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EFFETS DE POLLUTIONS PAR HYDROCARBURES SUR LES CAPACITES DE DEFENSE D'ORGANISMES MARINS

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CADRE GENERAL ET ENJEUX

Les littoraux Atlantiques sont des points névralgiques en matière de pollution marine du fait de la présence des routes de trafic maritime parmi les plus denses du monde. Depuis les dernières catastrophes majeures, comme celle de l'Erika et du Prestige, les Etats de l'Union Européenne se sont mobilisés contre les déficiences du transport maritime international. Dans ce cadre, la collaboration et les connaissances mutuelles entre les laboratoires de l'Union Européenne ont permis de progresser dans les mesures de prévention à prendre et dans l'analyse de leurs conséquences sur ces milieux aquatiques, à la fois nourricier et support d'activités économiques variées.

L'évaluation de l'impact des pollutions, la protection du milieu marin et la prévention des pollutions représentent donc des enjeux importants de l'action publique et font l'objet de propositions de nombreux programmes de recherche tant au niveau national qu'européen. A titre d'exemple, le programme INTERREG a pour objectif de favoriser la coopération transeuropéenne afin de développer un territoire européen équilibré et harmonieux. Il s'appuie sur l'idée que les frontières nationales au sein de l'Union Européenne ne doivent pas être un obstacle à l'essor économique et social des différentes régions transfrontalières. Il entend assurer un développement stable et durable de l'espace, répondre aux besoins des populations locales, élargir les réseaux d'échanges et les liens transfrontaliers ainsi que concilier le développement économique avec la protection de l'environnement. Dans le cadre de ce programme, conscient du défi que représente la pollution causée par la navigation pour les collectivités territoriales et leurs organismes d'intervention, le projet européen EROCIPS ("Emergency Response to coastal Oil, Chemical and Inert Pollution from Shipping") a été élaboré. Ce projet entend apporter une réponse opportune aux accidents engendrant une pollution pétrolière, chimique ou inerte des côtes. Son objectif est de présenter des outils de diagnostic transférables fournissant des informations pertinentes aux intervenants et aux décideurs engagés dans les opérations de lutte.

Mon sujet de recherche, qui est financé dans le cadre du projet EROCIPS suporté par la région Poitou-Charentes, se pose dans cette problématique écologique et économique d'intérêt mondial. D'un point de vue écologique, la compréhension générale des systèmes de défense des organismes marins face à une pollution représente un point important à maîtriser pour appréhender la gestion d'une situation de crise. D'un point de vue purement sanitaire les résultats obtenus permettront aux autorités de mettre en place, dans une certaine mesure, des actions préventives pour la préservation des espèces commerciales et des consommateurs.

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LISTE DES ABREVIATIONS

En chimie

HAP : Hydrocarbure Aromatique Polycyclique.

LCO : Light Cycle Oil.

US-EPA : United States Environmental Protection Agency.

En biologie

ACH_{50} : Activité hémolytique du complément voie alterne.

IL : Interleukine.

PO : Phénoloxydase.

TNF : Tumor Necrosis Factor.

Les laboratoires

Afssa : Agence Française de Sécurité Sanitaire des Aliments.

Cedre : CEntre de Documentation, de Recherche et d'Expérimentations sur les pollutions accidentelles des eaux.

Ifremer : L'Institut Français de Recherche pour l'Exploitation de la Mer.

IRIS : International Research Institute of Stavenger.

LEMAR : Laboratoire des sciences de l'Environnement MARin.

LIENSs : LIttoral, Environnement et Sociétés.

Les projets

DISCOBIOL : Dispersants et technique de lutte en milieux côtiers : effets biologiques et apports à la réglementation.

EROCIPS : Emergency response to coastal oil, chemical and inert pollution from shipping.

PRAGMA : A pragmatic and integrated approach for the evaluation of environmental impact of oil and chemicals spilled at sea: input to European Guidelines.

RESPIL : Response means to chemicals spilled at sea and environmental damage.

VALORISATION SCIENTIFIQUE

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Publiées

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Thomas-Guyon H., Gagnaire B., **Bado-Nilles A.**, Bouilly K., Lapègue S. and Renault T., accepté sous révision. Detection of phenoloxidase activity in early stages of the Pacific oyster, *Crassostrea gigas* (Thunberg). Developmental & Comparative Immunology, (IF : 3.155).

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[1] Numéro et page correspondant à l'article dans le manuscrit.

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Cuttlefish, *Sepia officinalis* and response to Ag and Cu exposure – SEB Metals Symposium, King's College, Londres, Grande Bretagne – 3 au 5 September 2007.

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Thomas-Guyon H., **Bado-Nilles A.**, Gagnaire B., Caillaud C., Bouilly K., Lapègue S. & Renault T. *In vitro* phenoloxidase activation detected during the development of the Pacific oyster, *Crassostrea gigas* (Thunberg) – 30th FEBS Congress- 9th IUBMB Conference, Budapest, Hungary – 2 au 7 Juillet 2005.**Bado-Nilles A.** & Thomas-Guyon H. Phenoloxidase activity in acellular fractions of haemolymph of the Pacific oyster, *Crassostrea gigas* – 29th FEBS Congress, Warsaw, Poland – 26 Juin au 1er Juillet 2004.

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CHAPITRE 1
CONTEXTE DE L'ETUDE

Le Lyria (Août 1993)
Source : Cedre

L'eau est un composant chimique omniprésent sur la Terre retrouvé essentiellement sous forme liquide mais également sous forme gazeuse (vapeur d'eau) et solide (glace). La surface de la terre est recouverte à 70 % par les milieux aquatiques dont 97 % par des eaux salées et uniquement 3 % par des eaux douces. En tant que composé essentiel à la vie, l'eau a une grande importance dans l'histoire de l'Homme. Cette importance explique que sa préservation soit considérée comme primordiale. Ainsi la Directive Cadre des Eaux (Directive 2000/60/CE – DCE), établie par le Parlement Européen et le Conseil de l'Union Européenne, rappelle que l'eau est un patrimoine qu'il faut protéger et traiter comme tel. Cette Directive Européenne cherche donc à favoriser une politique communautaire de gestion de l'eau dans un contexte de changements globaux et dans la perspective de la gestion durable des ressources et des milieux naturels. De ce fait, elle vise à définir les différents usages de l'eau par l'Homme au niveau domestique, agricole, industriel et également touristique (eaux de baignade), car une utilisation abusive et non contrôlée contribuerait à la dégradation de sa qualité. En effet, l'apport humain d'une grande quantité de substances polluantes biodégradables ou non, de type organique, biologique, physique et chimique n'est pas sans conséquence sur l'équilibre du milieu aquatique et donc de ses écosystèmes. Or, la pollution des milieux aquatiques est un problème majeur autant pour la population humaine utilisatrice des ressources en eau, que pour les populations végétales et animales. En effet, les écosystèmes aquatiques abritent une grande diversité d'espèces qui interagissent entre elles et qui puisent leur énergie et leur alimentation dans le milieu extérieur que représentent l'eau, le sol et l'atmosphère. Ces écosystèmes sont donc très dépendants des conditions de vie qui leur sont fournies et toute pollution y engendre des effets néfastes.

Ces pollutions peuvent être chroniques ou occasionnelles et elles concernent tous les réseaux hydrographiques continentaux et maritimes. Plus particulièrement, les estuaires et les milieux côtiers comptent parmi les zones les plus exposées à ces différents types de pollution notamment à celles liées au trafic maritime. En forte progression, le trafic maritime mondial d'hydrocarbures et autres substances chimiques provoque, sans être nécessairement la source principale en un endroit donné, d'importantes pollutions. En effet, le trafic maritime mondial induirait un rejet occasionnel d'environ 65.10^4 tonnes par an d'hydrocarbures dans l'océan mondial notamment par le biais des opérations de chargement et de déchargement des

cargaisons, du nettoyage des cuves des navires, des rejets opérationnels licites ou non, ou encore des marées noires, ainsi que des apports *via* les dépôts atmosphériques de composés forganiques volatils provenant des moteurs (GESAMP, 2007). L'ensemble de ces apports engendre une augmentation des risques pour la santé humaine et pour l'environnement dans sa globalité.

Liées au trafic maritime de ces dernières décennies, de nombreuses marées noires sont venues souiller le littoral Européen causant d'importants dommages économiques et environnementaux. Ce littoral a été marqué en décembre 1999 par le naufrage de l'*Erika*, qui laisse alors s'échapper une grande part des 31 000 tonnes de sa cargaison de fioul lourd (*Cedre*, 2000). Puis, le naufrage du chimiquier *Ievoli Sun* en octobre 2000, pour lequel sur les 150 tonnes de fioul de propulsion seulement 88 tonnes ont pu être pompées laissant 62 tonnes piégées dans l'épave ou perdues en Manche ; en plus, cet accident a soulevé le difficile problème de l'intervention face à un risque de pollution chimique. A ce jour, la dernière marée noire majeure survenue sur le littoral Atlantique a été causée par le naufrage du *Prestige* en novembre 2002. Le navire a déversé 63 000 tonnes de fioul lourd, générant des opérations de lutte en mer d'une ampleur sans précédent dans l'histoire et des impacts sur le littoral européen allant du Portugal jusqu'à la Hollande (*Cedre*, 2004).

