysis for the digestive gland of *B. ostreae* infected *O. edulis* showed that granulocytes were diffusely distributed in connective tissues and the parasite *B. ostreae* was detectable within some specifically labeled cells.

3.7. Granulocyte appearance during oyster development

In two sample batches of larvae 8–10 day old, no labeled cell was observed. For juvenile oysters 30–32 days after emission, labeled cells were detected in connective tissues in gills. They were also observed between gill epithelial cells. No labeled cell was demonstrated in other tissues.

4. Discussion

The main objective in preparing monoclonal antibodies against European flat oyster hemocytes was to develop specific markers for hemocyte sub-population identification. Since three hemocyte sub-populations

were obtained by density gradient centrifugation representing granulocytes, large hyalinocytes and small agranulocytes [13], the hemocyte fractions were used as antigen sources for mouse immunization. An indirect immunoperoxidase labeling procedure (ELISA) using fixed hemocytes in culture plates was developed to quantify hemocyte specific antibodies.

In all six clones selected, four clones produced antibodies reacting with granulocytes, though three of them were derived from fusion experiments using agranulocytes-immunized mice. Immuno-cytological observations have revealed differences in antigen epitope locations among these four hybridomas. These results may indicate that European flat oyster granulocytes and hyalinocytes share common antigens. Investigators have applied monoclonal antibodies to identify different hemocyte types or neoplastic cells [29,31–33,35–37]. Dyrynda et al. [34] reported also that some monoclonal antibodies recognize both basophilic granular cells and hyaline hemocytes in *Mytilus edulis*.

However, granulocytes and agranulocytes of European flat oyster can be distinguished using the monoclonal antibody GP_2E_{11}. The significant positive correlation between granulocyte percentages determined on centrifuged hemocytes stained with Hemacolor kit (Merck) and the percentages of labeled cells using indirect immunoperoxidase staining verifies that all granulocytes reacted with this antibody. In addition, granulocytes of the Pacific oyster, *Crassostrea gigas,* were also labeled by this antibody.

The monoclonal antibody GP_2E_{11} was therefore used to investigate granulocyte distribution and ontogenesis in European flat oysters by immunochemistry. The distribution of hemocytes in different tissues can provide important information concerning molluscan immune capabilities [38,39]. Immunohistological analysis using the monoclonal antibody GP_2E_{11} revealed that granulocytes were distributed in connective tissues of different European flat oyster organs. In gills and mantle granulocytes were present in the connective tissues just under the epithelial layer and between epithelial cells. As organs directly in contact with the environment such as gills and mantle have more opportunities to be invaded by pathogenic agents, it is essential to have an efficient defense barrier consisting of granulocytes. Ganulocytes observed between mantle epithelial cells probably participate in physiological mechanisms other than phagocytosis. Granulocytes are actively involved in food capturing, digestion and transportation [3,40]. The India ink injected into Eastern oyster cardiac cavity was rapidly detected in mantle cavity [41]. Some phagocytosed microorganisms may be also destroyed during transportation. However, for hemocyte-infecting parasites such as *Bonamia ostreae*, hemocytes [42,43] particule uptake by granulocytes may favour the pathogen spread [44]. The digestive gland may be the way for *B. ostreae* to enter in oysters.

The absence of reactivity of the monoclonal antibody GP_2E_{11} with oyster larvae suggests that the epitope detectable on adult granulocytes is not present during early larval stages. However, it appears early in juveniles. Bivalve larvae have been reported to present high susceptibility to bacterial infections [45–49]. Viral infections have also been observed in larval stages among different bivalve species [50–52]. While different mechanisms can be involved in these phenomena, the absence of granulocytes in early developmental stages in European flat oyster larvae may be one important cause of defense weakness at this stage of development. Previous research utilizing monoclonal antibodies to invertebrate hemocytes has involved little detailed work on immune ontogeny [34,53,54]. The results obtained by these authors as well those from the current study suggest that monoclonal antibodies may be highly effective tools in studying invertebrate hemocyte development.

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4. 1. 2. Phagocytose

Les hémocytes des bivalves marins peuvent phagocyter de nombreux microorganismes et particules (bactéries, levures, virus, globules rouges, protéines) (Auffret, 1985). Les particules phagocytées sont dégradées et les produits de cette dégradation peuvent être accumulés dans des vacuoles. Le processus de phagocytose est un mécanisme conservé au cours de l'évolution. Les mêmes caractéristiques sont retrouvées tant chez les vertébrés que chez les invertébrés et notamment les mollusques bivalves avec un processus en quatre étapes : attraction, adhérence, internalisation et destruction (Cheng, 1983). Les trois premières phases reposent sur des capacités de chimiotactisme, de locomotion, de transformation et de communication. La quatrième phase est celle de la destruction faisant intervenir deux types de mécanismes biochimiques différents.

Le premier mécanisme implique les enzymes lysosomales. Les particules étrangères sont internalisées dans une vacuole de phagocytose nommée phagosome primaire. Ce dernier fusionne avec les lysosomes contenant des enzymes hydrolytiques, la fusion de ces deux éléments forme un phagolysosome et permet l'action des enzymes comme les estérases (Auffret, 1985) sur les particules étrangères internalisées. Des enzymes hydrolytiques ont été ainsi mises en évidence chez différentes espèces de mollusques bivalves, dont *C. gigas* (**Xue & Renault, 2000**).

Le second mécanisme repose sur la production et la libération de radicaux toxiques de l'oxygène (flambée oxydative). Les hémocytes de mollusques sont capables de produire des dérivés réactifs comme des radicaux anions super oxyde (O_2^-), du peroxyde d'hydrogène (H_2O_2), des radicaux hydroxyles (OH^-) (Torreilles *et al.*, 1997). Ces radicaux jouent un rôle important dans l'activité anti-microbienne des cellules phagocytaires. Leur production peut être initiée par diverses substances comme les composants de la paroi bactérienne (Torreilles *et al.*, 1996). Ce type de mécanisme existe chez différentes espèces de mollusque bivalve. Pipe (1990a) et Schlenk *et al.* (1991) ont mis en évidence chez la moule *Mytilus edulis* Linné, la synthèse de métabolites réactifs de l'oxygène et de la myéloperoxydase. Cette enzyme a également été détectée chez la moule méditerranéenne, *M. galloprovincialis* Lamarck (Torreilles *et al.*, 1997).

Le parasite protozoaire *Perkinsus marinus* peut induire la production de radicaux oxygénés ou bien en inhiber la synthèse chez l'huître américaine, *C. virginica*, (Volety & Chu, 1995). Le parasite tué est capable de stimuler la production de ces radicaux alors que pour le parasite vivant, une inhibition peut être détectée (Anderson, 2001). Cette observation laisse suspecter que le parasite *P. marinus* est capable d'éviter la réponse immunitaire. D'autres agents infectieux ont été également décrits comme ne stimulant pas la flambée oxydative chez certains bivalves : le parasite *Bonamia ostrea* chez l'huître plate *O. edulis* et la bactérie *Vibrio tapetis* chez la moule *M. galloprovincialis* (Ordas *et al.*, 2000).

4. 1. 3. Agrégation hémocytaire (ou "clotting"), adhésion hémocytaire et chimiotactisme

L'agrégation hémocytaire peut avoir deux origines. La première est l'agrégation dite spontanée (en absence de corps étranger) qui a lieu suite à un stress et particulièrement *in vitro*. En général, le contact avec des objets ou l'agitation mécanique peuvent servir de stimulus (Auffret & Oubella, 1997).

La seconde permet la formation d'amas cellulaires impliqués dans la cicatrisation de plaies ou l'immobilisation de corps étrangers (Chen & Bayne, 1995). En effet, les amas hémocytaires formés permettent d'obstruer des lésions et d'empêcher la perte d'hémolymphe. Cependant, contrairement au mécanisme de coagulation observé chez les vertébrés, aucune fibre

extracellulaire n'est produite au cours du phénomène d'agrégation. De plus, il s'agit d'un processus réversible permettant aux cellules de retourner dans la circulation générale. Il se déroule en deux phases. La première phase, dite agrégation faible, correspond au changement rapide de forme des hémocytes et à leur association à faible échelle, tandis que la seconde phase (agrégation cohésive) produit des amas cellulaires denses et peu dissociables. Les structures assurant ce phénomène sont des prolongements cytoplasmiques appelés filopodes (Narrain, 1973). Le processus peut être inhibé à faible température ou encore par certaines substances tel que l'acide éthylènediaminetétraacétique (EDTA) (Auffret & Oubella, 1997).

L'adhésion hémocytaire correspond à une interaction entre les cellules et des substrats. C'est un processus différent de l'agrégation, mais qui intervient aussi de manière essentielle dans les phénomènes de réparation des blessures. Le chimiotactisme est un phénomène correspondant à l'attraction des hémocytes par des substances produites par les organismes étrangers ou par les organismes eux-mêmes. Cheng et Rudo (1976) ont montré l'existence de ce phénomène chez *C. virginica*, en présence de bactéries exclusivement vivantes. Les agents exogènes, dès leur entrée dans l'organisme, peuvent ainsi être directement repérés par l'organisme et pris en charge par les cellules hémocytaires.

La plasticité hémocytaire est une caractéristique des hémocytes qui peuvent s'étaler au contact d'une surface. Leur forme devient ameoboide, des filopodes et des pseudopodes apparaissent alors, permettant une certaine mobilité des cellules (Hinsch & Hunte, 1990). La mobilité des hémocytes est variable. Elle dépend de la saison, du site géographique et de la salinité.

4. 1. 4. Substances solubles présentes dans l'hémolymphe

Les mollusques bivalves marins ne possèdent pas de mémoire immunitaire et de réponse immunitaire adaptative. Bien que Brillouet *et al.* (1984) rapportent la détection d'une molécule dont la structure et les fonctions semblent proches de celles d'un anticorps ("antibody-like") chez l'étoile de mer *Asterias rubens*, aucune molécule de ce type n'a été détectée chez les bivalves. Cependant, différents travaux rapportent la présence de molécules intervenant dans les mécanismes de défense telles que le lysozyme, des enzymes lysosomales (Pipe, 1990a et b), des peptides antimicrobiens (Mitta, 2000a et b), et des cytokines (Leippe & Renwrantz, 1988 ; Hughes *et al.*, 1990 ; Beck & Habicht, 1991 ; Hughes *et al.*, 1991).

4. 1. 4 .1. Lysozyme et enzymes lysosomales

Le lysozyme est une enzyme bactériolytique retrouvée dans la fraction acellulaire de l'hémolymphe et dans les hémocytes de bivalves (Cheng & Rodrick, 1975). Des séquences de lysozyme sont aujourd'hui disponibles chez différentes espèces de mollusques (Bachali *et al.*, 2002). Les lysozymes de bivalves appartiennent à la famille des lysozymes de type invertébrés (type i)(Nilsen & Myrnes, 2001).

Des enzymes lysosomales (β-glucuronidase, phosphatase, estérase, peroxydase, aminopeptidase) sont également retrouvées dans les hémocytes et l'hémolymphe chez différentes espèces de bivalves comme l'huître creuse, *C. gigas*, et l'huître plate, *Ostrea edulis* (**Xue & Renault, 2000**). Ces enzymes sont contenues dans les granules, présents dans les cellules phagocytaires, mais elles peuvent aussi être libres dans l'hémolymphe. Les aminopeptidases catalysent la réaction d'hydrolyse de la liaison peptidique de l'acide aminé N-terminal des protéines et en particulier favorisent la dégradation des protéines de surface des parasites (Cheng *et al.*, 1978). La β-glucuronidase, les phosphatases acides, l'amylase et la lipase ont également été recensées dans l'hémolymphe de nombreux mollusques, tels *C. virginica* (Cheng & Downs, 1988), *Biomphalaria glabrata* (Rodrich & Cheng, 1974),

Anadara ovalis (Rodrich & Ulrich, 1984), *Dreissena polymorpha* (Giamberini & Pihan, 1996), *M. mercenaria* (Cheng & Rodrick, 1975 ; Moore & Gelder, 1983), *M. campechiensis* (Rodrich & Ulrich, 1984), *M. californianus* (Bayne *et al.*, 1979), *M. edulis* et *M. trossulus* (Noël, 1992), *M. galloprovencialis* (Carballal *et al.*, 1997) et *Mya arenaria* (Beckmann *et al.*, 1992).

4. 1. 4. 2. Peptides anti-microbiens

Des peptides anti-microbiens ont été mis en évidence chez les moules *M. edulis* et *M. galloprovincialis* (Mitta *et al.*, 2000a et b). Également dénommées mytilines ou myticines, ces molécules à propriétés anti-microbiennes ont été détectées dans les hémocytes circulants des moules. Elles interviennent dans la destruction des bactéries à l'intérieur des phagocytes, et sont re larguées dans l'hémolymphe pour prendre part aux réponses immunitaires systémiques.

Un peptide de type défensine a été isolé à partir d'extraits de banchies chez l'huître américaine, *C. virginica* (Seo *et al.*, 2005). Une approche de biologie moléculaire a permis également d'identifier une molécule de type défensine chez l'huître creuse *C. gigas* (Gueguen *et al.*, 2006 ; Gonzalez *et al.*, 2007).

4. 1. 4. 3. Cytokines

Deux approches ont été développés de manière simultanée : (i) la recherché d'effets de cytokines de mammifères sur les hémocytes de mollusques et (ii) le marquage de cellules de l'hémolymphe par des anticorps spécifiques de cytokines de vertébrés (Hughes *et al.,* 1990).

Différents travaux rapportent également la détection de molécules présentant des activités similaires à celles de l'IL-1, l'IL-6 ou le TNFα chez *M. edulis*, *A. forbesi et B. glabrata* (Beck & Habicht, 1986 ; Leippe & Renwrantz, 1988 ; Hughes *et al.*, 1990 ; Beck & Habicht, 1991 ; Ouwe-Missi-Oukem-Boyer *et al.*, 1994 ; Beck *et al.*, 2000). Si ces cytokines ont un rôle immunomodulateur chez les vertébrés, leur implication dans les mécanismes de défense chez les invertébrés reste encore mal connue.

Plus récemment, des approches en biologie moléculaire ont permis d'identifier des gènes codant pour des protéines présentant des homologies avec des cytokines de mammifères (Tanguy *et al.*, 2004).

4. 1. 4. 4. Lectines

Les lectines sont des protéines à forte affinité pour les sucres. Elles sont détectées chez la quasi-totalité des organismes vivants. Ces molécules sont classifiées dans plus de six familles dont certains membres de la famille C sont impliqués dans les mécanismes de défense (Vasta & Marchalonis, 1985). Fonctionnellement, on les divise en deux catégories, les lectines reconnaissant des ligands endogènes et les lectines reconnaissant des ligands exogènes. Elles peuvent intervenir dans les phénomènes d'agglutination et d'opsonisation notamment lors de la phagocytose d'organismes pathogènes (virus, bactéries, champignons et parasites) (Pipe, 1990b) du fait de leur spécificité pour certains oses (mannose, fucose ou N-acétyl-glucosamine).

4. 1. 4 .5. Protéines de stress ("heat shock proteins" : HSP)

Les protéines de stress contribuent à la défense chez les mollusques marins. Des protéines de type hsp70 ont été décrites chez la coquille *Argopecten irradians* (Song *et al.*, 2006) et chez le pétoncle *Chlamys farreri* (Wu *et al.*, 2003). Elles interviennent lors de blessure, d'infection et d'exposition à des molécules toxiques (métaux lourds) (Tirard *et al.*, 1995). Dans le règne animal, elles sont caractérisées par une grande conservation. Une augmentation de la température, un choc osmotique, une modification de la salinité induisent une augmentation significative de la synthèse de certaines protéines de stress permettant ainsi aux cellules de supporter ces variations environnementales.

4. 1. 4. 6. Oxyde Nitrique Synthétase (NOS)

Le NOS catalyse la production d'oxyde nitrique qui intervient en tant qu'agent anti-bactérien et anti-tumoral (Torreilles *et al*., 1999). L'oxyde nitrique n'est pas toxique par lui même, mais combiné avec les anions super oxyde produit lors de la phagocytose, il génère l'anion peroxynitrique (ONOO-). Ce dernier composé labile est très fortement toxique.

4. 1. 4. 7. Protéases et inhibiteurs de protéases

Dans les interactions hôte/agent pathogène, les inhibiteurs de protéases jouent un rôle important. De telles molécules ont été retrouvées dans la partie acellulaire de l'hemolymphe chez différentes espèces de bivalves. En effet, un inhibiteur de sérine protéase (serpin) et une protéine de type α_2-macroglobuline ont été rapportés chez l'huître creuse, *C. gigas*, et l'huître américaine, *C. virginica* (Faisal *et al.*, 1998). Le rôle protecteurs des inhibiteurs de serine protéases vis à vis des parasites protozoaires est bien documenté (Rosenthal, 1999). Chez les bivalves, une corrélation entre faibles niveaux d'infections à *Perkinsus marinus* et forts niveaux d'inhibiteur de sérine protéase a été observée chez l'huître américaine, *C. virginica.* Une augmentation de ce type d'inhibiteur a été rapportée en cas d'infection par le même parasite chez l'huître creuse, *C. gigas*, espèce peu sensible (Romestand *et al.*, 2002). Ces résultats laissent suspecter une implication directe de ces molécules dans les mécanismes de défense des huîtres vis à vis de parasites protozoaires.

4. 1. 4. 8. Activités cytotoxiques

Chez la moule, *M. edulis*, la production de molécules cytolytiques par les hémocytes a été rapportée par Leippe et Renwrantz (1988). Une activité vis à vis de cellules de vertébrés (érythrocytes et cellules tumorales de souris) et de parasites protozoaires a également été détectée chez la moule, *M. galloprovincialis* (Hubert *et al.*, 1996). La purification des composés actifs a permis de mettre en évidence un complexe protéique de 320 kDa (Roch *et al.*, 1996). Composé de trois protéines différentes, le complexe agit comme une perforine par polymérisation après fixation à la surface des cellules cibles (Hubert *et al.*, 1997).

4. 1. 4. 9. Activités anti-virales

Alors qu'aucune molécule de type interféron n'a été mise en évidence chez les invertébrés, des activités anti-virales ont pu être observées chez les bivalves. Ainsi, une activité vis à vis du virus d'amphibien LT-1 a été mise en évidence dans un extrait aqueux préparé à partir de la mye *Mya arenaria* (Li & Traxler, 1972). Chez l'huître creuse, *C. gigas*, il a été possible de montrer la présence d'une activité de neutralisation du bactériophage T3 dans l'hémolymphe (Bachère *et al.*, 1990). Récemment, chez cette même espèce, un composé a montré la capacité d'inhiber la réplication de HSV-1 et du virus de la nécrose pancréatique infectieuse (NPI) (**Olicard *et al.*, 2005**).

4. 1. 4. 10. Système pro phénol oxydase/phénol oxydase (Hellio C., Bado-Nilles A., Gagnaire B., Renault T. & H. Thomas-Guyon, 2007, Fish & Shellfish Immunology, 22: 433-40)

La phénol oxydase (PO) est une enzyme cuivre-dépendante (Cole & Pipe, 1994), détectée dans l'hémolymphe (partie acellulaire et/ou hémocytes) chez plusieurs espèces d'invertébrés. Cette enzyme est capable d'oxyder les monos et di-phénols : elle catalyse la réaction de conversion de la diphénylalanine (L-dopa) et de la dopamine en dopa quinone. Cette molécule peut à son tour être polymérisée en mélanine (Coles & Pipes, 1994 ; Söderhäll & Cerenius, 1998). La PO est présente chez de nombreuses espèces sous une forme inactive, la pro phénol oxydase (pro-PO). Certaines substances de la paroi bactérienne peuvent stimuler le clivage de la pro-PO par digestion protéolytique donnant la PO (Söderhäll & Cerenius, 1998 ; Sritunyalucksana *et al.*, 1999).

Ce système enzymatique est principalement observé chez les arthropodes (Söderhäll & Smith, 1983). La PO prend une part active dans les mécanismes de défense par le biais de la catalyse de la réaction d'oxydation intervenant dans les processus de réparation des blessures, d'encapsulation et de mélanisation (Söderhäll & Smith, 1983). Le système pro-PO-PO existe également chez les mollusques tels que la mye, *M. arenaria* Linné, la moule commune, *M. edulis*, la patelle commune, *Patella vulgata* Linné et l'huître creuse, *C. gigas*, (Smith & Söderhäll, 1991 ; Coles & Pipes, 1994 ; Luna-González *et al.*, 2003). Des travaux récents (**Hellio *et al.*, 2007**) ont permis de démontrer l'existence d'une activité PO dans la partie acellulaire de l'hémolymphe chez l'huître creuse, *C. gigas*, l'activité PO dans les hémocytes étant très faible. Ce résultat est différent de ce qui est observé chez les crustacés et chez certaines autres espèces de mollusques. En effet, chez les crevettes, l'activité PO est essentiellement détectée dans les hémocytes et ce sont ces cellules après stimulation qui re larguent la PO à partir de granules. Sur la base d'une purification biochimique, il a été montré chez la palourde japonaise, *R. philippinarum*, que la phénol oxydase était de type tyrosinase (Cong *et al.*, 2005). Les connaissances sur le rôle joué par le système de la pro-PO-PO dans les mécanismes de défense restent parcellaires chez les bivalves. Une corrélation a été cependant établie entre résistance au parasite *Marteilia sydneyi* et des fortes activités phénol oxydase chez l'huître australienne, *Saccostrea glomerata* (Newton *et al.*, 2004).

4. 5. Phénomène apoptotique chez les bivalves

L'apoptose est un mécanisme fondamental permettant de réguler les populations cellulaires dans un contexte physiologique, mais aussi pathologique. Depuis la mise en évidence de ce phénomène chez *Caenorabditis elegans* (Ellis & Horvitz, 1986), l'existence d'un tel mécanisme a été démontrée chez de nombreux êtres vivants, vertébrés ou invertébrés. Une littérature abondante existe pour les vertébrés et notamment en biologie humaine. Chez les invertébrés, la drosophile et d'autres insectes représentent probablement les modèles d'investigation les plus étudiés (Claveria *et al.*, 1998 ; Chubykin & Omel'ıanchuk, 1999 ; Greene *et al.*, 2003 ; Ishikawa *et al.*, 2003). Cependant, les mécanismes apoptotiques ont aussi été rapportés chez des organismes marins comme l'éponge (Wiens *et al.*, 2003) ou des mollusques tels que le calmar hawaïen *Euprymna scolopes* (Foster *et al.*, 2000), la moule *M. galloprovincialis* (Micic *et al.*, 2001 ; Micic *et al.*, 2002), chez la palourde japonaise *R. philippinarum* (**Renault *et al.*, 2001**) ou encore l'huître plate *O. edulis* et l'huître creuse *C. gigas* (**Renault *et al.*, 2000**).

Certains virus sont capables de moduler la machinerie cellulaire afin de perdurer chez leur hôte. Les herpès virus, les baculovirus et les poliovirus sont dotés de cette faculté. Les membres de chaque sous-famille chez les *Herpesviridae* peuvent induire et/ou inhiber l'apoptose en fonction du type cellulaire considéré et des conditions environnementales (Kieff & Shenk, 1998).

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Short communication

Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) in vitro

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Abstract

The prophenoloxidase (ProPO) system is the origin of melanin production and is considered to be an innate defence mechanism in invertebrates. In different bivalve species, phenoloxidase (PO) is present in the haemolymph as an inactive form of ProPO. The present study focuses on the Pacific adult oyster, *Crassostrea gigas,* an economically important bivalve species along French coasts. The results indicate that many factors may inhibit the PO-like activity. These include: phenylthiourea (PTU), sodium diethylthiocarbamate (DETC), β-mercaptoethanol and tropolone, which repressed the spontaneous PO activity. The activation of PO-like activity in *C. gigas* acellular fraction by lipopolysaccharide (LPS) involved participation of other factors, including at least one serine protease. PO was present as proPO in the acellular fraction of haemolymph and haemocytes of *C. gigas* and could be activated by an exogenous protease (trypsin-*N*-tosyl-L-phenylalanine chloromethyl ketone) when used at 1 g L^{-1}. Treatment of acellular fractions with other modulators/activators namely LPS (1 g L^{-1}), zymosan (0.6 g L^{-1}) or laminarin (0.6 g L^{-1}) also increased PO-like activity but to a less important way. The study demonstrated the evidence of a true phenoloxidase activity in Pacific oyster, *C. gigas* (Thunberg). The activation of a proPO system by non-self molecules suggests the role played by PO in vivo in the internal defence mechanisms. Understanding the activation of the ProPO system could enable the evaluation of the health of oyster stocks.
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Keywords: Crassostrea gigas; Defence; Immunity; Oyster; Phenoloxidase; Prophenoloxidase

1. Introduction

In invertebrates, haemocytes and some proteolytic pathways (coagulation, melanin synthesis and opsonisation) are involved in defence reactions toward pathogens. The prophenoloxidase (ProPO) system, at the origin of melanin

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production, has been suggested as an innate defence mechanism in invertebrates [1]. In arthropods, it has been demonstrated that phenoloxidase (PO) is present in the haemolymph as an inactive form of ProPO. ProPO is cleaved by proteolysis via an endogenous activator system or exogenous agents, to PO, the enzymatically active form [2]. PO is copper-dependent and catalyses the synthesis of o-diphenols from monophenols by ortho-hydroxylation, which are then dehydrogenated into o-quinones [3–5]. Non-enzymatic polymerisation of *o*-diphenols leads to the production of melanin, a common response of invertebrates to the entry of an infectious agent. The major function of the PO cascade is the deposition of the pigment, melanin derived from tyrosine-based substrates such as L-3,4-dihydroxyphenylalanine (L-dopa). Components of the PO cascade can also opsonise pathogens for phagocytosis, while others appear to be fungistatic or cytotoxic [6]. The activation of the enzymatic system by bacterial or fungal components is an additional clue of the involvement of this enzyme in invertebrate immune defence mechanisms. Many components of the system have recently been isolated in invertebrates, particularly from aquatic arthropods [7–9].

Phenoloxidase-like activity has also been detected in the haemolymph of molluscs such as *Mya arenaria* Linne, *Mytilus edulis, Patella vulgata* Linne, *Ruditapes philippinarum*, *Chamelea gallina*, *Tapes decussatus* and *Hyriopsis cumingii* [1,10–17] but until now, only few studies have focused on the adult Pacific oyster *Crassostrea gigas* [16–18]. Moreover, in all these studies, no real PO activity was demonstrated. PO activity was followed using the classical spectrophotometric technique based on L-dopa hydrolysis. However, several molecules are able to hydrolyse L-dopa. In these conditions, it appears necessary to demonstrate that the activity detected is a real PO activity. Understanding the activation of the pro-PO system in *C. gigas* oysters could be used as a probe for the evaluation of their health and could provide rapid inexpensive tests for environmental stress [6]. This is of particular importance as the aquaculture of oysters contributes significantly to the economic development of many tropical, subtropical and temperate countries [19]. France is currently one of the leading European shellfish producing countries, harvesting annually more than 150,000 metric tons of *C. gigas* [20]. But aquaculture is threatened by the repeated appearance of new diseases, non-infectious and infectious. Difficulties in controlling these diseases come mainly from the differences in susceptibility of the animals according to their developmental stage (from larvae to adults) and from the diversity of pathogens (parasites, viruses, fungi and bacteria) that affect them [21–26]. Antibiotics have been used intensively as preventive and curative measures but such practices are now questioned due to the appearance of drug-resistant bacteria and their harmful effects on the environment [21]. As an outcome, alternative treatments as well as animal health monitoring have to be established as preventing measures. Investigation of the innate immune systems may give new insights into the management and control of diseases in aquaculture [27–30].

In this study, in vitro experiments on the modulation of the PO-like activity have been conducted in the cellular and the acellular fraction of haemolymph, in adult Pacific oyster (*C. gigas*). The specific inhibitors of PO activity were tested: phenylthiourea (PTU), β-mercaptoethanol, sodium diethyldithiocarbamate (DETC) and tropolone. Some activators of PO activity, lipopolysaccharide (LPS), laminarin, zymosan and trypsin were used to modulate activation of oyster proPO system in vitro. This allowed the demonstration of a true phenoloxidase activity and the process of PO activation in *C. gigas* to be characterised.

2. Material and methods

2.1. Animals

One-year-old Pacific oysters, *Crassostrea gigas* were purchased during January-May 2002 from shellfish farms in Aytré Bay (Charente Maritime, France), on the French Atlantic coast and were processed immediately after their arrival in the laboratory.

2.2. Circulating haemolymph collection

After opening the oyster shells by cutting off the adductor muscle, haemolymph was withdrawn directly from the pericardial cavity with a 1-mL syringe equipped with a needle (0.9 × 25 mm). A quantity (0.5–1 mL) of haemolymph was withdrawn and the haemolymph from 30 oysters was pooled to reduce inter-individual variation.

2.3. Detection of phenoloxidase-like (PO-like) activity

Haemolymph samples were centrifuged (260 × *g*, 10 min, 4 °C), and PO-like activity was analysed in the acellular fraction. The cellular pellet and the haemocyte lysate supernatants of *C. gigas* were also used as sources of PO activity after 24 h incubation.

The cellular pellet was resuspended in cacodylate buffer (CAC buffer: 10 mM sodium cacodylate, 100 mM trisodium citrate, 0.45 M NaCl, 10 mM $CaCl_2$, 26 mM $MgCl_2$, pH 7.0) and agitated. This preparation was then centrifuged (40,000 × *g*, 25 min, 4 °C, Microfuge 12, Beckman), and the haemocyte lysate supernatants (HLS) removed. PO-like activity was analysed in the cellular pellet and the HLS too.

The detection of PO-like activity in haemolymph samples was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-dopa) transformation to dopachromes [14]. Transformation was monitored by spectrophotometry at 490 nm. Samples were distributed in 96-well microplates (Nunc, France). Various well-known modulators of PO activity (Sigma, France) were tested at concentrations ranging from 0.2 g L^{-1} to 10 g L^{-1}: LPS (*Escherichia coli*/0111:B4), laminarin, zymosan and purified trypsin (TPCK-treated, bovine pancreas, 7500 units mg^{-1}). PTU (10 mM), DETC (10 mM), β-mercaptoethanol (10 mM) and tropolone (16 mM) were also tested for their effects on PO-like activity (Sigma France). Sixty microlitres of cacodylate buffer, 20 μL of a PO activity modulator, 20 μL of L-dopa (Sigma) and 20 μL of acellular fraction of haemolymph were added in each well. The assay was run at 20 °C. Control wells and negative control contained only 120 μL of CAC buffer and 100 μL of CAC buffer plus 20 μL of L-dopa, respectively.

Each sample was tested in triplicate wells and A_{490} was measured after a 21 h incubation period [17,18,20].

2.4. Statistical analysis

Data were expressed as mean values ± SD. Each experiment involved three replicates. The values presented were the averages of nine replicates. The data were statistically analysed using XLStat Pro version 7.5.2 software. Normality was tested using the Anderson–Darling test [31]. One-way analysis of variance (ANOVA) was applied to analyse the differences among treatments and control. *P* values lower than 0.05 were used to identify significant differences. Where significant differences occurred, an a posteriori Student–Newman–Keuls (SNK) test was used.

3. Results

PO-like activity was detected in the acellular fraction of the haemolymph of adult *C. gigas*. The absorbance reached a maximum after 21 h of incubation (Fig. 1). Oxidation of L-dopa was significantly increased after incubation

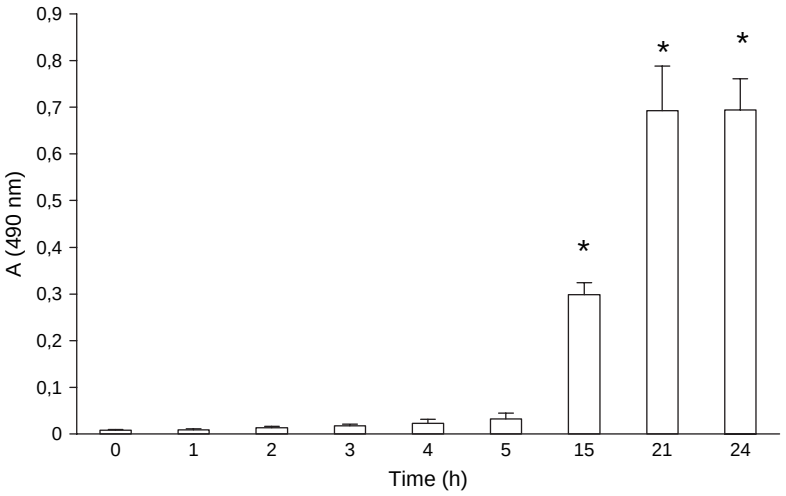

Fig. 1. In vitro PO-like activity (A_{490}) detected in acellular fraction of *C. gigas* haemolymph after 24 h incubation. Values are averages of 9 replicates. *Significant difference, $P < 0.05$.

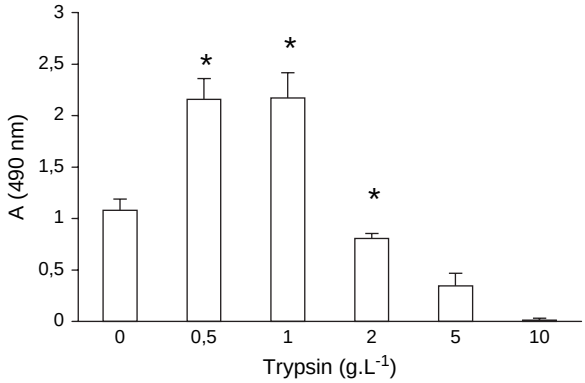

Fig. 2. In vitro PO-like activity (A_{490}) stimulated with Trypsin detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. For the control, Trypsin was replaced by CAC buffer. Values are averages of 9 replicates. *Significant difference, $P < 0.05$.

of the acellular fraction of oyster haemolymph with trypsin at 0.5 g L^{-1} or 1 g L^{-1}. From 2 g L^{-1}, PO-like activity was significantly lower than in the control and was not detected at a trypsin concentration of 10 g L^{-1} after 21 h incubation (Fig. 2).

A significant stimulation of PO-like activity was induced with zymosan at various concentrations. A maximum effect was observed at 10 g L^{-1} but below 1 g L^{-1}, highest stimulation was with a zymosan concentration of 0.6 g L^{-1} (Fig. 3). Stimulation was not dose-dependent.

Incubation of the acellular haemolymph of *C. gigas* with laminarin at concentrations ranging from 0.2 g L^{-1} to 1 g L^{-1} stimulated PO-like activity. At 0.5 and 0.6 g L^{-1}, laminarin induced the highest PO-like activity (Fig. 4). Stimulation was not dose-dependent.

PO-like activity in the acellular fraction was significantly enhanced with LPS concentrations of 0.5 g L^{-1}, 0.9 g L^{-1}, and 1 g L^{-1} (Fig. 5).

As shown in Table 1, in the cellular pellet and haemocyte lysate supernatant, PO activity is low compared to the acellular fraction. For the acellular fraction, the addition of trypsin significantly enhanced the PO-like activity for incubation from 4 h to 24 h. On the other hand, for the cellular pellet and the haemocyte lysate supernatant, the PO-like activity was always low despite addition of trypsin or not.

Phenylthiourea, a copper-binding compound, significantly inhibited PO-like activity compared to the control. PTU, β-mercaptoethanol and DETC completely abolished PO-like activity ($P < 0.05$). Tropolone, a specific inhibitor of PO

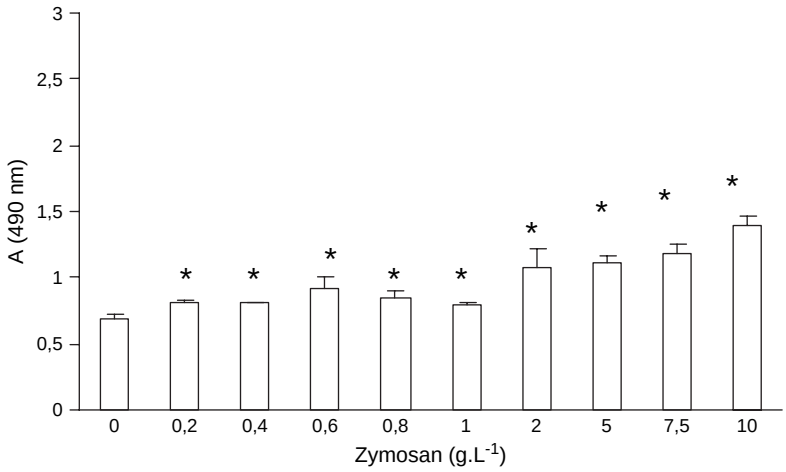

Fig. 3. In vitro PO-like activity (A_{490}) with zymosan detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. For the control, zymosan was replaced by CAC buffer. Values are averages of 9 replicates. *Significant difference, $P < 0.05$.

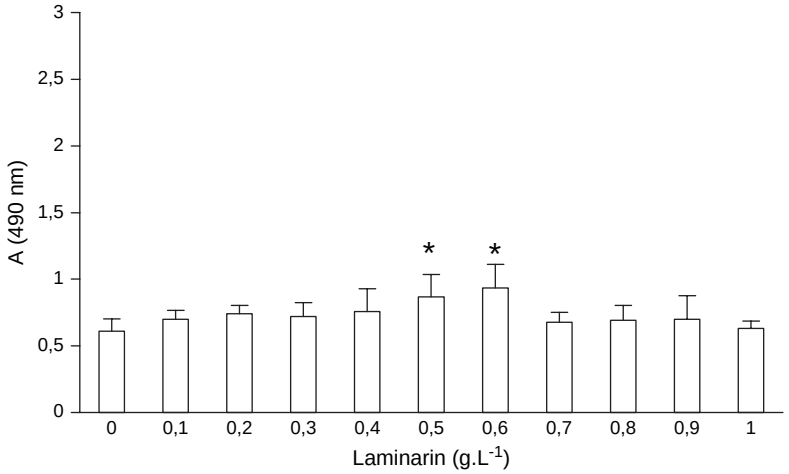

Fig. 4. In vitro PO-like activity (A_{490}) with laminarin detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. For the control, laminarin was replaced by CAC buffer. Values are averages of 9 replicates. *Significant difference, $P < 0.05$.

and a potential substrate for peroxidase, repressed the spontaneous activity and abolished the stimulating effect of LPS (Table 2).

4. Discussion

The present study confirmed that a PO-like activity can be detected in vitro in the acellular fraction of the haemolymph of the adult Pacific oysters, *C. gigas*. Maximal absorbance was observed in all samples after a 21 h incubation period with the substrate (L-dopa). The present study also establishes the effect of various factors on the phenoloxidase-like activity in the cellular fraction of the oyster haemolymph. Some molecules (tropolone, DETC, β-mercaptoethanol and PTU) inhibit the PO-like activity. This may be due to copper chelation by PTU, DETC and β-mercaptoethanol, as copper is essential to the functionality of the phenoloxidase. The activity estimated at 490 nm was not due to a peroxidase-like activity but definitely to a phenoloxidase activity, as tropolone, a specific

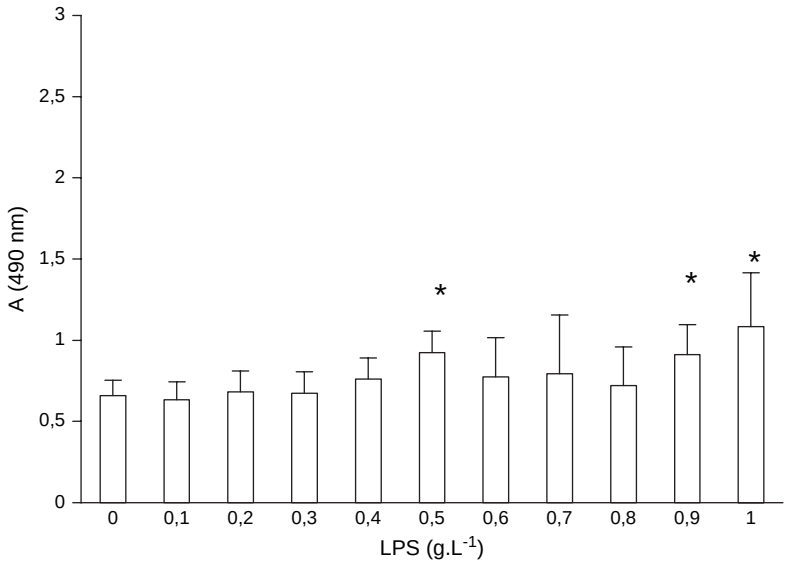

Fig. 5. In vitro PO-like activity (A_{490}) with LPS detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. In control, LPS was replaced by CAC buffer. Values are averages of 9 replicates. *Significant difference, $P < 0.05$.

Table 1
In vitro PO-like activity (A_{490}) with (stimulated activity) or without (spontaneous activity) trypsin in acellular fraction, supernatant and cellular pellet of *C. gigas* haemolymph after 24 h incubation

Fraction analysed		0 h	2 h	4 h	15 h	21 h	24 h
Acellular fraction	No trypsin	0.033 ± 0	0.055 ± 0	0.104 ± 0.038	0.235 ± 0.082	0.541 ± 0.102	0.684 ± 0.091
	Trypsin	−0.0006 ± 0.016	0.034 ± 0.0182	0.198 ± 0.053	0.849 ± 0.126	0.831 ± 0.062	0.925 ± 0.128
Haemocyte lysate supernatant	No trypsin	0.003 ± 0.002	0.005 ± 0.004	0.006 ± 0.005	0.002 ± 0.005	0.053 ± 0.017	0.052 ± 0.029
	Trypsin	0.002 ± 0.011	0.004 ± 0.007	0.004 ± 0.008	0.004 ± 0.007	0.030 ± 0.006	0.025 ± 0.005
Cellular pellet	No trypsin	−0.001 ± 0	0.009 ± 0.015	0.006 ± 0.014	0.006 ± 0.045	0	0.019 ± 0.002
	Trypsin	0.003 ± 0.006	0.008 ± 0.011	0.008 ± 0.011	0.004 ± 0.007	0.036 ± 0.015	0.025 ± 0.010

Control was pre-incubated in CAC buffer for 20 min before reaction with L-dopa. Values are averages ± SD of 9 replicates.

inhibitor of phenoloxidase but potential substrate of peroxidase, suppressed the activating effects of the LPS and also the oxidation of L-dopa.

The stimulation of PO activity by trypsin, a serine protease, suggests that PO may be present in the acellular fraction of the haemolymph in the form of a pro-enzyme and required a proteolytic cleavage for activation. Furthermore, this result suggests that endogenous serine-proteases may enhance the proteolytic cleavage of the pro-enzyme. However, the cleavage of inactive proPO into active PO may be earlier in *C. gigas* than in mussels since Coles and Pipe [10] showed that the activation of the proPO system in *Mytilus edulis* occurred after a 96 h incubation with L-dopa.

The PO activity in *C. gigas* is sensitive to in vitro activation by LPS, zymosan or laminarin. Increase of the enzymatic activity does not appear to be dependent on the concentrations of the exogenous molecules used. A maximum of PO activity was obtained with concentrations of trypsin at 0.5 g L^{-1} or 1 g L^{-1}. In the shrimp *Penaeus paulensis,* trypsin has been used at a concentration of 1 g L^{-1} and in the mussel *Perna viridis* at 0.7 g L^{-1} [11,24]. It has been shown that in the mussel *P. viridis,* the pro-PO system is 1000 times more sensitive to LPS than to laminarin [11]. Trypsin enhances PO activity in a more efficient way than LPS or β-glucans (laminarin and zymosan) in *C. gigas*.

Increase of the enzymatic PO activity by exogenous molecules in this study suggests that the pro-PO system plays a role in non-self recognition and defence reactions in the adult *C. gigas*. The mechanism of the activation of pro-PO system via exogenous molecules was studied before. Some proteins from serum capable of reacting with β-glucans (laminarin and zymosan) and initiating the activation of the pro-PO system have been identified in insects and shellfish [8,32–36].

Although PO is widely distributed throughout the animal kingdom, this study unambiguously demonstrated its presence as a proenzyme (proPO) in the acellular fraction of haemolymph of *C. gigas*. Microbial substances enhanced the enzyme activity in vitro suggesting its potential role in host defence. The activation responses of this proenzyme in *C. gigas* to exogenous proteases, microbial cell wall components, and its susceptibility to protease inhibitors in vitro were similar to the proPO activation system of arthropods. The similarities in activation responses of haemolymph proPO system in animals belonging to two different phyla tend to imply a unifying biochemical mechanism for immune recognition among invertebrates.

In aquaculture, animals are submitted to stressful environmental and ecological conditions and this has been linked to high-density animal populations, pollution and nutritional imbalances. As ProPO and PO are involved in the immune response, a biochemical test on PO or Pro-PO activities could be used as a probe to measure health conditions of animals.

Table 2
In vitro PO-like activity (A_{490}) with different inhibitor agents detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation

Treatment	PO-like activity (A_{490})	Significance
Control	0.693 ± 0.095	
PTU (10 mM)	0.001 ± 0.001	***
DETC (10 mM)	0.001 ± 0.001	***
β-Mercaptoethanol (10 mM)	0.001 ± 0.001	***
Tropolone (16 mM)	0.000 ± 0.000	***

Control was pre-incubated in CAC buffer for 20 min before reaction with L-dopa. Each experiment was repeated three times in triplicate. Values are averages of three replicates. ***Significant difference, $P < 0.001$.

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4. 2. Immunité et environnement chez les bivalves

Le développement de la conchyliculture peut être limité par des mortalités parfois massives. Ces mortalités sont liées pour certaines d'entre elles à la présence d'agents infectieux, bactéries, virus et parasites (Comps & Bonami, 1977; Lacoste *et al.*, 2001 ; **Renault *et al.*, 1994**). Les fortes densités d'animaux en élevage peuvent favoriser la propagation de ces agents pathogènes, de la même manière que les milieux contaminés par des xénobiotiques. Dans ces milieux, les coquillages peuvent se montrer plus sensibles aux maladies. L'environnement est en effet susceptible de fragiliser les animaux, notamment en réduisant leurs défenses immunitaires et les rendre ainsi plus vulnérables aux infections. Les espèces de mollusques bivalves élevées sont pour la plupart benthiques et sédentaires : ce mode de vie les expose aux fluctuations physico-chimiques de l'environnement (température, salinité, contaminants chimiques, etc.) sans possibilité de fuite. Leur mode de nutrition le plus souvent de type suspensivore les conduit à accumuler et concentrer dans leurs tissus les contaminants. Ainsi, l'étude de la modulation de la réponse immunitaire apparaît comme l'une des voies de recherche à privilégier pour l'évaluation des effets physiologiques de facteurs environnementaux chez les mollusques bivalves marins. Des expériences ont ainsi permis de mettre en évidence chez l'huître creuse, *C. gigas*, certains effets immunomodulateurs de micro polluants métalliques et organiques (Auffret & Oubella, 1994).

L'étude du système immunitaire des bivalves et de sa modulation en relation avec les fluctuations de l'environnement marin apparaît aujourd'hui comme une clé explicative aux phénomènes de mortalités observés chez les coquillages et indispensable au bon **développement de la conchyliculture**.

Le système immunitaire s'est développé au cours de l'évolution des espèces par de nombreuses interactions hôtes-agents infectieux. Ce système contribue au maintien de l'intégrité de l'organisme hôte en éliminant les bio-agresseurs (virus, bactéries et parasites). Cependant, plusieurs xénobiotiques, présents dans l'environnement, peuvent agir sur les composantes du système immunitaire et interférer ainsi avec leurs fonctions de protection de l'organisme (Descotes, 1988 ; Krzystyniak *et al.*, 1995 ; Dean et Murray, 1996 ; Kouassi *et al.*, 2001).

L'immunotoxicité peut être ainsi définie comme l'ensemble des effets délétères provoqués par un xénobiotique ou par toute autre constituant biologique ou physique de l'environnement sur le système immunitaire à la suite d'une exposition de l'organisme hôte. Différents types d'effets immunotoxiques sont possibles incluant l'immunosuppression qui peut favoriser le développement des infections et des tumeurs et l'immunostimulation. Comme discipline, l'immunotoxicologie est récente et l'analyse des nombreuses atteintes immunologiques induites par l'exposition à une substance chimique ne fait que commencer. Cependant, les données disponibles laissent entrevoir des conséquences néfastes pour les individus exposés quelles que soient les espèces considérées (Kaoussi *et al.*, 2001). Ainsi, l'épidémie due à un virus proche de celui de la maladie de Carré, qui a décimé plus des deux tiers des phoques de la Mer du Nord à la fin des années 80, s'explique probablement par le fort degré de pollution chimique des eaux marines (van Loveren *et a*l., 2001). De la même manière, la recrudescence de néoplasies et des cas d'infections virales à iridovirus chez certaines espèces de poisson a été reliée à la pollution des eaux marines et estuariennes (Grinwis *et al.*, 2001). Enfin, chez l'homme, une étude a montré que la susceptibilité aux otites moyennes était associée à l'exposition pré-natale aux composés organochlorés chez les enfants Inuit (Dewailly *et al.*, 2000). Certains métaux lourds (mercure, cadmium, plomb) possèdent également des

propriétés immunosuppressives (Zelikoff *et al.*, 1995) qui proviennent en partie de leurs effets cytotoxiques, par induction d'apoptose et/ou de nécrose dans les cellules du système immunitaire (Azzouzi *et al.*, 1994 ; Aten *et al.*, 1995 ; InSug *et al.*, 1997 ; Shenker *et al.*, 1997 ; Guo *et al.*, 1998). Les études *in vivo* dans différents modèles animaux corroborent les résultats des études *in vitro*. L'ensemble des travaux menés dans ce domaine démontre ainsi des altérations fonctionnelles plus ou moins importantes qui affectent le développement de la réponse immunitaire (Kaoussi *et al.*, 2001).

Cependant, s'il existe de nombreux exemples concernant les relations qui peuvent exister entre pollution et augmentation de la sensibilité aux maladies chez les mammifères et les vertébrés de manière plus générale, peu de travaux ont été réalisés chez les invertébrés (Galloway & Depledge, 2001). En effet, peu d'études ont porté sur l'immunosuppression induite par les polluants chez les différents groupes d'invertébrés alors qu'ils constituent 95% de l'ensemble des espèces animales recensées sur la terre. De plus, ils occupent souvent des rôles clés dans de nombreux écosystèmes, comme les zones d'estuaire. De nombreuses espèces d'invertébrés telles que les mollusques bivalves vivent dans des milieux soumis à diverses pollutions. Les rejets industriels et domestiques apportent de nombreux polluants dans certaines zones d'estuaire. La présence de sources de polluants chimiques dans la zone des pertuis charentais (Charente Maritime, France) est ainsi bien documentée (Cossa & Lassus, 1989 ; Scribe *et al.*, 1998) : cadmium, zinc, cuivre, provenant de la Gironde, mercure apporté probablement par la Charente et lindane présent sur tout le littoral dans cette zone. Il convient de s'interroger sur les effets néfastes de ces composés sur les animaux vivants dans les zones littorales, tout particulièrement les mollusques bivalves (huîtres) et leur impact sur les productions.

L'huître creuse, *C. gigas*, étant donné sa position phylogénique et son importance économique pour l'aquaculture tant au plan national (150 000 tonnes de production annuelle) qu'au plan international (plus de 4 millions de tonnes produites annuellement), apparaît comme **un modèle de choix** pour tenter de définir l'importance du **système immunitaire comme cible de polluants chez les invertébrés** et de montrer également l'intérêt d'utiliser des **bio marqueurs de type immunologiques** dans le cadre du **suivi et du contrôle de l'environnement** (Akcha *et al.*, 2000 ; Cosson, 2000 ; Donald *et al.*, 2001 ; Keppler & Ringwood, 2001 ; Lekube *et al.*, 2000 ; Minier *et al.*, 2000).

Les mortalités régulièrement observées chez cette espèce, tout particulièrement en période estivale, peuvent laisser suspecter un **rôle joué par les produits phytosanitaires** (carbamates, organophosphorés, organochlorés) d'utilisation saisonnière, associés à des arrivées intempestives d'eau douce dans les zones d'élevage. De plus, il est également possible d'envisager des relations de cause à effet entre l'**accumulation de polluants (métaux lourds) chez les huîtres et les mortalités observées**.

Dans ce contexte, les travaux menés au sein du LGP (Ifremer, La Tremblade, France), en particulier dans le cadre de la thèse de Béatrice Gagnaire (2002-2005) avaient pour objectif principal d'explorer les effets de différents polluants (cadmium, mercure, atrazine, carbamates, organophosphorés, etc.) à différentes concentrations sur le système immunitaire de l'huître creuse, *C. gigas*, en réalisant des expériences *in vivo* et *in vitro* et en effectuant des études de terrain (suivis *in situ*). Les effets individuels de molécules sélectionnées ont été étudiés ainsi que les effets combinés de certaines d'entre elles.

Les objectifs spécifiques étaient de :

- rechercher des effets sur les hémocytes d'huître maintenus *in vitro* et mis au contact de polluants,
- rechercher des effets sur les hémocytes chez des huîtres mises au contact de polluants dans des structures expérimentales,
- rechercher et quantifier les polluants dans les animaux et l'eau,
- rechercher la cause d'un effet de polluants sur les hémocytes (mort par apoptose/nécrose des hémocytes ou inhibition des activités de défense de ces mêmes cellules),
- valider l'hypothèse d'une sensibilité accrue aux infections,
- suivre certains paramètres hémocytaires chez des huîtres élevées dans différents sites.

Différents polluants ont été ciblés (cadmium, mercure, atrazine, carbamates, organophosphorés, …) afin d'étudier leurs effets sur les hémocytes de l'huître creuse, *C. gigas*. Les effets des composés considérés ont été analysés d'une part chez des animaux maintenus dans des structures d'élevage et mis au contact des polluants à différentes concentrations (concentrations correspondant au pic environnemental observé dans le milieu et concentrations supérieures), d'autre part dans des expériences *in vitro*. Dans ce dernier cas, les hémocytes provenant d'animaux possédant la même origine que ceux utilisés dans les expériences *in vivo* et élevés dans des conditions identiques, ont été mis au contact de différentes concentrations de polluants. Dans les expériences *in vivo* et *in vitro*, des lots témoins ont été incorporés afin de pouvoir réaliser des comparaisons. Les possibles modifications du système immunitaire ont été recherchées en suivant la mortalité cellulaire et différentes activités enzymatiques et fonctions des hémocytes chez des animaux traités et non traités et chez des hémocytes traités et non traités. L'ensemble de la démarche scientifique est synthétisé dans les Figures 19 et 20.

Si des effets des polluants étaient observés sur les capacités de défense chez les huîtres, il a été tenter d'élucider quel type d'action entrait en jeu dans les phénomènes détectés. Il a été en particulier vérifié si les polluants étaient capables d'induire une immunosuppression en tuant les hémocytes. De la même manière, si une immunosuppression était mise en évidence, des expériences ont été réalisées afin d'étayer d'éventuelles relations entre sensibilité aux infections chez les bivalves et présence de xénobiotiques dans l'environnement. Dans ce cadre, des expériences *in vivo* ont été réalisées.

Pour compléter cette double approche *in vitro* et *in vivo*, des études de terrain ont été également réalisées en suivant des animaux mis en élevage sur sites. De cette manière, il est possible d'établir des liens entre les observations de terrain et les résultats obtenus en laboratoire (Burgeot *et al.*, 1995). Les sites ont été choisis en fonction de leur typologie et des profils de contaminations par des polluants répertoriés (bassin versant de la Charente et de la Seudre par exemple). L'échantillonnage a été réalisé en période estivale (avril à août). Les analyses des paramètres hémocytaires ont été complétées et comparées avec la recherche d'effets neurotoxiques et génotoxiques (acétylcholine estérase et adduits à ADN) effectuée au Laboratoire d'Ecotoxicologie du Centre Ifremer de Nantes.

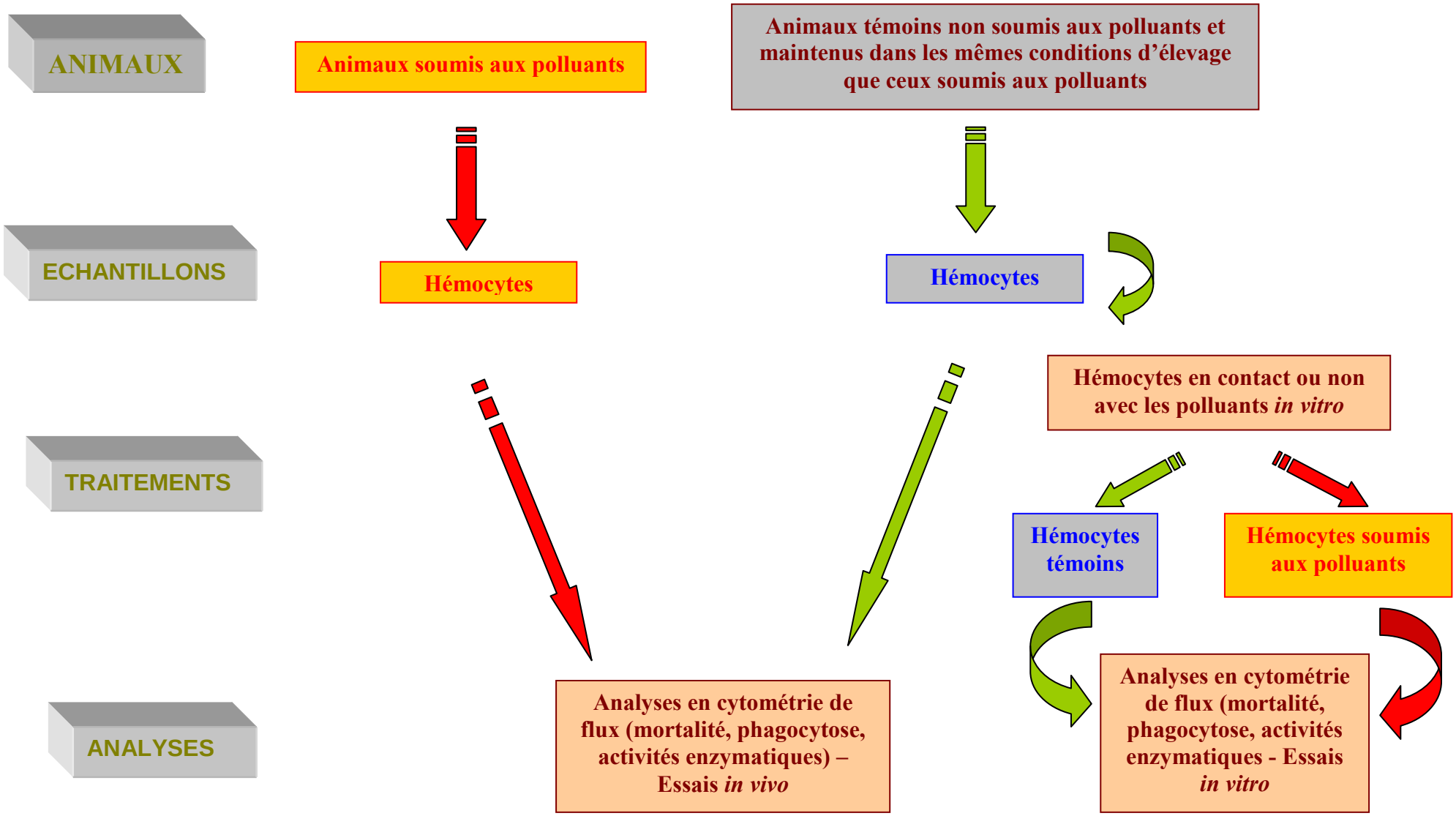

Figure 19. *Schéma général de l'approche scientifique proposée (paramètres en cytométrie en flux). N. B. : le dosage de certains polluants a été réalisé chez les animaux et dans l'eau*

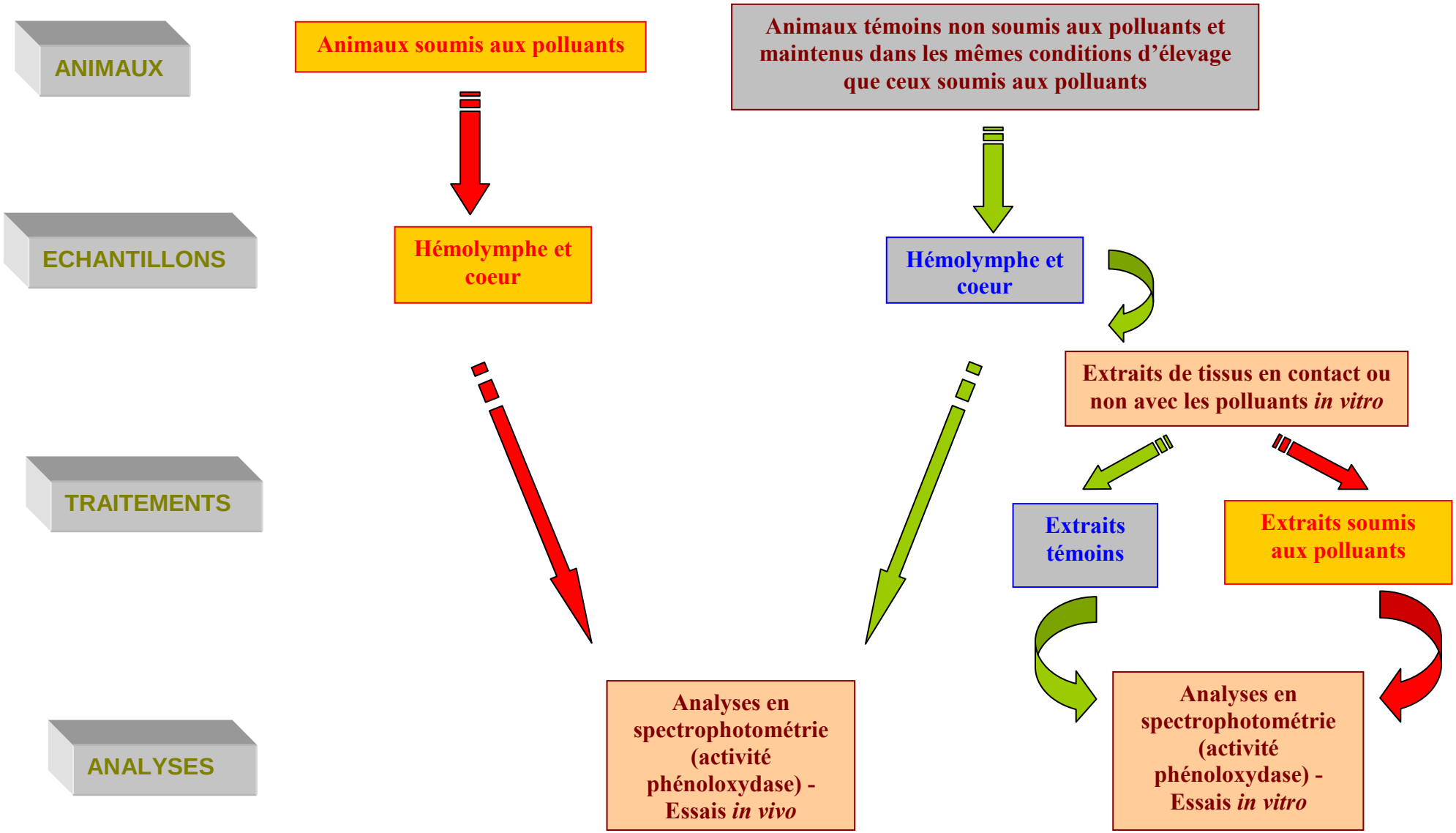

Figure 20. Schéma général de l'approche scientifique proposée (activité phénol oxydase suivie en spectrophotométrie). N. B. : le dosage de certains polluants a été réalisé chez les animaux et dans l'eau

La cytométrie en flux a été en particulier utilisée afin de caractériser les effets des polluants sur les hémocytes. Cette méthodologie apparaît adaptée aux études sur l'immunotoxicité (Burchiel *et al.*, 1999). Par ailleurs, le LGP (IFREMER, La Tremblade, France) a développé ces dernières années des protocoles de cytométrie en flux adaptés à l'analyse des hémocytes de bivalves (**Renault *et al.*, 2001** ; **Xue *et al.*, 2001** ; **Gagnaire *et al.*, 2001**)

La spectrophotométrie a également été utilisée dans le but de quantifier une activité de type phénol oxydase dans différents types d'échantillon (hémolymphe, hémocytes et cellules cardiaques) afin de différencier les animaux/hémocytes traités par les polluants par rapport aux témoins.

4. 2. 1. Des essais in vitro (Gagnaire B., Thomas-Guyon H., Burgeot T. & T. Renault, 2006, Cell Biology and Toxicology, 22: 1-14)

Des travaux ont été réalisés au Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France) afin de déterminer les effets de divers polluants retrouvés dans le milieu marin sur les hémocytes de l'huître creuse, *C. gigas*. La cytométrie en flux a été sélectionnée pour suivre les effets des polluants sur les cellules. En effet, cette technique permet rapidement de tester un grand nombre de constituants en intégrant de très nombreuses données. Cette approche a permis de suivre plusieurs paramètres pour un même échantillon d'hémocytes comme la taille, la complexité (granularité), la mortalité cellulaire, la présence d'estérases non spécifiques, l'activité de phagocytose, la production de radicaux libres, la présence de lysosomes et de mitochondries. Les analyses en cytométrie en flux ont été complétées par un dosage biochimique de l'activité phénol oxidase.

Les essais ont consisté à mettre en contact des hémcytes d'huître creuse, maintenus en culture, avec différents polluants. En effet, nous de disposons pas de lignées cellulaires de bivalves marins et les cellules de l'hémolymphe apparaissent comme de bons candidats dans la mesure où ce sont les cellules supportant l'immunité et que leur prélèvement est aisé (Anderson, 1994 ; Brousseau *et al.*, 2000 ; Sauve *et al.*, 2002).

Une première étape des travaux a été centrée sur la mise au point des conditions d'incubation (**Gagnaire *et al.*, 2006a**). Il a été ainsi choisi de travailler avec des temps de contact courts (24 heures maximum). Les hémocytes peuvent être conservés sur des périodes plus longues, mais dans ce cas des proliférations bactériennes sont très souvent observées. L'hémolymphe n'est en effet pas un milieu stérile. Il est possible d'ajouter des antibiotiques pour contrôler la multiplication des bactéries dans les puits de culture. Cependant, cette voie n'a pas été choisie dans la mesure où des interactions entre antibiotiques et polluants pouvaient être suspectées.

Des premiers travaux, entrepris dans le cadre d'une collaboration entre le Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France) et le Laboratoire de Biologie et Environnement Marins (Université de La Rochelle, France), ont permis de montrer que le mercure pouvait induire *in vitro* la mort des hémocytes, ainsi qu'une inhibition marquée de l'activité de type phénol oxydase, chez l'huître creuse, *C. gigas* alors que le cadmium, testé dans les mêmes conditions n'avait aucun effet (**Gagnaire *et al.*, 2004**).

Dans une seconde étape, 23 molécules, appartenant aux groupes majeurs de polluants (Hydrocarbures Aromatiques Polycycliques (HAP), Polychlorobiphényles (PCB) et pesticides), ont été testées *in vitro* sur des hémocytes d'huître creuse : 5 HAP (benzo(*a*)pyrène (BaP), phénanthrène, pyrène, anthracène et fluroanthène), 2 PCB (PCB 77 et PCB 153) et 16 pesticides (7 herbicides : metolachlor, alachlor, terbutylazine, glyphosate, diuron, atrazine et acide 2,4-dichlorophenoxyacetique (2,4D) ; 6 insecticides : DDT, carbofuran, chlordane, dieldrin, paraoxon et carbaryl ; 2 fongicides: chlorothalonil et fosteylaluminum ; pentachlorophénol (PCP)). Ces composés ont été utilisés individuellement. Cependant, les effets d'un mélange de huit pesticides (carbaryl, fosetyl, aluminum, alachlor, metolachlor, terbutylazine, atrazine, diuron et glyphosate) ont également été recherchés.

Les concentrations utilisées ont variées de 7 $pmolL^{-1}$ à 500 $\mu molL^{-1}$. Elles ont été choisies afin de détecter des effets rapides des polluants. Les paramètres hémocytaires analysés en cytométrie de flux étaient la taille, la complexité (granularité), la mortalité cellulaire, la présence d'estérases non spécifiques, la production de radicaux libres, la présence de lysosomes et l'activité de phagocytose.

Huit des 23 molécules testées ont montré des effets significatifs sur les paramètres hémocytaires suivis. Quatre HAP (BaP, phénanthrène, anthracène et fluoranthène) induisent de manière significative une augmentation du pourcentage de granulocytes et une réduction de la mortalité cellulaire et du pourcentage de cellules positives pour les estérases et les lysosomes après 4 et 24 heures de contact à une concentration de 200 µmol/L. Une réduction significative du pourcentage des hémocytes présentant un marquage des lysosomes est observée en présence du PCB 77 à 6 µmol/L et 60 µmol/L après une incubation de 4 heures. Trois des pesticides (2,4D, paraoxon et chlorothalonil) modulent également certains paramètres hémocytaires. Enfin, le mélange de huit pesticides est capable d'induire une augmentation significative de l'activité de phagocytose après une incubation de 4 heures.

Parmi les paramètres hémocytaires suivis, ce sont les activités estérases non spécifiques et la présence de lysosomes qui apparaissent comme les plus sensibles. Elles sont modulées par six composés. Les lysosomes sont de bons marqueurs de la viabilité des cellules (Moore *et al.*, 1978 ; Lowe & Fossato, 2000 ; Moore, 2002). La sonde utilisée dans ce travail permet de manière concomitante de donner une information sur le nombre de lysosomes et leur activité. Il est possible de suspecter qu'un plus grand nombre de lysosomes ou des lysosomes plus actifs peuvent être à l'origine d'une plus grande capacité de réponse vis à vis d'une agression.

Cette étude est une des premières à investiguer les effets de nombreux polluants de manière concomitante sur les activités hémocyanines chez l'huître creuse et a permis de ce fait de réaliser une comparaison entre polluants.

La démonstration d'un effet de polluants sur les capacités de défense (capacités immunitaires) nécessite de passer par des expositions simultanées ou séquentielles à des polluants et à des agents infectieux. Nous avons pris la précaution d'utiliser le terme d'**activités hémocytaires** et non pas le terme d'activités immunitaires pour l'ensemble des **essais réaliser en présence de polluants uniquement**. En effet, les **hémocytes** sont impliqués dans de **nombreuses fonctions comme l'ingestion et le transport de nutriments** et pas seulement dans des activités de défense.

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Pollutant effects on Pacific oyster, *Crassostrea gigas* (Thunberg), hemocytes: Screening of 23 molecules using flow cytometry

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Abstract

The shellfish industry is an important economic activity in France, occurring mostly in estuarine zones subject to pollution due to anthropogenic activities. The harmful effects of pollutants on species inhabiting these estuarine zones are not well known. Among marine species, bivalve mollusks—particularly Pacific oyster, *Crassostrea gigas*—may serve a model of interest. The species is sedentary and filter-feeding, which favors bioaccumulation of pollutants in their tissues. Oysters may be suitable for studies on disturbance by pollutants of physiological activities, among which defense mechanisms are poorly documented in bivalves. In this study, effects of pollutants on hemocyte functions were monitored in Pacific oyster, *C. gigas*. Hemocytes were exposed *in vitro* to selected pollutants. The strategy for investigating the effects of pollutants on hemocyte functions is based on several biomarkers, which is more relevant than that of published papers based on single-endpoint experiments. Pollutants belonging to the most important groups of xenobiotics (PAHs, PCBs, and pesticides) were selected and their effect on hemocyte activities was analyzed using flow cytometry. Twenty-three pollutants were tested and eight of them showed significant modulation of hemocyte activities. PAHs and PCB 77 induced a decrease of hemocyte activity after an incubation periods of 4 and 24 h at 200 μmol/L. Three pesticides (2,4D, paraoxon, and chlorothalonil) modulated hemocyte activities. A mixture of eight pesticides also decreased phagocytotic activity. This study is one of the first to investigate the effects of so many pollutants on hemocyte functions at the same time and therefore allows a real comparison of different pollutant effects.

Abbreviations: ASW, artificial seawater; BaP, benzo(*a*)pyrene; DHR123, dihydrorhodamine 123; 2,4D, 2,4-dichlorophenoxyacetic acid; FDA, fluorescein diacetate; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; PCP, pentachlorophenol; PI, propidium iodide; ROS, reactive oxygen species

Introduction

Shellfish farming is an ancestral activity in France. It has been developed intensively over the last century and now represents a major economic activity. The Pacific oyster, *Crassostrea gigas*, is the most cultivated species, introduced in France in the 1970s after the decline of the Portuguese

oyster, *C. angulata* (Grizel and Heral, 1991). This species is mostly reared in estuarine zones under the continual threat posed by contamination with pollutants. Natural and synthetic foreign compounds (xenobiotics) enter and are dispersed in aquatic ecosystems by various routes, including direct discharge, land run-off, atmospheric deposition, *in situ* production, abiotic and biotic movements, and food-chain transfer (Livingstone, 1998). Xenobiotics include polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, and heavy metals. Many of these compounds come from anthropogenic activities including industry, fuel transport, and agriculture.

Bivalve mollusks are often used as sentinel organisms in invertebrate immunotoxicology. Their worldwide distribution, their sedentary mode of life, and their filter-feeding behavior, susceptible bioaccumulation of pollutants make them ideal species for the assessment of environmental pollution (Wade et al., 1998; Wootton et al., 2003). Study of the modulation of the immune system, or immunomodulation, in marine mollusks has become one of the most important ways of evaluating the physiological effects of environmental factors (Oubella and Auffret, 1995). Physiological responses of bivalve mollusks to environmental and biological stresses are mediated, in part, by hemocytes circulating within the open vascular system and across epithelial boundaries (Cheng, 1981). Hemocytes are responsible for recognition, phagocytosis, and elimination by oxidation of nonself particles, including viruses, bacteria, and parasites (Cheng, 1981). In bivalve mollusks, it has been well established that hemocytes can be affected by environmental factors such as stress (Lacoste et al., 2002) or pathogens (Oubella et al., 1993; Anderson et al., 1995). In past decades, emerging diseases have been reported in marine species and outbreaks of disease have also increased considerably (Harvell et al., 1999). Anthropogenic compounds may be partly responsible by depressing defensive capacities of host animals and therefore increasing susceptibility to infections, as proposed by several authors (Coles et al., 1994; Pipe and Coles, 1995). Studies have demonstrated effects of PAHs (Coles et al., 1994; Pipe and Coles, 1995), PCBs (Pipe et al., 1995; Canesi et al., 2003), pesticides (Tripp, 1971; Auffret and Oubella, 1997), heavy metals (Cheng et al., 1987; Auffret et al., 2002; Gagnaire et al., 2004), and contaminated sediments (Sami et al., 1993; Oliver et al., 2001) on whole oysters or directly on hemocyte activities.

Flow cytometry is a routine tool in vertebrate biomedical research and it has been applied more recently to marine invertebrate research. This powerful tool has been used to characterize hemocyte population characteristics in oysters (Xue et al., 2001; Goedken and De Guise, 2004) or changes associated with environmental contaminants (Fournier et al., 2001; Sauve et al., 2002b). In the present work, we investigated the *in vitro* effects of 23 xenobiotics on several hemocyte activities of Pacific oyster, *C. gigas*, using flow cytometry. This is one of the first studies to investigate the effect of so many pollutants on hemocyte functions at the same time and therefore allows a real comparison of different pollutant effects. The strategy of using several biomarkers is more relevant than that of published papers based on single-endpoint experiments. The first step of the study focused on optimization of incubation conditions for the subsequent *in vitro* experiments. Toxic molecules were selected from among the major groups of xenobiotics: 5 PAHs, 2 PCBs and 16 pesticides (7 herbicides, 6 insecticides, 2 fungicides and pentachlorophenol (PCP)). Concentrations ranging from 7 pmol/L to 500 μmol/L were used for observation of rapid effects of toxic compounds on oyster hemocytes. The resulting data were based on autofluorescence light scattering parameters related to size and internal complexity (granularity). Cellular activities were monitored using appropriate fluorescent markers with respect to cell mortality, esterase activity, production of reactive oxygen species (ROS), presence of lysosomes, and phagocytosis.

Material and methods

Oysters

Pacific oysters, *Crassostrea gigas*, with a shell length of 7–10 cm, were produced in the IFREMER hatchery of La Tremblade laboratory (Charente-Maritime, France) in February 2002. They were held at Bouin (IFREMER, Vendée, France). Analyses were performed from August to December 2003.

Hemocyte collection

Hemolymphs were collected from the posterior adductor muscle sinus, with the use of a 1 ml syringe equipped with a needle (0.9 × 25 mm) after breaching the shell using pincers. For each oyster, 1–1.5 ml of hemolymph were collected. Hemolymph samples were filtered on a 60 µm mesh to eliminate debris and then maintained on ice to avoid aggregation. Hemocytes from 10 oysters were pooled to reduce inter-individual variation and to provide enough hemocytes for the various exposure protocols. After pooling, hemocytes were counted on a Malassez cell.