Parmi les pollutions occasionnelles, bien que plus discrets, les rejets opérationnels d'hydrocarbures, licites ou non, par les navires représentent approximativement les 3/4 des apports dus au transport maritime (*Cedre*, 1997). Cependant, la conscience environnementale des opinions publiques et des autorités qui augmente au rythme des dommages écologiques et économiques successifs, engendre une diminution du nombre de pollutions occasionnelles depuis 25 ans. Ces dernières années, des outils de mesure, essentiellement socio-économiques, de l'impact des rejets ont été développés. Il en ressort l'intégration au Code de l'Environnement (Article L.218-22) de sanctions liées aux pollutions occasionnelles des milieux aquatiques. Mais, l'évaluation des impacts écologique et sanitaire de tels déversements est encore peu maîtrisée. En effet, les réglementations actuelles s'adressent pour l'essentiel aux pollutions par hydrocarbures en eau douce, sans prendre en considération

l'influence des paramètres environnementaux sur leur toxicité[2]. De ce fait et faute de disposer de données fiables sur l'impact direct de ces substances sur les organismes, face à un déversement maritime d'hydrocarbures, il est nécessaire d'extrapoler ces informations au domaine marin et de se livrer à des suppositions qu'il est difficile de vérifier. Pour pallier cette difficulté, depuis l'accident de l'*Erika* de nombreux laboratoires ont décidé d'étudier les effets d'une contamination par hydrocarbures d'espèces marines appartenant à différents niveaux trophiques (Bocquené *et al.*, 2004; Budzinski *et al.*, 2004; Davoodi & Claireaux, 2007; Geffard *et al.*, 2004a; Tronczynski *et al.*, 2004). Il est reconnu que l'assimilation et la bioconcentration des molécules constitutives d'un pétrole sont complexes et dépendantes de nombreux facteurs comme les espèces considérées, leur mode de vie, leur stade de développement, leurs capacités métaboliques, le milieu ou encore la nature chimique du polluant (Baussant *et al.*, 2001). Ainsi, l'ensemble des agressions chimiques d'ordre anthropique (hydrocarbures aromatiques polycycliques ou HAPs, les dioxines, les polychlorobiphényles) ainsi que d'ordre naturel (fortes teneurs en matières en suspension, variation de salinité, de pH ou encore de température) qui oblige l'organisme à s'adapter à son nouveau milieu pour survivre, est nommé stress environnemental (Burgeot *et al.*, 1999).

S'inscrivant dans ce cadre général, comme une recherche scientifique à vocation pratique, ce travail a pour objectif d'étudier expérimentalement les conséquences d'une pollution occasionnelle par hydrocarbures sur un ensemble de phénomènes intervenant dans les mécanismes de défense des animaux vivant dans les zones littorales et son impact possible sur les productions aquacoles. Cette approche est menée dans des conditions de laboratoire *in vitro* et *in vivo*.

Suite aux nombreux accidents maritimes à l'origine d'arrivées de nappes d'hydrocarbures sur les côtes et plus particulièrement aux deux pollutions majeures récemment survenues dans les eaux françaises (*Erika* et *Prestige*), il a semblé intéressant d'étudier l'effet d'un produit pétrolier lourd, fioul lourd de type *Erika*, et de l'un de ces fluxants[3], le light cycle oil (LCO), sur les capacités immunitaires des organismes marins. Or, chaque produit pétrolier est constitué d'un ensemble complexe de molécules (hydrocarbures, résines, asphaltènes, métaux

[2] **Toxicité :** capacité inhérente à une substance de produire un effet délétère.
[3] **Fluxant :** distillats légers issus du raffinage du pétrole.

lourds…) présentant des propriétés physico-chimiques très variables à l'instar des limites de solubilité. Afin de palier le problème majeur de la quantification de chaque composé, dont certains ne sont pas encore identifiés, seules les molécules prioritaires identifiées par l'Agence de Protection Environnementale Américaine (les 16 HAPs US-EPA) (US.EPA, 1998) ont été étudiées. De plus, ces dernières sont connues pour être persistantes dans l'environnement (Shiaris, 1989) et fortement bioaccumulables par les organismes du fait de leur faible polarité (Walker *et al.*, 2001). D'autre part, ces 16 HAPs sont reprotoxiques (Diamond *et al.*, 1995), immunotoxiques (Yamaguchi *et al.*, 1996) et, plus leur nombre de cycles benzéniques est élevé et plus ils présentent d'effets cancérigènes et mutagènes.

D'un point de vue expérimental, divers modes de contamination peuvent être utilisés. Le plus fréquent consiste à exposer les organismes, notamment les poissons plats et les Invertébrés benthiques (Geffard *et al.*, 2004b), à du sédiment pollué naturellement ou par ajout d'une quantité connue de xénobiotiques[4]. Ce mode de contamination prend en compte l'exposition aux xénobiotiques par contact avec le sédiment et *via* la colonne d'eau du fait de la solubilité des molécules. La contamination par voie orale consiste à alimenter les organismes par des granulés ou du phytoplancton préalablement pollués (Akcha *et al.*, 2000). Ce mode de contamination ne permet pas de connaître précisément la dose de polluant ingérée à l'inverse de la contamination par injection intra-péritonéale ou intra-musculaire. Mais, cette dernière technique, très utilisée en écotoxicologie, ne tient pas compte de la cinétique d'intoxication et elle modifie les réponses des animaux (Lemaire-Gony *et al.*, 1995). A côté de ces modes, la technique du "caging" est de plus en plus employée puisqu'elle utilise une contamination "naturelle" en terme de produits et de concentrations. Les individus sont placés dans des enclos directement sur les sites expérimentaux. Cette méthode constitue une approche prometteuse dans l'évaluation de l'accumulation et des effets des polluants sur les poissons comme sur les Mollusques sans toutefois maîtriser le stress environnemental d'ordre naturel (Stien *et al.*, 1998). Enfin, la contamination *via* la colonne d'eau, choisie dans le cadre de ce travail, correspond à la mise en présence directe des organismes avec une eau polluée par contact passif avec une ou plusieurs molécules. Ce mode de contamination se rapproche

[4] **Xénobiotique :** substance non présente naturellement dans l'environnement.

également des conditions "naturelles" avec l'avantage de maîtriser l'ensemble des stress environnementaux (Burgeot *et al.*, 1995).

Ce travail a été réalisé sur des espèces qui tiennent compte des intérêts écologiques et des enjeux économiques du Bassin de Marennes-Oléron. L'huître creuse, *Crassostrea gigas*, dont la production est très importante dans ce bassin, a été choisie de par son mode de vie. En effet, il s'agit d'un Bivalve filtreur microphage[5] capable de fortement bioaccumuler les polluants. Un Vertébré pélagique a également été retenu, le bar commun, *Dicentrarchus labrax*. Ce poisson, migrateur actif, présente des zones de nourricerie dans des estuaires vaseux qui sont bien souvent contaminés (Parlier, 2006). Il s'agit également d'un poisson d'aquaculture dont le grossissement est souvent réalisé en cages flottantes, ce qui le rend particulièrement vulnérable à tout déversement occasionnel de polluants. Les animaux sauvages, représentatifs d'une population naturelle, permettent une meilleure interprétation écologique des effets d'une contamination. Cependant, leur passé d'exposition aux xénobiotiques étant inconnu, il devient un facteur limitant dans le cadre des connaissances actuelles pour la mise au point des biomarqueurs d'intérêt. C'est pourquoi, quel que soit le modèle biologique étudié, le choix s'est porté sur des animaux d'élevage, aux antécédents connus et dont l'homogénéité de la population, en âge, en poids et en taille, laisse envisager une meilleure "standardisation" de la réponse étudiée.

Suite à une pollution, l'homéostasie[6] et la capacité d'adaptation de ces organismes peuvent être altérées par la modification d'une ou plusieurs fonctions biologiques. En effet, les composés toxiques sont susceptibles de causer, directement ou indirectement, des effets nuisibles sur les principaux systèmes physiologiques comprenant les systèmes endocriniens, reproducteurs, nerveux et immunitaires (Fournier *et al.*, 2000). Ceci a pour conséquence la remise en cause de leur croissance, de leur reproduction, voire de leur survie. Le système immunitaire, qui contribue au maintien de l'intégrité de l'individu en éliminant les pathogènes (virus, bactéries et parasites…), peut ainsi interagir avec plusieurs xénobiotiques de l'environnement, provoquant une modification des fonctions de protection de l'organisme

[5] **Filtreur microphage :** se nourrissant de particules inférieures à 4 µm et en suspension dans la colonne d'eau comme les bactéries et le phytoplancton.

[6] **Homéostasie :** tendance des êtres vivants à maintenir constant et en équilibre leur milieu interne et leurs paramètres physiologiques.

(Krzystyniak *et al.*, 1995). L'immunotoxicité est alors définie comme l'ensemble des effets délétères induits par tout constituant biologique, chimique ou physique de l'environnement sur le système immunitaire à la suite d'une exposition. Afin d'évaluer qualitativement et quantitativement les modulations du système immunitaire, des biomarqueurs sont recherchés. Un biomarqueur correspond à un paramètre biologique susceptible de refléter l'interaction entre un système biologique et un agent environnemental (Lagadic *et al.*, 1997). En écotoxicologie, trois grands types de biomarqueurs existent : les biomarqueurs d'exposition témoignant d'un contact avec un polluant, les biomarqueurs d'effet caractérisant l'intensité de la réponse face à une pollution et les biomarqueurs de sensibilité traduisant une variation dans la sensibilité. Ils peuvent se situer à différents niveaux d'intégration allant de l'échelle moléculaire à celle de l'écosystème. En effet, la réponse biologique est la résultante d'une série de modifications allant de la modulation génétique à la modulation biologique.

Après une revue bibliographique rappelant l'état des connaissances (Chapitre 2), une première série d'expérimentations *in vitro* est décrite (Chapitre 3 – Partie 1). Des hémocytes d'huître et du plasma de bar ont été mis en contact avec trois produits pétroliers différents (fioul lourd, LCO et gasoil) et avec les 16 HAPs US-EPA dans le but de sélectionner les polluants et d'identifier les biomarqueurs d'intérêt, utilisables ensuite lors des contaminations *in vivo*. Suite à la mise en place d'un protocole expérimental adapté aux deux espèces, deux séries d'exposition *in vivo* des organismes à un fioul lourd et à un LCO sont réalisées (Chapitre 3 - Partie 2). Lors de ces expositions aux produits pétroliers, la modulation des biomarqueurs et des gènes de type immunologique précédemment sélectionnés est étudiée. Afin de répondre à la problématique du projet EROCIPS, des outils de diagnostic pertinents et capables d'aider les intervenants engagés dans les opérations de lutte sont identifiés (Chapitre 4).