Preliminary experiments

Cell treatments. Collected hemocytes were subjected to three different treatments and cell concentration was adjusted to 10^6 cells/ml. One pool received a volume of artificial seawater to adjust cell concentration (treatment 1). The second pool was centrifuged (40*g*, 15 min, 4°C, Microfuge 12, Beckman) and cells were resuspended in artificial seawater (ASW, Lewis composition; for 1 L distilled water: 234 g NaCl, 15 g KCl, 12 g $MgSO_4$. $4H_2O$, 1.5 g $CaCl_2{\cdot}2H_2O$, 1.5 g $CaCl_2$ anhydrous; use at dilution 1/10) (treatment 2). The third pool was centrifuged (40*g*, 15 min, 4°C, Microfuge 12, Beckman), supernatant (serum) was filtered, and the cell pellet was then resuspended in the 0.22 µm-filtered serum (treatment 3). Experiments were performed three times at 15°C or 20°C for 4 h and 24 h. Cell mortality was monitored using flow cytometry as described under "Flow cytometry analysis" below.

Effect of temperature and incubation time on hemocyte activities. For all these experiments, cell concentration was adjusted to 10^6 cells/ml by addition of ASW without centrifugation (treatment 1). Different temperatures (10°C, 15°C, or 20°C) and incubation periods (4, 24, 48, and 72 h) were tested. Experiments were performed three times. Cell mortality and esterase activity were monitored using flow cytometry as described below.

Xenobiotics

Twenty-three xenobiotics were selected on the basis of their immunotoxic potential for invertebrate models described in the literature. They belonged to five families of xenobiotics: PAHs (benzo(*a*)pyrene (BaP), phenanthrene, pyrene, anthracene, fluroanthene), PCBs (PCB 77, PCB 153), herbicides (metolachlor, alachlor, terbutylazine, glyphosate, diuron, atrazine, 2,4-dichlorophenoxyacetic acid (2,4D)), insecticides (DDT, carbofuran, chlordane, dieldrin, paraoxon, carbaryl) and fungicides (chlorothalonil, fosteyl aluminum). Pentachlorophenol (PCP) is a pesticide (herbicide, insecticide, and fungicide). A mixture of 8 pesticides (carbaryl, fosetyl aluminum, alachlor, metolachlor, terbutylazine, atrazine, diuron and glyphosate) was also tested. Solvents were used as recommended by the manufacturer (Table 1) and final solvent concentration was 0.5% in order to avoid disturbance of hemocyte parameters.

Pyrene, anthracene, fluoranthene, PCB 77, PCB 153, PCP, 2,4D, DDT, chlordane, dieldrin, paroxon and chlorothalonil were purchased from Fluka (Sigma-Aldrich). The eight compounds composing the pesticide mixture were purchased from LGC Promochem. BaP, phenanthrene and chlordane were kindly provided by J. Faucet

Table 1. For the 23 pollutants tested individually and the pesticide mixture. Final concentrations of the pollutants in presence of hemocytes and the solvents used are presented

	PAH						PCB	
	BaP (DMSO)	Phenanthrene (DMSO)	Pyrene (CH)	Anthracene (CH)	Fluoranthene (CH)		PCB 77 (TMP)	PCB 153 (TMP)
	200 µmol/L	300 µmol/L	2.5 µmol/L	180 µmol/L	500 µmol/L		60 µmol/L	70 µmol/L
	20 µmol/L	30 µmol/L	250 nmol/L	18 µmol/L	50 µmol/L		6 µmol/L	7 µmol/L
	2 µmol/L	3 µmol/L	25 nmol/L	1.8 µmol/L	5 µmol/L		600 nmol/L	700 nmol/L
	200 nmol/L	300 nmol/L	2.5 nmol/L					70 nmol/L
	20 nmol/L	30 nmol/L	250 pmol/L					
	2 nmol/L	3 nmol/L						
	Pesticides	Herbicides						
	PCP (MeOH)	Metolachlor (A80)	Alachlor (A80)	Terbutvlazine (A80)	Glyphosate (H_2O)	Diuron (A80)	Atrazine (A80)	2.4 D (AN)
	2 µmol/L	7 µmol/L	7 µmol/L	26 µmol/L	12 µmol/L	17 µmol/L	30 µmol/L	450 µmol/L
	200 nmol/L	700 nmol/L	700 nmol/L	2.6 µmol/L	1.2 µmol/L	1.7 µmol/L	3 µmol/L	45 µmol/L
	20 nmol/L	70 nmol/L	70 nmol/L	260 nmol/L	120 nmol/L	170 nmol/L	300 nmol/L	4.5 µmol/L
	2 nmol/L	7 nmol/L	7 nmol/L	26 nmol/L	12 nmol/L	17 nmol/L	30 nmol/L	
	200 pmol/L	700 pmol/L	700 pmol/L	2.6 nmol/L	1.2 nmol/L	1.7 nmol/L	3 nmol/L	
	20 pmol/L	70 pmol/L	70 pmol/L	260 pmol/L	120 pmol/L	170 pmol/L	300 pmol/L	
		Insecticides					Fungicides	
	DDT (MeOH)	Carbofuran (A80)	Chlordane (AN)	Dieldrin (AN)	Paraoxon (CH)	Carbaryl (A80)	Chlorothalonil (AN)	Fosteyl Al (H_2O)
	1.5 µmol/L	120 µmol/L	250 µmol/L	1.3 µmol/L	400 µmol/L	1 µmol/L	200 µmol/L	700 nmol/L
	150 nmol/L	12 µmol/L	25 µmol/L	130 nmol/L	40 µmol/L	100 nmol/L	20 µmol/L	70 nmol/L
	15 nmol/L	1.2 µmol/L	2.5 µmol/L	13 nmol/L	4 µmol/L	10 nmol/L	2 µmol/L	7 nmol/L
	1.5 nmol/L	120 nmol/L	250 nmol/L	1.3 nmol/L		1 nmol/L		700 pmol/L
	150 pmol/L	12 nmol/L				100 pmol/L		70 pmol/L
	15 pmol/L	1.2 nmol/L				10 pmol/L		7 pmol/L
		Pesticide mixture						
	Carbaryl	Fosteyl Al	Alachlor	Metolachlor	Terbutylazine	Atrazine	Diuron	Glyphosate
6	1 µmol/L	700 nmol/L	7 µmol/L	7 µmol/L	26 µmol/L	30 µmol/L	17 µmol/L	12 µmol/L
5	100 nmol/L	70 nmol/L	700 nmol/L	700 nmol/L	2.6 µmol/L	3 µmol/L	1.7 µmol/L	1.2 µmol/L
4	10 nmol/L	7 nmol/L	70 nmol/L	70 nmol/L	260 nmol/L	300 nmol/L	170 nmol/L	120 nmol/L
3	1 nmol/L	700 pmol/L	7 nmol/L	7 nmol/L	26 nmol/L	30 nmol/L	17 nmol/L	12 nmol/L
2	100 pmol/L	70 pmol/L	700 pmol/L	700 pmol/L	2.6 nmol/L	3 nmol/L	1.7 nmol/L	1.2 nmol/L
1	10 pmol/L	7 pmol/L	70 pmol/L	70 pmol/L	260 pmol/L	300 pmol/L	170 pmol/L	120 pmol/L

Solvents: DMSO, dimethyl sulfoxide; CH, cyclohexane; TMP, trimethylpentane; AN, acetonitrile, A80, ethanol 80%; MeOH, methanol. For the pesticide mixture, numbers 1 to 6 correspond to different concentrations of the mixture, and concentrations of the eight components are indicated.

(IFREMER Nantes, France) and F. Quiniou (IFREMER Brest, France).

Exposure protocol

For all exposure experiments, hemocyte concentration was adjusted to 10^6 cells/ml by addition of ASW without centrifugation (treatment 1). Pollutants were added individually at 5 µl per ml of hemocyte suspension. In each experiment, the same volume of corresponding solvent was used as negative control. Six concentrations were tested for BaP, phenanthrene, PCP, metolachlor, alachlor, terbutylazine, glyphosate, diuron, atrazine, DDT, carbofuran, carbaryl, fosteyl aluminum, and the pesticide mixture (Table 1), five concentrations for pyrene (Table 1) and four concentrations for PCB 153, chlordane and dieldrin (Table 1). In addition, three concentrations were tested for anthracene, fluoranthene, PCB 77, 2,4D, paraoxon, and chlorothalonil (Table 1). Hemocytes were incubated at 15°C. Analyses were carried out after a 4 and 24 h incubation periods, except for paraoxon and chlorothalonil for which analyses was carried out only after a 4 h incubation period. Cell mortality, granulocyte percentage, esterase activity, phagocytosis, production of superoxide anion, and presence of lysosomes were analysed using flow cytometry as described below. Experiments were carried out three times for BaP, phenanthrene, DDT, pesticide mixture, PCP and pyrene, twice for 2,4D, anthracene, chlordane, chlorothalonil, dieldrin, fluoranthene, paraoxon, PCB 77, and PCB 153, and once for each of the eight pesticides composing the mixture.

Flow cytometry analysis

Flow cytometry protocols were as previously described (Gagnaire et al., 2003, 2004). For each sample, 3000 events were counted using an EPICS XL 4 (Beckman Coulter). Results were depicted as cell cytograms indicating cell size (FSC value) and cell complexity (SSC value) and the fluorescence channel(s) corresponding to the marker used. A gate was defined on the basis of FSC value in order to eliminate cell debris. Recorded fluorescence depended on monitored parameters: enzymes, lysosomes, and phagocytosis were measured using green fluorescence, and cell mortality was measured using red fluorescence. Mortality was quantified using 200µl of hemocyte suspension. Hemocytes were incubated in the dark for 30 min at 4°C with 10 µl of propidium iodide (PI, 1.0 mg/L, Interchim). The EPICS XL 4 software allows differentiation between supposed populations of granulocytes and hyalinocytes based on their FSC and SSC values. Esterase activity was measured using the nonspecific liposoluble substrate fluorescein diacetate (FDA, Molecular Probes). FDA solution (1 µl of 400 µmol/L) was added to 200 µl of hemocyte suspension. Cells were incubated for 30 min in the dark at room temperature and the reaction was stopped on ice (5 min). Presence of lysosomes was measured using a commercial kit (LysoTracker® Green DND-26, 1 mmol/L in DMSO, Molecular Probes). A 1µl aliquot of LysoTracker® was added to 200 µl of hemocyte suspension. Cells were incubated for 2 h in the dark at room temperature and the reaction was stopped on ice (5 min). Production of ROS was measured using dihydrorhodamine 123 (DHR123, Molecular Probes), specific for superoxide anion O_2^-. DHR123 solution (1 µl of 145 µmol/L) was added to 200 µl of hemocyte suspension. Cells were incubated for 30 min in the dark at room temperature and the reaction was stopped on ice (5 min). Phagocytosis was measured by ingestion of fluorescent beads. Hemocyte suspension (200 µl) was incubated for 1 h in the dark at ambient temperature with 10 µl of a 1/10 dilution of Fluorospheres® carboxylate-modified microspheres (1 µm diameter, Interchim).

For esterase activity, ROS, and lysosomes, gates were defined on the cytograms to distinguish different populations according to fluorescence intensity (Figure 1). Events were previously gated in order to analyze only cells and not bacteria or debris. Analysis was performed on the whole

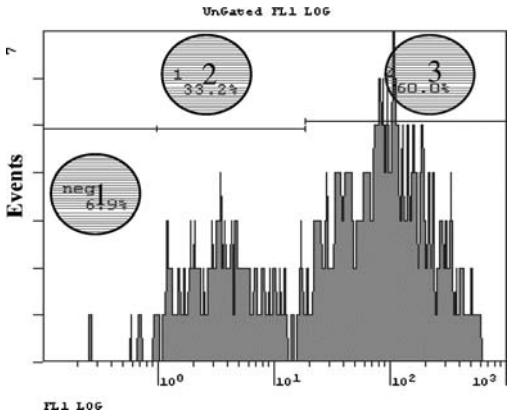

Figure 1. Flow cytometry cytogram of cells stained with FDA. Horizontal-axis, intensity of fluorescence; Vertical-axis, number of cells: (1) population of negative cells; (2) population of moderately stained cells; (3) population of strongly stained cells.

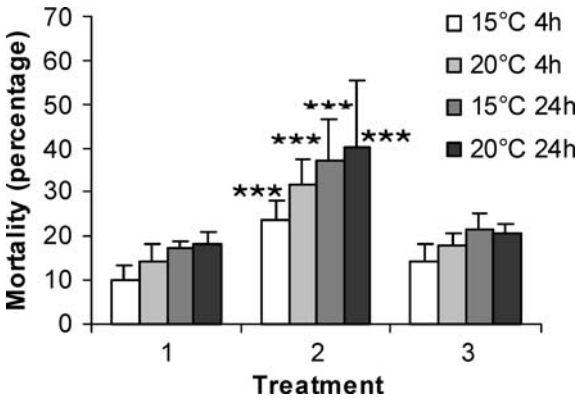

Figure 2. Cell mortality percentage after a 4 h incubation period at 15°C or 20°C. (1) Cells adjusted to 10^6 cells/ml by addition of ASW; (2) cells adjusted to 10^6 cells/ml by resuspension in ASW after centrifugation; (3) cells adjusted to 10^6 cells/ml by resuspension in hemolymph after centrifugation. Values are means of 3 replicates. Bars represent standard error. Asterisks indicate a significant difference (increase) between the three treatments for one given condition (temperature and incubation time);*** $p < 0.001$.

population of cells, live and dead. Gates obtained were similar for all cytograms. Three cell populations were defined: a population of negative cells, a population of moderately stained cells, and a population of strongly stained cells. For these three parameters, further analyses were realized only on the population of strongly stained cells.

Statistical analysis

Results were expressed as percentage of positive cells. Values were converted into *r* angular arcsine $\sqrt{}$(% of positive cells) before analysis. Values were normally distributed after transformation. ANOVA analysis was carried out using Statgraphics. Plus version 5.1 software to detect any effect of the tested pollutants. In the case of rejection of H_0, an *a posteriori* test was used. Significance was set at $p \leq 0.05$.

Results

Maintaining hemocytes in vitro

Cell treatments before culture. Among the three treatments, treatment 2 (centrifugation and cells resuspended in ASW) caused the highest mortality ($p < 0.001$) for both temperatures tested after 4 h and 24 h incubation periods (Figure 2). No significant difference was found between treatments for 1 and 3, or for effect of incubation temperature on percentage mortality (Figure 2). Treatment 1 was chosen for xenobiotic exposure.

Effects of temperature and time of culture on hemocyte activities. Cell mortality increased significantly after 48 h and 72 h incubation periods at 15°C compared to 4 h and 24 h. At 20°C, mortality increased significantly after 24 h and 48 h incubation periods compared to 4 h ($p < 0.01$, Figure 3). No variation of mortality percentage was observed at 10°C (Figure 3).

For all temperatures, percentage of esterase-positive cells was significantly lower after 48 h and 72 h incubation periods compared to 4 h and 24 h ($p < 0.01$, Figure 4). Cells incubated at 20°C also showed a significant decrease of positive cell percentage after a 24 h incubation period compared to 4 h ($p < 0.01$, Figure 4).

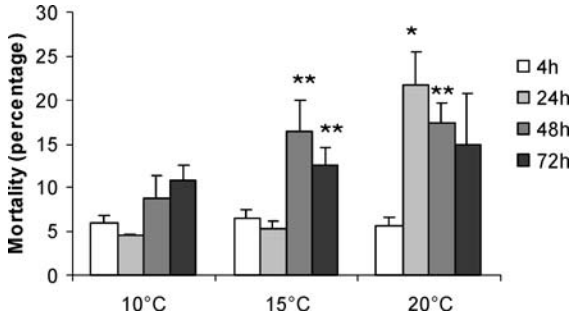

Figure 3. Cell mortality percentage after different incubation periods at 10°C, 15°C, or 20°C. values are means of 3 replicates. Bars represent standard error. Asterisks indicate a significant difference (increase) between the four incubation periods for one given temperature;** $p < 0.01$.

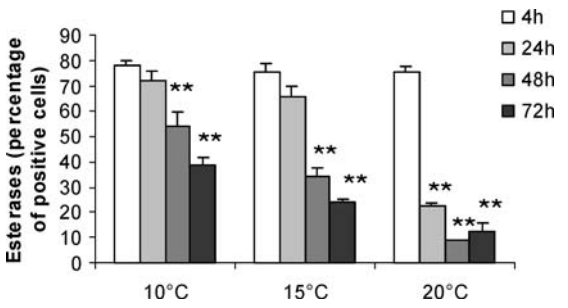

Figure 4. Percentage of esterase-positive cells after different incubation periods at 10°C, 15°C, or 20°C. Values are means of 3 replicates. Bars represent standard error. Asterisks indicate a significant difference between the four incubation periods for one given temperature;** $p < 0.01$.

On the basis of those results we chose a temperature of 15°C and incubation periods of 4 h and 24 h for further xenobiotic exposures.

Modulations of hemocyte parameters by pollutants

Among the 23 pollutants tested, eight showed significant modulations of at least one hemocyte parameter (Table 2). These eight compounds belong to different families of xenobiotics: four PAH (BaP, phenanthrene, antracene, fluoranthene), one PCB (PCB 77), one herbicide (2,4D), an insecticide (paraoxon) and a fungicide (chlorothalonil). The pesticide mixture also showed significant effects. However, there was no significant effect of PCP on hemocyte parameters.

PAHs. BaP and phenanthrene had similar effects on hemocyte parameters. They significantly increased granulocyte percentage but significantly decreased cell mortality and esterase- and lysosome-positive cells for the highest concentration tested compared to the control (200 µmol/L for BaP and 300 µmol/L for phenanthrene) after 4 h and 24 h incubation periods (Table 2). Anthracene at 180 µmol/L induced similar effects to BaP and phenanthrene, except for a decrease of cell mortality after a 24 h incubation period ($p < 0.01$, Table 2) and of esterase- and lysosome-positive cell percentage after a 4 h incubation period ($p < 0.01$, Table 2). Fluoranthene significantly decreased esterase-positive cells only at 500 µmol/L after a 4 h incubation period compared to control ($p < 0.05$, Table 2). Values for the negative control for both BaP and phenanthrene ranged between 66% and 70% after a 4 h incubation period and decreased significantly to 43.9% with 200 µmol/L BaP and to 11.1% with 300 µmol/L phenanthrene ($p < 0.01$, Figure 5). After a 24 h incubation period, values for controls for both pollutants were 27% and decreased to 16.8% with 200 µmol/L BaP and to 2% with 300 µmol/L phenanthrene (Figure 5).

PCBs. PCB 77 significantly decreased lysosome-positive cell percentage at 6 µmol/L and 60 µmol/L after a 4 h incubation period compared to control ($p < 0.05$, Table 2).

Herbicides. The presence of 2,4D increased cell mortality at the highest concentration of 450 µmol/L after a 4 h incubation period compared to control ($p < 0.05$, Table 2). The value was 7.8% in the control and increased to 11.8% with 450 µmol/L 2,4D (Figure 6).

Insecticides. Paraoxon exposure significantly decreased the percentage of esterase-positive

Table 2. Effects of pollutants on *C. gigas* hemocyte activities after an *in vitro* contact. For each pollutant, the concentration (s) and the period (s) of incubation for which an effect of the pollutant was observed are indicated

Group	Compound	Mortality	Granulocytes (percentage)	Esterases	Lysosomes	ROS	Phagocytosis
PAH	BaP	▼200 µmol/L 4 h and 24 h **	▲200 µmol/L 4 h and 24 h **	▼200 µmol/L 4 h and 24 h *	▼200 µmol/L 4 h and 24 h *		
	Phenanthren	▼300 µmol/L 4 h and 24 h *	▲300 µmol/L 4 h and 24 h *	▼300 µmol/L 4 h and 24 h *	▼300 µmol/L 4 h and 24 h **		
	Anthracen	▼180 µmol/L 24 h *	▲180 µmol/L 4 h and 24 h **	▼180 µmol/L 4 h **	▼180 µmol/L 4 h **		
	Fluoranthen			▼500 µmol/L 4 h *			
PCB	PCB 77				▼6 µmol/L and 60 µmol/L 4 h *		
Herbicides	2,4D	▲450 µmol/L 4 h *					
Insecticides	Paraoxon			▼400 µmol/L 4 h and 24 h **	▼40 µmol/L and 400 µmol/L 24 h **	▲400 µmol/L 4 h *	
Fungicides	Chlorohalonil	▲2 µmol/L 4 h *	▲200 µmol/L 4 h **	▼200 µmol/L 4 h *			
Pesticides	Pesticide mixture						▲ cc = 6 4 h *

▲ = increase; ▼ = decrease. * $p < 0.05$;** $p < 0.01$.

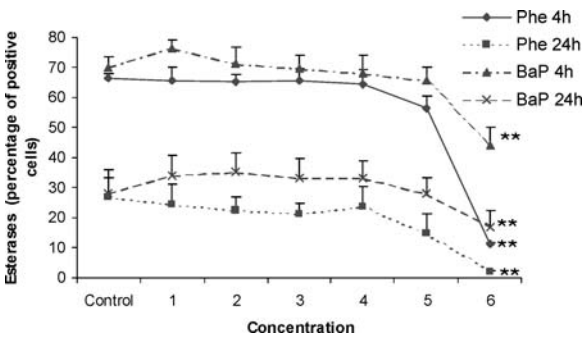

Figure 5. Percentage of esterase-positive cells after 4 h and 24 h incubation periods with increasing concentrations of BaP or phenanthrene (Phe). BaP:(1)2 nmol/L; (2) 20 nmol/L; (3) 200 nmol/L; (4) 2 µmol/L; (5) 20 µmol/L; (6) 200 µmol/L. Phe:(1) 3 nmol/L; (2) 30 nmol/L; (3) 300 nmol/L; (4) 3 µmol/L; (5) 30 µmol/L; (6) 300 µmol/L. Values are means of 3 replicates. Bars represent standard error.** $p < 0.01$.

cells after 4 h and 24 h incubation periods at 400 µmol/L compared to control ($p < 0.01$, Table 2). A similar effect was found on percentage of lysosome-positive cells at 40 µmol/L

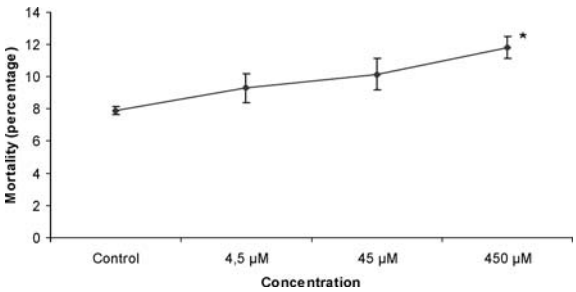

Figure 6. Cell mortality percentage after a 4 h incubation period with increasing concentrations of 2,4D. Values are means of 2 replicates. Bars represent standard error.* $p < 0.05$.

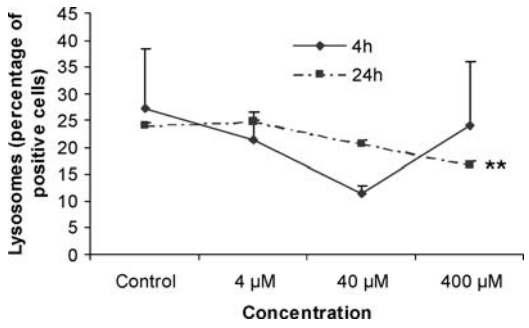

Figure 7. Percentage of lysosome-positive cells after 4 h and 24 h incubation periods with increasing concentrations of paraoxon. Values are means of 2 replicates. Bars represent standard error. ** $p < 0.01$.

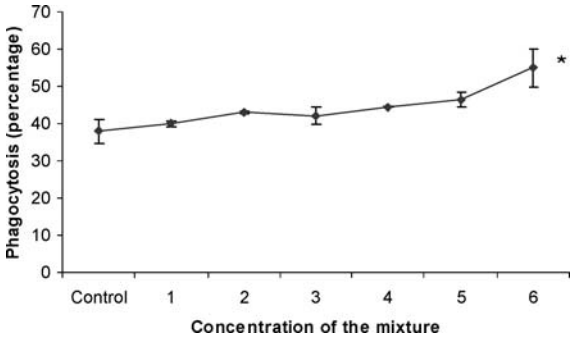

Figure 8. Phagocytosis percentage after a 4 h incubation period with increasing concentrations of the pesticide mixture. See Table 1 for the different concentrations of the eight pesticides in the mixture. Values are means of 3 replicates. Bars represent standard error.* $p < 0.05$.

and 400 µmol/L but only after a 24 h incubation period ($p < 0.01$, Table 2). ROS-positive cell percentage increased significantly at 400 µmol/L after a 4 h incubation period ($p < 0.05$, Table 2). Percentages of lysosome-positive cells were 24% in the negative control and decreased to 16.7% at 400 µmol/L after a 24 h incubation period (Figure 7). However, no significant effect was reported after a 4 h incubation period (Figure 7).

Fungicides. The presence of chlorothalonil significantly increased cell mortality at 2 µmol/L ($p < 0.05$) and granulocyte percentage at 200 µmol/L ($p < 0.01$) and significantly decreased esterase-positive cells percentage at 200 µmol/L ($p < 0.05$) after a 4 h incubation period (Table 2).

Pesticide mixture. Exposure to the pesticide mixture significantly increased phagocytosis activity after a 4 h incubation period at the highest concentration tested ($p < 0.05$, Tables 1 and 2). Values increased from 37.9% in the negative control to 54.8% at maximal concentration (Figure 8).

Discussion

In order to allow comparison of results, hemocyte concentrations must be adjusted (Brousseau et al., 2000; Fournier et al., 2001, 2002). However, techniques for cell adjustment are poorly documented. Our results showed that cell resuspension in ASW after centrifugation increased cell mortality. Cellular parameters including esterase activity were modified by centrifugation and were dependent on the medium used for cell resuspension (data not shown). For immunotoxicity studies, modification of cellular activities must be due only to pollutant tested and not to culture conditions. We thus added ASW to adjust cell concentration. Under these conditions, no change in cell parameters was observed.

In experiments concerning the effects of temperature and time of incubation on hemocyte activities, esterase activities decreased as cell mortality increased. This has been reported previously for phagocytosis and cell viability (Brousseau et al., 2000). Protocols of flow cytometry used in this study are therefore validated and allow also validations of further results on pollutant effects. Lysosomes have been reported to be a valid marker of cell viability (Moore et al., 1978; Lowe et al., 1995; Lowe and Fossato, 2000). A decrease of lysosome-positive cells could also have been related to an increase of cell mortality. However, in these experiments we tested only esterase activity.

In vitro exposure of cells to pollutants may provide an approach to evaluation of pollutant

toxicity, although several mechanisms, including mucus in the mantle cavity or alimentary canal, may be involved in the toxicity of pollutants under *in vivo* conditions (Fisher et al., 1987). *In vitro* conditions are frequently used for the assessment of pollutant toxcity in bivalve hemocytes (Larson et al., 1989; Alvarez and Friedl, 1992; Anderson et al., 1992; Baier-Anderson and Anderson, 2000; Brousseau et al., 2000; Gagnaire et al., 2004).

This study confirmed significant effects of PAHs on hemocyte viability. Our results also revealed that PAHs significantly decreased lysosomes and esterases. This is in accordance with literature reports (Wootton et al., 2003). Lysosomes appeared to be one of the most important target of PAHs (Moore et al., 1978; Lowe et al., 1995). There are reports that PAHs increased the release of peroxidase and ROS production, both mechanisms occurring in oxidative burst (Coles et al., 1994; Gomez-Mendikute et al., 2002). Our results did not support this hypothesis. However, our detection method permits quantification only of superoxide anion, O_2^-, but not of all components of the oxidative burst. Moreover, as PAHs significantly decreased lysosomes and esterases, we hypothesize that hydrolytic enzymes, which also act in oxidative burst, may be affected by PAHs. Granulocyte percentages were increased in the presence of PAHs. We can hypothesize that percentage of hyalinocytes decreased because they were killed by pollutants. Granulocytes may be more resistant.

We found an effect on PCB 77 on lysosomes but not on ROS release or esterase activity. Some studies have demonstrated the effects of PCBs to be congener-specific (Coteur et al, 2001). In mussels PCBs act on targets corresponding to those found in human neutrophils, i.e. signal transduction pathways involved in the immune response (Canesi et al., 2003). Moreover, PCBs might be more toxic in mixtures (Harper et al., 1995).

Lysosomes appear to be a more generalized target of toxic stress induced by PAHs and PCBs. Lysosomal enzymes are considered to play an important role in invertebrate defense reactions involving bacterial destruction (Moore et al., 1978; Moore and Gelder, 1985). Their functional impairment may affect host resistance to a pathogen infection (Anderson, 1981).

No effect was detected in this study with PCP. However, a previous study showed that PCP decreased ROS production in *C. virginica* (Baier-Anderson and Anderson, 1996). PCP is an uncoupler of oxidative phosphorylation and has been shown to inhibit NADPH, a component involved in ROS production (Anderson, 1981). The probe used in our study, DHR 123, is for detection of superoxide anion. NADPH production was reported to take place earlier in the oxidative burst mechanism (Anderson, 1981) and our probe may not be an effective indicator of NADPH production.

A previous study reported no effect of atrazine on hemocyte parameters in *C. gigas* (Gaganaire et al., 2003). Here, the range of herbicides tested showed no effect. Herbicides would act on photosynthesis. Studies on herbicides have mostly shown a genetoxic potential of herbicides such as glyphosate (Lioi et al., 1998) and atrazine (Bouilly et al., 2003) in both vertebrates and invertebrates.

Organophosphorus compounds and carbamates including paraoxon and carbaryl are known to be acetylcholinesterase and carboxylesterase inhibitors in vertebrates (Cooreman et al., 1993) and in musels (Orzetic and Krajnovic-Ozretic, 1992; Galloway et al., 2002). However, cholinesterases in *C. gigas* were insensitive to insecticides (Bocquene et al., 1997). We found a decrease of esterase- and lysosome-positive cell percentages with paraoxon. Esterases measured in this study were nonspecific esterases.

Dieldrin and chlordane induced a decrease of phagocytosis of *C. virginica* hemocytes (Larson et al., 1989). We found no effect on phagocytosis at equivalent concentrations for both compounds. However, the previous authors used chemiluminescence to measure phagocyotic activity.

A few studies have explored effects of fungicides on oysters. Exposure of hemocytes from *C. virginica* to chlorothalonil and triforine induced a

decrease of ROS production and of cell viability and phagocytotic activity (Alvarez and Friedl, 1992; Baier-Anderson and Anderson, 2000). Our results showed effects on percentages of esterase- and lysosome-positive cells at similar concentrations. These compounds thus appear to modulate hemocyte activities.

The pesticide mixture (alachor, metolachlor, terbutylazine, glyphosate, diuron, atrazine, carbaryl and fosteyl aluminium) is representative of pollutants in the surface waters of the Marennes-Oleron Basin (Charente-Maritime, France). Concentrations of these compounds in the environment are between 0.25 nmol/L and 4 nmol/L (Léonard, 2002). Interestingly, none of these eight compounds generated significant effects when tested individually on *C. gigas* hemocytes. However, as a mixture, the same compounds increased phagocytotic activity. The vast majority of available toxicity data is related to single substances, not to mixtures. However, in the environment, molecules are usually present concomitantly (Moore, 2002). Moreover, a study on joint toxicity of multiple-component triazine mixtures demonstrated that the toxic effects of the mixtures exceeded those of each component alone (Faust et al., 2001). This synergistic effects (or concentration addition) may be enhanced by the fact that the different compounds possess a same specific target, and also by their additional effects on different sites (Faust et al., 2001).

Among the 23 pollutants tested (belonging to five groups of pollutants), at least one compound in each group showed effects on at least one hemocyte parameter. It may therefore be concluded that all groups of pollutants (PAHs, PCBs, herbicides, insecticides, and fungicides) may be hazardous to bivalve defense systems. However, PAHs appeared to be the group inducing more modulations, since four of the five PAHs tested showed significant effects. This work is the first to study such large number of pollutants from different groups on hemocytes of the Pacific oyster, *C. gigas*. However, only one mixture of pesticides was tested. This work needs to be extended by testing mixtures of different pollutants by groups. Moreover, several of the xenobiotics selected in this study need to be bioactivated in order to become toxic. In this *in vitro* type of exposure, we can never be assured that hemocytes have the ability to perform this activation. It is therefore difficult to compare the toxicity of these chemicals. We may hypothesize that under *in vivo* exposure conditions these xenobiotics could be more toxic.

This study showed that pollutants can increase or decrease hemocyte parameters. However, we cannot conclude whether an increase in a given hemocyte activity is related to a positive or a negative effect on immunity. Hemocytes are involved in other physiological processes (Cheng, 1981; Fisher, 1986) and hemocyte activities are therefore not indicators only of defensive activities. We may hypothesize that an increase or a decrease of hemocyte activities correspond to a perturbation of the immune system and, as other authors have advanced, that pollutants induce modulation of the immune system via toxic effects (Oubella and Auffret, 1995). Moreover, several studies have demonstrated that pollutants can have inhibitory or stimulatory effects depending on the dose (Cheng and Sullivan, 1984; Sauve et al., 2002a). In vertebrates, an increase of phagocytotic activity may be related to an enhancement of cell sensitivity and may lead to the development of autoimmune diseases (Christin et al, 2004). An increase of hemocyte activity is not necessarily related to a better defensive capacity of the organism.

Demonstration of the link between hemocyte parameters and immune activities requires experiments involving exposure to pollutants and pathogens. Several authors have already demonstrated that alteration of hemocyte parameters in bivalves exposed to pollutants could be associated with an increase of disease susceptibility (Chu et al., 2002; Coles et al., 1994, 1995; Fournier et al., 1988; Pipe et al., 1999).

No bivalve cell lines are available and experiments have therefore to be conducted on cells

maintained *in vitro* (Anderson, 1994; Brousseau et al., 2000; Sauve et al., 2002a). Hemocytes are of interest because of their ease of collection.

Among the hemocyte parameters tested, the presence of lysosomes and esterase activity appeared to be the most sensitive. These were modulated by six pollutants. As demonstrated previously, lysosomes are biomarkers of cellular viability (Moore et al., 1978; Lowe and Fossato, 2000; Moore, 2002). The probe used in this study allowed us to measure the presence of lysosomes. We may hypothesize that a high number of lysosomes is correlated with a greater capacity of the cell to respond to insult. A few studies have reported evolution of esterase activity, other involving acetyl- and carboxylesterases (Cooreman et al., 1993).

Production of superoxide anion as measured by DHR123 was affected bonly by paraoxon. However, this parameter may also be modulated by PAHs (Gomez-Menkikute et al., 2002). Other studies used different methods for flow cytrometric measurement of superoxide anion with DCFH-DA (Lambert et al., 2003). Further comparative studies on both of these components are required.

Conclusion

This study is the first to analyze the *in vitro* effects of 23 xenobiotics on six oyster hemocyte parameters. All groups of pollutants had an effect on one or more hemocyte parameters. These results need to be confirmed by *in vivo* experiments in order to demonstrate the effects of these potential immunomodulators in the whole animal.

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4. 2. 2. Des essais in vivo (Gagnaire B., Renault T., Bouilly K., Lapègue S. & H. Thomas-Guyon, 2003, Current Pharmaceutical Design, 9: 193-199)

Afin de compléter les résultats obtenus *in vitro*, des essais *in vivo* ont été réalisés. Les expérimentations effectuées ***in vitro*** sur hémocytes de coquillages permettent de tester un **grand nombre de molécules** et de faire de cette manière un **screening rapide**. Cependant, cette approche présente l'inconvénient majeur de ne pas prendre en compte les **interactions complexes** qui peuvent exister dans un organisme.

Dans ce contexte, afin de mieux comprendre la capacité de polluants à moduler les activités des hémocytes, des huîtres creuses maintenues dans des bacs au Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France) ont été mises en contact avec diverses molécules (**cadmium, diuron et atrazine**). Le choix des concentrations testées dans les expérimentations menées *in vivo* a été basé sur les concentrations observées dans l'environnement pour les polluants sélectionnés (pics environnementaux).

Par ailleurs, un autre objectif pour ces expérimentations était de développer **un modèle de modulation des capacités hémocytaires chez l'huître creuse** afin d'étudier les relations qui peuvent exister entre **polluants, réponse immunitaire et sensibilité aux maladies infectieuses**. En effet, si de nombreux travaux rapportent les effets de contaminants sur les capacités de défense chez les mollusques, il existe peu de références bibliographiques confirmant la corrélation entre immunité compromise et sensibilité aux maladies infectieuses.

Un herbicide, l'atrazine, a été utilisé dans une première expérimentation (**Gagnaire *et al.*, 2003**). Des huîtres adultes ont été soumises à deux concentrations d'atrazine (46,5 et 465 nM) pendant trois semaines. La dose la plus faible correspond à la concentration maximale observée dans le bassin de Marennes Oléron (Charente Maritime, France). Plusieurs paramètres hémocytaires ont été analysés en cytométrie en flux (mortalité cellulaire, proportion de hyalinocytes, présence d'enzymes (estérases, cathepsines, peroxydases et aminopeptidases) et activité de phagocytose). Aucun de ces paramètres n'a été affecté par le polluant pour les deux concentrations testées chez les animaux *in vivo*.

Deux expérimentations ont aussi été menées respectivement avec du cadmium (**Bouilly *et al.*, 2006**) et du diuron (**Bouilly *et al.*, 2007**) afin de tester leurs effets respectifs sur l'aneuploïdie et certains paramètres hémocytaires. Pour ces deux polluants, aucun effet sur les activités cellulaires suivies en cytométrie de flux (mortalité hémocytaire, activité de phagocytose, production de radicaux libres et présence de lysosomes) n'a été détecté pour les concentrations testées incluant les concentrations maximales rapportées dans le bassin de Marennes Oléron (Charente Maritime, France). Ces deux molécules induisent par contre une augmentation du nombre d'animaux aneuploïdes (huîtres présentant 17, 18 ou 19 chromosomes au lieu de 20).

L'ensemble de ces expérimentations n'a pas permis de montrer d'effets sur les hémocytes pour les trois polluants testés (cadmium, diuron et atrazine). **Ces composants ne sont donc pas des candidats d'intérêt pour définir un modèle d'immunomodulation par un polluant chez l'huître creuse, *C. gigas***. Sur la base de résultats obtenus *in vitro* (**Gagnaire *et al.*, 2006**), il a été décide de travailler avec un mélange de huit pesticides (cf. 4. 3. Avec une pincée de bactéries).

Study of Atrazine Effects on Pacific Oyster, *Crassostrea gigas*, Haemocytes

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Abstract: Shellfish farming is an important economic activity around the world. This activity often takes place in areas subjected to various recurring pollutions. The recrudescent use of herbicides in agriculture including atrazine implies pollutant transfer towards aquatic environment in estuarine areas. Harmful effects of such substances on animals in marine environment, particularly on cultured bivalves, are poorly documented. Bivalve molluscs such as mussels and oysters have been postulated as ideal indicator organisms because of their way of life. They filter large volumes of seawater and may therefore accumulate and concentrate contaminants within their tissues. Moreover, development of techniques allowing effect analysis of such compounds on bivalve biology may lead to the development of diagnosis tools adapted to analyze pollutant transfer towards estuarine areas. In this context, influence of atrazine on defence mechanisms was analyzed in Pacific oysters, *Crassostrea gigas*. Atrazine was tested *in vitro* and *in vivo* on oyster haemocytes, and its effects were analyzed by flow cytometry. Haemocyte viability, cell cycle and cellular activities were monitored. Atrazine induced no significant effect in oyster under tested conditions except for peroxidase activity.

Key words: *Crassostrea gigas*; Haemocytes; Flow cytometry; Atrazine; Cellular activities; Toxicity.

INTRODUCTION

In the last decades shellfish culture became particularly important economic activity around the world. The most economically important bivalve species is the Pacific oyster, *Crassostrea gigas*, with about 95% of the production in the world. This species is primarily reared in estuarine areas, which are subject to various pollutants from agricultural or industrial wastes. Bivalve molluscs are considered as indicator organisms for assessing environmental pollution levels. They filter large volumes of seawater and thus may bioconcentrate environmental contaminants within their tissues. The vulnerability of aquatic species to chemical pollution depends of pollutant properties, pollutant concentrations entering ecosystems and capacity of ecosystems to resist to pollutants [1].

Our work focused on atrazine effects on *Crassostrea gigas* immune response. Atrazine, a selective herbicide, is widely used in agriculture even though real effects of this substance have been little defined. Studies on atrazine were essentially carried out on genotoxicity and physiological changes.

Bivalve molluscs have an open circulatory system which is continually exposed to contaminants. These pollutants may directly interact with haemocytes that are involved in oyster defence system (phagocytosis, production of hydrolytic enzymes and reactive oxygen metabolites). Atrazine was tested *in vitro* by direct contact with haemocytes freshly collected and *in vivo* by contact with adult oysters using different concentrations. Our study was carried out at cellular level using flow cytometry. This technology allows rapid analysis of morphological characteristics of free cell suspensions. Resulting data are acquired by autofluorescence light scattering parameters related to cell size and granularity. Cellular activities may also be quantified with corresponding fluorescent markers. Proportion of granulocytes and hyalinocytes, cell viability, cellular cycle, presence of hydrolytic enzymes, and phagocytosis activity were monitored.

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MATERIALS AND METHODS

Experimental Animals

Two years-old Pacific oysters, *Crassostrea gigas*, 8-10 cm in shell length were purchased from a shellfish farm located in Marennes-Oleron Bay (La Tremblade, Charente-Maritime, France) on the French Atlantic coast from February to April 2001. All animals originated from the same resource. They were acclimated during two weeks in the Ifremer hatchery.

Circulating Haemocyte Collection

After opening oyster shells by cutting off the adductor muscle, haemolymph was withdrawn directly from the

pericardial cavity by puncture with a 1-mL syringe equipped with a needle (0,9525 mm). For each oyster, 0.5-1 mL of haemolymph was withdrawn. Haemolymphs from 30 oysters were pooled to eliminate possible inter-individual variation and to provide haemocytes to fulfil requirements of pollutant exposure protocol assays.

In Vivo Exposure Protocol

Oysters were exposed to water containing atrazine supplied by INRA (Saint-Laurent-de-la-Prée, Charente-Maritime, France). Two concentrations of atrazine were used (46.5 and 465 nM). For each concentration and for control, two batches were established. Oysters were acclimated six days in raceways. Then animals received pollutant and were maitained three weeks in raceways without seawater recirculation. Water samples from raceways were analyzed (CEMAGREF, Bordeaux, France) all experience long in order to control atrazine levels. Each raceway contained 157 L of seawater including atrazine. Water was changed every day. Raceways and biologic filters were cleared out and filled up with water maintained at 19.5°C+/-1°C. A volume of 157 mL of atrazine at 46.5 and 465µM diluted in 5 L of seawater was added in corresponding raceways. Oysters were daily fed with 8 L of *Isochrysis galbana* (6.10^6 cells per mL) and 3.5 L of *Tetracelmis suecica* ($1.5.10^6$ cells per mL) for each raceway.

Haemolymphs from four oysters were pooled in order to realise all the tests in flow cytometry.

In Vitro Exposure Protocol

Atrazine was tested using various concentrations (0-9.3-93-930µM respectively). Atrazine dilutions were prepared in dimethylsulfoxide (DMSO). Atrazine was added on

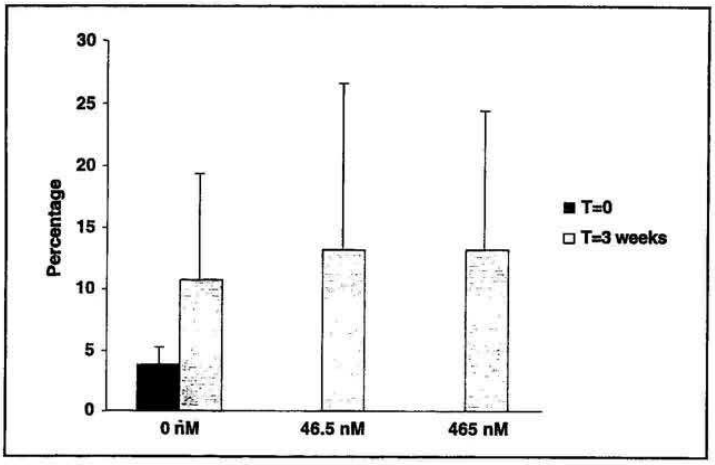

Fig. (1). Haemocyte mortality monitored by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

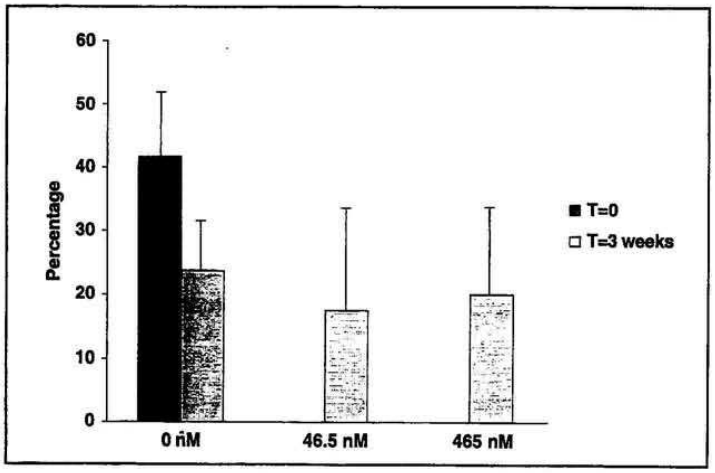

Fig. (2). Peroxidase activity monitored by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

haemocytes in low quantities : two µL of pollutant per mL of pooled haemolymph. Four and 24 hour incubations were carried out at ambient temperature in the dark.

Cellular Activity Analysis by Flow Cytometry

Haemocytes were analyzed with a flow cytometer (Coulter EPICS XL 4). For each haemocyte samples, 10 000 events were counted. Results were expressed as cell cytograms indicating size (FSC value), complexity (SSC value) and fluorescence channel(s) corresponding to the marker used. Fluorescence depended of monitored activities : enzymatic activities and phagocytosis were measured using FL1 (green fluorescence) and mortality using FL3 (red fluorescence).

Esterase, aminopeptidase, cathepsin and peroxidase activities were evaluated with commercial kits (Cell Probe™ Reagents, Beckman Coulter). Each analysis required 200µL of haemolymph and 10µL of the corresponding kit (FDA•Esterase, TP•Cathepsin, A•Aminopeptidase M and DFCH, PMA•Oxidative Burst). Haemocytes were then incubated in the dark at ambient temperature 15 minutes.

Mortality was quantified using 200µL of haemolymph. Haemocytes were incubated in the dark 30 minutes at 4 °C with 5µL of propidium iodide (PI, 1.0 $mg.mL^{-1}$, Interchim).

Cell cycle was analyzed using 200µL of haemolymph. Haemocytes were incubated in the dark two hours at 4°C with 20µL of PI and 200µL of lysing buffer. Percentages of haploïd cells (gamets), diploïd cells (G_0G_1) and tetraploïd cells (G_2M) were determined.

Phagocytosis was measured by ingestion of fluorescent beads (Fluorospheres® carboxylate-modified microspheres, diameter 1µm, Interchim). Five hundred µL of haemolymph were incubated one hour in the dark at ambient temperature with 10µL of a 1/10 dilution of beads.

Morphological characteristics of haemocytes were also analyzed. Two hundred µL of haemolymph were analyzed without any additional product. Proportion of hyalinocytes and granulocytes were defined by FSC and SSC parameter analysis.

Statistical Analysis

All cellular parameters were analyzed three times after four and 24 hour incubations. Results were analyzed using a non-parametric Kruskall-Wallis test for independent samples. In the case of the reject of H_0, an *a posteriori* Student-Newman-Keuls (SNK) test was used.

RESULTS

Atrazine Effects on Haemocyte Viability and Cellular Activities *in Vivo*

Haemocyte viability and peroxidase activity were similar in control and treated cells after a three week-atrazine exposure (Figures 1 and 2). Statistical analysis confirmed that atrazine had no effect on haemocyte viability and peroxidase activity after treatment, whatever concentration used. However, both parameters were different in controls at time 0 and after three weeks. Haemocyte mortality increased in controls after a three week period (Figure 1), while percentages of peroxidase positive cells decreased (Figure 2).

Esterase positive cells and number of dividing cells increased with atrazine concentrations (Figures 3 and 4). However, statistical analysis indicated that increase of dividing cells and esterase positive cells was not significant. Furthermore, esterase positive cell percentages did not seem to be affected by a three week period in raceways (Figure 3),

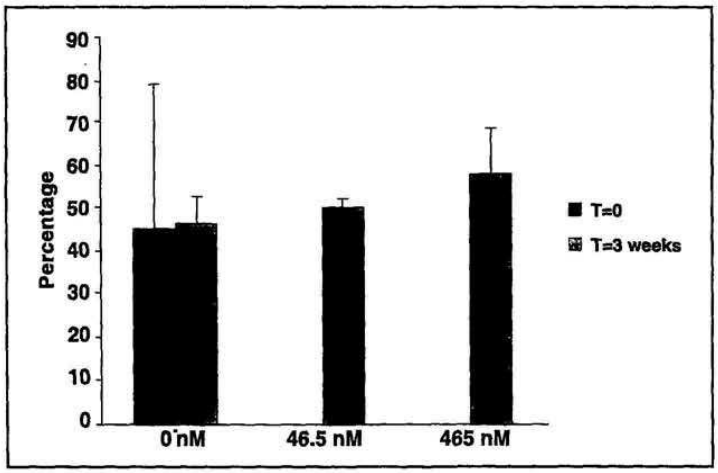

Fig. (3). Esterase activity monitored by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

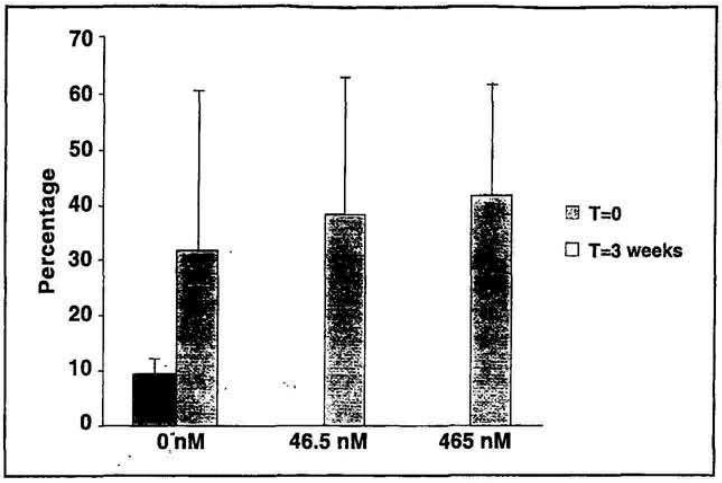

Fig. (4). Dividing cell number (G_2M) determined by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

while number of dividing cells was higher in controls after three weeks than in controls at time 0 (Figure 4).

No change was reported after atrazine treatment for all other tested parameters (cathepsins, aminopeptidases, phagocytosis activity and hyalinocyte percentage).

Atrazine Effects on Haemocyte Viability and Cellular Activities *in Vitro*

Esterase activity and haemocyte viability were similar in control and treated cells after a four hours incubation in presence of atrazine (Figures 5 and 6). Same results were reported after 24 hours incubation. Statistical analysis confirmed that atrazine had no effect on haemocyte viability and esterase activity after four and 24 hours incubations, whatever concentration used. However, no esterase activity was detected in control and treated cells after 24 hours incubation (Figure 5). Furthermore, haemocyte mortality was higher in controls after 24 hours incubation than in controls after a four hour incubation (Figure 6).

Percentages of peroxidase positive cells increased and hyalinocyte percentage decreased after 24 hours incubation in haemocytes treated with 930µM of atrazine (Figures 7 and

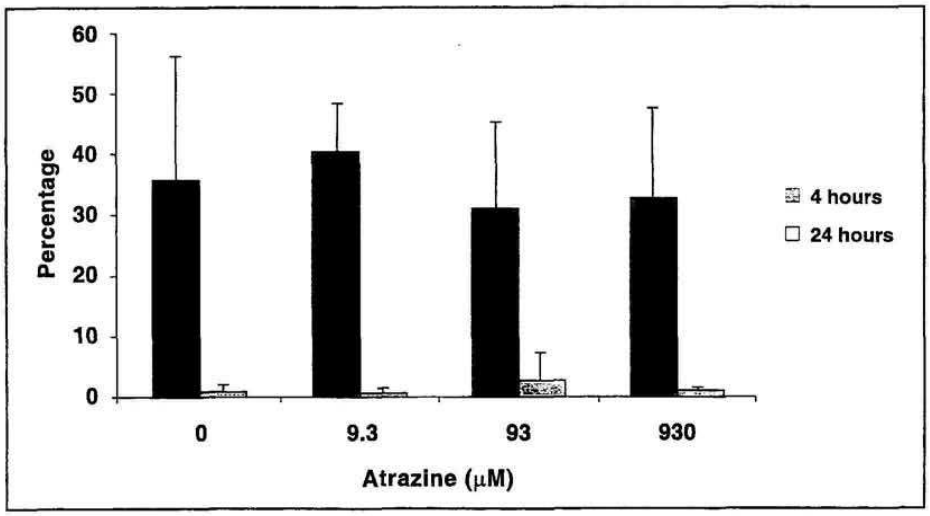

Fig. (5). Esterase activity monitored by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930µM). Values are means of three replicates.

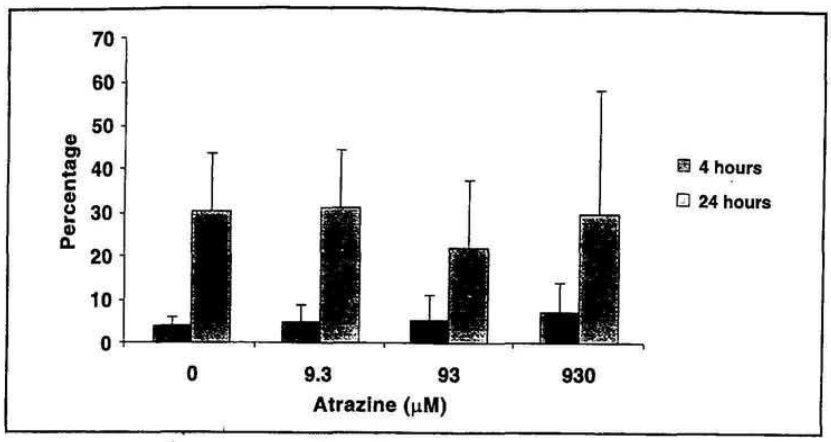

Fig. (6). Haemocyte mortality monitored by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930μM). Values are means of three replicates.

8). No effect was detected in haemocytes treated with both other atrazine concentrations. Although statistical analysis indicated that decrease of hyalinocyte percentage was not significant, increase of peroxidase positive cell percentages was statistically significant ($\alpha = 0.75$).

No change was reported after atrazine treatment for all other tested parameters (cathepsins, aminopeptidases, phagocytosis activity, cell cycle and hyalinocyte percentage).

DISCUSSION

Atrazine is a herbicide belonging to the triazine family, which is one of the most used around the world [2]. Atrazine inhibits photosynthesis. It interacts with photosystem II, which is an essential link of the electron transport. Atrazine enters into plants by leaves and roots and penetrates also through unicellular algal membranes [3]. In marine ecosystems, atrazine may affect phytoplancton. Furthermore, atrazine is classified as a potential human carcinogen by the International Agency for Research on Cancer (IARC, 1991) and the United States Environmental Protection Agency (USEPA, 1991). However, studies on atrazine effects (ecotoxicology, genotoxicity) provided contradictory results. Atrazine is not a real inducer of aneuploïdy in human cells [4] and no mutagenic effect was also reported [5]. Furthermore, a study carried out on chronically exposed populations revealed no increased incidence of benign or malignant disease attributable to atrazine [6]. On the

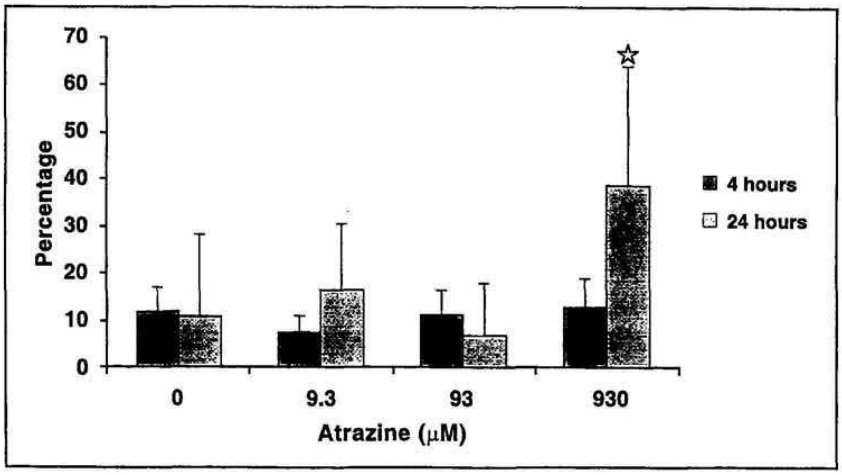

Fig. (7). Peroxidase activity monitored by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930μM). Values are means of three replicates.
☆ = statitical difference for α=0.75.

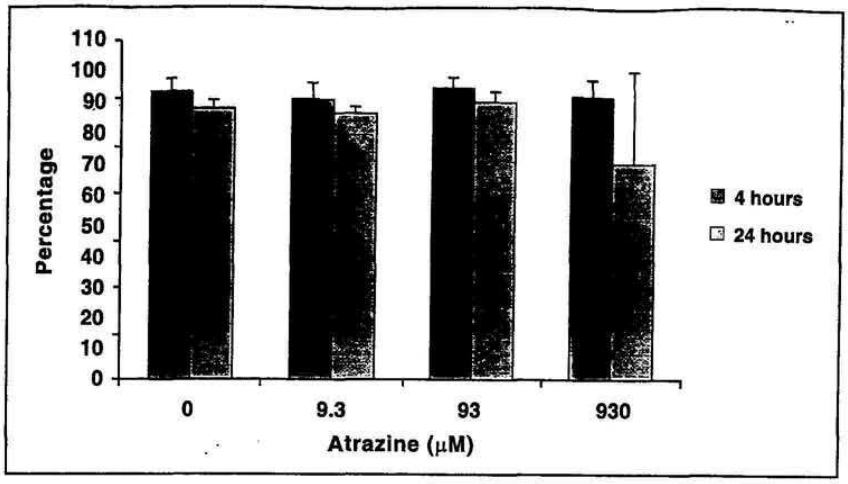

Fig. (8). Proportion of hyalinocytes defined by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930µM). Values are means of three replicates.

contrary, a study [7] showed that atrazine induced sister chromatid exchanges and chromosome aberrations in human lymphocyte cultures exposed to 5 to 51µM of atrazine during 72 hours. However, gliphosate and vinclozolin showed a higher cytogenetic effect with increase of structural aberrations, sister chromatide exchanges and G6PD activity in exposed bovine lymphocyte cultures [8].

No significant effect of atrazine on most of the selected cellular parameters (phagocytosis activity, cell viability, cell cycle, enzymatic activities, hyalinocyte proportion) was detected in *Crassostrea gigas*. However, high concentrations of atrazine induced an increase of peroxidase positive haemocyte percentages *in vitro*.

Most studies about atrazine effects in *C. gigas* concerned physiology, ecotoxicology or genetic. A study [9] showed atrazine toxicity on growth of *C. gigas* larvae and two feeder-algae, *Isochrysis galbana* and *Chaetoceros calcitrans*. At 2.3µM, 5 % of larvae were abnormal and at 46.5µM, 75 % of larvae died in one week. Moreover, atrazine caused 60 to 70 % of mortality of *C. gigas* adults after two month *in vivo* exposure at 465 and 930nM [10]. Atrazine can also induce moderate changes of clotting of *C. gigas* haemocytes at 46.5 and 465nM [11]. Some works are in progress at the LGP laboratory on aneuploïdy induced by atrazine (Bouilly, personnal communication). However atrazine is not currently detected in bivalve tissues[12].

Effect of atrazine has also been studied in freshwater molluscs, which inhabit polluted areas. Atrazine induced a behavioural change of freshwater molluscs, *Physa acuta* and *Ancylus fluviatilis*, after a 18 day exposure at 70nM. Cellular lysis appeared after a 10 day exposure at 465 nM without significant effect on mortality and biomass [13]. Some studies [14] showed an increasing number of circulating haemocytes in *Lymnaea palustris* after atrazine exposure. They reported also a decrease of phagocytosis and oxidative burst metabolites in treated haemocytes.

In our experiments, atrazine induced an increase of peroxidase positive haemocyte percentages *in vitro* when high concentrations are used. Thus, atrazine is not a highly immunotoxic pollutant in *C .gigas* oysters. However, in *in vitro* experiments haemocytes were undoubtedly weaken after 24 hours incubation. *In vitro* maintenance of haemocytes is poorly documented and substances which may help it can have unknown effects on cellular activities. Furthermore, for *in vivo* experiments, results show that animal haemocytes had adapted to maturation conditions: cellular activities were different in controls after three weeks of maturation.

These results show the interest of using flow cytometry. This technique allows to analyze haemocyte viability and cellular functions after four and 24 hours *in vitro* incubations. It also permits to show how oysters react at cellular level after a maturation maintenance.

ACKNOWLEDGEMENTS

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4. 2. 3. Des suivis in situ (Gagnaire B., Soletchnik P., Madec P., Geairon P., Le Moine O. & T. Renault**,** 2006, Aquaculture, 254: 606-616)

Des mortalités anormales d'huîtres creuses, *C. gigas*, sont observées régulièrement en France en fin de printemps et début d'été. Elles sont désignées sous le vocable de « **mortalités estivales** » et représentent un problème économique important pour l'ostréiculture française. Des mortalités de même type ont également été rapportées dans d'autres régions du monde (Japon, USA,…).

Les causes de ces mortalités restent encore aujourd'hui mal connues. Une origine multi-factorielle est largement suspectée (Cheney *et al.*, 2000 ; 2004) pour laquelle certains agents infectieux comme des bactéries du genre *Vibrio* et le virus OsHV-1 semblent jouer un rôle important (Lacoste *et al.*, 2001 ; Le Roux *et al.*, 2002 ; Waechter *et al.*, 2002 ; **Renault *et al.*, 1994a**). Dans ce phénomène, les pathologistes sont particulièrement intéressés par éclaircir les relations qui peuvent exister entre **les mortalités, la présence d'agents pathogènes et les effets de facteurs de l'environnement sur la sensibilité des animaux à ces agents infectieux**. Des études de terrain doivent permettre de disposer de données sur l'état de l'environnement et des animaux au moment où surviennent les mortalités.

Depuis 1996, les mortalités estivales chez l'huître creuse, *C. gigas*, font l'objet de travaux de recherche dans le sud du Bassin de Marennes-Oléron (Charente-Maritime, France) (Lodato, 1997). Les animaux élevés près du sédiment présentent des taux de mortalité plus élevés que les individus placés sur des tables ostréicoles (Soletchnik *et al.*, 1999). Des travaux ont montré que l'allocation d'énergie pour la reproduction, la croissance et le gain de coquille sont plus faibles pour les huîtres proches du sédiment que pour celles élevées sur des tables à 50 ou 70 cm au dessus du sol (Goulletquer *et al.*, 1998 ; Soletchnik *et al.*, 1999 ; 2003).

L'huître creuse, *C. gigas*, est l'espèce de bivalve la plus cultivée dans le monde. Pour répondre à une demande croissante pour ce coquillage, l'utilisation d'animaux à fécondité réduite (triploïdes) est vite apparue comme un atout (Hawkins *et al.*, 2000). Les huîtres triploïdes peuvent être produites, sur la base d'un traitement chimique ou physique, par rétention d'un globule polaire après fécondation (Beaumont & Fairbrother, 1991). Elles peuvent être aussi obtenues en croisant des mâles tétraploïdes et des femelles diploïdes (Nell, 2002). Les animaux triploïdes sont aujourd'hui largement utilisés par les ostréiculteurs pour leur croissance plus rapide que celle des diploïdes. Ils présentent une gamétogenèse réduite et ils consacrent donc moins d'énergie dans la production de gamètes que les huîtres diploïdes (Nell, 2002). Leur hétérozygotie est aussi plus grande, ce qui pourrait avoir une influence positive sur le niveau d'alimentation, l'efficacité d'absorption et la croissance (Magoulas *et al.*, 2000). Les coûts métaboliques sont moins élevés chez les triploïdes, ce qui peut rendre ces animaux plus résistants à des conditions stressantes (Garnier-Gere *et al.*, 2002). Cependant, les différences en terme de survie entre diploïdes et triploïdes restent controversées.

Une étude de terrain a ainsi été conduite dans le Bassin de Marennes-Oléron (Charente-Maritime, France) avec pour objectif principal de suivre certains paramètres hémocytaires, la gamétogenèse et les mortalités pour trois stocks d'animaux mis en élevage sur l'estran (**Gagnaire *et al.*, 2006b**). Les huîtres ont été suivies pendant une période de sept mois de mars à septembre 2002. Trois lots d'animaux ont été placés sur l'estran en début d'expérimentation (mars 2002) : des huîtres diploïdes issus de captage naturel, des coquillages diploïdes et triploïdes produits à l'écloserie Ifremer de La Tremblade (LGP, France). Les huîtres ont été mises en élevage sur le même site à deux niveaux, à 15 cm et à 70 cm au

dessus du sédiment. Les mortalités ont été suivies et le contenu lipidique a été mesuré chez les animaux afin de définir leur état de maturité sexuelle.

Plusieurs paramètres hémocytaires (taille, complexité, mortalité cellulaire, présence d'estérases non spécifiques et activité phagocytaire) ont aussi été analysés en cytométrie en flux. Les relations entre maturation sexuelle et activités hémocytaires ont été étudiées pour les trois types de coquillages élevés à deux niveaux au dessus du sol.

Les **huîtres triploïdes** ont présenté les **plus faibles taux de mortalité cumulée** (6,5% à 11,3%) par rapport aux animaux diploïdes (14,3 % à 43,8%). Les plus fortes mortalités (43,8%) ont été rapportées pour les diploïdes issus d'écloserie et élevés à 15 cm au dessus du sédiment. Les diploïdes de captage naturel ont également montré des mortalités plus fortes à 15 cm au dessus du sol qu'à 70 cm (36,2% versus 21,5%). Pour les huîtres triploïdes, le même constat a pu être fait : les mortalités observées à 15 cm sont significativement supérieures à celles rapportées à 70 cm, respectivement 11,3% et 6,5%. Dans cette étude, la hauteur à laquelle les animaux ont été élevés permet ainsi d'expliquer en partie les mortalités. Pour les trois types d'animaux, les **mortalités sont plus importantes à 15 cm qu'à 70 cm**. Un résultat comparable a été rapporté chez l'huître australienne, *Saccostrea glomerata*, (Smith *et al.*, 2000). Il est possible de suspecter que la **proximité par rapport au sédiment** soit un **facteur stressant** pour les huîtres avec en particulier le **re larguage de composés toxiques** (polluants).

Les **huîtres triploïdes** ont aussi montré des niveaux d'**activité plus élevés pour plusieurs paramètres hémocytaires** (granulocytes, estérases non spécifiques, peroxydase et activité de phagocytose). Les **différences entre animaux diploïdes et triploïdes** sont les plus marquées durant la **période de maturation avancée** (présence de gamètes chez des animaux en attente de ponte). Une différence entre les deux types de diploïdes (captage naturel versus écloserie) n'a été observée que pour les activités de type peroxydase. Une différence significative a aussi été rapportée pour les deux types de site (15 cm et 70 cm au dessus du sédiment) sur la base du pourcentage de cellules positives pour les activités estérases non spécifiques.

Certains paramètres hémocytaires (estérases non spécifiques et activité de phagocytose) ont permis de différencier les animaux présentant de fortes mortalités (diploïdes) de ceux avec de faibles mortalités (triploïdes) durant la période de pleine maturité et au moment où les mortalités ont été observées. La gamétogenèse pourrait rendre les animaux diploïdes plus sensibles à différents facteurs de stress que les triploïdes. Une des principales caractéristiques des huîtres triploïdes utilisées dans cette étude était l'absence de maturation sexuelle durant le printemps et l'été 2002. Les diploïdes quant à eux présentaient des gonades développées indiquant que ces animaux avaient alloué de l'énergie à la production de gamètes. Les triploïdes n'ayant pas utilisé d'énergie à la production de gamètes, ils ont pu alloué de l'énergie à d'autres processus comme les activités hémocytaires.

Des expériences complémentaires apparaissent nécessaires afin de tester la sensibilité des huîtres triploïdes à différents micro-organismes et de démontrer que des **valeurs élevés pour les paramètres hémocytaires** peuvent être la **marque d'une plus faible sensibilité aux agents pathogènes**.

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Diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg), reared at two heights above sediment in Marennes-Oleron Basin, France: Difference in mortality, sexual maturation and hemocyte parameters

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Abstract

Summer mortality is an important economic concern for the Pacific oyster, *Crassostrea gigas*, industry all over the world and particularly in France. Outbreaks appear when oysters are in gonadal maturation. Bivalve defence mechanisms against pathogen invasion are assumed by circulating cells, the hemocytes. A field experiment was conducted in the Marennes-Oleron Basin (Charente-Maritime, France) in order to monitor hemocyte parameters, gametogenesis and mortality rates among *C. gigas* during 7 months from March to September 2002. Diploid (from natural bed and from hatchery) and triploid oysters were placed at 15 cm or 70 cm above sediment at Marennes-Oleron Basin. Triploid animals were included because they are considered as sterile animals. Mortality rates were monitored and lipid contents were analysed in order to define sexual maturation stages. Hemocyte parameters (cell mortality, enzymatic activities and phagocytosis) for the three oyster groups were monitored by flow cytometry. Results were analysed by pooling data for the whole sampling period and by separating data in three periods related to the gametogenesis process (A, B and C). Mortality outbreak occurred during the ripe gamete period (B). Results showed that triploids presented the highest values for several hemocyte parameters and the lowest mortality rates. Differences between triploids and diploids were highest during the ripe gamete period. Relationships between oyster mortality, gonadal maturation and hemocyte parameters are discussed. This is the first study monitoring hemocyte parameters of wild diploid, hatchery bred diploid and triploid oysters reared in the field.
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Keywords: Pacific oyster; *Crassostrea gigas*; Hemocyte activities; Flow cytometry; Diploids; Triploids; Summer mortality; Sexual maturation

1. Introduction

Important mortality outbreaks have been reported in *Crassostrea gigas* populations since the 1940s in Japan in different locations (Koganezawa, 1974), on the west coast of North America (Glude, 1974; Cheney et al., 2000) and on the French coasts since 15 years (Renault

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et al., 1994; Goulletquer et al., 1998; Soletchnik et al., 1999). The syndrome is known as summer mortality. As worldwide bivalve production is mainly based on the Pacific oyster, summer mortality appears as a major concern for the shellfish industry (Mori, 1979; Perdue et al., 1981; Beattie, 1988). Factors such as food limitation, oxygen depletion, salinity and temperature do not appear as single direct causes of the syndrome (Soletchnik et al., 1998). Moreover, outbreaks occur during oyster gametogenesis. Some authors suggest that mortalities occurring in Pacific oysters are the result of multiple factors, including elevated temperatures, physiological stress associated with gonadal maturation, aquaculture practices, pathogens and pollutants (Goulletquer et al., 1998). Therefore, it can be assumed that the background rate of mortality due to environmental conditions, and the physiological and genetic origin of the oysters, can be modified by infectious agents. As many filter-feeding benthic invertebrates, oysters are permanently exposed to various microorganisms. Efficient humoral and cellular defence mechanisms normally help to limit the proliferation of microorganisms in animals (Harris-Young et al., 1995; Cheng, 1996). Stress and disease outbreaks appear to be linked in molluscs (Friedman et al., 1999; Lacoste et al., 2001b). In vitro investigations reveal that noradrenaline, the main catecholamine released in oyster hemolymph during stress, exerts an inhibitory effect on immune functions such as phagocytosis and reactive oxygen species production (Lacoste et al., 2001a, 2002). As a consequence, possible relationships between environmental factors, physiological state of oysters, immune system and pathogens need further investigations.

Since 1996, summer mortality of Pacific oyster, *C. gigas*, has been studied in the south of Marennes-Oleron Bay (Charente-Maritime, France) (Lodato, 1997). Mortality of oysters reared near the sediment was significantly higher than mortality of oysters reared on racks (Soletchnik et al., 1999). Previous studies also showed allocation of energy to reproduction, and gain in shell and somatic tissue weight, to be lower near the sediment than on racks at 50 cm or 70 cm above the sea bed (Goulletquer et al., 1998; Soletchnik et al., 1999, 2003).

The Pacific oyster, *C. gigas*, possesses an open circulatory system (Cheng, 1981). Internal defence mechanisms of bivalves involve circulating blood cells, the hemocytes. These cells are considered to be the equivalent of vertebrate phagocytic cells because of their morphology and functions. Hemocytes are involved in the recognition of invading pathogens and in their elimination by phagocytosis and encapsulation (Cheng, 1981; Fisher, 1986). Hemocyte activities have already been used for monitoring oyster immune capacities. Moreover, hemocyte parameter values can depend on oyster physiological status including sexual maturation.

Pacific oysters, *C. gigas*, are among the most widely cultured of all shellfish species. To help meet demand, advantages of increased sterility are currently being exploited during commercial growing of triploid *C. gigas* (Hawkins et al., 2000). Triploidy may be induced in shellfish by chemical or physical treatment which retain a polar body during early division of the egg, with the result that each cell nucleus contains one additional set of chromosomes (Beaumont and Fairbrother, 1991). Triploids can also be produced by crossing tetraploid males and diploid females (Nell, 2002). Triploidy is now widely used to obtain faster growth than diploids in shellfish. Gametogenesis is reduced in these animals. Triploid oysters invest less energy in gamete production, so more energy is available for somatic growth (Nell, 2002). Heterozygosity is higher, which could have positive influences on feeding rate, absorption efficiency and growth efficiency (Magoulas et al., 2000). Metabolic energy costs are lower than diploids, rendering triploids more resistant to stressful conditions (Garnier-Gere et al., 2002). However, difference in survival between triploids and diploids has been controversial.

As a part of a multiyear French research programme on summer mortalities in *C. gigas* oysters called MOREST, a focal study has been conducted on diploid and triploid oysters reared at two heights above sediment in Marennes-Oleron Bay (Charente-Maritime, France). Mortality rates were daily monitored and lipid contents analysed in order to define sexual maturation stages. Hemocyte parameters including granulocyte percentage, phagocytosis and percentages of cells possessing hydrolytic enzymes (esterases, peroxidases) were investigated using flow cytometry. This method appears well suited to morphological and functional characterisation of oyster hemocytes (Renault et al., 2001; Xue et al., 2001). These parameters are usually monitored in studies on bivalve hemocytes (Cheng et al., 1978; Beckmann et al., 1992; Carballal et al., 1997). Daily mortality was also monitored on all oyster groups. Lipid contents were also measured for all oyster groups and their analysis allowed to define sexual maturation status.

In the present paper, we have analysed the possibility that environmental conditions and physiological state of the oysters may alter hemocyte functions,

rendering the oysters more susceptible to infectious agents. Relationships between maturation stages and hemocyte parameters in three oyster groups (triploids, diploids from natural bed and hatchery diploids) and rearing levels above the sea bed were studied. Results were analysed firstly by pooling all data for the whole sampling period and, secondly, by separating data in three periods related to the gametogenesis process. The aim of this study was to identify possible relationships between gonadal maturation, oyster group, rearing level, hemocyte parameters and oyster mortality.

2. Materials and methods

2.1. Experimental set

The three groups of oysters : natural bed diploid oysters (D), hatchery diploid (Dh) and triploid (T) oysters. D were 1.5-year-old wild oysters from Marennes-Oleron Bay (French Atlantic coast). Dh were hatchery reared oysters from experimental hatchery of IFREMER (La Tremblade Charente Maritime, France). Triploid oysters (T) were produced by crossing tetraploid males and diploid females from a private hatchery. Oysters were grown in the south of the Marennes-Oleron Bay in bags placed on tables. Oysters were reared on "off bottom" culture racks, 15 cm (L1) and 70 cm (L2) above the sea bed from March to September 2002. Sampling dates were: May 14 and 28; June 6, 12, 20 and 26; July 3 and 25; August 8, 21 and September 11. Seawater temperature was recorded by "YSI" probe in 15-min steps from March to September 2002.