CHAPITRE 2
ETAT DES CONNAISSANCES

L'Erika (Décembre 1999)
Source : Marine Nationale

Le pétrole étant la principale source d'énergie des pays industrialisés, il est sujet à un commerce très important dont l'exploitation et le transport peuvent induire des pollutions accidentelles plus ou moins massives (les marées noires) et des pollutions opérationnelles plus ou moins fréquentes (les déballastages ou dégazages). Ainsi, le trafic maritime mondial représente le facteur unitaire le plus important de pollutions occasionnelles par hydrocarbures. En effet, chaque année, les rejets occasionnels lié au transport maritime représentent une contamination d'environ 65.10^4 tonnes dans l'océan mondial.

En parallèle, la protection du milieu marin et la prévention des pollutions sont des enjeux importants de l'action publique. De ce fait, les autorités s'interrogent sur le devenir de ces polluants dans les milieux aquatiques, ainsi que sur leurs toxicités au niveau des écosystèmes touchés. En effet, les polluants produisent des stress environnementaux capables d'induire des déstabilisations importantes chez certains organismes voire leur mort en cas d'arrivages massifs. De façon générale, le premier impact d'une modification d'un paramètre environnemental se produit à l'échelle cellulaire avec une action directe sur la respiration, les échanges ioniques et plus spécialement sur les capacités de défense.

Dans l'état des connaissances, trois thèmes seront traités afin de situer le travail de recherche effectué. Le premier intègrera les différents types de pollutions occasionnelles rencontrées et les produits étudiés. Puis les modèles biologiques, qui sont des espèces marines à forts enjeux écologiques et économiques, et leurs systèmes de défense seront décrits. Le dernier thème abordera le risque environnemental lié à ce type de pollution sur l'état de santé, et plus particulièrement, sur les capacités de défense des organismes aquatiques.

1. PETROLE ET TRAFIC MARITIME MONDIAL

1.1. Le pétrole

Le pétrole est une roche liquide carbonée, ou huile minérale, constituée d'une multitude de molécules composées majoritairement d'atomes de carbone et d'hydrogène appelés hydrocarbures. Energie fossile, son exploitation est l'un des piliers de l'économie industrielle contemporaine, car il fournit la quasi totalité des carburants liquides. Le pétrole est aussi souvent appelé « or noir » en référence à sa couleur noire et à son prix élevé.

1.1.1. Sa formation

Suite à la sédimentation de matières organiques végétales et animales et à leur enfouissement sous d'autres couches sédimentaires, la pétrogénèse commence. La décomposition de ces matières organiques provoque une perte d'oxygène dans le milieu induisant des réactions réductrices donnant du kérogène. Puis, sous l'action combinée de la chaleur (60 °C) et de la pression (enfouissement d'environ 1 500 à 2 000 mètres), la transformation en hydrocarbures débute. Ce pétrole en formation est moins dense que la roche qui l'entoure, il va donc migrer vers la surface. Si le pétrole est arrêté dans sa progression par des roches imperméables, il se concentre pour former des poches qui sont à l'origine des réservoirs actuels.

1.1.2. Sa composition

Il est possible de distinguer les différents types de pétrole selon leur densité, leur fluidité, leur teneur en soufre et autres impuretés (vanadium, mercure, sels…) et leur teneur en différentes classes de molécules chimiques (HAPs, *n*-alcanes, cycloalcanes…). Le pétrole est alors paraffinique, naphthénique ou aromatique. Il est aussi possible de les classifier selon leur provenance, car les pétroles issus de gisements voisins ont souvent des propriétés proches. Cependant chaque poche de pétrole a des caractéristiques particulières dues à l'histoire géologique de sa formation.

1.1.3. Ses secteurs d'utilisation et son commerce

Le pétrole brut est un produit stratégique, qui après raffinage est utilisé dans un grand nombre de secteurs différents (e.g. domaines énergétiques, industries pétrochimiques, voirie…), ce qui en fait un produit vital et central dans l'économie mondiale. Bien que servant dans de nombreux domaines, l'utilisation du pétrole raffiné est surtout dominant au niveau des activités liées aux moyens de transport. En effet, selon le Fond Monétaire International, 48 % des produits pétroliers sont employés dans ce secteur en 2002, et cette part continue à augmenter (http://www.imf.org/).

1.1.4. Son transport

Deux voies de transport existent pour le pétrole brut et ses produits de raffinage : le transport par terre et le transport par mer. Les oléoducs sont la principale voie d'acheminement transcontinental, car ils restent le moyen de transport terrestre le plus sûr et le moins coûteux par rapport aux voies ferroviaires ou routières. Ils relient les sites de production aux ports de chargement ou les raffineries aux centres de distribution ou d'utilisation. Le second type est le transport maritime. Les mers et les océans sont les principales voies de transport du pétrole et de ces produits dérivés, et ceci malgré les risques inhérents au trafic maritime. En 2000, environ 1,9 milliard de tonnes ont ainsi été transportées sur les océans, ce qui représente 62 % de la production mondiale (http://r0.unctad.org/infocomm/).

Un de ses avantages est sa souplesse : les pétroliers peuvent changer de trajet selon les besoins, transporter n'importe quel type de pétrole et répondre à la demande saisonnière en augmentant leur capacité. La plupart des convois pétroliers suivent un ensemble de routes maritimes dont environ la moitié a comme point d'origine le Moyen Orient et comme destination l'Asie, les Etats-Unis ou l'Europe (Figure 2 - 1).

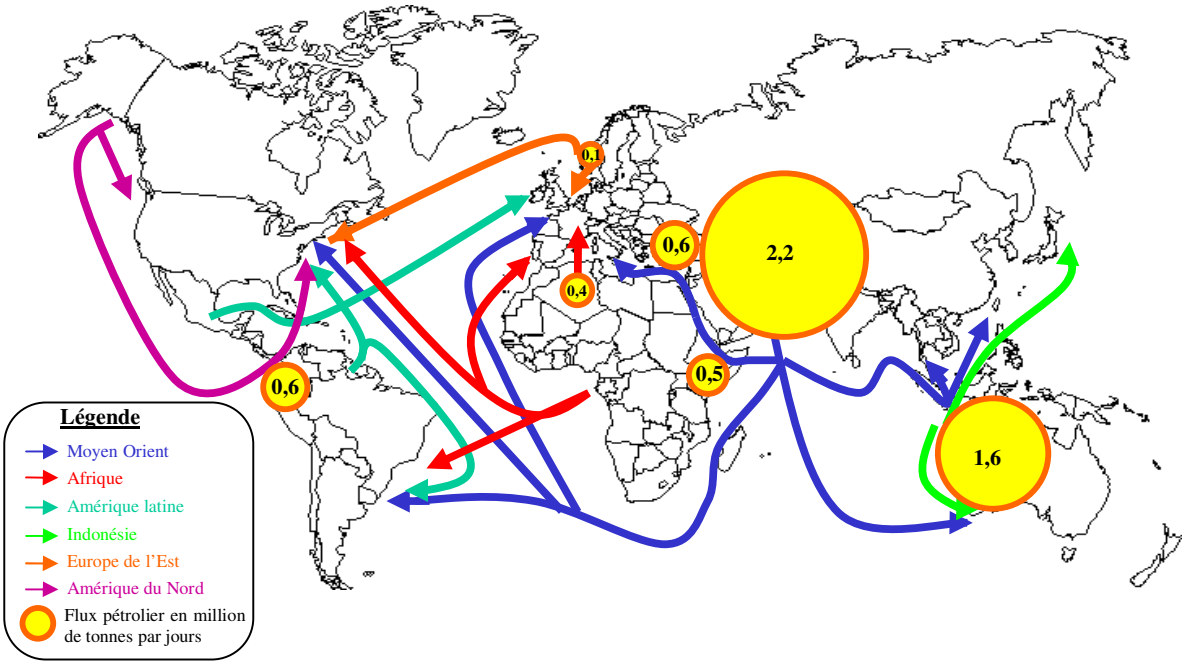


Figure 2 - 1 : Carte des principales routes maritimes du pétrole brut et de ces produits de raffinage en 2006 (d'après : http://r0.unctad.org/infocomm/).

1.2. Différentes pollutions occasionnelles rencontrées

Au cours du transport du pétrole, une probabilité de déversement accidentel ou opérationnel existe et la nature du produit déversé sera alors fonction de son origine et de l'étape de raffinage auquel il se trouve (Figure 2 - 2).

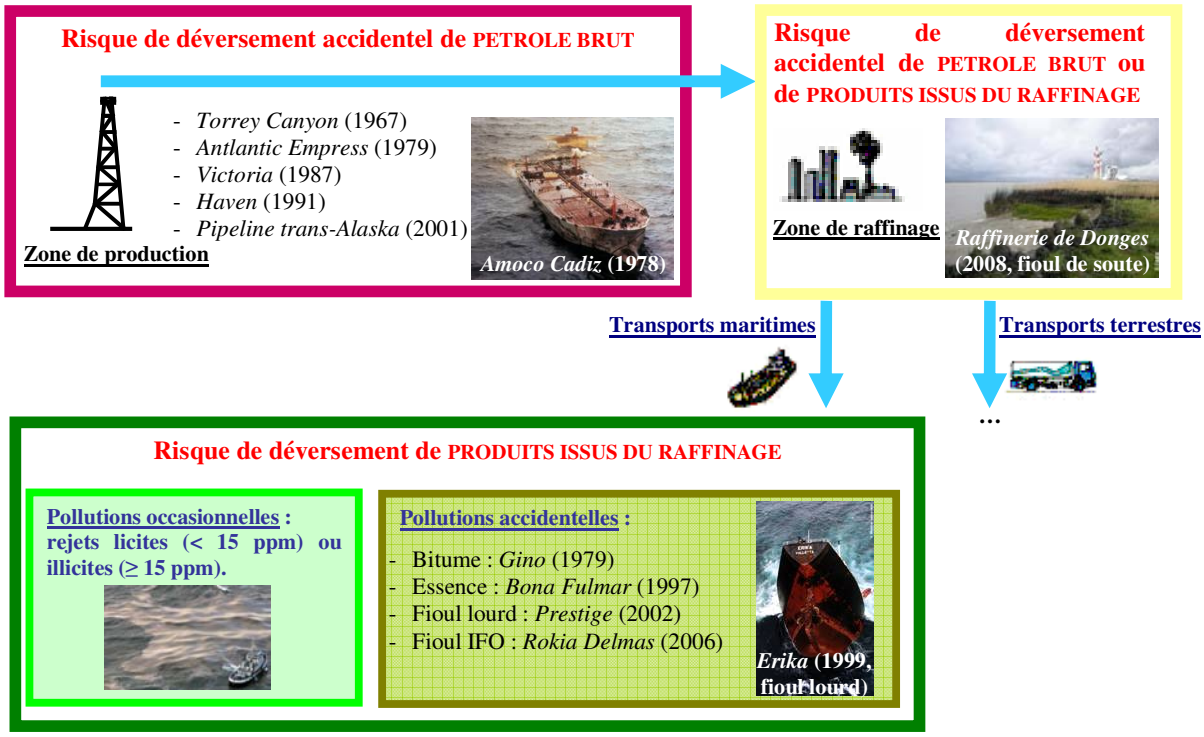


Figure 2 - 2 : Représentation schématique des différents cas de pollution occasionnelle pouvant être observés.

1.2.1. Rejets au niveau des zones d'exploitation et de raffinage

Les zones d'exploitation et de raffinage du pétrole sont des zones à risque en ce qui concerne les fuites d'hydrocarbures vers les milieux aquatiques (rivières, nappes phréatiques et mers) et les sols. En effet, le dernier accident grave en date concerne la Raffinerie de Donges (Loire-Atlantique) où, en mars 2008, une fuite de canalisation sur un canal de chargement provoque un déversement estimé à 400 tonnes de fioul de soute (IFO 380) (http://www.cedre.fr/). Afin de réguler les rejets licites, une législation stricte a été adoptée par la Commission d'Oslo et de Paris (OSPAR), il s'agit des lignes directrices concernant les rejets provenant de raffineries nouvelles (PARCOM II/11,1 annexe VII)

(http://www.ospar.org). Cette législation définit en plusieurs points les rejets opérationnels licites dans le milieu pour les zones d'exploitation et les raffineries :

- Concentrations inférieures à 5 $mg.L^{-1}$ pour l'eau de traitement et les autres effluents liquides pollués.
- La quantité totale d'hydrocarbures susceptible d'être déversée doit être inférieure à 3 g par tonne de capacité de raffinage avec une fourchette de 5 - 15 $mg.L^{-1}$.

Lorsque les hydrocarbures dans l'eau passent au dessus de ces limites, le rejet n'est pas autorisé.

1.2.2. Les rejets licites et illicites lors du transport maritime

La principale source de pollution occasionnelle de l'environnement marin par les navires est attribuée à ce jour aux machines de propulsion, et s'applique à tous les types de navires qui sont utilisateurs de fioul en tant que carburant. La réglementation autorise un rejet à la mer à travers un dispositif réglementaire de séparation d'eau huileuse à 15 $cm^3.m^{-3}$ (15 ppm) (http://www.afcan.org/). Cependant ce séparateur est souvent peu fiable et les opérations de maintenance des moteurs et du navire induisent régulièrement une hausse conséquente de l'apport en hydrocarbures présents dans les eaux de cale des machines à laquelle le séparateur ne peut pas faire face. De plus, la législation oblige les capitaines des navires, lors d'escale dans un port maritime, d'y déposer leurs déchets d'exploitation et leurs résidus de cargaison (Articles L. 343-1 à L. 343-3 du code des ports maritimes). Pour échapper aux frais des débarquements d'eau souillée en escale, le rejet illicite est assez largement répandu, et ceci en dépit des textes de loi l'amendant fortement (http://www.assemblee-nationale.fr). Selon certaines estimations, ces pollutions dites occasionnelles pourraient représenter chaque année une pollution équivalente à dix fois celle due à l'*Erika*. Environ 750 observations de pollutions par rejet illicite sont faites en moyenne par an dans la zone Manche, mer du Nord et mer Baltique, et entre 1 000 et 1 500 en Méditerranée. Ce sont ainsi quelques 100 000 à 150 000 tonnes d'hydrocarbures qui sont déversées chaque année en Méditerranée, soit douze fois l'équivalent de la cargaison du *Prestige*. En outre, le volume des résidus rejetés (fioul, eaux de déballastage, eaux et huiles de vidange) est de plus en plus conséquent. Le rapport, paru en 2005,

intitulé « les perspectives du Plan bleu sur l'environnement et le développement » estime une augmentation globale de ces rejets de 5 % par an.

1.2.3. Les marées noires

L'exploitation et le transport du pétrole font régulièrement l'objet d'accidents responsables des marées noires. En 1990, la quantité estimée de pétrole déversé annuellement suite aux accidents dans le milieu marin était de 5 millions de tonnes.

La protection du milieu marin et la prévention des pollutions représentent des enjeux importants de l'action publique, en effet, pas moins de 34 marées noires d'importance variée ont touché les côtes Européennes, et plus particulièrement les côtes Bretonnes qui restent les zones internationales les plus touchées, depuis les années 60 (http://www.cedre.fr/).

Néanmoins, une diminution du nombre de marées noires liée à l'apparition de réglementations plus strictes a été constatée au cours de ces 20 dernières années (http://www.itopf.com). Ainsi le nombre d'accidents majeurs est passé de 25 par an dans les années 70, à 9 dans les années 80, puis à 8 dans les années 90 et pour l'instant 4 pour les années 2000. De plus, une tendance à la baisse des quantités de pétrole déversé est également observable et ceci malgré les 63 000 tonnes de fioul lourd du *Prestige* (2002).

1.3. Produits pétroliers étudiés

Il est constaté qu'une fois déversé en mer, un pétrole brut est sujet à des processus de vieillissement à court terme (étalement, évaporation, dissolution, dispersion, émulsification…) et à long terme (sédimentation, photo-oxydation, biodégradation…). Ces différents processus de vieillissement définissent le comportement, puis le devenir du pétrole une fois déversé dans l'environnement marin. De ce fait, l'US-EPA a établi une classification basée sur les propriétés physico-chimiques des produits pétroliers afin de les répertorier en fonction des problématiques posées lors de la gestion d'une pollution par hydrocarbures (http://www.epa.gov/). Dans cette classification sont distingués les produits pétroliers légers et volatils (classe A), les non-collants (classe B), les lourds et collants (classe C) et les non-fluides (classe D).

Dans ce travail de recherche, l'intérêt s'est porté sur un produit pétrolier de classe C du fait de son impact médiatique lors des dernières grandes marées noires (*Erika*) : un fioul lourd. Ce produit peut provoquer des dommages irrémédiables à la faune et à la flore marine, notamment par engluement. Le second produit sélectionné correspond à un LCO (classe A) qui est très toxique pour l'environnement à cause de sa teneur élevée en composés aromatiques légers. Les produits de la classe B semblaient moins intéressants de par leur évolution rapide vers la classe A lors d'une augmentation de température, ou vers les classes C et D selon les processus de vieillissement. De même, les produits de la classe D n'ont pas été retenus du fait de leur viscosité élevée qui leur confère une fraction soluble quasi nulle.

1.3.1. Fioul lourd

Les diverses opérations de raffinage conduisent à la formation entre autre de produits lourds composés d'un mélange des résidus des procédés de distillation et de craquage. Lorsqu'il est nécessaire de réduire leur viscosité, ces résidus sont mélangés avec des distillats plus légers appelés fluxants. Ce mélange résidus/fractions légères, obtenu en fin de chaîne de raffinage, est appelé communément fioul lourd (Jézéquel, 2005).

Le fioul lourd est donc constitué d'un mélange complexe de différents produits chimiques liés non seulement à la nature du (ou des) pétrole(s) brut(s) utilisé(s), mais également aux procédés de raffinage dont il est issu et à la nature du (ou des) fluxant(s) additionné(s). De ce fait, l'effet de ce produit de classe C sur l'environnement dépendra, en plus de la quantité déversée, de la nature exacte de sa composition chimique.

Les analyses sont focalisées sur le fioul lourd issu de la marée noire de l'*Erika* (transmis par la Raffinerie TotalFina de Dunkerque, http://www.cedre.fr). Il est composé d'un mélange de 10 % de fluxant léger, 30 % de fluxant lourd et 60 % de produits de distillation sous vide. Ce fioul a une densité très proche de celle de l'eau de mer avec une viscosité élevée (20 000 cSt à 10 °C). Sa composition chimique et son bulletin d'analyse sont présentés dans le Tableau 2 - 1.

Bulletin d'analyse du fioul lourd de l'*Erika* (source TotalFina)	
Densité	1.0025
Point d'écoulement	3 °C
Viscosité	38 cSt (100 °C) ; 55 cSt (50 °C) ; 20 000 cSt (10 °C)
Soufre	2.28 %
Vanadium	82.7 ppm
Nickel	45 ppm
Asphaltènes	3.78 %
Composition chimique du fioul lourd de l'*Erika* (http://www.cedre.fr)	
Hydrocarbures saturés	22 – 30 %
Hydrocarbures aromatiques	42 – 50 %
Résines et asphaltènes	21 – 36 %

Tableau 2 - 1 : Bulletin d'analyse et composition chimique du fioul lourd de l'*Erika*.

1.3.2. Light cycle oil

Lors du raffinage des produits pétroliers, des distillations atmosphériques et sous vide sont entreprises afin d'optimiser la production des composés légers à forte valeur économique, tels que les diesels et les kérosènes. Les produits issus de ces distillations sont ensuite catalysés par le biais des systèmes de craquage catalytique fluide. Les LCOs, aussi nommés fluxants, correspondent aux produits issus des résidus de ce craquage catalytique (Poveda Jaramillo *et al.*, 2004). Ce fluxant est régulièrement utilisé pour réduire la viscosité des résidus lourds et ainsi en favoriser leur transport (Ding *et al.*, 2007).

Le LCO utilisé dans cette étude a été fourni par la Raffinerie Total de Donges (Tableau 2 - 2). Il correspond à un produit de faible viscosité, riche en HAPs, en aromatiques soufrés et en composés nitrogénés.