2.2. Oyster mortality

Oysters were deployed in mesh bags, at a starting density of 200 per bag, placed on iron racks. Live and dead oysters were counted at each sampling date in three bags per condition. Dead oysters were removed from bags. Percentage of mortality between each sampling date was afterwards transformed in daily rate by dividing per the number of days.

2.3. Lipid content analysis

Measurement of lipid content was carried out on the same animals used for hemocyte collection. Biochemical analysis of tissues was carried out on each batch in 3 replicated pools of 10 oysters for each level (L1 and L2) and oyster group (D, Dh and T). Lipids were extracted and purified according to a protocol previously described (Bligh and Dyer, 1959) and the analytical procedure was from Marsh and Weinstein (1966). Spectrophotometric analysis was conducted on an aliquot of ground tissues of each pool. Results are expressed in milligrams of lipids per gram of dry tissues. Dry tissue lipids have previously been shown to indicate the maturation level at the different sampling dates (Deslous-Paoli et al., 1981; Soletchnik et al., 1999, 2002). Maturation stages refer to a relative scale previously defined corresponding to gametogenesis in progress, ripe stage and post-spawning stage (Soletchnik et al., 1997).

2.4. Circulating hemocyte collection

After removing the shell by severing the adductor muscle, hemolymph was withdrawn directly from the pericardial cavity by puncture with a 1 mL syringe equipped with a needle (0.9×25 mm). For each oyster, 0.5 mL of hemolymph was withdrawn without any buffer. Hemolymph samples were conserved on ice during collection to prevent hemocyte aggregation (25). For each group (TL1, DL1, DhL1, TL2, DL2 and DhL2), 30 oysters were sampled at each date. The samples were combined into 3 pools of 10 oysters each to reduce inter-individual variation and to provide sufficient hemocytes to fulfil assay requirements.

2.5. Cell analysis by flow cytometry

Hemocytes were analysed with an EPICS XL 4 flow cytometer (Beckman Coulter) after hemocyte collection using previously described protocols (Xue et al., 2001; Auffret et al., 2002). For each hemocyte pool, 3000 events were counted. Results were depicted as cell cytograms indicating that the relative size (FSC value), the granularity (SSC value) and the fluorescence channel(s) corresponded to the marker used. Presence of enzymes and phagocytosis were measured using FL1 (green fluorescence) and cell mortality using FL3 (red fluorescence).

Esterase and peroxidase activities were evaluated using commercial kits (Cell Probe™ Reagents, Beckman Coulter). Percentages of cells presenting enzymatic activities were defined on the basis of fluorescent cells among all cells. Each analysis required 200 μL of hemolymph and 20 μL of the corresponding kit reagent (FDA (fluorescein diacetate)·esterase and DFCH (dichlorofluorescein diacetate), PMA (phorbol-12-myristate-13-acetate)·oxidative burst). Hemocytes were then incubated in the dark at ambient temperature for 15 min for esterase and peroxidase detection. Hemocyte mortality (Hm) was quantified using 200 μL of hemolymph. Hemocytes were incubated in the dark for 30 min at 4 °C

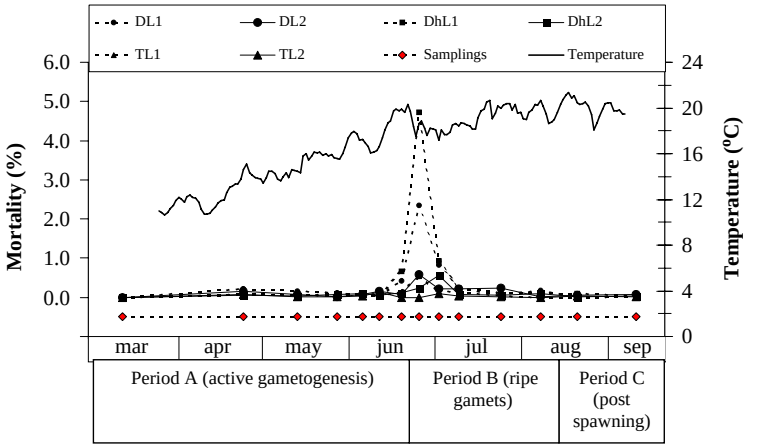

Fig. 1. Daily mortality rate of oyster groups: wild diploid oysters (D), hatchery bred diploid (Dh) and triploid (T) oysters reared on culture racks, L1 level (15 cm) or L2 level (70 cm) above the sediment.

with 10 μL of propidium iodide (PI, 1.0 mg mL^{-1}, Interchim). Morphological characteristics of hemocytes were also recorded: gates were realised on the SSC/FSC plot on the most granular and largest cells in order to determine a granulocyte population. Phagocytosis was measured in vitro as the proportion of cells that had ingested three or more fluorescent beads. Two hundred microliters of hemolymph were incubated for 1 h in the dark at ambient temperature with 10 μL of a 1/10 dilution of Fluorospheres® carboxylate-modified microspheres (diameter 1 μm, Interchim). The final concentration of beads was 10^8 beads mL^{-1}.

2.6. Statistical analysis

Statistical multi-way analysis of variance and least significant differences (LSD) post hoc test were realised using Statgraphics version 5.1 software. Hemocyte parameters and cumulated mortalities were converted into *r* angular arc sinus √(value) before analysis. However, figures were realised using non-transformed percentage values.

3. Results

3.1. Oyster mortality

A daily mortality rate, ranging from 2% to 5%, occurred in the two groups of diploid oysters at L1 level between June 21 and 26 (Fig. 1). Daily mortality of diploid oysters did not exceed 0.5% at L2. Mortality occurred after temperature had reached 18–19 °C for a few days. After a 7-month rearing period (March to September 2002), the highest cumulative mortality,

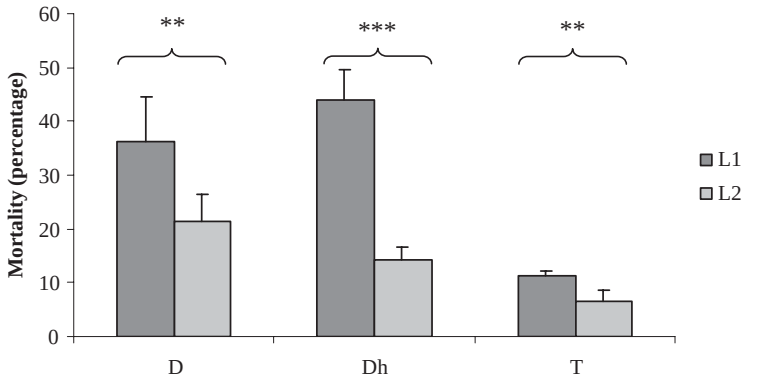

Fig. 2. Cumulative mortality of wild oysters (D), hatchery diploids (Dh) and triploids (T) reared on culture racks at 15 cm (L1) or 70 cm (L2). Values are means of three replicates. Bars are confidence limit at 95%. The post-hoc test gives TL2<(TL1-DhL2)<(DhL2-DL2)<(DL1-DhL1). $^{**}p<0.01$; $^{***}p<0.001$.

36.2% and 43.8%, affected the DL1 and DhL1 groups, respectively (Fig. 2). Significant differences were reported between L2 and L1 cumulative mortality for each oyster population: 21.5% at L2 and 36.2% at L1 for wild diploids, 14.3% at L2 and 43.8% at L1 for hatchery diploids, and 6.5% at L2 and 11.3% at L1 for triploids (Fig. 2). Whatever the rearing levels, cumulative mortality in triploid oysters was significantly lower (Fig. 2).

3.2. Lipid content and definition of maturation stages

From March to mid May 2002, the mean lipid content was about 80–85 mg g^{-1} for diploid and triploid oysters (Fig. 3). Values then reached ~150 mg g^{-1} at the end of June 2002 and remained at this level until spawning for the two groups of diploid oysters (D and Dh) (Fig. 3a and b). Lipid content did not exceed ~100 mg g^{-1} for triploid oysters (Fig. 3c). After spawning in August 2002, lipid contents fell in diploids to 90 mg g^{-1}. Three periods A, B and C were defined on the basis of lipid contents in diploids. Period A was defined as the active gametogenesis (before mortality; May to June 20th), period B was defined as the ripe gamete period (after mortality; June 20th to August 8th), and period C was defined as the post-spawning period (August 8th to September 11th) (Fig. 3). The evolution of lipid contents was directly related to maturation

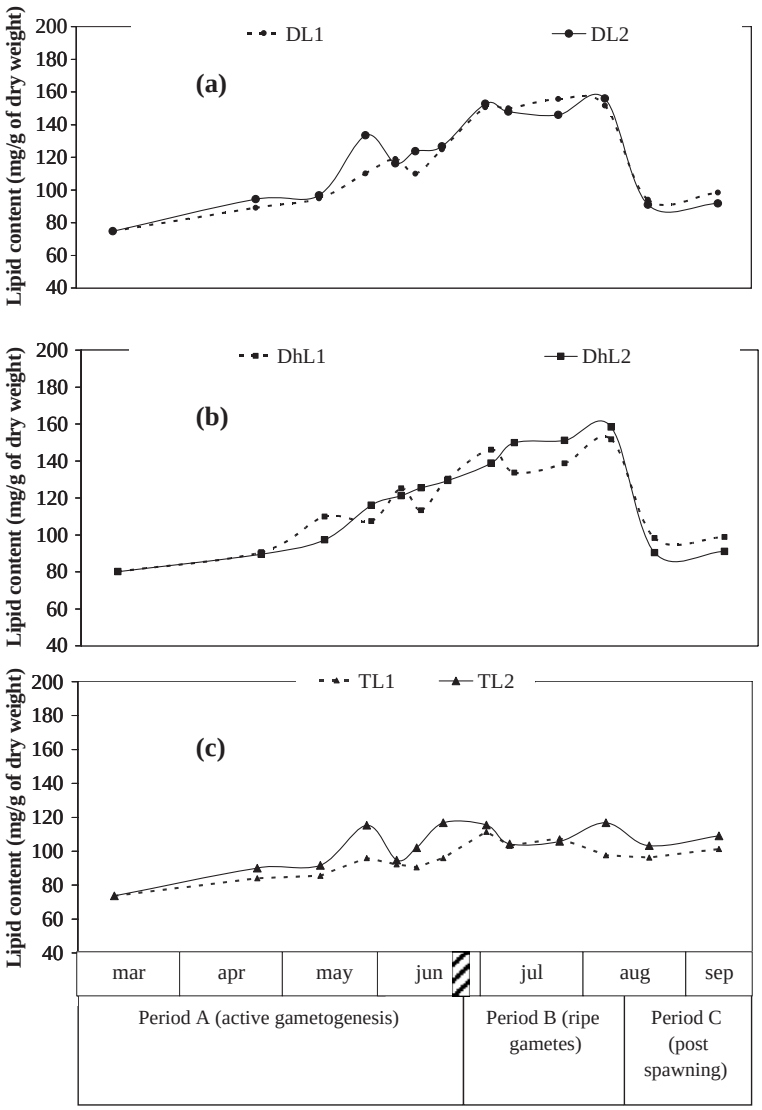

Fig. 3. Lipid content (mg g^{-1}) in the dry flesh along the experiment for wild diploids (D) (a), hatchery bred diploids (Dh) (b) and triploids (T) (c) reared at L1 and L2. Values are means of 3 replicates of 10 oysters. Hatched surface indicates the mortality period.

stage, with a maximal value just before spawning. Values for triploid oysters remained about 100 mg g^{-1} (Fig. 3c).

3.3. Hemocyte parameters: effect of sites and oyster groups

The first analysis was realised on the whole data, in order to study a potential difference between oyster groups or an effect of rearing level on the basis of hemocyte parameters. Two-way analysis of variance of each parameter was carried out. Thirty-three values were available for each hemocyte parameter. Results showed no effect of level ($p>0.05$) for any of the hemocyte parameters (Fig. 4, Table 1). On the contrary, some parameters varied significantly among the three oyster groups. Phagocytosis values were significantly higher for triploids than for diploids ($p<0.0001$) (Fig. 4a, Table 1). For triploids, 36.7% (L1) and 29.5% (L2) of hemocytes presented phagocytosis activity. For diploids D, 21.8% (L1) and 25.0% (L2) of hemocytes presented phagocytosis activity. For diploids Dh, 20.2% (L1) and 16.4% (L2) of hemocytes presented phagocytosis activity (Table 1). Percentages of cells presenting a peroxidase activity differentiated T, D and Dh oysters (T>D>Dh, LSD test, $p<0.0001$) (Fig. 4c, Table 1). Percentages of cells presenting an esterase activity and the granulocyte percentage were also significantly higher for triploids than for diploids ($p<0.05$) (Fig. 1b,d, Table 1). Cell mortality permitted differentiation between triploid and diploid groups (T<D=Dh, Fig. 4f). However, this result was not significant ($p<0.1$).

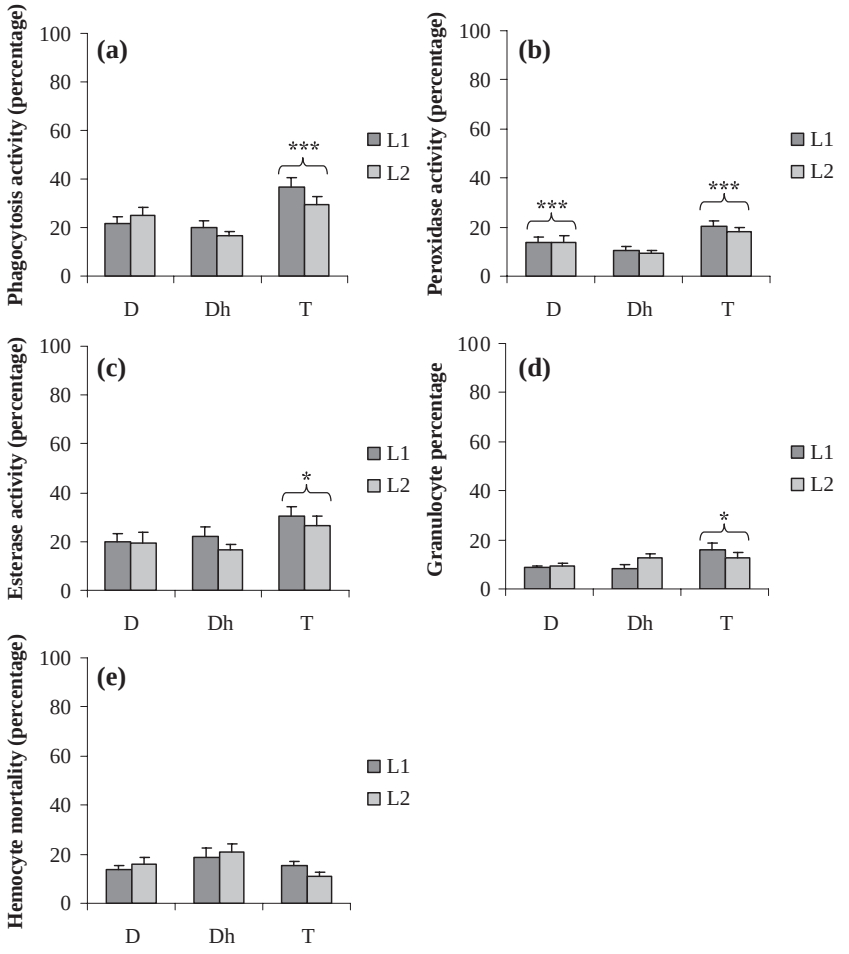

Fig. 4. Means and standard errors of the means of hemocyte parameters for the three oyster groups (D=diploids from natural bed, Dh=hatchery diploids and T=triploids) and both distances above sediment (L1=15 cm, L2=70 cm). (a) Percentage of cells that phagocytosed beads in vitro; (b) percentage of cells presenting a peroxidase activity; (c) percentage of cells presenting an esterase activity; (d) granulocyte percentage; (e) hemocyte mortality. Significant differences between groups are indicated with stars: *$p<0.05$; ***$p<0.001$.

3.4. Relationships between hemocyte activities and gonadal maturation, oyster groups or rearing levels

The multi-way analysis of variance of hemocyte activities versus levels (L1 and L2), oyster groups (D, Dh and T) and maturation stages (A, B and C, Figs. 1 and 3) demonstrated a major effect of maturation. All the hemocyte activities showed lowest values during period A compared to B or C periods (Table 2, $p<0.001$). "Oyster group" had a significant effect on phagocytosis, esterase and peroxidase activities and granulocyte percentage (Table 2, $p<0.01$). Highest hemocyte activities were reported in triploid oysters. The rearing level had an effect only on esterase activity: values were significantly higher at L1 (Table 2, $p<0.05$).

Phagocytosis values were maximal for triploid oysters during the A and B periods (21.3% and 48.1%, respectively) (Fig. 5a, Table 2). Phagocytosis values for natural bed diploids were 13.1% and 24.7% during the A and B periods and for hatchery diploids 9.0% and 22.0% during the A and B periods, respectively (Fig. 5a, Table 2). After spawning, in period C, triploids and diploids presented similar values of phagocytosis. The highest percentages of peroxidase and esterase positive cells were recorded for triploids in periods A and B (Fig. 2c and e, Table 2). Triploids presented the highest percentages of granulocytes during the three periods (A, B and C), with a maximum value (21.7%) in period C (Fig. 5b, Table 2).

Values were not statistically different between triploids and diploids for hemocyte mortality, but were lower for triploids in periods A and B (10.9% and 13.4%, Fig. 5d, Table 2) than for hatchery diploids in period B (32.%, Fig. 5d, Table 2). Hatchery diploids presented the lowest activities for hemocyte parameters during period A and period C: phagocytosis activity (Fig. 5a, Table 2), percentage of peroxidase, esterase positive cells (Fig. 2c, e and f, Table 2) and percentage of granulocytes (Fig. 5b, Table 2). Hatchery diploids also presented the lowest activities for two parameters in period B: the lowest phagocytosis activity (Fig. 5a, Table 2) and percentage of peroxidase positive cells (Fig. 5c, Table 2).

Table 1
Multi-way analysis of variance of hemocyte parameters versus "level" (L1=15 cm and L2=70 cm) and "group" (D=diploids from natural bed, Dh=hatchery diploids and T=triploids)

Hemocytes activities	Level	Group
Phagocytosis activity	NS $p=0.3108$	T>Dh, D $p<0.0001$
Peroxidase activity	NS $p=0.5192$	T>D>Dh $p<0.0001$
Esterase activity	NS $p=0.1680$	T>Dh, D $p=0.0130$
Granulocyte percentage	NS $p=0.8780$	T>Dh, D $p=0.0203$
Hemocytes mortality	NS $p=0.9195$	NS $p=0.0522$

Significant effects are reported at 5% (*), 1% (**) and 1‰ (***) probability level. NS=no significant difference. Related LSD (least significant difference) post hoc tests are reported.

Table 2
Multi-way analysis of variance of hemocyte parameters versus "level" (L1=15 cm and L2=70 cm), "group" (D=diploids from natural bed, Dh=hatchery diploids and T=triploids) and maturation stage (gametogenesis=A, ripe gametes=B, post-spawning=C)

Hemocytes activities	Level	Group	Maturation stage
Phagocytosis activity	NS	T>Dh, D ***	A<B<C ***
Peroxidase activity	NS	T>D>Dh ***	A<B<C ***
Esterase activity	L1>L2 *	T>Dh, D **	A<B<C ***
Granulocyte percentage	NS	T>Dh, D **	A<B, C ***
Hemocytes mortality	NS	NS	A<B, C ***

Significant effects are reported at 5% (*), 1% (**) and 1‰ (***) probability level. NS=no significant difference.

4. Discussion

Field studies on summer mortality of *C. gigas* have been conducted in France for several years (Lodato, 1997; Goulletquer et al., 1998; Soletchnik et al., 1999, 2003, 2005). A mortality model comparing "on" and "off" bottom culture has been developed (Soletchnik et al., 2005). This model demonstrated an effect of proximity to sediment on oyster survival, called "sediment effect". This result was confirmed in the present experiment. Mortality of diploid oysters from natural bed was higher at rearing level L1 (15 cm) than at L2 (70 cm) (36.2% cumulative mortality versus 21.5% after a 6-month grow-out period). Mortality of wild diploid oysters was also nearly doubled in 1997 on the same site, with 8–19% and 23–33% for L2 and L1, respectively (Soletchnik et al., 1999). Mortality rates for "off" and "on" bottom culture were 8–15% and 40–50% in 2000 and 10% and 28% in 2001, respectively (Soletchnik et al., 1997). In 2000 and 2001, mortality outbreaks occurred in June after an increase of seawater temperature above 19 °C. Other studies conducted in USA reported mortality outbreaks during the same period (June and July) (Perdue et al., 1981; Cheney et al., 2000).

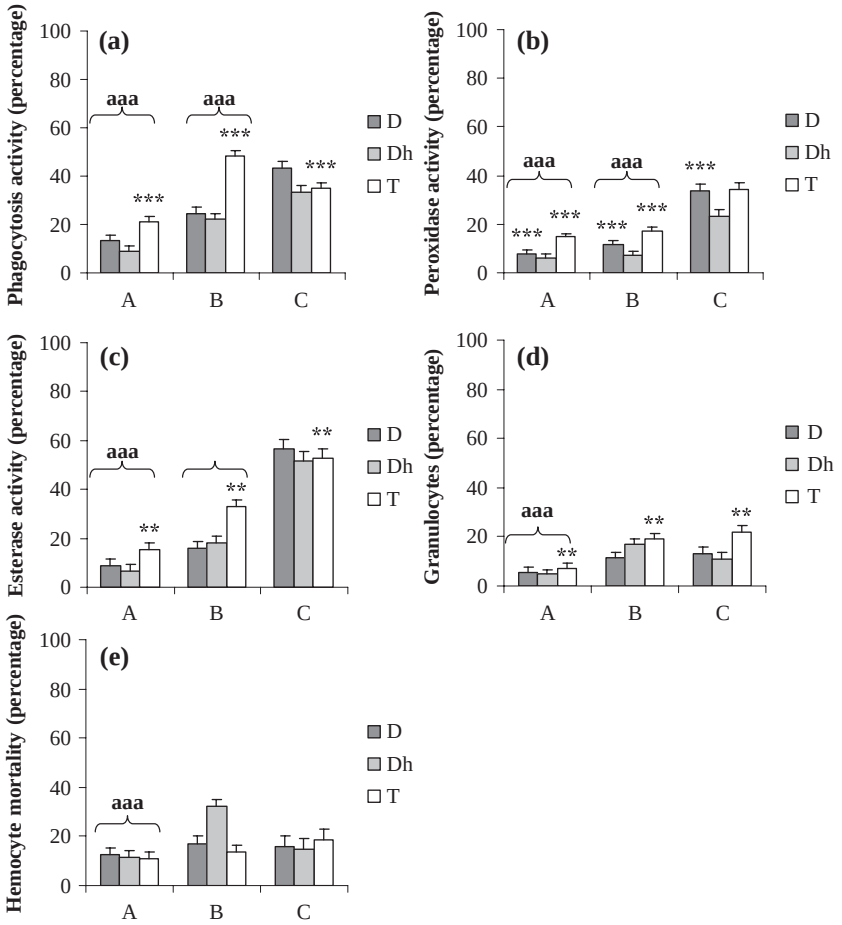

Fig. 5. Means and standard errors of the means of hemocyte parameters over the three periods (A=gametogenesis, B=ripe gametes and C=post-spawning) for the three oyster groups (D=diploids from natural bed, Dh=hatchery diploids and T=triploids). (a) percentage of cells that phagocytosed beads in vitro; (b) percentage of cells presenting a peroxidase activity; (c) percentage of cells presenting an esterase activity; (d) granulocyte percentage; (e) hemocyte mortality. Significant differences between groups (*) or periods (a) are indicated: aa=$p<0.01$; *** and aaa=$p<0.001$.

In the present study, hatchery diploids presented the highest cumulative mortality at L1. Moreover, cumulative mortality was as in wild diploids higher at L1 than at L2 (43.8% and 14.3%, respectively). Triploids presented the lowest mortality rates, but cumulative mortality was also statistically different between L1 and L2 (11.3% and 6.5%, respectively). This is the first field study in France including diploid and triploid oysters from hatchery. In this "on–off" bottom model, the rearing level allows to explain partly mortality outbreaks: cumulative mortality was higher at L1 than at L2 for the three oyster populations. A study on the Sydney rock oyster, *Saccostrea glomerata*, reported a higher mortality at low height compared to high height (Smith et al., 2000).

In this study, we also measured oyster lipid contents in order to define different stages of sexual maturation. This technique has already been use before (Deslous-Paoli et al., 1981; Soletchnik et al., 1999, 2002). Diploids presented the highest mortality rates and mortalities were observed during the ripe gamete period (B). As recorded by other authors (Imai et al., 1965; Perdue et al., 1981), mortality occurred during gametogenesis. On the contrary, the lowest mortality rates were reported for triploid oysters, presenting low lipid contents. Another study on Hiroshima Bay also reported low mortality rates for *C. gigas* triploids (Akashige and Fushimi, 1992). A study on *C. gigas* in Australia also reported lower mortality of triploids compared to diploids (Nell and Perkins, 2005). Another study on the Sydney rock oyster, *S. glomerata*, reported a higher survival rate for triploids than diploids (Hand et al., 1998). However, on this same oyster, another study reported no difference of mortality between triploids

and diploids (Smith et al., 2000). Other studies in USA always reported a mortality rate twice higher for triploids compared to diploids (Cheney et al., 1998, 2000, 2004).

A major aim of this study was also to monitor certain hemocyte parameters in oysters placed in the field over a long period. Our results underscore the utility of flow cytometry in monitoring hemocyte functions in oysters (Goedken and De Guise, 2004). To our knowledge, this is the first study evaluating hemocyte activities in triploid oysters reared in the field. The analysis of the whole data (without separation by periods on the basis of sexual maturation) showed that triploid oysters presented statistically higher hemocyte activities than diploid oysters from hatchery and from natural bed for several parameters (phagocytosis, esterase and peroxidase activities and granulocyte percentages). When analysing the data by periods related to the gametogenesis status (A, B and C), statistically significant differences for the same hemocyte parameters were reported between diploids (low values) and triploids (high values). Moreover, the highest differences for esterase and phagocytosis activities were reported between animals presenting the lowest mortality (triploids) and animals presenting the highest mortality (diploids) during period B (ripe gamete period) where mortality events were reported. We can therefore hypothesize that gametogenesis process makes diploid oysters more susceptible to several factors and then lead to mortality. The principal characteristic of triploid oysters was the absence of sexual maturation during Spring and Summer 2002. During the ripe gamete period (B), triploid oysters presented no gamete development. We can assume that, during the ripe gamete period B, diploid oysters allocated energy to gamete production and, on that account, a minimum of energy was allocated to the defence system. Triploids do not use energy in gamete synthesis and so may allocate energy for other processes including hemocyte activities and defence mechanisms. A previous study reported a decrease of the activity of complement and the number of lymphocytes during sexual maturation of the sockeye salmon, *Oncorhynchus nerka* (Alcorn et al., 2002). Sexual maturation is a sensitive period and physiological activities such as defence mechanisms can be disrupted.

Peroxidase activities allowed differentiation between diploids from hatchery and from natural bed, with lowest values for hatchery diploids (Dh<D). Moreover, esterase activity was higher at L1 level, where highest mortality was reported for both diploid populations. This difference appeared only when data for the three gonadal maturation periods (A, B and C) were analysed separately. We may assume that, at L1, bacterial biomass related to turbidity is more important (Lodato, 1997). The environment at the lower level (15 cm) induced a greater stimulation of the immune system.

Hemocyte parameters monitored in this study allowed differentiation between oysters presenting high mortality (diploids: from 14.3% to 43.8% of cumulative mortality) and low mortality (triploids: 6.5% and 11.3% of cumulative mortality). However, only one parameter allowed differentiation between both diploid populations and only one parameter allowed to differentiate both levels, although mortality was higher at L1. As hemocyte parameter values are different between triploids and diploids, we can hypothesize that abnormal mortalities account for more than 12% of cumulative mortality. Beyond this threshold, different oysters presenting different percentages of cumulative mortality cannot be differentiated on the basis of the hemocyte parameters monitored. We can therefore hypothesize that only abnormal mortality, and not genetic origin or rearing level, explains values obtained for hemocyte parameters.

However, the factors implicated in *C. gigas* mortality are not identified yet. Some authors hypothesised the impact of physico-chemical factors in seawater (Cheney et al., 2000), toxicity of sediment, presence of pollutants, the role of bacterial infections (Lacoste et al., 2001b; Le Roux et al., 2002; Waechter et al., 2002) and herpesviral infections following temperature elevation (Renault et al., 1994, 1995). However, no difference has been found in the resistance of triploid or diploid *C. virginica* (Gmelin) oysters to infection by MSX (*Haplosporidium nelsoni*) or *Perkinsus marinus* (Meyers et al., 1991; Barber and Mann, 1991; Matthiessen and Davis, 1992). Further experiments should be conducted in order to test the susceptibility of triploids to different microorganisms and to demonstrate that higher values of hemocyte parameters correspond to lower susceptibility to infectious diseases.

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4. 3. Avec une pincée de bactéries (Gagnaire B., Gay M., Saulnier D. & T. Renault, 2007, Aquatic Toxicology, 84: 92-102)

La démonstration d'une **corrélation entre modulation des activités hémocytaires et capacités de défense** nécessite de réaliser des expériences impliquant une **exposition simultanée ou séquentielle à des polluants et à des agents pathogènes**. Plusieurs auteurs ont ainsi rapporté qu'une altération de paramètres hémocytaires chez des bivalves exposés à des polluants pouvait être associée à une plus grande sensibilité à des maladies infectieuses (Chu *et al.*, 2002 ; Coles *et al.*, 1994 ; 1995 ; Fournier *et al.*, 1988; Pipe & Coles, 1995).

Dans ce cadre, afin d'étudier les effets de pesticides sur l'état de santé des huîtres creuses en France, les effets combinés d'une exposition à un mélange de pesticides et d'une injection de bactéries ont été testés d'une part en cytométrie en flux sur différentes activités hémocytaires et d'autre part en PCR en temps réel pour l'expression de gènes d'intérêt. Les objectifs de cette étude étaient de développer un modèle expérimental de contamination avec des pesticides et d'étudier les effets de ces polluants lors d'un « challenge » bactérien.

Au cours de ce travail, dans une première étape, un **modèle de contamination expérimentale** (*in vivo*) a été développé chez l'huître creuse. Un mélange de **huit pesticides** (atrazine, glyphosate, alachlore, métolachlore, fosetyl-alumimium, terbuthylazine, diuron et carbaryl) a été utilisé sur la base de concentrations observées dans l'environnement, avec une exposition de sept jours. Dans une étude précédente, des essais *in vitro* avaient permis de démontrer des effets de ce mélange de pesticides sur l'activité de phagocytose, alors qu'aucune des molécules composant le mélange n'avait montré d'effet lorsqu'elles étaient testées seules (**Gagnaire *et al.*, 2006**). L'expérience a été répétée trois fois. Différents paramètres cellulaires (mortalité des hémocytes, présence d'estérases non spécifiques, production de radicaux libres et activité de phagocytose) ont été suivis en cytométrie en flux et 19 gènes analysés en PCR quantitative en temps réel. Pour les trois essais réalisés, une **réduction significative de la phagocytose** et une **sous-expression des 19 gènes** sélectionnés (impliqués dans les activités hémocytaires) ont été observées après sept jours de contamination.

Dans une seconde étape, après une contamination de sept jours avec le mélange de pesticides, des huîtres ont reçu une injection intra-musculaire de deux souches de *Vibrio splendidus*, isolées lors d'un épisode de mortalité et pour lesquelles il a été démontré qu'elles coopéraient pour induire une infection et des mortalités (**Gay *et al.*, 2004**). La mortalité des animaux a été suivie quotidiennement et l'expression de dix des 19 gènes précédemment sélectionnés a été analysée en PCR en temps réel 4 et 24 heures après injection. Un taux de **mortalité plus élevé** a été observé pour les **huîtres traitées par les pesticides** par rapport **aux individus non contaminés**. Une **sur-expression de plusieurs gènes** (galectine, LPS Binding Protein, C-Src kinase, ankyrine, précurseur de la cathepsine L, lysozyme et défensine) a été détectée pour les **animaux contaminés après l'injection des bactéries**. La sur-expression de gènes impliqués dans l'immunité pourrait laisser suspecter une meilleure capacité à se défendre pour les animaux contaminés préalablement à l'injection des bactéries. Cependant, cette interprétation est en contradiction avec les plus fortes mortalités observées pour les huîtres mises au contact des pesticides. Dans ce contexte, il est possible de supposer que cette **sur-expression** soit impliquée dans le **développement de l'infection et des lésions**.

Le **contact préalable des huîtres avec les pesticides** induit une **aggravation des effets de l'injection de bactéries** et montre qu'il peut exister des interactions complexes entre polluants, huîtres et agents infectieux.

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Combination of a pesticide exposure and a bacterial challenge: *In vivo* effects on immune response of Pacific oyster, *Crassostrea gigas* (Thunberg)

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Abstract

To assess the impact of pollution induced by pesticides on Pacific oyster, *Crassostrea gigas*, health in France, *in vivo* effects of combined pesticide exposure and bacterial challenge on cell activities and gene expression in hemocytes were tested using flow cytometry and real-time PCR. As a first step, an *in vivo* model of experimental contamination was developed. Pacific oysters were exposed to a mixture of eight pesticides (atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl) at environmentally relevant concentrations over a 7-day period. Hemocyte parameters (cell mortality, enzyme activities and phagocytosis) were monitored using flow cytometry and gene expression was evaluated by real-time PCR (RT-PCR). The expression of 19 genes involved in *C. gigas* hemocyte functions was characterized using RT-PCR. After 7 days of exposure, phagocytosis was significantly reduced and the 19 selected genes were down-regulated in treated animals. As a second step, the experimental contamination method previously developed was used to study interactions between pesticide exposure and bacterial challenge by intramuscular injection of two *Vibrio splendidus*-related pathogenic strains. Oyster mortality and expression of 10 of the 19 selected genes were followed 4 and 24 h post-injection. Oyster mortality was higher in pesticide-treated oysters compared to untreated oysters after the bacterial challenge. Gene expression was up-regulated in pesticide-treated oysters compared to untreated oysters after the bacterial challenge. We hypothesize that gene over-expression due to an interaction between pesticides and bacteria could lead to an injury of host tissues, resulting in higher mortality rates. In conclusion, this study is the first to show effects of pesticides at environmentally relevant concentrations on *C. gigas* hemocytes and to hypothesize that pesticides modulate the immune response to a bacterial challenge in oysters.
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Keywords: Pacific oyster; *Crassostrea gigas*; Bivalve immunity; Pesticides; Hemocyte; Phagocytosis; Pathogenesis; *Vibrio splendidus*; Flow cytometry; Gene expression

1. Introduction

Shellfish exploitation is an historical world-wide activity and has intensified over the last century. The Pacific oyster, *Crassostrea gigas*, is the most frequently cultivated bivalve species and is typically reared in estuarine environments that have become increasingly threatened by exposure to pollutants. Among pollutants, pesticide contamination of shellfish has become more common in estuarine areas over the past several decades due, in part, to chemical run-off from terrestrial agriculture (Banerjee et al., 1996). Pesticides are introduced into rivers by ground "scrubbing" when rainfalls occur and then may enter marine areas, particularly estuarine and coastal zones. These pollutants may have major ecological consequences and could endanger organism growth, reproduction or survival (Banerjee et al., 1996).

Among physiological processes possibly disturbed by pollutants, the immune system is likely to be one of the more sensitive physiological systems (Fournier et al., 2000). The immune system contributes to host homeostasis by eliminating foreign

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particles (viruses, bacteria or parasites), killing abnormal cells and rejecting "non-self" components (Fournier et al., 2000). Pollutants or xenobiotics can interact with immune system components and interfere with protection functions and are therefore referred to as immunotoxics (Colosio et al., 2005). Xenobiotics can induce immunosuppression or stimulation, auto-immunity and decrease of disease resistance (Wong et al., 1992). In the past decades, the emergence of infectious diseases has been reported in marine species and disease outbreaks have also increased (Harvell et al., 1999). According to Snieszko (1974), the development of an infectious disease results from an unbalance between the host and the pathogen due to external factors (including pollutants) and/or internal factors of both protagonists (virulence of the pathogen, susceptibility of the host). Animals with impaired defence mechanisms may be more susceptible to infectious diseases. Demonstration of the relationship between pollution and increase of susceptibility to infectious diseases exist in vertebrates. Harbour porpoises that died from infectious diseases had a higher polychlorobiphenyl level than those that died from physical trauma (Jepson et al., 2005). Some studies have also attempted to link contaminant presence and susceptibility to infectious diseases in bivalves. The immunological defence of bivalves is mediated, in part, by hemocytes responsible for recognition, phagocytosis, and elimination by oxidation of non-self particles (Cheng, 1981). Several studies have demonstrated harmful effects of pollutants in bivalves, including PAHs (Wootton et al., 2003), PCBs (Canesi et al., 2003), pesticides (Alves et al., 2002) and heavy metals (Gagnaire et al., 2004) in particular in the Pacific oyster, *C. gigas* (Gagnaire et al., 2006). Contamination of the eastern oyster, *Crassostrea virginica*, by polluted sediment and tributyltin increased the intensity of *Perkinsus marinus* infection, but no cellular or humoral parameters were modulated (Chu et al., 2002). In contrast, contamination of the blue mussel, *Mytilus edulis*, followed by a *Vibrio tubiashii* challenge resulted in an increased hemocyte count when cadmium was used (Pipe and Coles, 1995) and in an increased phagocytosis with low concentrations of copper (Parry and Pipe, 2004). However, no clear relationship has been established between contaminants and immune response, especially in *C. gigas*. This phenomenon is nevertheless of interest because of its potential impact on the shellfish farming economy.

In this context, the Pacific oyster, *C. gigas*, was used to evaluate the impact of a pesticide mixture on some immune-related capabilities in an economically important bivalve species and to demonstrate a relationship between infectious diseases, defence capacities and pollutants. In a previous study, we have demonstrated in *in vitro* assays the effect of a mixture of eight pesticides on phagocytosis on *C. gigas* hemocytes, while none of these eight pesticides induced any effects when tested separately (Gagnaire et al., 2006). The aims of the present study were (i) to develop an *in vivo* model of experimental contamination using a mixture of pesticides similar to that used previously and representative of concentrations experienced in natural settings where oysters are cultured and (ii) to study effects of this mixture on the immune response to a bacterial challenge.

Oysters were exposed over a 7-day period with a mixture of eight pesticides (atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl). The pesticides were selected on the basis of spread amounts in the Marennes-Oleron Basin (Charente-Maritime, France), one of the most important oyster producing areas in France (Léonard, 2002; Munaron et al., 2006). As a first step, oysters were only contaminated in three distinct experiments and hemocyte biomarkers (cell mortality, esterase activity, production of reactive oxygen species (ROS) and phagocytosis) were monitored using flow cytometry. The expression of 19 genes involved in hemocyte functions was also analysed using real-time PCR. In a second step, after a 7-day contamination period, two pathogenic *Vibrio splendidus*-related strains were simultaneously injected into the adductor muscle of oysters. Oyster mortality was monitored daily. The expression of 10 genes among the 19 genes involved in hemocyte functions was followed after bacterial injection using real-time PCR.

2. Material and methods

2.1. General methods

2.1.1. Animals

Pacific oysters, *C. gigas*, with a shell height of 3–5 cm, were produced and held at the Ifremer's laboratory in La Tremblade (Charente-Maritime, France) in February 2003. Analyses were performed from February 2004 to March 2005.

2.1.2. Xenobiotics

Eight pesticides were selected: atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl (Promochem®). A stock solution of each solid pesticide was separately prepared in an appropriate solvent (distilled water for glyphosate and terbuthylazine, 80% alcohol for the six other pesticides). The eight pollutant stock solutions were then each mixed in distilled water and 10 mL of this mixture were diluted in 1990 mL of seawater to create a working solution. This working solution was prepared just before assays and 2000 mL were added to individual experimental (contaminated) tanks (100 L). Oysters were only exposed to the mixture of the eight pesticides. The final concentrations in tanks were: 0.7 μg/L for atrazine, 0.7 μg/L for glyphosate, 0.8 μg/L for alachlor, 0.6 μg/L for metolachlor, 0.6 μg/L for fosetyl-alumimium, 0.6 μg/L for terbuthylazine, 0.5 μg/L for diuron and 0.05 μg/L for carbaryl.

2.1.3. Exposure protocol

Experiments were conducted at Ifremer's laboratory in La Tremblade (Charente-Maritime, France). Oysters were acclimated over a 1-week period by increasing water temperature from 11–12 to 19–20 °C using a thermostatic control (1–2 °C increase per day). Seawater temperature was maintained at 19–20 °C during the trial. Two tanks were used: one received 100 oysters in 100 L of seawater (untreated control) and one received 100 oysters in 100 L seawater added with pesticides (experimental treatment). Oysters were fed daily with *Chaeto-*

ceros gracilis (addition of 10 L of phytoplankton solution at 5×10^6 cells/mL in both tanks). Seawater was changed daily in both tanks. The pesticide solution was added every day after the seawater renewal in the experimental tank for 7 days.

2.1.4. Bacterial challenge

Bacterial strains LGP31 and LGP32 (*V. splendidus*-related) used in this study were isolated from oyster hemolymph during a mortality outbreak (Gay et al., 2004a). Both bacterial strains were separately grown at 20 °C for 24 h in solid Zobell media that contained 2 g pastone, 0.5 g yeast extract, 6 g agar, 500 mL of autoclaved (121 °C for 20 min) artificial seawater (ASW: 234 g NaCl, 15 g KCl, 12 g $MgSO_4 \cdot 7H_2O$, 2 g $CaCl_2 \cdot 2H_2O$, 1 L distilled water, use at 1/10 dilution, pH 7.4). After 24 h, bacterial strains were maintained separately in 5 mL of liquid Zobell (2 g pastone, 0.5 g yeast extract, 500 mL sterile ASW, pH 7.4) and cultivated with agitation for 24 h at room temperature. An equal volume of each bacterial suspension was collected and the two strains were mixed. Bacterial concentrations were evaluated using a spectrophotometer at 600 nm (UV-160, Shimadzu®). If the optical density value was 1.1, cell concentration was considered as 10^9 Colony Forming Units (CFU) mL^{-1}. Bacteria were centrifuged (30 min, 2000 × *g*) and re-suspended in ASW.

Before injection, oysters were anaesthetised by the addition of a 50 g L^{-1} $MgCl_2$ solution (250 g $MgCl_2$, 2 L seawater, 3 L freshwater) with phytoplankton (*Isochrysis* sp.) to stimulate feeding over a 2–3 h period with oxygenation. Subsequently, 50 μL of the mixed bacterial strains were injected into the adductor muscle. After injection, oysters were maintained in tanks (30 oysters in 2.5 L) in aerated seawater filtered to 0.45 μm at 20 °C. Animals were fed with phytoplankton daily.

These two *V. splendidus*-related strains have a growth optimum of 19–20 °C. These strains were isolated from a mortality episode which occurred at similar temperatures. We, therefore, chose to conduct our bacterial challenge experiments at these temperatures.

2.1.5. Hemocyte collection

Hemolymph (0.5–1.0 mL) was collected from the pericardial cavity with the use of a 1-mL syringe equipped with a needle (0.9 mm × 25 mm) after opening carefully the shell. Hemolymph samples were filtered through a 60 μm mesh to eliminate aggregates and then maintained on ice to limit hemocyte aggregation for further flow cytometry analysis and RNA extraction.

2.1.6. Flow cytometry analysis

Flow cytometry protocols were previously described (Gagnaire et al., 2006). For each sample, 3000 events were counted using an EPICS XL 4 flow cytometer (Beckman Coulter). Results were analysed on cytograms indicating cell size (FSC value) and cell complexity (SSC value) with the fluorescence channel(s) corresponding to the marker used. A gate was defined on the basis of FSC value in order to eliminate cell debris and bacteria. Excitation was performed by an argon laser at 488 nm.

Four hemocyte parameters (cell mortality, esterase activity, ROS production and phagocytosis) were monitored using flow cytometry. Analyses were conducted using 200 μL of hemocyte suspension. For cell mortality, hemocytes were incubated in the dark for 30 min at 4 °C with 10 μL of propidium iodide (PI, 1.0 mg L^{-1}, Interchim); fluorescence emission was measured at 600 nm. The EPICS XL 4 software allows differentiation between supposed populations of granulocytes and hyalinocytes based on FSC and SSC values. Esterase activity was measured using the non-specific liposoluble substrate fluoresceine diacetate (FDA, Molecular Probes). The non-fluorescent FDA diffuses in most cells due to its liposoluble nature. Non-specific esterases hydrolyse it into a charged fluorescent product which is impermeable and trapped in viable cells (Dive et al., 1990). One microliter of a FDA solution (400 μM) was added to 200 μL of hemocyte suspension; fluorescence emission was measured at 514 nm. Production of ROS was measured using dihydrorhodamine 123 (DHR123, Molecular Probes). DHR123 is a non-fluorescent reduced product derived from rhodamine 123. DHR 123 diffuses into the cells and is oxidized to fluorescent rhodamine 123 by superoxide anion O_2^- during oxidative burst (Rothe et al., 1988). One microliter of a DHR123 solution (145 μM) was added to 200 μL of hemocyte suspension; fluorescence emission was measured at 528 nm. For esterase and ROS detection, cells were incubated for 30 min in the dark at room temperature and the reaction was stopped on ice (5 min). For esterase activity and ROS, gates were defined on cytograms in order to distinguish different populations according to fluorescence intensity and statistical analysis was only conducted on the population of strongly stained cells (Gagnaire et al., 2006). For phagocytic assay, 200 μL of hemocyte suspension were incubated for 1 h in the dark at ambient temperature with 10 μL of a 1/10 dilution of Fluorospheres® carboxylate-modified microspheres (1 μm diameter, Interchim); fluorescence emission was measured at 515 nm. Phagocytosis was measured as the proportion of cells that gave a fluorescent signal equal to or higher than the fluorescence of three beads as previously published (Gagnaire et al., 2006).

2.1.7. Gene selection

Nineteen genes involved in hemocyte functions were selected from *C. gigas* cDNA databases. Sixteen genes encode cell signalling proteins (ficolin, galectin, LBP/BPI, LPS/β-1,3 glucan, Vav, importin α, ECSIT, DOCK180 and C-Src kinase), cytoskeleton proteins (ankyrin, cofilin, filamin and Rho) and proteins involved in post-phagocytosis degradation and cellular protection mechanisms (Isocitrate dehydrogenase, proCL and SOD). Three other genes related to defence mechanisms in general were also monitored (Tissue Inhibitor of MetalloProteinases (TIMP), lysozyme and defensin) (Table 1).

2.1.8. Total RNA extraction

Total RNA was extracted from hemocytes using Trizol (Trizol® reagent, Invitrogen™; 1 mL Trizol for 0.5–1 × 10^6 hemocytes) and treated with 1 unit DNase I (deoxyribonuclease) (Sigma) per μg of total RNA to prevent DNA contamination. RNA concentrations were measured before and after DNase

Table 1
The 19 *C. gigas* genes selected for real-time PCR analysis; these genes are involved in hemocyte functions

Cell signaling	Cytoskeleton proteins	Post-phagocytosis degradations and cellular protection mechanisms	Defence mechanisms
Ficolin	Ankyrin	Isocitrate deshydrogenase	TIMP
Galectin 4	Cofilin	pro-Cathepsin L	Lysozyme
LBP/BPI protein	Filamin	SOD	Defensin
LPS/β-1,3-glucan protein	Rho protein		
Vav-3 protein			
Importin-α			
ECSIT			
DOCK 180 protein			
c-Src kinase			

treatment at 260 nm using the conversion factor 1 OD = 40 μg RNA.

2.1.9. Reverse transcription

Reverse transcription (RT) was carried out as previously described (Huvet et al., 2003) using oligo(dT)23 anchored as a primer, 200 U moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma), and 2 μg of total RNA PCR samples from each condition.

2.1.10. Real-time PCR analysis

The relative levels of gene transcripts in untreated (U) and pesticide-treated (T) oysters were investigated by real-time PCR using an Icycler (Bio-Rad Laboratories Inc.). Amplification of a specific cDNA (elongation factor, coefficient of variation < 5%) was performed to confirm the steady-state expression of a housekeeping gene, allowing an internal control for gene expression. Primer sequences and GenBank Accession numbers for all studied and housekeeping genes are reported in Table 2. Real-time PCR was performed in triplicates in a total volume of 15 μL with 5 μL of cDNA (1/10 dilution), 0.5 μL of each primer (final concentration of 0.33 μM), 1.5 μL distilled water and 7.5 μL of 2× "iQ™ SYBR® Green supermix" (BioRad). The cycling conditions consisted of Taq polymerase thermal activation for 3 min at 95 °C, then 40 cycles of denaturation at 95 °C for 30 s and annealing/elongation at 60 °C for 1 min. For every individual sample, a melting curve program was carried out from 95 to 55 °C by decreasing temperature by 0.5 °C each 10 s in order to confirm production of a single product. Each run included the cDNA control, negative controls (total RNA treated with DNase I) and blank controls (distilled water). PCR efficiency (E) was determined for each primer pair by constructing standard curves from serial dilutions to ensure that E ranged from 99% to 100%. Cycle threshold (C_t) value corresponded to the number of cycles at which the fluorescence emission monitored in real-time exceeded the threshold limit. C_t and E were determined using the iCyclerTMiQ, Optical System Software, v. 3.0a (Bio-Rad). The relative expression ratio of every gene was calculated using REST-384© software (Relative Expression Software Tool, http://www.gene-quantification.de/rest-384.html) (Pfaffl et al., 2002). The relative expression ratio for a considered gene is based on the PCR efficiency (E) and the C_t of the pesticide treated (T) sample versus the untreated (U) sample expressed in comparison to the reference gene, according to Pfaffl's mathematical model (Pfaffl, 2001):

$$\text{Ratio} = \frac{(E\ \text{studied gene})^{\Delta C_t\ \text{studied gene(untreated}-\text{treated)}}}{(E\ \text{reference gene})^{\Delta C_t\ \text{reference gene(untreated}-\text{treated)}}}.$$

2.2. Schedule of trials

2.2.1. Pesticide contamination

The contamination experiment was performed three distinct times during February and March 2004. Flow cytometry analyses were performed for these three experiments to assess the four hemocyte parameters outlined in the general methods section above. At days 1, 3 and 7 after pesticide contamination, hemocytes from 20 oysters per condition (untreated (U) and treated (T)) were collected. Twenty untreated oysters were also analysed at Day 0 (before adding pesticides). For both conditions (U and T), hemolymph from four pools of five oysters were used to reduce inter-individual variation and to provide enough hemocytes. Pooled hemocytes were counted on a Malassez cell and were adjusted to 10^6 cells mL^{-1} with ASW. After 7 days of contamination for the first experiment, hemolymph from 20 oysters per condition (U and T) were collected and pooled for RNA extraction and characterization of gene expression using real-time PCR analysis of the 19 selected genes (Table 1).

2.2.2. Bacterial injection

Experiments were run in January 2005 without pesticide contamination in order to define the mortality percentages induced by different concentrations of LGP31 + LGP32 *Vibrio* strains in control (without pesticides) oysters. Equal concentrations of each strain were inoculated into oysters (total concentrations of 2×10^8, 4×10^7 and 4×10^6 CFU/oyster). Experiments were carried out three times for each bacterial concentration and two replicates were employed in each of the three conditions (injected with 2×10^8, 4×10^7 and 4×10^6 CFU/oyster). Oyster mortality percentages were recorded for every bacterial concentration at several post-injection times (2, 4, 24, 48, 72, 120, 144 and 168 h).

2.2.3. Pesticide contamination followed by bacterial injection

In order to observe the impact of pesticide contamination on oyster susceptibility to bacterial disease, exposure to the pesticide mixture was followed by a bacterial injection trial in

Table 2
Information (primer sequences, GenBank Accession numbers and references) related to the 19 genes selected for real-time PCR analysis

Gene	5′-3′ primer sequence		GenBank number	Reference
Ficolin 3	Forward	Gca aag gct gtg ttc tgt ga	BQ426875	Gueguen et al. (2003)
	Reverse	Ctt gta atc cgt cca gtt acg g		
Galectin 4	Forward	Aga aca gac cta cca tgc cac t	BQ426390	Gueguen et al. (2003)
	Reverse	Atc cgt ctt gtc cag agc ac		
LBP/BPI I	Forward	Acg gca cag aac gga tct ac	AY165040	Gueguen et al. (2003)
	Reverse	Tgg ttg aca tcg ttg ctg ac		
LPS/β-1,3-glucan	Forward	Tgc ggt gaa ctc tga ctt gt	CB617438	Boutet et al. (2004)
	Reverse	Aat gta gct gtg gga ggt gtg		
Vav-3 oncogene homologue	Forward	Gcg ttg act ggc tcg tta g	BQ427355	Gueguen et al. (2003)
	Reverse	Gca ctc cat ctc gca aag tt		
Importin α	Forward	Acg cag cag att gaa cga c	CB617497	Boutet et al. (2004)
	Reverse	Gca gga gac agt gca gaa tg		
ECSIT	Forward	Gtg tga ttc ccg atg agg ag	BQ427193	Gueguen et al. (2003)
	Reverse	Act tgg gca tcc agt aca gc		
DOCK 180 protein	Forward	Caa cga ctc cgt tca aca ac	BQ426954	Gueguen et al. (2003)
	Reverse	Cgc tgg aaa caa cga aca		
c-Src kinase	Forward	Aca aca gga ggc tga agg tg	BQ426966	Gueguen et al. (2003)
	Reverse	Gtg tgg cgt gtt tca tcg t		
Ankyrine brank-2	Forward	Gtt cgg agc taa cgt gaa cc	BQ426701	Gueguen et al. (2003)
	Reverse	Tag ctg gac gat cag gga gt		
Cofilin	Forward	Cgt ggg tga cgg ata gtc tt	BQ426293	Gueguen et al. (2003)
	Reverse	Tca gtg gcg tcg tta cac tg		
Filamin	Forward	Gtg acc ttt gat ggc tgt ga	BQ426716	Gueguen et al. (2003)
	Reverse	Ggt acg aag tcc gtc tcc tg		
Rho protein	Forward	Agc aaa gac cag ttc cca ga	BQ426365	Gueguen et al. (2003)
	Reverse	Ccc ata atg cca act caa cc		
Isocitrate dehydrogenase	Forward	Ccg acg gaa aga ctg tcg	AY551096	Huvet et al. (2004)
	Reverse	Ctg gct acc ggg ttt gtg		
Precursor of Cathepsine L	Forward	Cca gcc aga agc tgt agt cc	CF369221	Boutet et al. (2004)
	Reverse	Gag cgg aag acg aag cta ga		
SOD-like	Forward	Atg tca cag gaa ccg tcc a	BQ426796	Gueguen et al. (2003)
	Reverse	Tcc atg ctg tcc agg tgt ta		
TIMP	Forward	Att gcc gtg gtg aga act g	BQ427105	Gueguen et al., 2003
	Reverse	Tag cgt agc agt cgt tcg tg		
Lysozyme-like	Forward	Gca gcc gga ttc aac ct	AB179775	Nakamura et al. (unpublished)
	Reverse	Cgt tgt ggt ctt tgc ttc ac		
Defensin	Forward	Tgt cct tct gat ggt ttc tgc	AM050547	Gueguen et al. (2006)
	Reverse	Gcc cgc tct aca act aat gg		
Elongation factor I	Forward	Acc acc ctg gtg aga tca ag	BQ426516	Gueguen et al. (2003)
	Reverse	Acg acg atc gca ttt ctc tt		

Elongation factor I gene was used as reference (Gueguen et al., 2003, 2006; Boutet et al., 2004; Huvet et al., 2004).

March 2005. After a contamination period of 7 days as outlined above, treated oysters as well as untreated oysters were injected with 4×10^7 CFU/oyster. Negative controls consisted of 10 oysters (U and T) that were injected with sterile ASW. Oyster mortality was monitored for each of the four conditions (U injected with ASW, T injected with ASW, U injected with 4×10^7 CFU/oyster, T injected with 4×10^7 CFU/oyster) at several times post-injection (2, 4, 24, 48 and 72 h). Two replicate tanks were included for all the four conditions.

Hemolymph from 10 oysters for the four conditions were collected 4 and 24 h post-injection for RNA extraction and gene expression analysis. In this trial, the expression of the given 10 genes was followed by real-time PCR: ficolin, galectin and LBP/BPI, C-Src kinase, ankyrin, proCL, SOD, TIMP, lysozyme and defensin (Table 1).

2.3. Statistical analysis

For flow cytometry analysis, results were expressed as a percentage of positive cells. Results were expressed as percentages for mortality measurements. Values were arcsine square root transformed to achieve normality. ANOVAs were carried out

using Statgraphics® Plus Version 5.1 software. Significance was set at $p \leq 0.05$.

For real-time PCR analysis, statistical differences in gene expression between conditions were evaluated in group means by randomisation tests (Pfaffl et al., 2002) using REST-384© software and Pair Wise Fixed Reallocation Randomisation Test©. Two thousand random allocations were performed and significant differences were considered at $p < 0.05$.

3. Results

3.1. Effects of pesticide exposure on oysters

3.1.1. Hemocyte parameters

No oyster mortality occurred in any of the three experiments (data not shown). Cell mortality, esterase and ROS production showed no significant differences between U (untreated oysters) and T (treated oysters) (Fig. 1). Phagocytosis was significantly lower in T compared to U (i) after 7 days of contamination for experiment 1, 1 and 7 days for experiment 2, and 3 and 7 days for experiment 3 ($p < 0.05$, Fig. 2), (ii) for the whole data treated independently of time for each experiment and (iii) after 7 days of contamination when pooling data of the three experiments (data not shown).

3.1.2. Gene expression

Relative expression of the 19 selected genes involved in hemocyte functions was significantly lower for all genes from treated (T) compared to those from untreated (U) animals ($p < 0.001$, Fig. 3). Decreases in gene expression of T compared to U ranged from 60.8% for defensin (2.5 times lower) to 96.9% for filamin (32.6 times lower) (Fig. 3).

3.2. Effects of bacterial challenge on oyster mortality

The injection of 4×10^7 CFU/oyster induced $55 \pm 8.8\%$ of mortality at 168 h post-injection (Fig. 4). Injection of 4×10^6 CFU/oyster induced significantly lower mortalities relative to other concentrations: mortality percentage was $20 \pm 10.5\%$ 168 h post-injection (Fig. 4). Injection of 2×10^8 CFU/oyster induced the highest mortality: mortality rates were $23.3 \pm 6\%$ and $90 \pm 2.6\%$ at 2 and 168 h post-injection, respectively (Fig. 4).

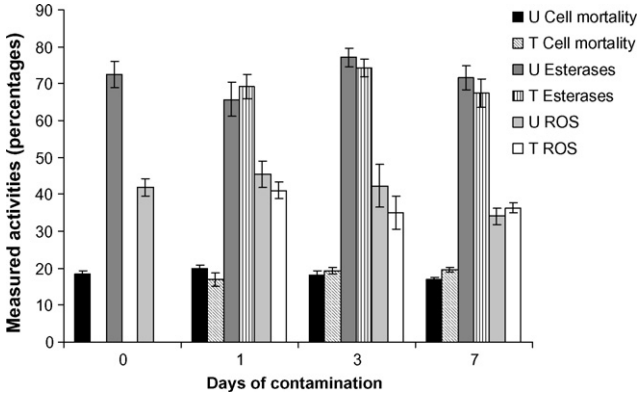

Fig. 1. Hemocyte parameter results obtained after pooling results from three replicates of the *in vivo* pesticide contamination (4 pools per replicate). U: untreated oysters, T: treated oysters. ROS: Reactive Oxygen Species. Means ± standard errors are presented. $N = 12$ replicates.

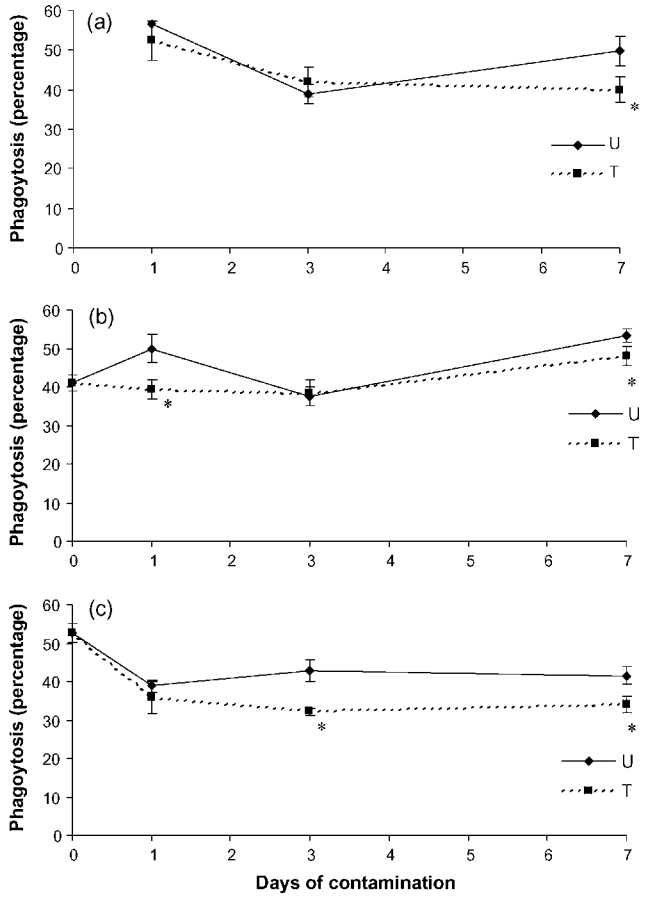

Fig. 2. Phagocytosis percentages obtained for each of the three *in vivo* pesticide contamination experiments. U: untreated oysters, T: treated oysters. (a) Experiment 1; (b) experiment 2; (c) experiment 3. Means ± standard errors are presented. $N = 4$ replicates for each experiment. $^*p < 0.05$.

3.3. Combined effects of pesticide exposure and bacterial challenge on oysters

Preliminary experiments using only bacteria showed that 2×10^8 CFU/oyster, induced high losses 24 h post-injection. In contrast, 4×10^6 CFU/oyster induced low mortality. Both bacterial concentrations were not selected for further studies because pesticide effects on mortality would have been difficult to detect under very high or low mortality conditions. The concentration of 4×10^7 CFU/oyster was selected to carry out assays including pesticide contamination and a bacterial challenge.

3.3.1. Mortality

After bacterial inoculation, mortality was significantly higher for treated oysters (T) compared to untreated oysters (U) at 48 h, 72 h post exposure and when cumulative mortality was examined (three way ANOVA, $F = 19.62$, $p = 0.0001$, Fig. 5). Seventy

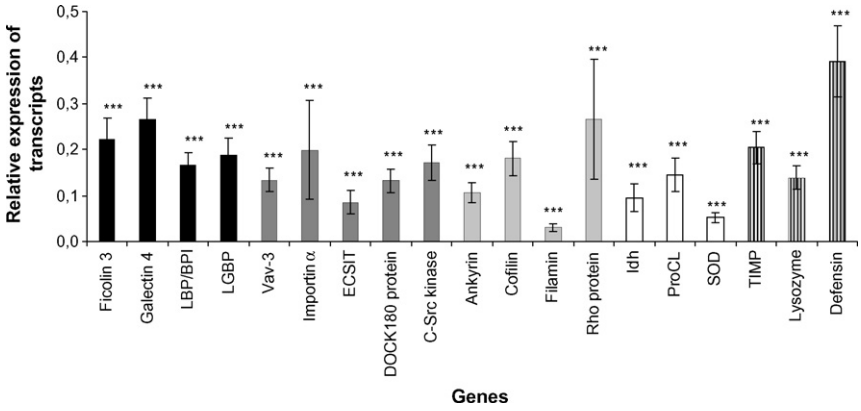

Fig. 3. Relative expression of the 19 gene transcripts (normalised to elongation factor I) in pesticide exposed oysters compared to controls after 7 days of contamination. RNA was extracted from hemocytes of 20 oysters for each condition. $N = 3$ intra-experimental replicates. Standard error is presented. Value < 0.5 shows a significant decrease of relative expression of transcripts in treated oysters (T) compared to untreated oysters (U). All histograms presented a value < 1 ($p < 0.001$). See Table 1 for the category of the genes.

percent (±5%) of T injected with 4×10^7 CFU/oyster died by 72 h post-injection, while only 25 ± 3.7% of U injected with the same dose died (Fig. 5). At 24 h post-injection, mortality rates were not significantly different between pesticide T and U injected with bacteria ($p > 0.05$, Fig. 5). A few of control oysters, T or U injected with ASW, died during this trial (8% cumulative mortality 72 h post-injection) and values were not significantly different between T and U (Fig. 5). Mortality was highest at 72 h post-injection in all conditions ($F = 50.75$, $p = 0$, Fig. 5).

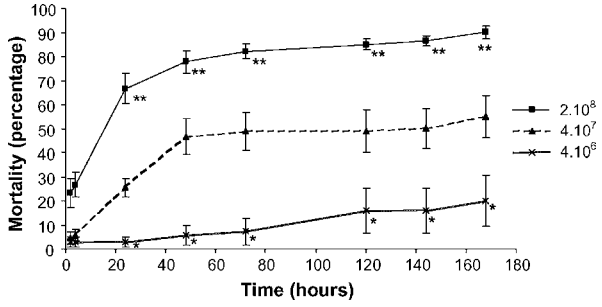

Fig. 4. Mortality percentages induced by several concentrations of LGP31+LGP32 strains (2×10^8, 4×10^7 and 4×10^6) at several post-injection times (2, 4, 24, 48, 72, 120, 144 and 168 h). $N = 6$ replicates. Standard error is presented. $^*p < 0.05$; $^{**}p < 0.01$.

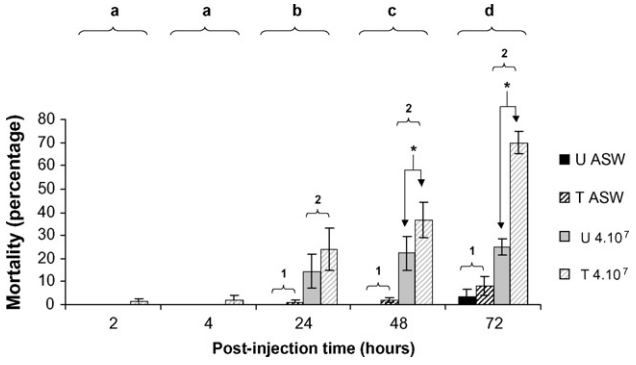

Fig. 5. Mortality (in percent) obtained after injection of artificial seawater or 4×10^7 CFU/oyster in 7-day controls and pesticide exposed oysters. $N = 2$ replicates. Bars represent standard error. *Represents a difference between U (untreated oysters) and T (treated oysters) (U < T, $p < 0.05$). Numbers 1 and 2 represent a difference between ASW and 4×10^7 CFU injected oysters (1 < 2, $p < 0.05$). Letters a, b, c and d represent a difference between post-injection times (a < b < c < d, $p < 0.05$).

3.3.2. Gene expression

In our experiments, gene expression from pesticide-treated animals injected with ASW or bacteria were compared to untreated animals injected with ASW or bacteria. Our experiment design allowed to follow pesticide effects, and not bacterial effects alone, on gene expression after a bacterial challenge.

We monitored the expression of only 10 of the 19 genes followed in pesticide contamination experiments (Table 1). We had to make a selection because of the limited quantity of RNA extracted from hemocytes for this experiment. We monitored gene expression 4 and 24 h post-injection because mortality rates were not significantly different between treated injected oysters and untreated injected oysters. Samples at this time were therefore comparable.

Experimental treatments impacted gene expression. Both down and up-regulation were observed.

(i) When injected with seawater, the relative expression of five genes (galectin, C-Src kinase, ankyrin, lysozyme and defensin) was down-regulated in pesticide treated oysters (T) compared to untreated oysters (U) 4 h post-injection (1.9, 2.7, 1.8, 1.7 and 2.6 times lower, respectively, $p < 0.05$, Fig. 6). The relative expression of three genes (ficolin, SOD and TIMP) increased 24 h post-injection of ASW (3.0, 4.6 and 2.4 times higher, respectively, $p < 0.05$, Fig. 6).

(ii) When injected with bacteria, the relative expression of ficolin, galectin, C-Src kinase, ankyrin, SOD, lysozyme and defensin decreased in T compared to U 4 h post-injection of 4×10^7 CFU/oyster (8.9, 6.6, 9.1, 3.3, 4.4, 23.6 and 2.2 times lower, respectively, $p < 0.05$, Fig. 7). The expression of the 10 genes (ficolin, galectin, LBP/BPI, C-Src kinase, ankyrin, proCL, SOD, TIMP, lysozyme, defensin) was up-

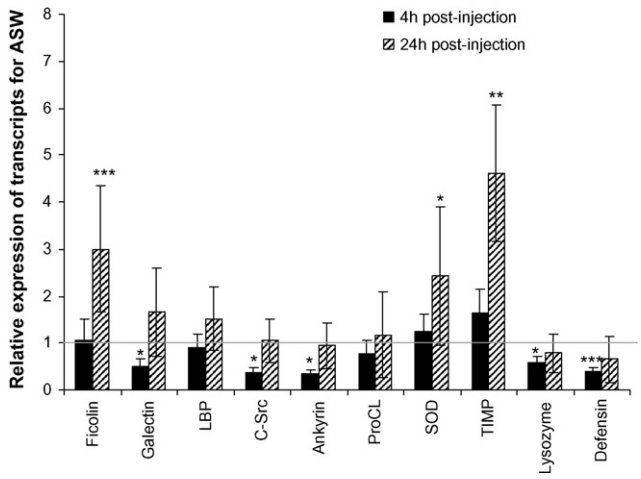

Fig. 6. Relative expression of the 10 gene transcripts (normalised to elongation factor I) in 7 days pesticide treated oysters injected with ASW compared to untreated oysters injected with ASW 4 and 24 h post-injection. RNA was extracted from hemocytes of 10 oysters for each condition. $N = 3$ intra-experimental replicates. Bars represent standard error. The grey line (value = 1) corresponds to an identical relative expression of transcripts in U (untreated oysters) and T (treated oysters). When statistically different from 1, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

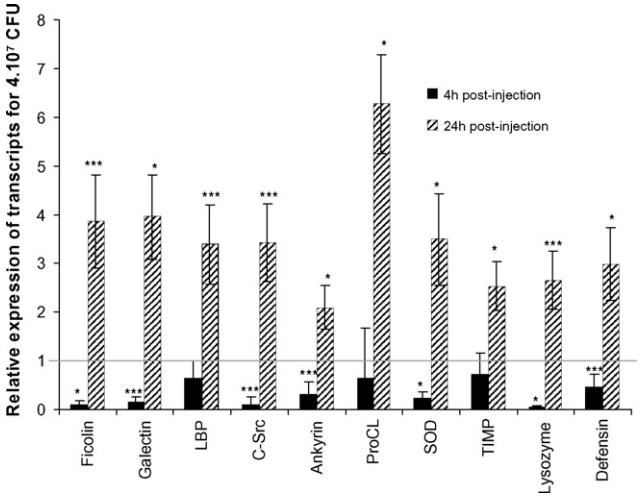

Fig. 7. Relative expression of the 10 gene transcripts (normalised to elongation factor I) in 7 days pesticide treated oysters injected with 4×10^7 CFU/oyster compared to untreated oysters injected with 4×10^7 CFU/oyster 4 and 24 h post-injection. RNA was extracted from hemocytes of 10 oysters for each condition. $N = 3$ intra-experimental replicates. Standard error is presented. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. The grey line (value = 1) corresponds to an identical relative expression of transcripts in U (untreated oysters) and T (treated oysters).

regulated 24 h post-bacterial injection (3.8, 3.9, 3.4, 3.4, 2.1, 6.3, 2.5, 3.5, 2.6 and 2.9 times higher, respectively, $p < 0.05$, Fig. 7). Among these 10 genes, the expression of seven genes (galectin, LBP/BPI, C-Src kinase, ankyrin, proCL, lysozyme and defensin) was not changed 24 h after ASW injection. Results are summed up in Table 3.

4. Discussion

Although often employed in successful modern agriculture, the use of pesticides leads to severe environmental pollution and can be dangerous for human safety. Dangerous amounts of pesticides and pesticide residues have been found in vegetables, milk and meat (Banerjee et al., 2001). The effects of pesticides have been evaluated most commonly by quantifying enzymatic alterations, and pathological, carcinogenic and mutagenic effects. Despite the intensive use of pesticide in coastal areas, few studies have investigated their effects on marine animals in laboratory trials (Galloway and Depledge, 2001) and have focused primarily on the effects of pesticide mixtures (Faust et al., 2001; Tanguy et al., 2005; Gagnaire et al., 2006). Although animals are exposed to complex mixtures of pollutants in the environment, most of laboratory experiments are based on the use of a unique pollutant (Banerjee et al., 2001). Studies on herbicides (Faust et al., 2001) have shown some positive interactions: toxicity of a mixture of pollutants was higher than the toxicity of individually tested chemicals. A pesticide mixture modulated *C. gigas* hemocyte phagocytosis *in vitro* whereas no modulation was reported for each molecule tested individually (Gagnaire et al., 2006). Antagonist effects have also been shown for heavy metals (Breittmayer et al., 1984).

4.1. Effects of pesticides on hemocyte parameters and defence gene expression

Although cell mortality, esterase and ROS activity were not modified by the pesticide mixture, phagocytic ability decreased after 3 and 7 days of contact in the three experiments. Some of the selected pesticides present in the mixture have already been tested individually on phagocytosis in *in vivo* assays. Atrazine decreased phagocytosis in the pond snail, *Lymnaea stagnalis* (Russo and Lagadic, 2004) but had no effect on that activity in *C. gigas* (Gagnaire et al., 2003). Diuron increased phagocytosis of *C. gigas* hemocytes after 4 weeks of exposure (Bouilly et al., 2007). The combined effects of these molecules affected phagocytosis in *C. gigas*.

Table 3
Summary of gene expression results obtained with injection of ASW and 4×10^7 CFU/animal at 4 and 24 h post-injection for the 10 genes followed

		Ficolin	Galectin	LBP	C-Src	Ankyrin	ProCL	SOD	TIMP	Lysozyme	Defensin
ASW	4 h	NS	▼	NS	▼	▼	NS	NS	NS	▼	▼
	24 h	▲	NS	NS	NS	NS	NS	▲	▲	NS	NS
4×10^7	4 h	▼	▼	NS	▼	▼	NS	▼	NS	▼	▼
	24 h	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲

NS: non significant; arrows: up- or down-regulation.

The expression of all 19 genes monitored by real-time PCR in the present study showed a decrease in mRNA levels in samples exposed to pesticides compared to untreated samples. These results support the observed decrease in phagocytosis activity reported using flow cytometry after 3 and 7 days of contamination; some of these genes are involved in defence mechanisms and phagocytosis process. Indeed, ficolin-3 and galectin-4 are two lectins that bind β-galactoside residus (Mitta et al., 2005). LBP/BPI complex and LGBP bind LPS and activate the pro-phenoloxidase cascade (Mitta et al., 2005). LGPB is over-expressed upon bacterial infections (Tanguy et al., 2004) and was over-expressed in *C. gigas* after 30 days of contamination with a mixture of atrazine, diuron and isoproturon (Tanguy et al., 2005). Our results, in contrast, showed a down-regulation of these genes. Isoproturon was not used in our experiments and concentrations of other molecules were lower than in the study by Tanguy et al. (2005). Vav-3 protein, cofilin and importin-α are involved in signal cascade of lymphocytes in vertebrates (Lee et al., 2000; Andrade et al., 2003; Fujikawa et al., 2003). DOCK180 protein and c-Src kinase are involved in macrophage activation (Suzuki et al., 2000; Cote and Vuori, 2002). ECSIT is involved in the Toll/IL-1 and Rel/NF-κB signal cascade, important in innate immune response to infectious agents by the regulation of MAPK (Kopp et al., 1999), which exists in bivalves (Canesi et al., 2002). Our experiments showed a down-regulation of these six genes which suggest a disruption of cell activation pathways, and may be related to the reduced phagocytic ability observed in the present study. Ankyrin is important in cytoskeletal organisation (Lambert and Bennett, 1993). Filamin, an actin binding protein, and Rho protein, a GTPase, are involved in the regulation of actin organisation and adhesion (Chimini and Chavrier, 2000; Van der Flier and Sonnenberg, 2001). In our study, these three genes were down-regulated by pesticide exposure, which could modify cytoskeleton activities and render hemocytes unable to phagocytose particles. Isocitrate dehydrogenase and SOD protect cells against reactive oxygen species (Kim and Park, 2003). We showed a down-regulation of these both genes, which could lead to a decrease of protection against free radicals, one of the known action mode of several pesticides (Banerjee et al., 2001). However, no modification of ROS production monitored by flow cytometry was found after the pesticide exposure (Fig. 1). Cathepsin L is present in lysosomes and is involved in protein degradation (Hu and Leung, 2004). Our results showed a down-regulation of this gene by pesticides which could lead to a decrease of post-phagocytotic degradation. However, cathepsin L over-expression has been observed in oysters contaminated with glyphosate after 30 days of contamination (Tanguy et al., 2005). TIMP is a protease inhibitor over-expressed after injuries and bacterial challenges in *C. gigas* (Montagnani et al., 2001). Lysozyme is a bactericidal enzyme largely present in bivalves (Cheng, 1981). Defensin is an anti-bacterial peptide (Gueguen et al., 2006). These genes were down-regulated in our experiments. Pesticides may lead to decrease of bactericidal activities.