Bulletin d'analyse du LCO (source Total France)	
Densité	0.93
Intervalle de distillation	180 - 440 °C
Viscosité	1.24 cSt (100 °C)
Distillats légers (pétrole), craquage catalytique	100 %
Composition chimique du LCO	
Hydrocarbures aromatiques	85 – 86 %
Sulfures	1 – 2 %
Composés polaires	4 – 2 %

Tableau 2 - 2 : Bulletin d'analyse et composition du light cycle oil (source Total France).

2. MODELES BIOLOGIQUES ETUDIES

Le Bassin de Marennes-Oléron représente une zone littorale sous influence estuarienne bénéficiant d'une forte productivité biologique. De ce fait, cette zone est le siège d'activités humaines variées. Une étude effectuée dans le Bassin de Marennes-Oléron, financée par l'Agence de l'eau Adour-Garonne, a montré que la macrofaune benthique est contaminée par des HAPs et que ces molécules sont également présentes dans le sédiment à l'état de trace (Miramand *et al.*, 2002). Les HAPs présents dans ce bassin ont des origines chroniques (moteurs hors-bord et tourisme) et occasionnelles (rejets illicites et marées noires). Les modèles biologiques d'étude choisis prennent en compte à la fois les enjeux économiques et écologiques du Bassin de Marennes-Oléron, ce qui explique le choix d'étudier en parallèle des organismes benthiques et pélagiques.

2.1. Enjeux économiques et écologiques

L'huître creuse, *Crassostrea gigas*, a été choisie car elle constitue un bon modèle écotoxicologique de la zone littorale du Bassin de Marennes-Oléron. En effet, son mode de

vie sédentaire et son mode de nutrition par filtration l'amènent à bioaccumuler les polluants présents dans son environnement proche (Fochtman, 2000). De plus, la filière conchylicole représente à l'échelle internationale une activité à enjeux économiques majeurs, 25 % de la production aquacole mondiale en 2004 provient de la conchyliculture, soit 20 millions de tonnes. La FAO (Food and Agriculture Organisation), l'Union Européenne et le CIEM (Comité International pour l'Exploitation de la Mer) attestent du rôle primordial occupé par cette activité. La filière conchylicole française est le pôle d'aquaculture le plus important au niveau national avec 72 % du chiffre d'affaire. Cependant, l'élevage de coquillages concerne un faible nombre d'espèces qui sont majoritairement deux Mollusques Bivalves : l'huître creuse, *Crassostrea gigas*, 99 % de la production ostréicole française et 59 % de la production conchylicole française, soit 113 750 tonnes en 2004 (FAO, 2006a) ; et dans une moindre mesure la moule commune, *Mytilus edulis*, 75 % de la production mytilicole française et 29 % de la production conchylicole française, soit 55 575 tonnes en 2004 (FAO, 2006a). La production française d'huître creuse de la région Poitou-Charentes, dont principalement celle du Bassin de Marennes-Oléron, fournit 30 % de la production nationale.

Concernant le bar commun, *Dicentrarchus labrax*, il s'agit d'un Téléostéen à haute valeur ajoutée. Ce poisson migrateur actif colonise les slikkes[7] à marée haute et possède notamment dans le Bassin de Marennes-Oléron des zones de nourricerie (Parlier, 2006). Parmi toutes les espèces produites en aquaculture, en 2004, les poissons représentent 44 % de la production mondiale soit 34,9 millions de tonnes, avec une faible proportion de la production marine, 10 % (FAO, 2006a). Bien qu'en France, les poissons représentent une part du marché économique plus faible que celui des Mollusques, 28 % versus 72 %, ils restent néanmoins fortement élevés en zone Atlantique, avec 58 % du chiffre d'affaire français (FAO, 2006b). Parmi eux, *D. labrax* correspond à un chiffre d'affaire important (FAO, 2006b) provenant des pêcheries et d'une faible production en élevage, inférieure à 1 %, alors qu'en Méditerranée l'aquaculture est majoritaire (FAO, 2006a). Ainsi, la plus grande part de ce marché repose non pas sur l'aquaculture (42 % du chiffre d'affaire) (FAO, 2006a) mais sur les pêches (58 % du chiffre d'affaire) (FAO, 2006c).

[7] **Slikke :** vasière d'estuaire.

2.2. Système immunitaire

L'immunité a pour vocation le maintien de l'intégrité du soi. Ce terme s'adressait initialement à la résistance des individus vis-à-vis des infections microbiennes. Aujourd'hui, cette définition concerne l'ensemble des réactions qui tendent à éliminer les substances étrangères. Par extension, il s'agit de l'ensemble des facteurs humoraux et cellulaires, spécifiques ou non de la substance introduite, qui protège l'organisme contre les substances infectieuses, parasitaires et les proliférations malignes (Bach, 1999).

Parallèlement à la diversité et à la complexité progressive du monde animal, une sophistication des stratégies de défense est observée. Ainsi, les éponges sont capables de synthétiser des composés bioactifs aux activités antimicrobiennes diverses, alors que les annélides et les Mollusques, systématiquement plus élevés, interagissent à la fois par le biais de produits humoraux et cellulaires. A ces mécanismes de défense non spécifique s'ajoute, chez les Vertébrés, une immunité spécifique adaptative donnant naissance à des systèmes très organisés (Cooper, 2006). Les composantes indispensables du système immunitaire spécifique des Vertébrés proviendraient de deux paralogies[8] successives qui ont eu lieu chez les Chordés (Kasahara, 1999). La première serait survenue il y a environ 515 millions d'années chez un ancêtre commun à tous les Vertébrés et la seconde aurait eu lieu chez un ancêtre commun à tous les Gnathostomes. Ces duplications ont généré quatre ensembles de gènes paralogues qui ont ensuite évolué indépendamment et ont donné naissance aux principaux systèmes spécifiques de reconnaissance du soi et du non-soi. Ainsi, les poissons Téléostéens, sont dotés d'un système immunitaire proche de celui des Vertébrés supérieurs (Secombes *et al.*, 1983), alors que les Invertébrés, auxquels appartiennent les Mollusques Bivalves, ne présentent qu'un système immunitaire non spécifique, sans mémoire immunitaire (Galloway & Depledge, 2001).

Avant d'aborder plus spécifiquement l'exposé des capacités immunitaires développées pour cette étude, un résumé des différentes lignes de défense des Mollusques Bivalves marins et des poissons Téléostéens est présenté (Figure 2 - 3) :

[8] **Paralogie** : étape de duplication d'un bloc de gènes.

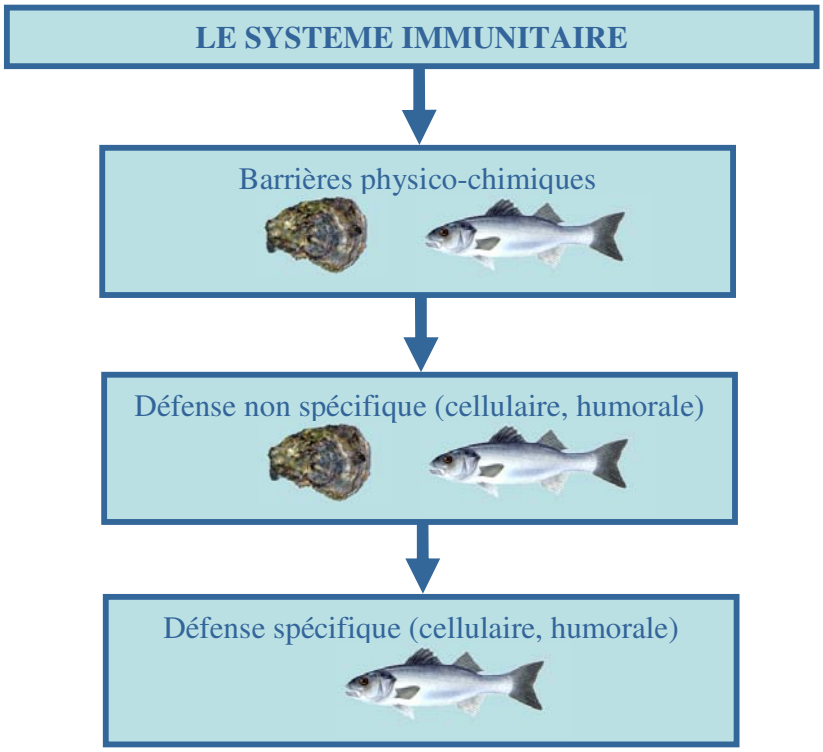


Figure 2 - 3 : Représentation schématique des différentes lignes de défense.

- *Les premières lignes de défense* des poissons et des coquillages sont les barrières physico-chimiques telles que le mucus, les épithéliums cutanés, branchiaux et intestinaux, et de façon plus spécifique les écailles pour les poissons et la cuticule et la coquille pour les Bivalves.
- *Les réponses immunitaires non spécifiques* constituent la deuxième ligne de défense des Téléostéens et la dernière ligne de défense des Mollusques Bivalves. Cette réponse immunitaire met en jeu les cellules circulantes, les hémocytes chez les Mollusques et les leucocytes chez les Téléostéens, et dans le milieu acellulaire, l'hémolymphe chez le coquillage et le sérum plus les liquides extracellulaires pour le poisson. Certains mécanismes cellulaires, comme la capacité phagocytaire et les activités lysosomales, des Vertébrés sont assimilables à ceux observés chez les Invertébrés (Secombes, 1996). De plus, certaines activités antibactériennes retrouvées dans les hémocytes des Bivalves, comme *C. gigas* et l'huître plate, *Ostrea edulis*, rappellent les activités cytotoxiques des cellules cytotoxiques non spécifiques des Téléostéens et des cellules Natural Killer des Mammifères (Hubert *et al.*, 1996). De

même, les mécanismes humoraux peuvent avoir des activités similaires chez les Vertébrés et Invertébrés : le lysozyme est une enzyme bactéricide ubiquitaire chez les poissons (Holloway *et al.*, 1993), mais également retrouvée dans le milieu acellulaire de nombreux Bivalves tels que l'huître creuse (Mon *et al.*, 1984) ou la moule commune, (Pipe, 1990) ; les lectines, qui permettent entre autre la reconnaissance des microorganismes avant phagocytose, sont détectées dans la quasi-totalité des êtres vivants sous forme libre (Pipe, 1990; Yano, 1996) ; le système du complément des poissons (Zarkadis *et al.*, 2001) et le système prophénoloxydase-phénoloxydase des Invertébrés aquatiques (Cerenius *et al.*, 2008) sont tous deux des systèmes biologiques constitués d'un ensemble de protéines solubles acellulaires, qui de par leurs propriétés enzymatiques, s'activent en cascade. Néanmoins, les activités innées des Vertébrés sont plus développées et différenciées que celles des Invertébrés.

- *La réponse immunitaire spécifique*, dernière ligne de défense des Vertébrés, revêt également deux formes, une réponse de type humoral avec la production d'anticorps et une réponse à médiation cellulaire cytotoxique confirmée par le phénomène de rejets de greffe (Romano *et al.*, 2005). Toutes deux découlent de la stimulation respective des lymphocytes B et T dont l'existence a été démontrée chez le poisson. La différenciation entre ces deux sous-populations lymphocytaires est réalisée par la mise en évidence de leurs marqueurs de surface dont les plus caractéristiques sont les immunoglobulines de surface chez les lymphocytes B (De Luca *et al.*, 1983) et le récepteur T chez les lymphocytes T (Partula *et al.*, 1995). L'existence de cellules présentatrices de l'antigène (Vallego *et al.*, 1991; Vallego *et al.*, 1990) et des molécules du complexe majeur d'histocompatibilité (CMH) (Grimholt *et al.*, 2002; Hordvik *et al.*, 1993), qui jouent un rôle crucial dans la reconnaissance de l'antigène par les cellules T, a également été établie chez le poisson. Les poissons sont donc capables de reconnaître un antigène et de développer une réponse immunitaire spécifique. Cependant, leur réponse mémoire est moins élaborée que celles des Vertébrés supérieurs. Ils ne produisent que deux classes d'immunoglobulines (Ig), des IgM et des IgD dont l'affinité est plus faible que celle des Mammifères.

D'autre part, des messagers intercellulaires, appelés cytokines, sont présents dans les milieux cellulaires et acellulaires. Ces cytokines sont des glycoprotéines à effets pléïotropes[9]. En effet, elles induisent, contrôlent ou inhibent l'intensité de la réponse immunitaire, mais également des mécanismes d'hématopoïèse[10] et certaines différenciations et proliférations tissulaires. Elles se distinguent des hormones par leur mode d'action à la fois autocrine[11], paracrine[12] et endocrine[13]. En se liant à des récepteurs spécifiques présents sur les cellules cibles, elles déclenchent des voies de transduction de signal modifiant l'expression des gènes de ces cellules. Elles sont très nombreuses chez les Mammifères où elles sont actuellement regroupées en quatre grandes familles : les hématopoïétines, les interférons, les chémokines et les Tumor Necrosis Factor (TNF). Grâce essentiellement à des essais biologiques basés sur leurs similarités fonctionnelles avec l'activité des cytokines mammaliennes ou à des réactions croisées avec ces dernières, un certain nombre d'entre elles ont été identifiées chez le poisson (interleukine (IL) 1, 2, 3, 6, interférons, Transforming Growth Factor, Chemotactic Factor, Macrophage Migration Inhibition Factor, TNF…). Sécrété par les macrophages, le TNF couvre chez le poisson, comme chez les Mammifères, une large gamme d'activités : effets cytotoxiques, activité anti-bactériennes, virales et parasitaires (Secombes, 1996). D'autre part, l'existence d'un récepteur spécifique au TNF-alpha chez les poissons indique une bonne conservation évolutive de ce constituant membranaire (Manning & Nakanishi, 1996). Chez les Invertébrés, des molécules assimilables aux IL-1 (anti-tumoral, modulateur de l'apoptose) et IL-6 (facteur hématopoïétique) et au TNF-alpha ont été détectées dans le milieu acellulaire et cellulaire de *M. edulis* (Hughes *et al.*, 1991). Une autre molécule de type cytokine a également été découverte dans les hémocytes de *C. gigas* (Peatman *et al.*, communication personnelle).

Suite à ce rappel concernant le système immunitaire dans son ensemble, un développement plus conséquent des activités humorales et cellulaires non spécifiques, étudiées au cours de la thèse, est présenté ci-dessous.

[9] **Effet pléïotrope :** effet influençant différentes fonctions.
[10] **Hématopoïèse :** formation des érythrocytes.
[11] **Autocrine :** action des cellules sécrétrices sur elles-mêmes.
[12] **Paracrine :** action des cellules sécrétrices sur des cellules proches.
[13] **Endocrine :** sécrétion dans le sang.

2.2.1. Défense non spécifique à médiation cellulaire

Les cellules

- *Les hémocytes* : chez les Mollusques Bivalves, les hémocytes se situent dans un système circulatoire de type semi-ouvert (Cheng, 1981). Plusieurs sous-populations hémocytaires coexistent dans l'hémolymphe et dans les tissus des Bivalves, donnant lieu à différentes classifications indépendamment des espèces et des auteurs (Auffret, 1988; Hine, 1999). Néanmoins, tous s'accordent à dire que les hémocytes se divisent en deux grand types cellulaires majeurs : les cellules granuleuses, granulocytes, et les cellules non granuleuses, hyalinocytes (Cheng, 1981). Leur proportion dans l'hémolymphe change selon la période de l'année, le site de résidence des organismes et les variations physico-chimiques de l'environnement (Auffret & Oubella, 1995). Les hémocytes accomplissent diverses fonctions dont certaines participent à la défense de l'organisme, vis-à-vis des agressions diverses qu'il subit (Cheng, 1981). Ils sont notamment impliqués dans la réparation des blessures en formant un clou hémostatique (Cheng, 1983) et dans la réparation et l'entretien de la coquille (Fisher, 1986) par le biais du transport du calcium et de protéines (Cheng, 1996). Ils sont capables de détruire les microorganismes par phagocytose et les fluides par pinocytose (Auffret, 1985; Xue & Renault, 2000). Enfin, les hémocytes peuvent détoxiquer les polluants. En effet, ils piègent les polluants, notamment les métaux lourds, dans leurs lysosomes (George *et al.*, 1982) où les métallothionéines mettent en route des processus de détoxication intracellulaire (Simkiss *et al.*, 1982) avant excrétion vers le milieu extérieur (Coombs & George, 1977; George & Pirie, 1980).
- *Les leucocytes* : les leucocytes se trouvent dans le sang, système circulatoire fermé, et dans un grand nombre d'organes chez les poissons Téléostéens. Ils présentent une forte hétérogénéité morphologique et fonctionnelle, qui ont permis leur classification en trois grandes familles : les lymphocytes, les granulocytes et les monocytes/macrophages. Parmi les lymphocytes certains ont une activité non spécifique comme les cellules cytotoxiques non spécifiques, équivalentes des cellules Natural Killer des Mammifères. Elles exercent une activité cytotoxique non spécifique vis-à-vis des cellules tumorales ou infectées par des organismes

intracellulaires dont les virus (Secombes, 1996). Les granulocytes, essentiellement neutrophiles, les monocytes circulants ou les macrophages tissulaires jouent un rôle important dans l'inflammation et la phagocytose permettant ainsi la destruction des corps étrangers ou des tissus altérés. Cependant, contrairement aux Mammifères où la phagocytose est principalement effectuée par les granulocytes polynucléaires et neutrophiles, les monocytes/macrophages apparaissent comme les cellules phagocytaires les plus actives chez les poissons dont le bar commun (Esteban & Meseguer, 1997).

La phagocytose

La phagocytose, décrite en Annexe 1, est un système clef de la défense interne des Mollusques Bivalves et des poissons Téléostéens. La première étape correspond à la reconnaissance par les phagocytes du microorganisme par chimiotactisme qui migrent vers la zone infectée (Fisher, 1986). Le microorganisme est alors reconnu et immobilisé par adhérence cellulaire. Cette phase d'adhérence correspond à des interactions non spécifiques, liées aux propriétés physico-chimiques de la membrane plasmatique et de la particule à ingérer, ou spécifiques, par l'intermédiaire de récepteurs ou de lectines. Puis se succèdent l'internalisation de la particule grâce à la formation de pseudopodes qui l'englobent totalement et la dégradation et la destruction du corps étranger. Cette dernière étape est effectuée sous l'effet combiné d'enzymes hydrolytiques lysosomiales (e.g. estérase), de protéines bactéricides et de mécanismes de flambée oxydative (Auffret, 1985; Secombes, 1996). Chez les poissons, la quantité d'enzymes lysosomiales comme les protéines cationiques et les cytokines cytotoxiques, est cependant plus importante que celle observée chez les Invertébrés. Chez les Bivalves, une coopération cellulaire particulière est mise en place lorsque le microorganisme ne peut être phagocyté : l'agrégation cellulaire. Des « filopodes » se forment par prolongation cytoplasmique à la surface des hémocytes et vont encapsuler les microorganismes dans des kystes de fibres protéiques (Chen & Bayne, 1995).

2.2.2. Défense non spécifique à médiation humorale

Le lysozyme

Le lysozyme est une enzyme retrouvée, chez les Téléostéens comme chez les Bivalves, dans la plupart des tissus et des fluides corporels, et tout particulièrement, dans les zones qui sont les plus exposées aux bactéries comme le mucus, les branchies, le milieu extracellulaire, la rate, le rein et le tractus digestif (Cheng *et al.*, 1975; Holloway *et al.*, 1993). Il joue un rôle dans les mécanismes de défense vis-à-vis des infections à bactéries Gram positif en clivant la liaison β(1,4) entre l'acide N-acétyl-glucosamine et l'acide N-acétyl-muramique du peptidoglycane des membranes bactériennes (Annexe 2). Il provoque ainsi la lyse cellulaire.
Plus particulièrement, chez les poissons, et contrairement aux Mammifères, il agit également mais à un moindre degré sur les bactéries Gram négatif, même en absence de l'activation du système du complément (Yano, 1996). En plus de son action directe sur les membranes bactériennes, il aide à la phagocytose des corps étrangers en tant qu'opsonine ou directement en activant les macrophages et les granulocytes (Yano, 1996).