The down-regulation of these 19 genes by pesticides may therefore decrease the capacity of oysters to respond to an infectious agent.

4.2. Effects of pesticides and bacterial injection on oyster mortality and gene expression

V. splendidus-related strains (LGP31 + LGP32) used in this study were previously isolated from moribund oysters during a mortality episode. These bacteria induced a higher mortality when injected simultaneously than when inoculated separately (Gay et al., 2004a). Intramuscular injection was selected in the present study to induce high mortality rates (∼50%) (Gay et al., 2004a). This technique bypasses the first lines of defence. Although bath exposure using the same bacterial strains induced mortality, losses were lower than via injection, and with high variations between experiments (Gay et al., 2004b).

The present study provides the first demonstration of the effects of a pesticide mixture at environmentally relevant concentrations on *C. gigas* exposed to a bacterial challenge. After the bacterial challenge, mortality rates in pesticide treated animals were higher than in untreated animals at 48 and 72 h post-injection, indicating that pesticide exposure increased oyster susceptibility to the bacterial injection. Chou et al. (1998) previously demonstrated an interaction between heavy metal exposure and viral infection in the Oriental clam, *Meretrix lusoria*. Higher losses were observed in *M. lusoria* contaminated with heavy metals and exposed to a birnavirus relative to animals that were exposed to single stressors (heavy metals or birnavirus).

An increase of the mRNA expression of the 10 selected genes was reported 24 h after the bacterial challenge in pesticide treated oysters. However, three of these 10 genes (ficolin, TIMP and SOD) were also up-regulated 24 h after ASW injection. We could hypothesize that the up-regulation of these three genes is due to the mechanical aggression due to injection. The higher expression of the seven other genes (galectin, LBP, C-Src kinase, ankyrin, proCL, lysozyme, defensin) could be first interpreted as a more efficient defence response of the oysters to the bacterial injection. Transcriptional up-regulation of these genes has been reported in invertebrates and vertebrates during the course of infectious diseases (Montagnani et al., 2001; Ge et al., 2004; Allam et al., 2006). However, higher mortality rates were reported in pesticide-treated injected oysters compared to untreated injected oysters at 48 and 72 h post-injection. Moreover, high expression of some of these seven genes observed during the course of infectious diseases has been demonstrated to correlate with host damage in vertebrates. High levels of cathepsins B, D and L were observed after a *Pseudomonas aeruginosa* infection in mice and resulted in corneal lesions (Dong et al., 2001). In another study, defensin concentrations were higher in human patients infected with lung diseases than in healthy human patients and have been demonstrated to contribute to disease pathogenesis (Ashitani et al., 1998). In vertebrates, when mechanisms initially developed for the host defence are not correctly controlled, these complex defence mechanisms could create pathological disorders (Haslett et al., 1989).

Pesticides modified the oyster response to a bacterial challenge by inducing an up-regulation of genes involved in hemocyte functions. Some of these genes are involved in defence mechanisms. We could therefore expect that oyster

would present higher defence capacities and therefore reduced disease susceptibility. However, by analogy with vertebrates, and considering the fact that oyster mortality was higher in treated animals compared to untreated animals after the bacterial challenge, we therefore hypothesize that this up-regulation of defence genes could become harmful for the host by causing tissue damages and could be associated to pathogenesis of the disease. Pesticides could modify the regulation capacity of the response to infectious agents in oysters. To support this concept and to substantiate correlation among over-expression of genes and host damage, some tissue morpho-histological observations are needed.

5. Conclusion

This study showed that a pesticide mixture used on Pacific oyster, *C. gigas*, in laboratory conditions at environmentally relevant concentrations (i) decreased hemocyte phagocytosis in a reproducible manner, (ii) down-regulated genes involved in hemocyte functions, particularly phagocytosis, and (iii) increased susceptibility to a bacterial challenge. This is the first study to combine two different techniques (flow cytometry and real-time PCR) to study the effects of pollutants on defence mechanisms in Pacific oyster, *C. gigas*, and to demonstrate, at molecular level, a link between pesticide effect and susceptibility to pathogenic bacteria in oysters. However, our results were obtained using an experimental model of infection based on intramuscular injection of bacteria. This could be useful to reproduce these experiments in conditions closer of those encountered in natural infection (bath exposure). Our study showed that Galectin, LBP, C-Src kinase, ankyrin, proCL, lysozyme and defensin may be considered as markers of a bacterial infection and could be involved in disease pathogenesis. These experiments allowed establishing a reproducible model of contamination and immunosuppression in *C. gigas* capable of weakening the organism in *in vivo* conditions. This model could be suitable for studying interactions between host/pathogen in other models. It would also be interesting to study the expression of the same genes after a sub-lethal injection (4×10^6 CFU/animal) of *V. splendidus*-related strains. Moreover, pollutants could also have an effect on pathogen virulence. Several genes potentially involved in the virulence of these bacterial strains have been identified (Saulnier et al., personal communication). The joint study of host and pathogen response to pollutants would be of interest to identify the conditions needed for a disease to be established.

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5. Amélioration génétique et maladies infectieuses chez les huîtres

5. 1. Huîtres plates et bonamiose (Naciri-Graven Y., Martin A.-G., Baud J.-P., Renault T. & A. Gérard, 1998. Journal of Experimental Marine Biology and Ecology, 224: 91-107)

L'histoire de l'ostréiculture française met en évidence la fragilité de cette activité économique face à l'apparition de maladies. La production d'huître plate, *O. edulis,* a fortement diminué suite à l'apparition de deux maladies parasitaires : la marteiliose et la bonamiose. Depuis sa détection en association à des mortalités d'huîtres plates de l'Ile Tudy en Bretagne en 1979 (Pichot *et al.*, 1979), le parasite protozoaire *Bonamia ostreae,* agent étiologique de la bonamiose (maladie hémocytaire) a fait l'objet de nombreux travaux de recherche pour clarifier sa position taxonomique, développer des outils de diagnostic spécifiques et comprendre son impact sur son hôte.

L'étude ultra structurale et l'analyse moléculaire de la petite sous-unité ribosomale ont permis de confirmer l'intégration du parasite *B. ostreae* dans le phylum des Haplosporidia. Par ailleurs, les techniques d'histologie et d'apposition de tissu cardiaque sont les techniques de référence retenues par l'Office Internationale des Epizooties (OIE) pour le diagnostic de la bonamiose. Cependant, le développement de techniques moléculaires telles que PCR et HIS (Cochennec *et al.*, 2000 ; Carnegie *et al*., 2003) offre de nouvelles possibilités de part leur sensibilité et leur spécificité notamment pour l'étude et la compréhension du cycle du parasite.

Malgré certaines études sur le cycle d'infection du parasite (Van Banning, 1990 ; Montes *et al.* 1994), celui-ci reste inconnu avec toutefois une constante : aucune spore n'a jamais été décrite. En outre, la possibilité pour le parasite d'être transmis à des huîtres saines par simple contact (cohabitation) avec des huîtres infectées a été démontrée expérimentalement, prouvant qu'un hôte intermédiaire n'est pas indispensable.

L'obtention de parasites purifiés (Miahle *et al*. 1988) a largement aidé à reproduire et modéliser la bonamiose au laboratoire (Hervio *et al.*, 1995). Les possibilités de purification du parasite et la primo culture d'hémocytes ont permis l'étude *in vitro* des interactions entre le parasite *B. ostreae* et les cellules hôtes (Mourton *et al.*, 1992 ; Perroquin, 1995). Enfin, la possibilité de purifier le parasite a permis également d'entreprendre des programmes de sélection.

Les moyens de lutte contre la bonamiose sont relativement restreints et l'obtention d'animaux présentant une sensibilité réduite à la maladie apparaît comme une voie à privilégier. Un programme de sélection a été ainsi initié à l'Ifremer en 1985 sur la base d'une pression de sélection reposant en particulier sur l'injection de parasites purifiés. Deux lignées sélectionnées ont été obtenues, respectivement en 1985 (S85) et en 1989 (S89). Il a été ainsi possible de montrer que le **pourcentage de survie** de la troisième génération de la lignée S85 était **significativement supérieur** à celui observé pour les animaux non sélectionnés (contrôles) après un test de 20 mois dans le milieu où le parasite est endémique (**Naciri *et al.*, 1998**). Des différences significatives ont également été observées entre huîtres sélectionnées et non sélectionnées pour la détection du parasite (**Naciri *et al.*, 1998**). L'approche choisie basée sur la sélection massale a montré ainsi son intérêt pour obtenir des huîtres présentant des caractéristiques de résistance accrue à une maladie parasitaire.

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Selecting the flat oyster *Ostrea edulis* (L.) for survival when infected with the parasite *Bonamia ostreae*

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Abstract

In the early 1970s, the appearance of the protozoan parasite *Marteilia refringens* restricted the production of the European flat oyster *Ostrea edulis* to subtidal areas. Ten years later a second protozoan parasite, *Bonamia ostreae*, caused a further serious decline in flat oyster farming in subtidal areas. Prophylactic measures were taken to sustain oyster farming, but the continuing presence of the two parasites led to the initiation of a programme to select for resistant oysters. Efforts were concentrated on *B. ostreae* and two selected strains were obtained in 1985 (S85) and 1989 (S89). The programme was based on mass selection and parasite inoculation or natural infections. This paper focuses on the survival and weights of the third generation of S85 (S85-G3), the second generation of S89 (S89-G2) and a cross between S85-G2 and S89-G1. The % survival of S85-G3 oysters was more than 4-times higher than the control group after a 20 month experiment in the wild, and significant differences in parasite prevalence were recorded. No significant differences between S89-G2 and the control group were observed for either survival or parasite prevalence. This was attributed either to a less powerful experiment or to the effect of genetic load clearing as a result of inbreeding. The % survival of the cross between the two strains was more than twice as high as the control group and significant differences were recorded for parasite prevalence. Good evidence for additivity of the resistance was provided by both the intermediate behaviour of a cross between S85-G2 and the controls and the rapid improvement that was obtained from the first generations. The selected strains showed a tendency toward higher weights and higher weight variances when compared to controls. These populations are suspected to have undergone bottlenecks, which would explain the increase in phenotypic variance. No measurable natural resistance has arisen in the wild. This could be due first to the relatively short time that has elapsed since the first infections, secondly to the early sale of resistant oysters as soon as they reach a marketable size, and thirdly to the slow kinetics of *B. ostreae* infection that

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allows both susceptible and potentially resistant oysters to participate in recruitment. Improved resistance seems to be related to the delayed mortality of selected oysters. This last result is discussed in the light of a previous study conducted in the laboratory which showed that resistant and susceptible oysters have different haemograms. © 1998 Elsevier Science B.V.

Keywords: Ostrea edulis; *Bonamia ostreae*; Disease resistance or tolerance; Inoculation; Selection

1. Introduction

In the past 25 years two protozoans, *Marteilia refringens* and *Bonamia ostreae*, have caused a sharp decline in the commercial production of the European flat oyster *Ostrea edulis* (L.) in France. Present production is down to only 10% of the level attained before the arrival of these protozoans. *M. refringens* first appeared in France in the late sixties and caused high mortality in oyster populations grown in estuaries (Grizel et al., 1974). *B. ostreae* was first reported in France in 1979 (Comps et al., 1980) and was probably introduced with the importation of *O. edulis* spat from California in the late seventies (Elston et al., 1987; Grizel et al., 1988). *B. ostreae* is indeed suspected to have caused mortality in *O. edulis* in California much earlier than in France (Katkansky et al., 1969; Elston et al., 1986). It has subsequently been reported in other parts of North America (Elston et al., 1986; Friedman et al., 1989; Friedman and Perkins, 1994) and also in the Netherlands, Spain, Denmark, England and Ireland (Bannister and Key, 1982; Polanco et al., 1984; Van Banning, 1985; Elston et al., 1987; Figueras, 1991; Culloty and Mulcahy, 1996). *B. ostreae* usually causes mortality in both intertidal and subtidal areas. In France, various management practices have allowed oyster farming to continue in subtidal areas but due to the presence of the two parasites a study to select for oysters resistant to *B. ostreae* was undertaken in 1985 as part of a programme on parasite purification and characterisation. Selecting for *B. ostreae* resistance has been considerably facilitated by the successful purification of *B. ostreae* (Mialhe et al., 1988), since the inoculation of purified parasites allows stronger selection pressures to be used (Hervio et al., 1995). Two different strains, namely S85 and S89, have been developed since 1985 and 1989, respectively, and showed a significant increase in resistance to *B. ostreae* when compared to controls (Martin et al., 1992). In 1993, the programme was completely reorganised and based for each selection cycle on pair mating within and between strains and tests in the wild or in controlled conditions (Baud et al., 1997). This article gives an overview of the *B. ostreae* resistance obtained during the ten years since the beginning of the selection programme and provides an example of selected disease resistance in oysters. Unfortunately, since the selection against *M. refringens* is still difficult and time consuming for several technical reasons, no strain resistant to this parasite has as yet been developed.

2. Materials and methods

Our use of the term ‘‘resistance’’ refers only to improved survival and implies nothing about host–parasite interactions.

2.1. Historical record of the B. ostreae resistant oyster strains

The programme presented in this paper was initiated by pathologists trying to isolate and purify the *B. ostreae* parasite (Mialhe et al., 1988). It was then converted into a selection programme per se in 1990 using the strains already developed (Martin et al., 1992) and the techniques perfected for *B. ostreae* purification and inoculation (Hervio et al., 1995). Consequently, the first experiments were not designed to test for heritable resistance and the corresponding data are therefore presented in this section.

Two selected strains were produced using old animals from Quiberon Bay (Brittany, France) that were thought to have survived *B. ostreae* infections. Up until 1990, control oysters of the same age were collected from the wild in Quiberon Bay and therefore named ''wild Quiberon''. After 1990, controls were produced at the IFREMER hatchery of La Tremblade (Charente Maritime) using wild animals and named ''Quiberon control'' (Fig. 1).

B. ostreae is usually detected in oysters that are at least 18 months old (Cáceres-Martinez et al., 1995; Culloty and Mulcahy, 1996). Therefore, 4–5 year-old oysters that were expected to have developed parasite resistance were used to produce the first generation of strain 85 (S85-G1, Fig. 2). The S85-G1 spat was produced in 1985 at the SATMAR hatchery (Normandie, France) and was reared under different conditions in four sites (Quiberon and Paimpol Bays, Morbihan Gulf and Penze River) where *B. ostreae* is known to be present. The resistance level to natural infections was tested in the Penze River from February 1988 to March 1989. S85-G1 survived significantly better than the wild control population (59 and 33%, respectively, $P < 0.001$) but no significant difference in parasite prevalence was observed (22 and 20%, respectively, $P > 0.05$). Inoculation was performed for the first time with the first generation, as previous to this a technique had not been developed for large samples (Mialhe et al., 1988; Hervio et al., 1995). Only some of the S85-G1 animals from Quiberon Bay were inoculated in 1988. A S85-G2 generation was derived in 1990 using S85-G1 animals selected from the survivors at each site. The S85-G2 spat was reared in two different locations (La Trinite and Quiberon) and resistance was tested in Quiberon Bay from February 1992 to November 1993 (Fig. 2). On average, survival was higher among the S85-G2 oysters (44%, $P < 0.05$) than among the Quiberon control (29%) but the cohorts produced from oysters under selection in the wild (in Quiberon Bay: 55%, $P < 0.001$; in Paimpol River: 45%, $P < 0.01$) performed better than animals produced from inoculated parents (33%, $P > 0.05$). No parasite prevalence data is available for this test. Parasite inoculations were performed on 2 year-old oysters either to test the resistance (data not shown) or to produce new selected parents. A third generation (S85-G3) and a backcross (BC) were produced in 1993 and tested in the wild from July 1993 to March 1995. The remaining animals were kept in La Tremblade quarantine and monitored for survival from March to September 1995.

For Strain 89 (Fig. 2), wild Quiberon oysters were chosen in 1987 and some of them inoculated. Parents were later selected from the surviving inoculated and non-inoculated animals. The first generation (S89-G1) was produced in 1989 at La Tremblade hatchery and spat were reared in Quiberon Bay under two conditions (sowing and bags). Resistance was assessed from March to October 1991 both to natural infections in the wild and to parasite inoculation in La Tremblade quarantine (Martin et al., 1992).

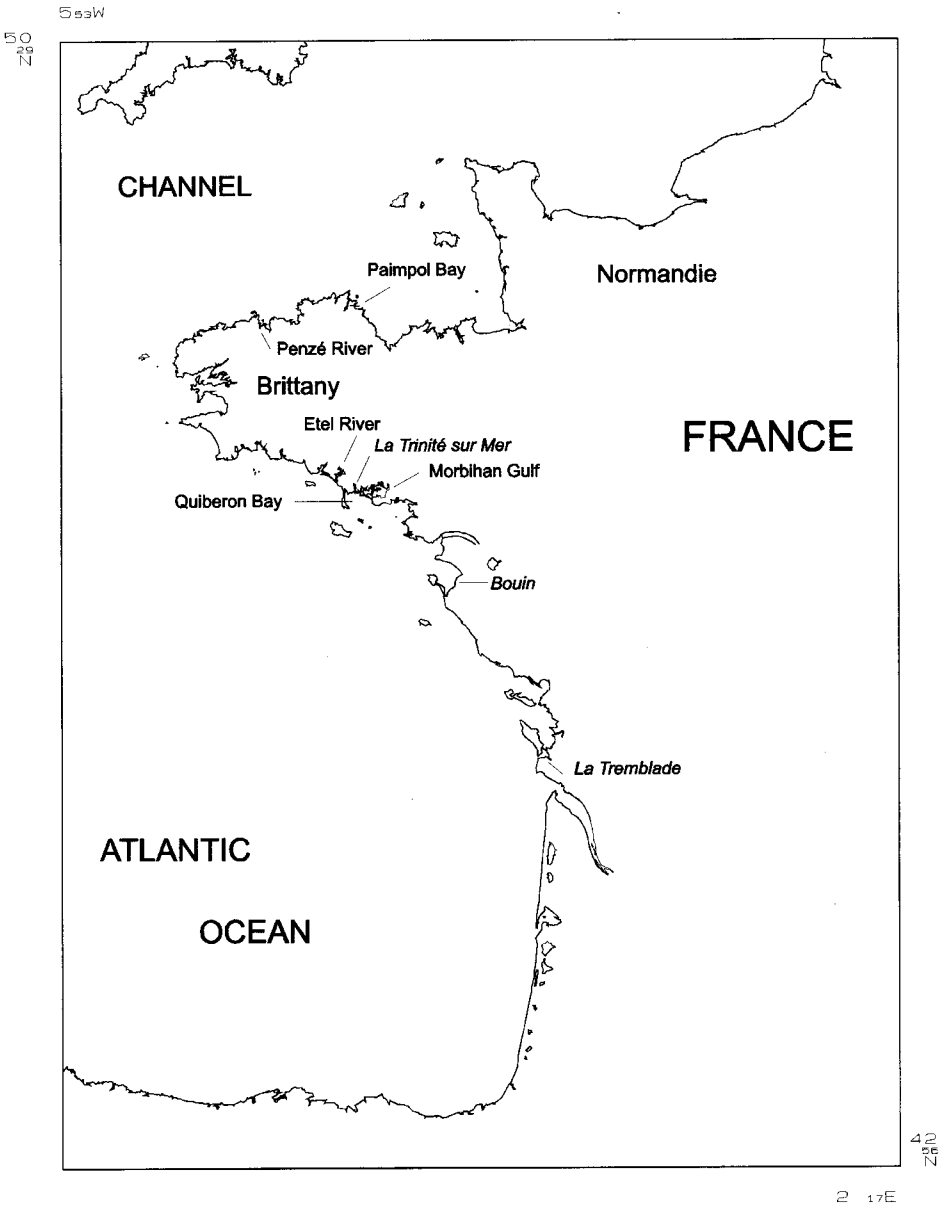

Fig. 1. Map of the different experimental sites.

S89-G1 performed significantly better in both survival tests (94 and 72%, respectively, $P < 0.001$) than the wild Quiberon controls (66 and 48%, respectively). A significant difference in parasite prevalence was observed in the wild (2% versus 20% for the control, $P < 0.01$) but not after inoculation (32% versus 33% for the control, $P > 0.05$).

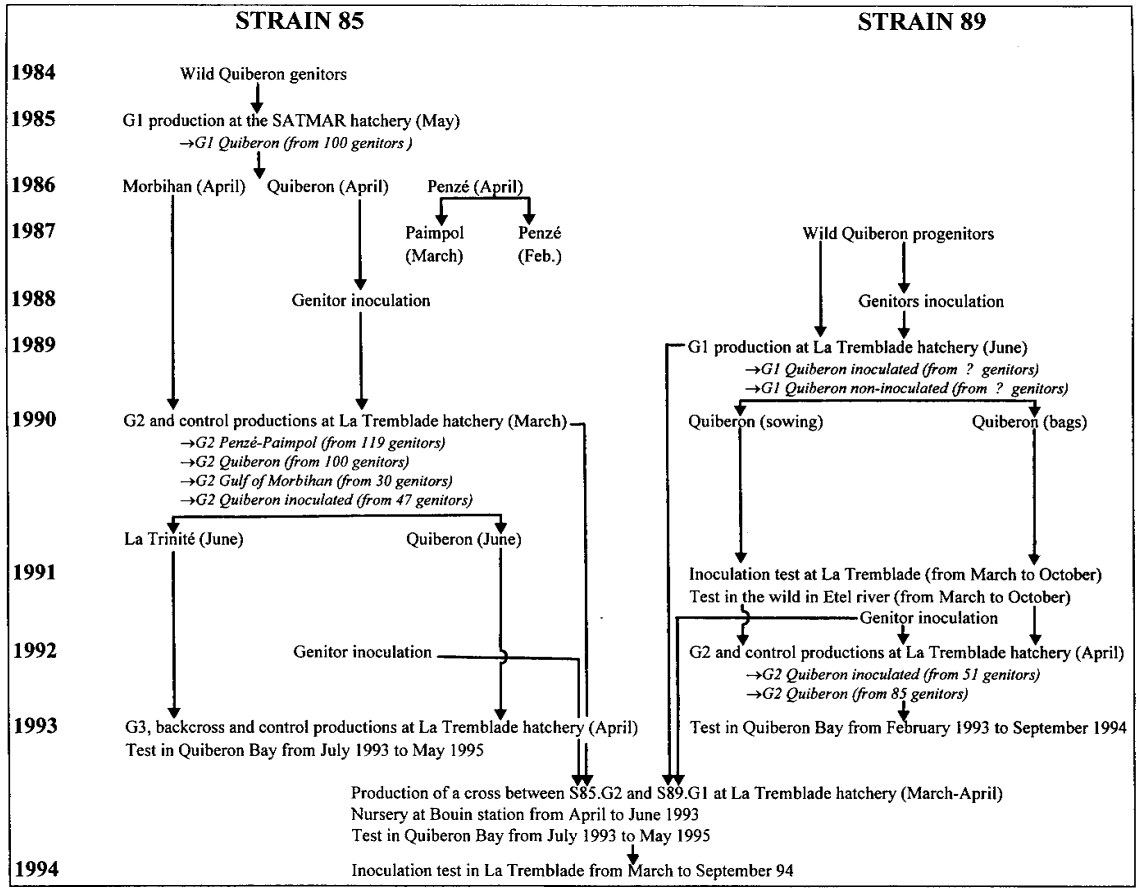

Fig. 2. Chronological account of *O. edulis* strain 85 (S85) and strain 89 (S89).

S89-G2 oysters were produced from inoculated and non-inoculated G1 animals in 1992 (S89-G2I and S89-G2W, Table 1) and tested from February 1993 to September 1994 in Quiberon Bay (Fig. 2).

2.2. Biological material and culture conditions

This section focuses on the tests for resistance of three different populations, namely S85-G3, S89-G2 and the cross between S85-G2 and S89-G1. For these generations and their respective controls, oysters were conditioned in an open circulating system at 20°C at La Tremblade hatchery. Oysters were usually placed together in a raceway for mass spawning. It was therefore impossible to know the exact number of parents. As a consequence, the numbers in parentheses in Fig. 2 give only the potential number of parents that participated in spawning. At least two different broods were reared from

Table 1
Mean weights and survivals of *O. edulis* S85-G3, S89-G2, the cross between S85-G2 and S89-G1 and their respective controls in Quiberon Bay

Populations and period of experiment	Survival (%)	Prevalence (%)	Final Weight (g)
Strain 85, third generation from 07/93 to 03/95 (20, 4)[1]	Population*** C.V. = 13.3%	Population*	Population[ns2] C.V. = 29.2%
S85-G3 (3, 263)[3]	59.1a[4]	22.7* (10/44)[5]	35.4a
S85-G2×Quiberon Controls (3, 276)	23.7b	28.9[ns] (13/45)	37.4a
Quiberon Controls (3, 288)	13.5c	48.9 (22/45)	34.8a
Strain 89, second generation from 02/93 to 09/94 (19, 11)	Population[ns] Rope[ns] Interaction[ns] C.V. = 13.5%	Population*	Population*** Rope[ns] Interaction[ns] C.V. = 35.5%
S89-G2I, (9, 227)	49.7a	28.7[ns] (27/94)	62.2a
S89-G2W, (9, 239)	53.4a	12.5[ns] (12/96)	51.6b
Quiberon controls (9, 231)	48.3a	21.1 (20/95)	47.5b
Cross between S85-G2 and S89-G1 from 07/93 to 05/95 (22, 5)	Population*** Rope[ns] Interaction[ns] C.V. = 26.1%	Population*	Population[ns] Rope[ns] Interaction[ns] C.V. = 35.3%
S85-G2×S89-G1 (12, 223)	39.4a	47.2* (51/108)	39.6a
Quiberon Controls (12, 269)	16.1b	64.0 (69/108)	40.3a

[1] The two figures indicate the length of the experiment and the spat age at the beginning of the experiment (in months).
[2] Significance levels are given by [ns] for $P>0.05$, * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$.
[3] The two figures in each set of brackets indicate the initial numbers of bags and the number of oysters within each bag.
[4] Figures with different letters are significantly different at $\alpha = 0.05$ with the Game and Howell method or the Student–Neuman–Keuls test.
[5] Figures in brackets indicate the number of infested oysters over the number of analysed oysters. Each test compares a given percentage with the corresponding percentage of the control.

each population. The cross between the two strains (Fig. 2) was obtained by controlled spawning. Therefore, oysters from each strain were individually conditioned in 2-l beakers and spawning was induced by heat shocks (up to 30°C) as described by Baud et al. (1997). The S85-G2 × S89-G1 cross was a mixed population of full-sib and half-sib families with 13 different females and 10 males as parents. The same protocol was used to produce a backcross generation between S85-G2 and a control using five females and four males.

Larvae and spat were reared following the protocol described by Walne (1974) in enriched sea water. Spat cultured to a minimum size of 4 mm were transferred to the IFREMER facilities at Bouin (Vendée, Fig. 1) where they were grown in an upwelling system with *Skeletonema costatum* enriched sea water (Bacher and Baud, 1992). Oysters were transferred to the open sea in Quiberon Bay as soon as they reached the minimum size necessary for growth in mesh bags (about 16 mm).

2.3. Experimental designs

The initial number of oysters within each bag ranged between 220 and 290 depending on their size (Table 1). Each mesh bag was inserted in a metallic structure that allowed the bed of the bay to be brushed aside and also served to reduce fouling and attack by predators. Up to 12 metallic structures were fixed on a single rope that was laid underwater (−5 m). Monitoring and maintenance were ensured by skin divers four times during the course of the experiment. Only one rope was used for the S85-G3 experiment for which three replicates per population were used. Three and four ropes, respectively were used to test the survivals of the S89-G2 and the cross between S85-G2 and S89-G1. Three replicates per population were distributed at random within each rope. Weights were measured at the end of the experiment. At the end of the S85-G2 × S89-G1 experiment in the wild, oysters were transferred to La Tremblade hatchery and survival was monitored for another six months under controlled conditions.

2.4. Parasite examination

Parasite examinations were usually performed at the end of each experiment on sub-samples of surviving oysters. Heart or gill smears were examined for the presence of *B. ostreae* by light microscopy. Smear coloration was performed using the Merck Hemacolor kit. Parasite prevalence was calculated as the number of infected oysters over the number of analysed oysters.

2.5. Statistical analyses

Differences between parasite prevalence ratios were tested using two tests for percentage comparisons using a method described by Schwartz (1969). For each experiment, the within population variances were compared using $F_{\max}$ statistics (Sokal and Rholf, 1995). When homoscelasticity was verified, analyses of variance were performed on final weights and survival percentages using the GLM procedure reported in SAS Institute Inc. (1988) software in order to test population, rope and interaction

effects depending on the experiment. Mean comparisons were performed using the Student–Neuman–Keuls test. If non-normality and/or heteroscelasticity of variance was found, a bootstrap method was preferred to analysis of variance for testing the mean squares. The data were resampled 1000 times without replacement and mean squares were calculated for each resampling. The true mean squares were declared significant at the α percent level when they had a higher value than $(1-\alpha)$ percent of the resampled mean squares. Calculations were performed using the Resampling Stat software for personal computers (Bruce, 1993). Mean comparisons were performed using the minimum significant differences (MSD_{ij}) between pairs (i, j) of populations based on the Game and Howell Method (Sokal and Rholf, 1995) which is appropriate when the variances are heterogeneous. MSD_{ij} is given as:

$$MSD_{ij} = Q\alpha_{[k,\nu]}\sqrt{(S_{Yi}^2 + S_{Yj}^2)}$$

where α is the significant level, k the number of populations and ν is the special weighted average degrees of freedom calculated as:

$$\nu = (Sy_i^2 + Sy_j^2)^2 / \left[(sy_i^2)^2/(n_i - 1) + (Sy_j^2)^2/(n_j - 1)\right]$$

The critical value $Q\alpha_{[k,\nu]}$ is obtained from the table of the studentized range.

The S85-G3 experiment was analysed using a one fixed effect model as follows:

$$Y_{ij} = \mu + p_i + E_{ij} \tag{1}$$

where p_i is the population effect ($i=1$ to 3), and E_{ij} the residual ($j=1$ to 3 replicates for survival rates, $j=1$ to 45 individual weight measurements). For the two other experiments, the population effect was tested using the following two-fixed model:

$$Y_{ijk} = \mu + p_i + r_j + i_{ij} + E_{ijk} \tag{2}$$

where r_i is the rope effect, i_{ij} the interaction effect between populations and ropes and E_{ijk} the residual (S89-G2: $i=1$ to 3, $j=1$ to 3 and $k=1$ to 3 for survival records and $k=1$ to 33 for individual weight measurements; S85×S89: $i=1$ to 2, $j=1$ to 4, $k=1$ to 3 for survival records and $k=1$ to 27 for individual weight measurements).

3. Results

Our strategy was to test the resistant oysters in conditions as similar as possible to those found on oyster farms. This is why, for populations produced in 1993, the monitoring began on 4 month-old oysters. The experiments were stopped after a 20 month period when the survival of controls became very low. For S89-G2, which was produced in 1992, monitoring began with older oysters (11 month-old) and again lasted 20 months. At the end of the 20 months these oysters were heavier than S85-G3 and S85-G2×S89-G1 (50–60 g versus 35–40 g).

3.1. Strain 85

The spat was 4 month-old at the beginning of the experiment. After a 20 month test in the wild, the survival rates of S85-G3, its backcross and the controls were significantly different from each other ($P<0.001$, Table 1). Table 1 shows that S85-G3 had a higher survival rate (59.1%) than the backcross population, whose survival rate (23.7%) lies between those of the third generation and the controls (13.5%). The three populations were also significantly different in terms of the percentage of parasite prevalence ($\chi^2=7.496$, df$=2$, $P=0.024$). When tested by pairs, the difference in parasite prevalence between the backcross and the controls was no longer significant ($\chi^2=3.787$, df$=1$, $P=0.052$) whereas the difference between S85-G3 and the control remained significant ($\chi^2=6.612$, df$=1$, $P=0.010$). The prevalence results for the backcross population were again in between those of the S85-G3 oysters and the Quiberon controls. Fig. 3 illustrates survivals for the three populations monitored in the wild from July 1993 to March 1995 (Table 1) and Fig. 4 shows the results obtained at La Tremblade quarantine from March to September 1995. In the latter figure, monitoring was conducted on small populations (30 to 67 oysters) and mortality that occurred before March 1995 was not taken into account. Both figures show that the backcross group had an intermediate survival when compared with the other two populations, S85-G3 (delayed mortality) and the controls (early mortality). No significant differences in weight was observed at the end of the experiment. The backcross was, however, slightly heavier (37.4 g) than both S85-G3 (35.4 g) and the Quiberon controls (34.8 g). The variance for weight within each population was highest in the S89.G3 population ($\sigma^2=134.9$) followed by the backcross population ($\sigma^2=129.8$) and the Quiberon controls ($\sigma^2=64.8$) but no significant difference was detected ($F_{\max[\sim,44]}=2.08$,

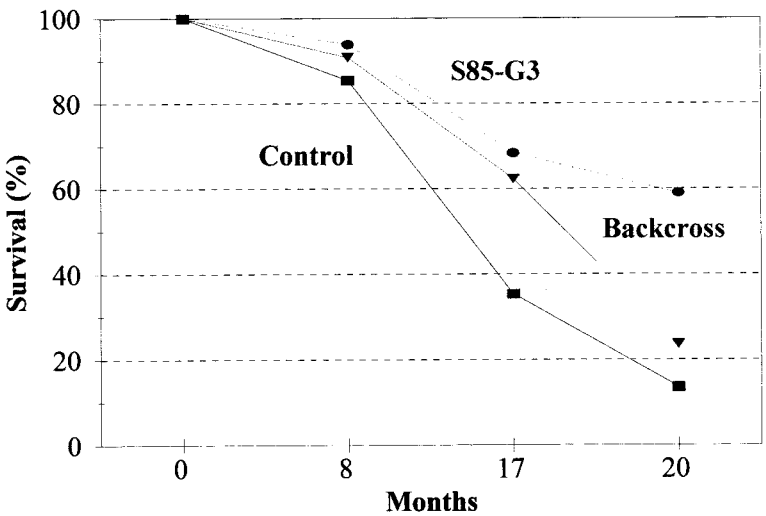

Fig. 3. *O. edulis*. Survival of the third generation of strain 85 (S85-G3), its backcross (S85-G2×Quiberon control) and a Quiberon control from July 1993 to Marsh 1995 in Quiberon Bay.

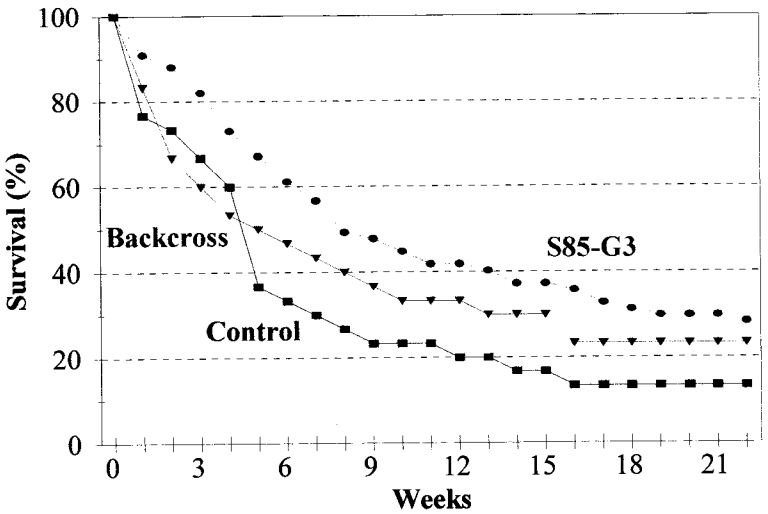

Fig. 4. *O. edulis*. Survival of the third generation of strain 85 (S85-G3), its backcross (S85-G2×Quiberon control) and a Quiberon control from March to September 1995 at La Tremblade quarantine.

$P>0.05$). The same trend was observed for the coefficients of variation (C.V.=33% for S89.G3, 31% for the backcross and 23% for the Quiberon controls).

3.2. Strain 89

S89-G2 populations produced from inoculated parents (S89-G2I) and from parents under selection in the wild (S89-G2W) were tested in Quiberon Bay for 19 months. The spat was 11 months old at the beginning of the experiment (Table 1). Population, rope and interaction effects were not significant at the 5% level for survival rates. S89-G2W survived slightly better than S89-G2I which in turn survived as well as the Quiberon controls (53.4, 49.7 and 48.3%, respectively). Significant differences were observed for parasite prevalence between the three populations ($\chi^2=7.625$, df=2, $P=0.022$). However, when parasite prevalence was compared by pairs the difference between the selected populations and the controls was no longer significant (G2I against Quiberon control: $\chi^2=1.488$, df=1, $P=0.223$; G2W against Quiberon control: $\chi^2=2.504$, df=1, $P=0.114$). Significant differences were observed between populations for final weights: S89-G2I had a significantly higher weight (62.2 g) than both S89-G2W and the Quiberon controls (51.6 and 47.5 g, respectively). Significant differences were observed for weight between the population variances ($F_{\max[3,99]}=2.32$, $P<0.05$). These variances were highest for S89-G2I ($\sigma^2=518.2$) followed by S89-G2W ($\sigma^2=370.2$) and the Quiberon controls ($\sigma^2=223.0$). The coefficients of variation were higher for the selected populations (37%) than for the Quiberon controls (31%).