Le système prophénoloxydase-phénoloxydase

Les enzymes cuivre-dépendantes (tyrosinases, laccases et phénoloxydase) représentent une classe de protéines ayant probablement évoluées à partir d'une protéine ancestrale dont la fonction était de protéger les organismes primitifs à métabolisme anaérobie de l'oxygène toxique issu de la photosynthèse (Canfield & Teske, 1996; Van Holde *et al.*, 2001, Durán *et al.*, 2002; Decker & Jaenicke, 2004). Plus particulièrement, la phénoloxydase (PO) a été détectée dans l'hémolymphe et les hémocytes de plusieurs espèces d'Invertébrés (Coles & Pipe, 1994). Bien que principalement observée chez les arthropodes (Cerenius *et al.*, 2008), elle existe également dans l'hémolymphe des Mollusques Bivalves tels que la moule commune (Coles & Pipe, 1994) et l'huître creuse (Hellio *et al.*, 2007), et ce, dès les niveaux précoces du développement larvaire (Luna-González *et al.*, 2003; Thomas-Guyon *et al.*, acceptée). Cette enzyme est modulée par les xénobiotiques (Bouilly *et al.*, 2006; Coles *et al.*, 1994; Gagnaire *et al.*, 2004). Le système prophénoloxydase-phénoloxydase (proPO-PO) prend une part active dans les mécanismes de défense grâce aux réactions d'oxydation intervenant dans les processus de réparation des blessures, d'encapsulation et de mélanisation

(Söderhäll & Smith, 1983). La PO est capable d'oxyder les monos et diphénols induisant ainsi la formation d'éléments bactéricides (Carballal *et al.*, 1997) et de mélanine insoluble (Söderhäll & Cerenius, 1998) par une voie non enzymatique (Coles & Pipe, 1994). Le système proPO-PO est activé par divers composants de membranes bactériennes comme les β-1,3-glucanes (Asokan *et al.*, 1997), les peptidoglycanes (Cerenius *et al.*, 2008) et les lipopolysaccharides (LPS) (Asokan *et al.*, 1997 ; Hellio *et al.*, 2007) (Annexe 3), suite au clivage de la proPO par une hémoline nommée *Molluscan Defense Precursor* (Terenius *et al.*, 2007). L'hémoline peut servir de médiateur entre les microorganismes et les hémocytes, stimulant ainsi la phagocytose des pathogènes (Bettencourt *et al.*, 1997; Ladendorff & Kanost, 1991).

Le système du complément

Le système du complément regroupe plus d'une trentaine de protéines plasmatiques et cellulaires chez les Mammifères. Bien que la majorité des composants de ce système ait été identifiée chez les poissons, la cascade enzymatique complète n'a pas encore été déterminée pour une espèce donnée (Zarkadis *et al.*, 2001). Cette caractérisation structurale et/ou les similarités fonctionnelles observées entre chaque élément du système du complément des Vertébrés supérieurs et des poissons laissent à penser qu'il existe également, chez ces derniers, trois voies d'activation comparables à celles des Mammifères (Annexe 4) :

- *La voie classique* activée par le complexe antigène-anticorps en présence de calcium et de magnésium.
- *La voie alterne* avec une activation spontanée du composé C3 en présence de magnésium, par notamment des lipopolysaccharides et du zymosan.
- *La voie des lectines* qui nécessite l'interaction de lectines comme la *mannose-binding lectin* (MBL) avec les sucres de la surface des microorganismes étrangers induisant ainsi la formation du complexe *MBL-associated serine proteases* (MASPs). Encore mal connue chez le poisson, des molécules « MAPS-like » viennent d'être clonées chez la carpe suggérant l'existence de cette voie (Nagai *et al.*, 2000).

Ces trois voies conduisent à l'activation du composant C3, élément clef du système du complément qui lui même va activer le C5 initiant ainsi la formation du complexe d'attaque membranaire (MAC) entraînant la lyse des cellules cibles.

Pouvant aussi bien être bénéfique que néfaste pour l'organisme, ce système biologique nécessite, chez les Mammifères, une régulation très structurée. Cette régulation existe probablement également chez le poisson Téléostéen chez qui une première protéine de régulation a été identifiée (Boshra *et al.*, 2006). D'autre part, le système du complément des poissons Téléostéens est adapté au milieu aquatique avec une activation dès les faibles températures, 0,5 °C, une voie alterne 5 à 10 fois plus importante que celle des Mammifères et une production de plusieurs isoformes de certains de ces composants comme le C3 et le facteur Bf (Zarkadis *et al.*, 2001).

Son activation se traduit par de nombreuses conséquences biologiques (Boshra *et al.*, 2006; Holland & Lambris, 2002) :

- Neutralisation virale, bactérienne et parasitaire par altérations fonctionnelles et structurelles irréversibles des membranes, puis lyse des cellules présentant sur leur membrane les composants activés du complément.
- Activation de certaines fonctions cellulaires spécialisées telles que l'attraction de polynucléaires par l'intervention des C3a et C5a (Annexe 4) qui sont des anaphylatoxines puissantes, la phagocytose par les macrophages, la libération d'enzymes lysosomales par les granulocytes et macrophages ou encore la stimulation des lymphocytes B…

3. IMPACT DES POLLUANTS SUR LES CAPACITES DE DEFENSE DES ORGANISMES AQUATIQUES

La présence dans l'ensemble des écosystèmes aquatiques de polluants, dont les hydrocarbures particulièrement répandus (Chapitre I), n'est pas sans conséquence pour la flore et la faune qui les constituent. Ils sont retrouvés à différents niveaux (surface colonne d'eau, sédiments…) sous différentes formes, (intacts ou biotransformés, solubles, particulaires…) et sont pour la plupart susceptibles de contaminer les organismes qui y vivent. L'exposition d'un organisme à un contaminant chimique dépend de plusieurs facteurs

exogènes ou endogènes à l'espèce. Les premiers correspondent aux caractéristiques liées au polluant (solubilité des composés, origine de la contamination), au sédiment (adsorption plus ou moins forte des composés sur les particules, taux de carbone organique des particules, présence de colloïdes dans la colonne d'eau) et à l'hydrologie de la zone (courantologie, cartographie). Quant aux seconds, les facteurs endogènes, ils se rapportent aux caractéristiques liées à l'espèce considérée (mode d'alimentation et de vie, état physiologique) (Baumard *et al.*, 1998; Meador *et al.*, 1995; Swartz *et al.*, 1990, Budzinski *et al.*, 2004). Les polluants, notamment les hydrocarbures, sont des composés inertes non toxiques en tant que tels, mais qui peuvent le devenir après avoir subi une étape d'oxydation ou de biotransformation. Ces polluants biodisponibles peuvent alors être bioaccumulés en fonction de l'espèce considérée, de son état physiologique (alimentation, reproduction, métabolisme) (Baumard *et al.*, 1999) et aussi de son âge (Varanasi *et al.*, 1987).

Les perturbations structurales et fonctionnelles engendrées par les polluants et leurs métabolites se traduisent par des modifications des performances physiologiques et comportementales. L'impact au niveau des populations à court et à long terme est recherché grâce à des études visant à observer d'éventuelles altérations dans la survie (Lowe & Pipe, 1987), la croissance (Lindén *et al.*, 1979), la reproduction (Lowe & Pipe, 1986) et les paramètres physiologiques comme la sensibilité aux maladies (Carlson *et al.*, 2002). La quantification chimique des polluants bioaccumulés (D'Adamo *et al.*, 1997) constitue également un bon indicateur de pollution. L'ensemble des données ainsi recueillies reflète à la fois la qualité de l'environnement aquatique et l'état de santé des animaux présents qu'ils soient sauvages ou d'aquaculture et qui pour beaucoup sont, *in fine,* destinés à la consommation humaine. Cette connaissance est donc primordiale et une surveillance permanente du milieu aquatique a été mise en place grâce à la constitution de réseaux tel que le Réseau National d'Observation de la qualité du milieu marin (RNO) en France. Ce réseau, créé en 1974 par le Ministère chargé de l'Environnement et coordonné par l'Ifremer, a pour objectif d'évaluer les niveaux et les tendances des concentrations en contaminants chimiques et des paramètres généraux de la qualité du milieu, ainsi que de surveiller les effets biologiques de ces contaminants (http://www.ifremer.fr/lerlr/surveillance/rno.htm). Ainsi des analyses *in situ* sont réalisées, elles donnent des informations précises sur l'écosystème et la population avec cependant un fort impact non contrôlé de tous les facteurs

physico-chimiques. C'est pourquoi des expériences *in vivo* sont également effectuées. Menées en laboratoire, elles transmettent des informations sur l'individu et ses fonctions physiologiques grâce à des contaminations bien définies et dans des conditions maîtrisées. Enfin, les analyses *in vitro* renseignent sur les mécanismes d'actions des polluants au niveau des paramètres moléculaires et cellulaires en conditions expérimentales strictement contrôlées et identiques. Parmi les impacts ainsi recherchés, le système immunitaire, qui est particulièrement vulnérable face aux facteurs environnementaux (Wong *et al.*, 1992), représente une cible privilégiée de l'action de ces contraintes (Fournier *et al.*, 2000). De ce fait, l'immunotoxicité a rapidement été utilisée pour la mise en place de diagnostics de risque lors d'un apport dans le milieu de substances chimiques potentiellement toxiques. Cette altération du système immunitaire se détecte soit par une diminution de sa performance, qui est perçue comme un effet néfaste pour l'individu, soit par une augmentation de ses paramètres, qui peut également être interprétée comme un déséquilibre du fonctionnement interne de l'organisme.