3.3. S85-G2×S89-G1

The cross between the two strains was tested in the wild for 22 months and the experiment began when the oysters were 5 month-old (Table 1). S85-G2×S89-G1 survived significantly better than the Quiberon controls (39.4 and 16.1%, respectively, $P<0.001$). The rope and the interaction effects were not significant at the 5% level. A significant difference was also found for parasite prevalence between the cross and its control (47.2 and 64.0%, respectively, $\chi^2=6.075$, df=1, $P=0.014$). No difference was observed between populations for final weights ($P=0.36$) or for weight variances ($F_{\max[2,108]}=1.05$, $P>0.05$). The rope and interaction effects again were not significant.

3.4. Variation in the survival of controls

The S85-G3 and S85-G2×S89-G1 tests gave very similar results in terms of the survival of controls (13.5% and 16.1%, respectively). These two experiments were conducted at the same time (July 1993 to March or May 1995), in similar conditions with regard to the duration of the experiment and the age of the oysters. The S89-G2 experiment was conducted earlier (from February 1993 to September 1994) and for a similar period of time but on older oysters (Table 1) and control survival was higher (48.3%).

4. Discussion

4.1. Strain 85

The survival rate of S85-G3 was more than four-times higher than the Quiberon controls (4.38) and its standardised parasite prevalence rate was 46% of parasite prevalence in the control group. The experiment was stopped when oysters were 24 months old as survival for the Quiberon controls was very low, indicating that *B. ostreae* pressure had been strong throughout the experiment. The three populations have indeed undergone two summers in an infected area and the experiments were stopped in the Spring, well after the period of *B. ostreae* induced mortality (late Summer and Fall). Cáceres-Martinez et al. (1995); Culloty and Mulcahy (1996) have shown that *B. ostreae* usually infects wild oysters that are at least 18 months old. This figure does not fit with the hatchery spat, which are produced in spring and usually reach an average size of 4 mm when wild oysters begin to spawn.

The backcross survived less well than S85-G3 but better than the Quiberon controls (1.76). This intermediate survival suggests that the resistance determinism is mainly additive (Figs. 3 and 4). Hypothesising a strict additivity is however not enough to explain the results, as the backcross performance is significantly lower than the mean performance of S89-G3 and the Quiberon controls (36.3%) which suggests the presence of dominance or duplicate epistasis. In a two alleles per locus model that assumes independence between loci, the additive effect over loci Σa (Falconer, 1989) would be 22.8% and the dominance effect Σd would be −12.6%. In the absence of dominance

and assuming interactions between loci, the difference between the observed mean for the backcross and the expected mean assuming strict additivity could be the consequence of duplicate epistasis, which is known to lower hybrid performance. For parasite prevalence percentages, a strict additivity can be hypothesised as the mean performance of S89-G3 and the Quiberon controls (35.8%) is not significantly different from the backcross performance (28.9%).

So it would seem that additive effects of alleles are important and this is also supported by the rapid response to selection that was observed in both strains.

4.2. Strain 89×strain 85

The results obtained for the cross between the two strains are very similar to those obtained with S85-G3. The S85-G2×S89-G1 cross survived more than twice as well as the Quiberon controls (2.44) and standardised parasite prevalence was 74% of control prevalence. The experiment was stopped when oysters were 27 months old and weighed about 40 g. As for the S85-G3 experiment, Quiberon control survival was low, indicating once more that *B. ostreae* pressure had been strong during the experiment.

The standardised survival of S85-G2 when crossed with S89-G1 (2.44) was higher than the standardised survival of the same S85-G2 when crossed with the Quiberon controls (1.76) in the S85-G3 experiment, indicating a positive influence of S89-G1. Assuming additivity, the marginal contribution of S89-G1 in improving the standardised survival might then be 0.68, which, not surprisingly, is less than the marginal contribution of S89-G2.

4.3. Strain 89

The results are less impressive for S89-G2 even though S89-G1 showed a significant improvement (see Section 2). Different hypotheses have been put forward to explain this decline in resistance.

The first explanation suggests that the time period of the experiment was not long enough to reveal significant differences between the populations: although monitoring began on older oysters (11 months old instead of 4 or 5 months old), and lasted for approximately the same period as for the two previous experiments, it was stopped at the beginning of the second period of *B. ostreae* induced mortality (late Summer and Fall). As a consequence, these populations had undergone only one critical period (1993) instead of two (1993 and 1994) as was the case for S85 and the cross between the two strains. The observed survival rates might then underestimate the differences in mortality that might have been recorded in the Spring of 1995. This hypothesis is supported by the lack of a significant difference in parasite prevalence, the high survival rate of the Quiberon controls when compared to the other experiments and finally, by unpublished data of A.G. Martin, who observed that high parasite prevalence rates were found in late 1994 in wild oysters.

The second hypothesis underlines the behaviour of the two strains which both showed a decrease in resistance between G1 and G2 and suggests that this could be the consequence of inbreeding depression or deleterious hitch-hiking effects associated with

the high selective pressures. Both phenomena could have resulted in reduced overall performances. Because of the mating technique, the first generations are indeed suspected to derive from a small or even a very small number of individuals. Interestingly, the performance of the G2s was poorer for populations produced from inoculated parents. Inoculations were always performed on a limited number of animals (not more than 150) and the number of surviving animals was usually lower than for populations produced from parents selected in the wild (Fig. 2). Inbreeding depression or deleterious hitch-hiking effects might then have affected the populations produced using inoculated parents to a higher extent than the other populations. The inbreeding hypothesis is supported by the recent findings of McGoldrick and Hedgecock (1997) who showed that the genetic load can be very high in molluscs. This is not in conflict with the good performance of S85-G3 if one assumes that a large part of the genetic load has been cleared in G2.

4.4. Weights

Weights were significantly different only between S89-G2 and the Quiberon controls. The length of the experiment and especially the age of the oysters at the end of monitoring might be the decisive factor that led to the significant differences. However, there is an interesting tendency in the two strains toward a higher weight for the selected populations. This is not the case for the cross between the two strains, even though a significant difference was found for the same populations after one year of monitoring in a controlled environment with a high selective pressure (Baud et al., 1997).

The variances and the coefficients of variation for weight were, for both selected populations, higher than for their respective controls. The difference in variance was significant for S89-G2 but not for S85-G3. This result indicates that a large variation in weight still exists within the strains, although selective pressures were high and inbreeding might also be high. This variation is even larger than that found in the Quiberon controls. Whitlock and Fowler (1996) have demonstrated that inbred lines cannot be regarded as necessarily more uniform than outbred lines and that results on changes in variance due to inbreeding should be treated with caution unless enough replicates are available. Moreover, Goodnight (1987) has shown that founder events redistribute the genetic variance within the original population into genetic variance within the newly founded deme, whereas they shift the overall mean of the deme. Under specific conditions, including the presence of epistasis and/or dominance, a small number of founders and successive bottlenecks (Goodnight, 1988; Willis and Orr, 1993; Cheverud and Routman, 1996), the additive variance within a deme can overwhelm the additive variance within the initial population. This seems to be the case for the two selected populations and the increase in variance might then be due to reduced numbers of parents at each generation and the presence of epistasis and/or dominance.

4.5. A hypothetical mechanism for the resistance

Mortality in selected strains appears to be delayed when compared to the controls, as already noted by Martin et al. (1992). It follows that the resistance could be due to a better tolerance of the selected strains to *B. ostreae* infections. The same phenomenon

has been recorded by Ford and Haskin (1987) on MSX-selected strains. We still do not have any clear explanation for such a resistance to *B. Ostreae*. Preliminary results obtained by Cochennec et al. (1995) indicate, however, that the selected strains differ from the Quiberon controls for total and differential haemocyte counts, whether they are infected or not. Total haemocyte counts showed that the cell density of the haemolymph was lower in selected oysters than in susceptible animals. Moreover, differential haemocyte counts showed that agranular cells were highly significantly less numerous in resistant oysters than in susceptible ones. Interestingly, in *Tiostrea chilensis* these agranular cells are known to be preferentially infected by *Bonamia* sp. (Hine and Wesney, 1994) and Cochennec et al. (1995) have confirmed this finding on a small number of infected *O. edulis*. This might indicate that selection against *B. ostreae* susceptibility has resulted in a decrease in the proportion of the haemolymph cells which are most exposed to the parasite. If such a hypothesis were to be confirmed, the selected resistance would in fact be the result of tolerance and we might then predict that the genetic improvement would end more rapidly than expected because it is not possible to decrease the proportion of one of the haemolymph cell types indefinitely. These results are quite different from those obtained by Ford et al. (1993) who found a decrease in granular haemocytes in MSX-susceptible oysters compared to resistant ones. In their study, the loss of granulocytes was attributed to heavier MSX infections in susceptible oysters. Until these findings can be confirmed by further studies, we will continue to use the term ''resistance'' to mean an improvement in survival rates.

4.6. Why is there no natural increase in resistance in wild populations?

It is remarkable that until now no measurable enhancement of resistance has been observed in the wild. This is not in agreement with Elston et al. (1987) who tested a naturally acquired resistance to *B. ostreae* in a population of *O. edulis* from Washington state (USA). In this case, *B. ostreae* was suspected to have appeared in the early sixties. Consequently, the good survival performance measured by Elston et al. (1987) might be due to a resistance selected in the wild over a period of more than 23 years. However, no confirmation of this resistance has been published since the article by Elston et al. (1987). Different hypotheses have been put forward to explain the apparent lack of acquired resistance in wild oysters in France. The first explanation is that the resistance in the wild is too weak or that it is too early for the signs to be clearly detected. The second explanation relies on the dynamics of *B. ostreae* infections, which in Quiberon Bay are slow until oysters are 18 months old. As already noted above, serious infections are usually limited to oysters that are at least 24 months old. Robert et al. (1991) found similar results in Arcachon Bay. Flat oysters are able to spawn during their first summer after settlement and are fully reproductive at 24 months old. The parasite pressure might by then be ineffective since mortality due to *B. ostreae* occurs mainly on oysters that are at least two years old. As a consequence, susceptible oysters have had plenty of time to spawn before they die and ''resistance'' genes may be diluted in the wild population. The third explanation is that cultured resistant oysters may not contribute to future generations because they are sold as soon as they reach a marketable size. These three hypotheses would explain why no natural resistance has as yet arisen in the wild. Thus, the interest of hatchery-selected resistance is quite obvious.

5. Conclusions

There is strong evidence that an heritable increase in resistance to *B. ostreae* has been obtained in *O. edulis*, although we had some difficulty comparing the first and last generations since, for the reasons already mentioned, they were not tested in similar conditions. This increase in resistance was achieved using classical mass selection techniques and very high selective pressures. The quick response to selection was already significant in the first generation of S85 and S89 and, together with the intermediate behaviour of the backcross population, supports the idea of additivity of resistance. This resistance is all the more interesting because no natural resistance has clearly arisen in the wild since *B. ostreae* appeared in France. As for MSX resistance (Ford and Haskin, 1987), it seems that the improved survival of the selected strains is due to a delayed mortality, which might also be related in *O. edulis* to the lower probability that the parasite will find an appropriate target cell in the haemolymph. This hypothesis is supported by the recent findings of Cochennec et al. (1995) on the differential haemocyte counts in selected and control oysters. The clear differences recorded between selected and control oysters might be explained as a consequence of the selection. Differential haemocyte counts might, in the future, be a good way to screen populations for resistant oysters.

The high selective pressures that were applied, together with the suspected small numbers of effective parents, might explain the decrease in survival observed in both G2s. There is some evidence that the combination of both of these phenomena may have resulted in a significant inbreeding depression resulting in a genetic load clearing. However, the phenotypic variance for weight remained high in the selected populations, which might be attributed to the fact that the populations may have undergone bottlenecks. Considering the good performance of S85-G3, it has been decided to continue the within-strain selection and the crosses between strains using full-sib families, in order to further develop both the improved additive effects and the dominance effects, as suggested by Toro (1993). The results obtained on *Crassostrea gigas* by Hedgecock et al. (1995) have shown that it is possible to obtain significant heterosis for growth by crossing inbred families. We might therefore expect similar results with our two strains. As no allozyme or molecular data are available at the present time, another development from this programme will be to compare the variability and inbreeding level of the two selected strains with natural populations using allozymes and microsatellites (Naciri et al., 1995). This will allow us to test some of the hypotheses put forward in this paper, particularly the severe bottleneck hypothesis.

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5. 2. Une base génétique pour la sensibilité à OsHV-1 chez l'huître creuse

La découverte de virus chez les mollusques marins est un fait assez récent. Plusieurs épisodes de mortalités ont été corrélés à des maladies virales chez différentes espèces de coquillages. En France, des infections dues à des virus présentant les caractéristiques des iridovirus ont été associées à la disparition de l'huître portugaise, *C. angulata*, le long du littoral français au début des années 70 (Comps, 1970 ; Comps & Duthoit, 1978). Depuis 1991, des mortalités sporadiques récurrentes sont également reliées à la détection d'un herpès virus chez l'huître creuse, *C. gigas*.

Malgré l'impact que les maladies virales peuvent avoir sur les coquillages, nous ne savons que très peu de chose sur comment les conchyliculteurs peuvent se prémunir et éviter ces infections. Les données concernant les moyens de défense mis en œuvre par les mollusques vis à vis des virus manquent aussi. Comme il a été mentionné auparavant, il n'est pas possible d'utiliser la vaccination chez les mollusques qui ne possèdent pas de mémoire immunitaire. De plus, les traitements préventifs ou curatifs ne sont pas envisageables pour des animaux élevés la plupart du temps en milieu ouvert.

Dans ce contexte, la compréhension des mécanismes de défense dirigés contre les virus chez les coquillages apparaît comme une étape clé. Elle devrait permettre d'aider au développement de programmes de sélection en général, et de programmes de sélection à des maladies virales en particulier, par le biais de l'identification de marqueurs de sélection.

Le Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France) a assuré la coordination d'un projet de recherche financé par l'Union Européenne (AVINSI), dont l'objectif principal était d'étudier les réponses immunitaires vis à vis de virus chez différentes espèces d'intérêt en aquaculture (poissons, mollusques et crustacés). Trois approches ont été mises en œuvre :

- la recherche des substances à activité anti-virales dans des extraits tissulaires,
- la recherche de gènes viro-induits par la technique d'Hybridation Suppressive Soustractive,
- la recherche de gènes codant pour des protéines inhibitrices de l'apoptose.

Le laboratoire a en particulier développé des travaux en hybridation soustractive afin d'identifier des **gènes induits par le virus OsHV-1 chez l'huître creuse, *C. gigas***. Pour cela, des hémocytes maintenus *in vitro* ont été mis en contact (12 heures) avec un broyat de larves infectées (contact avec le virus) ou un broyat de larves saines (contrôle). Des ARN ont été extraits des hémocytes et ont servi à la construction d'une banque soustractive sur la base de l'utilisation de kits du commerce (Clontech). Les clones correspondant à des ARN présents spécifiquement dans les hémocytes mis au contact du virus ont été identifiés par hybridation sur membrane et ont été séquencés (362 clones). Les séquences ont été analysées afin de rechercher des homologies. Une classification a pu être ainsi obtenue:

- 138 séquences correspondant à des gènes codant pour des protéines de fonction connue (38,1%),
- 102 séquences correspondant à des gènes codant pour des protéines de fonction inconnue (28,2%),
- 122 séquences non exploitables (33,7%).

Parmi les 138 séquences qui présentent des homologies avec des gènes codant des protéines connues, 109 sont uniques et peuvent être regroupées dans cinq catégories :

- signalisation cellulaire, cycle cellulaire et protéines de structure (42%),
- métabolisme général et énergétique (27%),
- protéines ribosomales et synthèse protéique (11%),
- réplication, réparation et transcription de l'ADN (11%),
- immunité (9%).

Parmi les gènes d'intérêt, il a été possible d'identifier des séquences codant des protéines présentant de fortes homologies avec **des molécules effectrices de l'immunité** comme la Macrophage-expressed Protein, la Molluscan defence molecule, l'IK cytokine et la laccase 1 (Tableau IX) jusqu'alors non décrites chez l'huître creuse, *C. gigas*.

Tableau IX - Analyses de séquence des clones correspondant à des ARN exprimés dans des hémocytes au contact du virus OsHV-1 (protéines pouvant être impliquées dans la réponse de l'hôte, l'huître creuse, au virus)

Homologies	**E-value**	**Espèces**
Macrophage-expressed protein	4,00E-89	Ormeaux
Molluscan defense molecule precursor	3,00E-06	*Lymnaea stagnalis*
IK cytokine, down regulator of HLA	2,00E-21	*Dario rerio*
Laccase 1	3,00E-21	*Tribolium castaneum*
Bcl2-Like 2	3,00E-04	Xénope
Apoptosis-related protein PNAS2	3,00E-22	Homme
Similar to Acheron	1,00E-46	*Strongylocentrotus purpuratus*
Glypican 6 precursor	7,00E-33	Homme - Souris
Myeloid differenciation factor 88	1,00E-07	*Oncorhynchus mykiss*
Notch homolog 5	2,00E-07	*Dario rerio*
Protein tyrosine phosphatase	1,00E-13	Souris
NDR kinase	2,00E-46	Insectes
C phospolipase	3,00E-26	Homme
Phosphatidylinositol 4-kinase	2,00E-35	*Strongylocentrotus purpuratus*

Molécules effectices de l'immunité

Molécules impliquées dans l'apoptose

Récepteur membranaire

Molécule adaptateur dans la voie d'activation des TLR

Molécules impliquées dans l'activation cellulaire

La protéine appelée **Macrophage-expressed Protein** est une molécule produite par les macrophages chez les vertébrés (homme, souris) (Ambach *et al.*, 2004). Un homlogue a été récemment rapporté chez l'ormeau *H. corrugata* (Mah *et al.*, 2004) et chez l'éponge *Suberites domuncula* (Wienst *et al.*, 2005). Ces protéines possèdent un domaine conservé de type perforine. Les molécules de type perforine sont impliquées dans la défense immunitaire contre les micro-organismes. Il a été démontré que chez l'éponge *S. domuncula*, une protéine recombinante de type Macrophage-expressed protein avait un effet toxique sur les bactéries Gram négatives (Wienst *et al.*, 2005). Chez les vertébrés, la perforine contenue dans les granules des cellules NK intervient dans l'immunité anti-virale, en particulier par le biais de la destruction des cellules infectées (Young *et al.*, 1986a). La perforine est capable de s'intégrer à la membrane des cellules cibles et d'y former par polymérisation des pores. L'eau, des ions et des protéases peuvent alors pénétrer dans la cellule et entraîner sa mort. Cette molécule présente des homologies structurales et fonctionnelles avec le composé C9 du système du complément (Young *et al.*, 1986b).

La protéine **Molluscan Defence Molecule** (MDM) a été identifiée chez la limnée, *Lymnaea stagnalis*, un gastéropode d'eau douce (Hoek *et al.*, 1996). Cette molécule présente des homologies avec une protéine décrite chez les insectes, appelée Hemolin qui appartient à la superfamille des immunoglobulines. Elle est attachée à des bactéries (Sun *et al.*, 1990). Elle jouerait en effet le rôle de Pattern Recognition Protein (PRP) et se fixerait sur le peptidoglycan (Pathogen-Associated Molecular Pattern, PAMP) porté par les bactéries. Son expression est fortement induite par les bactéries et les composants bactériens, mais également les virus.

L'**IK cytokine** (down regulator of HLA) est une molécule qui inhibe l'expression des antigènes d'histocompatibilité de classe II induite par l'IFNγ chez l'homme (Krief *et al.*, 1994). Elle intervient également comme protéine régulatrice inhibant l'expression constitutive de ces mêmes antigènes (Vedrenne *et al.*, 1997).

La **laccase 1** est un enzyme cuivre dépendante. Elle appartient à la famille des oxydases cuivre dépendantes dont le système pro phénol oxydase/phénol oxydase fait partie. Elle est impliquée chez les invertébrés, en particulier les insectes, dans les mécanismes de défense.

Il est par ailleurs important de noter que pour certaines des séquences identifiées sur la base de leur homologie avec des gènes codant des protéines connues, il est possible de suspecter des interactions moléculaires et l'existence de cascades d'activation chez l'huître creuse, *C. gigas,* en réponse au virus OsHV-1.

Un travail récent, chez l'éponge, *S. domuncula*, a permis de montrer que la reconnaissance du LPS par un récepteur membranaire se traduisait dans un premier temps par une sur-expression du Myeloid Differentiation Factor 88 (MyD88) et était suivie d'une augmentation significative de l'expression de la molécule Macrophage-expressed Protein (Wienst *et al.*, 2005). Il a été possible d'identifier à partir des ARN extraits des hémocytes d'huître mis au contact du virus une séquence présentant des homologies avec le gène codant pour le Myeloid Differentiation Factor 88 (Tableau IX). Chez les vertébrés et les invertébrés, les récepteurs de type Toll (TRL) sont des éléments clés dans la reconnaissance des micro-organismes (Akira, 2003). Les TRL sont capables de reconnaître des motifs conservés portés par divers agents pathogènes (PAMP) et d'induire après transduction d'un signal l'expression de gènes impliqués dans les mécanismes de défense. La molécule MyD88 est un maillon (protéine

adaptateur) dans la cascade d'activation. Elle possède en effet un domaine de type TIR (Toll/Interleukin-1 Receptor) qui lui permet de se fixer sur la partie intracytoplasmique des TRL (McGettrick & O'Neill, 2004). Dans ce contexte, il apparaît indispensable de poursuivre les travaux de recherche concernant l'expression des homologues MyD88 et Macrophage-expressed Protein chez l'huître creuse.

Dans une autre étude récente, il a été démontré chez le lépidoptère, *Hyalophora cecropia*, qu'il était possible d'induire une diminution de l'activité phénol oxydase par interférence ARN (Terenius *et al.*, 2007). Une injection d'ARN double brin correspondant au gène codant pour l'hemolin dans des pupes réduit de manière significative l'activité phénol oxydase mesurée au spectrophotomètre. L'hemolin (PRP) interviendrait dans l'activation de la cascade enzymatique de cette enzyme impliquée dans la défense vis à vis des infections bactériennes (Terenius *et al.*, 2007). Les séquences identifiées sur la base de leur homologie avec des gènes codant pour la laccase 1 et le Molluscan defence molecule apparaissent comme des candidats de choix pour pousser les investigations plus avant chez l'huître creuse.

Enfin, une autre séquence mérite aussi notre attention. En effet, une séquence a montré une forte homologie avec le gène codant pour le **Glypican 6** (Tableau IX). Cette molécule fait partie de la famille des héparane sulfate protéoglycans, récepteurs membranaires pour des facteurs de croissance, des protéines de la matrice extracellulaire, des protéases et des anti-protéases (Veugelers *et al.*, 1999). Ces molécules servent aussi de récepteurs (contact initial) à différent virus incluant des herpès virus (Spear, 2004)

Afin de confirmer l'induction de transcrits de gènes identifiés en présence du virus OsHV-1, des analyses en RT PCR quantitative en temps réel ont été réalisées en utilisant des ARNprovenant d'hémocytes ou d'huîtres mis au contact du virus et d'ARN contrôles. De plus, pour certains gènes d'intérêt, la séquence complète de la protéine correspondante a été obtenue par PCR RACE en 5'et 3' (Tableau X).

L'ensemble des résultats obtenus dans le cadre du projet de recherche AVINSI est en cours de valorisation scientifique. Un premier manuscrit est en cours d'écriture : **"Suppression substraction hybridization (SSH) and real time PCR techniques reveal differential gene expression profiles in the Pacific oyster *Crassostrea gigas* challenged with OsHV-1" par Renault T., Faury N., Barbosa-Solomieu B. & Moreau K.**

Tableau X - Clones différentiellement exprimés pour lesquels la séquence codant la protéine a été obtenue par PCR RACE

Homologies	Taille (pb)
Glypican 6, precursor [*Homo sapiens*] 3e-105	2973
Macrophage-expressed protein [*Haliotis rufescens*] 4e-95	2826
Molluscan defence molecule precursor [*Lymnaea stagnalis*] 2e-10	541
IK cytokine, down-regulator of HLA II [*Danio rerio*] 5e-86	1009
Laccase-like multicopper oxidase 2 [Tribolium castaneum) 1e-31	3000
Bcl2-like 2 [*Xenopus tropicalis*] 8e-04	635
Similar to Acheron [*Strongylocentrotus purpuratus*] 1e-61	662

Récepteur membranaire

Molécules effectices de l'immunité

Molécules impliquées dans l'apoptose

6. Conclusion et Perspectives

Au cours de mon parcours professionnel, dans les années 90, une part importante de mon activité a été consacrée à la **description d'agents pathogènes chez différentes espèces d'intérêt en aquaculture** et au **developpement d'outils de diagnostic**. A partir des années 2000, cette activité s'est plus orientée vers la **validation des outils développés, leur utilisation et leur transfert vers divers utilisateurs** (réseau de surveillance REPAMO, professionnels de la conchyliculture). Elle s'est aussi recentrée autour de l'étude des **interactions complexes entre les agents infectieux, leurs hôtes et l'environnement**.

Il est rapporté dans ce chapitre un certain nombre de perspectives (de manière non exhaustive) en matière de pathologie des mollusques, projets dans lesquels je serai directement impliqué en tant que chercheur, mais aussi en tant que responsable de laboratoire. Les travaux seront développés au sein du Laboratoire de Génétique et Pathologie (LGP, La Tremblade).

Le LGP avec 30 agents statutaires est un lieu d'**intégration entre recherche et surveillance**. Des travaux de **recherche dans le domaine de la génétique et de la pathologie chez les mollusques d'intérêt commercial** y sont développées depuis plus de 20 ans. Ces travaux sont aujourd'hui reconnus mondialement et ont permis des avancés certaines (huîtres triploïdes, développement d'outils de diagnostic, détection de nouveaux agents infectieux …). Le laboratoire a été et est aujourd'hui aussi très fortement impliqué dans la **surveillance de la ressource**. Il assure en particulier pour le compte du Ministère de l'Agriculture la surveillance des coquillages en terme de santé animale (REPAMO). Il opère également la surveillance des sites nationaux de captage d'huîtres en terme de ploïdie (Réseau Biovigilance: réseau de suivi ciblant la recherche d'huîtres polyploïdes dans l'environnement.

Cette double implication en recherche et en surveillance est un atout majeur pour le laboratoire et pour l'Ifremer. Un dialogue incessant et un échange permanent ont lieu sur le site entre les acteurs de la surveillance et ceux de la recherche. Ils représentent une vraie force de proposition en terme de recherche, cette dernière se nourrissant des données recueillies par le biais des réseaux assurant la surveillance (REPAMO, Réseau Biovigilance). La surveillance quant-à elle profite en toute première intention des avancées émanant de la recherche. Cette complémentarité dans le domaine de la conchyliculture est une originalité reconnue au niveau internationale.

Il apparaît clairement aujourd'hui que la mise en place et l'entretien de systèmes de surveillance, de veille et de prévention en santé animale sont beaucoup moins couteux que le traitement des crises. Le coût de l'éradication d'une épizootie augmente de manière exponentielle en fonction de la rapidité de la détection et de la réponse apportée. Permettre **une détection précoce et une réaction rapide** doit être **l'objectif majeur en terme de santé animale**. La détection précoce des agents infectieux ne peut se faire qu'avec des conchyliculteurs avertis, un bon maillage vétérinaire territorial et un réseau adéquat de laboratoires de diagnostic. Une réaction rapide demande quant-à elle une législation adaptée et une administration préparée et efficace. Cependant, ces réseaux ne peuvent pas exister et perdurer sans **recherche cognitive sur les agents pathogènes eux-mêmes, les réactions de l'hôte et les effets de l'environnement sur les protagonistes**.

6. 1. Outils de diagnostic et autocontrôles

La production de mollusques s'est fortement développée ces dernières décennies et est devenue une des plus importantes activités d'aquaculture dans le monde. Cette production repose sur un nombre limité d'espèces et est souvent basée sur l'exploitation de populations sauvages. Ainsi, en France, l'ostréiculture se fait en grande partie à partir d'animaux issus de captage naturel (juvéniles provenant de la reproduction naturelle des huîtres dans le milieu). Cependant, les stocks naturels ne suffisent pas toujours pour répondre à la demande du marché. Il est en effet possible d'observer des variations importantes du captage naturel d'une année à l'autre. De plus, ce type d'approvisionnement en huîtres ne peut pas se faire dans les pays du nord de l'Europe (Irlande, Royaume Uni, Pays Bas, …) dans la mesure où jusqu'alors l'huître creuse ne s'y reproduisait pas.

Dans ce contexte, les écloseries de coquillages qui peuvent également fournir aux conchyliculteurs du naissain se sont développées. De plus, une demande croissante existe pour des animaux sélectionnés et des huîtres polyploïdes que seules les écloseries peuvent fournir. En France, 80% des professionnels ostréiculteurs utilisent des huîtres provenant de ces structures de production et 20% à 30% des 150 000 tonnes produites annuellement sont aujourd'hui constitués d'animaux triploïdes. Les écloseries contribuent ainsi de manière significative à la production de coquillages et aux échanges et transferts d'animaux vivants. Elles produisent en Europe plus de trois milliards d'individus par an dont plus de 30% sont destinés à l'exportation (Y. Le Borgne, SATMAR, communication personnelle).

Les transferts d'animaux sont bien identifiés comme une cause majeure de dissémination de maladies infectieuses. Ces dernières peuvent avoir des conséquences désastreuses en terme économique. Les écloseries de mollusques doivent donc être considérées comme un segment à risque dans le système de production. Bien que des progrès technologiques aient été réalisés ces dernières décennies dans ces structures d'élevage, un certain nombre de problèmes incluant les maladies infectieuses subsistent. Les écloseries possèdent cependant un atout majeur représenté par leurs systèmes confinés qui peuvent permettre un contrôle plus aisé des maladies infectieuses et la production d'animaux de statut infectieux connu et certifié. Ces structures de production peuvent aussi avoir un rôle pivot dans la diffusion du progrès génétique et dans la lutte contre les agents pathogènes par la distribution d'animaux sélectionnés pour leur résistance à certains agents pathogènes.

L'implication des écloseries de mollusques dans la détection précoce d'agents pathogènes dans leurs propres structures d'élevage offre la possibilité de contribuer de manière active au contrôle des maladies infectieuses. Il est possible de cette manière de prendre des mesures adaptées avant qu'aucun problème majeur ne survienne, de réduire en retour un possible impact économique et d'améliorer ainsi la compétitivité. La disponibilité de tests de diagnostic spécifiques, sensibles et de mise en œuvre aisée est indispensable pour initier une telle démarche d'autocontrôle. De tels outils peuvent permettre de contrôler les animaux à différents stades de développement (géniteurs, juvéniles ou larves) avant transaction commerciale et de fiabiliser les échanges d'animaux vivants et de leurs produits.

Une première étape autour d'un projet financé par l'Union Européenne (Recherche au profit des PME, 7e PC, Capacités) devrait se focaliser sur la validation d'un mini-array pour le diagnostic spécifique du virus OsHV-1 et son transfert à des écloseries professionnelles. Ce projet a été déposé en septembre 2007. La validation impliquerait plusieurs laboratoires européens possédant une expertise dans le domaine du diagnostic des maladies infectieuses chez les coquillages. Elle devrait permettre de démontrer la reproductibilité de la technique de mini-array et de préparer ainsi son transfert aux écloseries. Le projet prévoit en particulier pour le Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France) l'organisation d'une session de formation pour les laboratoires impliqués ainsi que la fourniture de matériel de référence pour les essais inter laboratoires. Cette étape de validation réalisée, il est planifié un transfert aux écloseries de coquillages impliquées dans le projet avec l'utilisation du mini-array pour l'analyse de leurs propres productions. Cependant, afin de confirmer les résultats obtenus directement sur les sites de production, il est prévu que les analyses soient réalisées de manière concomitante sur les mêmes échantillons par des laboratoires de diagnostic. Pour cette partie du travail, le laboratoire LGP serait impliqué dans l'organisation d'une session de formation pour les écloseries françaises participant au projet et réaliserait les analyses sur les échantillons provenant de ces structures de production.

Si le transfert du mini-array spécifique du virus OsHV-1 vers des structures européennes de production de coquillages se fait de manière effective à l'issue de ce projet, cela devrait permettre d'ouvrir la voie au développement et à l'utilisation d'outils similaires ciblant d'autres agents infectieux tels que des bactéries du genre *Vibrio* ou des parasites protozoaires dont certains sont à déclaration obligatoire. Des discussions ont déjà été initiées avec la société SkuldTech sur le sujet.

6. 2. Résistance à la bonamiose chez l'huître creuse et mécanismes de défense

La bonamiose représente un modèle d'intérêt pour l'étude des interactions hôtes bivalves/pathogènes de part la possibilité de purification du parasite et la reproduction de la maladie en laboratoire. Enfin, le développement d'animaux résistants/tolérants (**Naciri *et al.*, 1998**) a ouvert de nouvelles perspectives dans l'étude des mécanismes cellulaires de défense. La bonamiose représente également un modèle d'étude de parasitisme particulier du fait du double rôle joué par les hémocytes dans cette maladie. En effet, ils sont à la fois les cellules effectrices des mécanismes de défense et les cellules hôtes du parasite.

C'est dans ce contexte qu'un travail de thèse a été initié en octobre 2006 par B. Morga au sein du Laboratoire de Génétique et Pathologie (Ifremer, La Tremblade, France). J'en assure la direction (dispense d'HDR accordée par l'Ecole Doctorale de l'Université de La Rochelle). L'objectif principal de ce travail de thèse est d'étudier et de comprendre les interactions entre l'huître plate et le parasite *B. ostreae,* et, plus particulièrement les bases moléculaires de la résistance/tolérance au parasite. Les approches de génomique sont de plus en plus couramment utilisées dans l'étude des interactions hôte-parasite, en particulier chez les mollusques (Tanguy *et al.*, 2004). L'étude de l'expression de gènes du parasite *B. ostreae* et de son hôte, l'huître plate*, O. edulis*, au cours de l'infection et son éventuelle modulation par des facteurs environnementaux ou zootechniques paraît aujourd'hui essentielle pour proposer des mesures adaptées au maintien et à la relance de la culture de l'huître plate.

Des essais réalisés *in vitro* devraient permettre de décrire, à l'aide de la microscopie photonique et de la cytométrie en flux, les interactions entre hémocytes et parasites ainsi que d'étudier par hybridation soustractive suppressive l'expression de gènes impliqués dans ces interactions. Il est en particulier prévu d'effectuer une comparaison entre hémocytes issus d'huîtres plates sensibles et hémocytes issus d'huîtres plates résistantes/tolérantes (sélectionnées) afin de mieux comprendre les bases de la résistance à la maladie. La mise au point d'outils de quantification et d'étude de l'expression des gènes d'intérêt préalablement identifiés (RT-PCR, micro-array, hybridation *in situ*, …) et leur utilisation au cours d'expérience de reproduction de l'infection devrait permettre également de valider *in vivo* les résultats acquis *in vitro*.

6. 3. Bases génétiques de la résistance au virus OsHV-1 chez l'huître creuse

Une approche comparable a été également initiée chez l'huître creuse, *C. gigas*, dans le cadre du projet européen AVINSI (5. 2. Une base génétique pour la sensibilité à OsHV-1 chez l'huître creuse). Il a été possible par une approche d'hybridation soustractive suppressive d'identifier des gènes d'intérêt exprimés par les hémocytes d'huître mis en contact du virus OsHV-1.

De plus, dans le cadre d'un autre projet européen (AQUAFIRST), il a été observé des taux de mortalité significativement différents entre familles d'huîtres issues de croisements bi parentaux (un mâle et une femelle) au cours d'une expérimentation menée en juillet 2006. Il a été possible de corréler les fortes mortalités recensées à la détection du virus OsHV-1 par PCR quantitative en temps réel. Ces données laissent fortement suspecter qu'il soit possible d'entreprendre de sélectionner des huîtres vis à vis de la résistance au virus. Cette voie est une voie prometteuse à explorer dans la mesure où lutter contre les maladies virales reste difficile chez les bivalves marins et que les professionnels conchyliculteurs sont aujourd'hui en attente de solutions pratiques pour maîtriser les pertes liées au virus OsHV-1.

Par ailleurs, nous disposons pour le virus OsHV-1 de la séquence totale de son génome. Il est donc possible de suivre l'expression de différents gènes viraux en relation avec l'expression de certains gènes de l'hôte (gènes dont le produit est impliqué dans les mécanismes de défense). Cependant, un travail préalable concernant la cinétique d'expression des gènes viraux est nécessaire dans la mesure où très peu d'homologies ont été trouvées avec les autres herpès virus et que de nombreux cadres de lecture (ORF) identifiés dans le génome d'OsHV-1 codent pour des protéines de fonction inconnue.

Il devrait être proposé dans ce cadre un projet de thèse au Conseil Scientifique et Technique (CST) de l'Ifremer en début d'année 2008. L'objectif principal sera d'étudier les interactions qui peuvent exister entre le virus OsHV-1 et son hôte, *C. gigas*, afin en particulier de définir des marqueurs génétiques d'intérêt et d'aider ainsi par sélection à l'obtention d'individus plus résistants à l'infection virale.

6. 4. Maladies infectieuses chez les ormeaux

Des collaborations ont été initiées avec le Dr Chang (Department of Veterinary Medicine, College of Bio-Resources and Agriculture, Université de Taipei, Taiwan) et le Dr Corbeil (Australian Animal Health Laboratory, CSIRO, Geelong, Victoria, Australie) concernant le virus de type herpès infectant les ormeaux dans le Pacifique. Elles devraient être poursuivies en particulier sur les aspects de développement d'outils de diagnostic spécifiques (un accueil du Dr Corbeil est prévue au laboratoire en 2008) et de travaux sur la phylogénie des virus herpès infectant les invertébrés.

Par ailleurs, le laboratoire (LGP, Ifremer, La Tremblade, France) a été sollicité par un professionnel produisant des ormeaux pour participer à un projet financé par l'Union Européenne (Recherche au profit des PME, 7^{e} PC, Capacités). Ce projet a été déposé en septembre 2007 et a pour objectif principal de répertorier toutes les difficultés rencontrées en élevage d'ormeaux en Europe (zootechnie, nutrition, santé, réglementations,…) et de tenter d'y apporter des solutions. Dans ce projet, les maladies infectieuses sont bien sûr prises en considération et une première étape sera de faire un bilan des agents pathogènes présents aussi bien dans le milieu que dans des structures de type écloserie/nurserie. Sur la base de ces informations, il est proposé de développer des outils de diagnostic ciblant les agents infectieux les plus importants et d'étudier également les interactions de ces agents avec leur hôte.

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