3.1. Chez les Mollusques

Les effets des hydrocarbures sont largement recherchés chez les Invertébrés et plus particulièrement chez les Mollusques Bivalves. Qu'ils soient sauvages ou d'aquaculture, ces animaux sont considérés comme des espèces sentinelles du fait de leur présence massive dans les zones côtières particulièrement soumises aux pollutions de tout type, de leur mode de vie benthique et de leur mode de nutrition par filtration. Ces particularités font que les coquillages bioaccumulent dans leur chair de grande quantité de polluants même faiblement concentrés dans le milieu. Une bioaccumulation différentielle est présente chez les Invertébrés en fonction notamment des conditions d'exposition. En effet, Neff *et al.* (1976) observent une bioaccumulation préférentielle de naphtalène, fluorène et phénanthrène suite à une exposition à la fraction soluble d'un pétrole chez l'huître américaine, *Crassostrea virginica*. A l'inverse, chez la palourde, *Mercenaria mercenaria*, suite à une contamination par de la matière en suspension, des HAPs plus lourds sont retrouvés dans les tissus alors que les HAPs plus légers comme le phénanthrène sont bien moins représentés (Boehm & Quinn, 1976). Cette présence de polluants dans l'organisme peut affecter entre autre différents systèmes physiologiques, reproductifs ou encore immunologiques.

Dans ce contexte, l'impact des hydrocarbures sur les activités cellulaires de l'immunité non spécifique a largement été décrit. Suite à une contamination, les caractéristiques morphologiques et la quantité des différentes sous-populations hémocytaires peuvent varier différemment en fonction de l'espèce, du polluant et de l'intérêt écologique considérés (*in situ*, *in vivo* ou *in vitro*). Ainsi, lors d'une contamination au phénanthrène, bien qu'aucune modification ni dans le nombre ni dans la proportion des hémocytes de la coque, *Cerastoderma edule*, de la moule commune et des couteaux, *Ensis siliqua* (Wootton *et al.*, 2003) n'a été détectée *in vivo*, une diminution, chez l'huître creuse, de la mortalité hémocytaire concomitante à une augmentation du pourcentage de granulocytes est observée *in vitro* (Gagnaire *et al.*, 2006). Lors de contaminations par d'autres types d'HAPs, des échantillonnages *in situ* et *in vivo* peuvent notamment montrer une diminution du nombre d'hémocytes circulants (Dyrynda *et al.*, 1997; Fournier *et al.*, 2002; Oliver *et al.*, 2001; Sami *et al.*, 1992) pouvant provenir de la lyse cellulaire, d'une diminution du recrutement ou du départ des cellules du système circulatoire vers les tissus (Pipe & Coles, 1995). A l'inverse, une exposition *in vivo* au fluoranthène provoque une élévation du nombre d'hémocytes chez la moule commune (Coles *et al.*, 1994). Cette augmentation de la quantité totale d'hémocytes circulants pourrait provenir selon Pipe & Coles (1995) soit d'une prolifération cellulaire soit du mouvement des cellules des tissus vers le système circulatoire. Ces modifications observées sur le nombre d'hémocytes circulants suggèrent une modulation possible de l'activité phagocytaire. Une diminution de cette activité hémocytaire est observée notamment suite à des expositions *in vivo* aux hydrocarbures chez la praire (Fournier *et al.*, 2002), la coque (Wootton *et al.*, 2003), l'huître creuse (Jeong & Cho, 2005) et l'huître américaine (Sami *et al.*, 1993). Selon les auteurs différentes hypothèses ont été avancées. Ainsi Sami *et al.* (1993) envisagent des modifications phénotypiques dues à une altération des récepteurs hémocytaires entraînant une incapacité des hémocytes à lier une lectine, la concanavaline A, annihilant de ce fait la reconnaissance du non-soi. Par ailleurs, une déstabilisation membranaire des lysosomes directement reliée à la durée d'exposition (Jeong & Cho, 2005) et à la structure moléculaire du xénobiotique (Grundy *et al.*, 1996b; Moore & Farrar, 1985) est suggérée. Cette déstabilisation membranaire a été initialement signalée par Moore *et al.* (1988), qui observent lors d'une accumulation de lipides neutres insaturés types esters et glycérols, un élargissement important des lysosomes. Cette augmentation lipidique

proviendrait d'une perte de l'autophagie lysosomale liée à la concentration d'hydrocarbures dans les lipides (Moore, 1989). Enfin, Grundy *et al.* (1996b) proposent une rupture membranaire des lysosomes résultant de l'altération des glycoprotéines et protéines lysosomales lors de la fixation des HAPs sur les composés lipophiles des membranes. Cette rupture de la membrane lysosomale entraînerait une modification du pH interne, une destruction des phagocytes (Grundy *et al.*, 1996b; Pipe & Moore, 1986) et l'inhibition du transfert vers l'hémolymphe de certaines enzymes lysosomales telles que les chymotrypsines (Coles *et al.*, 1994).

En revanche, peu d'études ont été consacrées aux activités hémolymphatiques. Coles *et al.* (1994) démontrent cependant qu'une contamination *in vivo* de 7 jours dans une eau contaminée par 400 $\mu g.L^{-1}$ de fluoranthène résulte en une augmentation significative de l'activité de type PO chez la moule commune. De la même façon, des expositions *in vivo* de l'huître creuse au cadmium (Bouilly *et al.*, 2006) et du tunicier, *Styela plicata*, au tributylétain (Tujula *et al.*, 2001) augmentent cette activité phénoloxydasique. Selon Tujula *et al.* (2001), cette modulation résulterait d'un accroissement important du recrutement dans l'hémolymphe de cellules de type morula, connues pour présenter une importante activité PO (Ballarin *et al.*, 1998). Par contre, *in vitro,* l'action directe de ces mêmes polluants induit une diminution de cette activité chez l'huître creuse (Gagnaire *et al.*, 2004) et chez le tunicier (Tujula *et al.*, 2001), qui pourrait refléter l'impact du polluant sur le calcium. En effet, l'exocytose de la proPO par les hémocytes est dépendante à la fois de l'activité Ca^{2+}-ATPase et de l'assemblage des microtubules de tubuline. Or, le tributylétain inhibe la calmoduline, qui est un régulateur du calcium cellulaire, impliquant ainsi une perturbation des systèmes biologiques liés à l'activité Ca^{2+}-ATPase, tels que les mouvements du cytosquelette associés à la tubuline chez une ascidie, *Botryllus schlosseri* (Cima & Ballarin, 2000).

3.2. Chez les Téléostéens

Quel que soit leur mode de vie, les poissons vont pouvoir bioaccumuler les polluants par les branchies et les téguments cutanés et intestinaux et par la nourriture. Néanmoins, les types de produits biodisponibles seront très différents selon qu'ils soient en contact direct avec le sédiment ou non. En effet, les sédiments marins correspondent à un réservoir potentiellement

important de produits chimiques du fait de la capacité des particules à adsorber les polluants organiques. De par leur mode de vie, les poissons benthiques sont donc en contact constant avec les sédiments pollués expliquant les nombreuses études entreprises sur les poissons plats d'origine sauvage comme d'aquaculture tels que la limande, *Limanda limanda* (Hutchinson *et al.*, 2003), le flet, *Pleuronectes flesus* (Beyer *et al.*, 1996), le turbot, *Scophthalamus maximus* (Hutchinson *et al.*, 1999), ou encore la sole commune, *Solea solea* (Budzinski *et al.*, 2004). Ces études ont notamment démontré qu'un organisme en contact direct avec du sédiment contaminé et ne l'ingérant pas, va essentiellement refléter l'interaction entre l'organisme et l'eau interstitielle (Meador *et al.*, 1995). Ainsi, les poissons benthiques, comme le rouget barbet, *Mullus barbatus* (Baumard *et al.*, 1998), vont essentiellement bioaccumuler et biotransformer les composés légers retrouvés dans l'eau interstitielle. Cependant, le contact avec le fond favorise également la bioaccumulation des hydrocarbures hydrophobes à haut poids moléculaire quantifiés dans les sédiments, mais ces xénobiotiques sont peu retrouvés dans les chairs du fait de leur rapide métabolisation. En effet, l'élimination des HAPs de fort poids moléculaire (3-, 4- et 5- cycles) est reliée au métabolisme du poisson (Varanasi *et al.*, 1985) qui va principalement former des métabolites hydrophobes rapidement excrétés par l'organisme (Van der Oost *et al.*, 1994). Il semblerait qu'il n'advient aucune différence notable pour la quantification des polluants dans les tissus des poissons benthiques et pélagiques, car il n'est retrouvé que majoritairement des HAPs de 2- ou 3-cycles benzéniques chez le poisson (Van der Oost *et al.*, 1994; Varanasi *et al.*, 1985). La distribution des contaminants dans la chair des poissons est fonction de phénomènes complexes : la biodisponibilité du polluant (eau, sédiment), le type d'alimentation ingéré et les capacités de biotransformation (Baumard *et al.*, 1998). Malgré le peu d'informations disponibles à l'encontre des poissons pélagiques, la truite arc-en-ciel, *Oncorhynchus mykiss*, est l'espèce la plus testée d'un point de vue réglementaire pour l'homologation d'une nouvelle substance chimique, en accord avec l'Article L253-1 du Code Rural. Des investigations sont donc menées, notamment sur l'état de santé des poissons, afin d'identifier les paramètres d'intérêt pour diagnostiquer les impacts des polluants étudiés. Pour ce faire, des analyses *in situ*, *in vivo* et *in vitro* sont entreprises avec des intérêts écologiques et mécanistiques similaires à ceux des Invertébrés.

Dans ce contexte, ce sont des études portant sur l'activité immunitaire cellulaire non spécifique qui sont essentiellement documentées. Ainsi, lors d'une exposition *in vivo* aux hydrocarbures, les Téléostéens voient leurs nombres de lymphocytes et de cellules phagocytaires circulants diminuer. Chez les poissons plats, comme la limande, les fortes doses en hydrocarbures sont responsables de cette réduction cellulaire (Tahir *et al.*, 1993), alors que chez les poissons pélagiques, comme la truite arc-en-ciel (Hoeger *et al.*, 2004), et le medaka, *Oryzia latipes* (Carlson *et al.*, 2004), ce sont les faibles doses qui l'induisent. Suite à une injection intrapéritonéale de benzo[*a*]pyrène chez le tilapia du Nil, *Oreochromis niloticus*, cette réduction des lymphocytes serait due à la présence de lésions dans les organes lymphocytaires réduisant ainsi la prolifération cellulaire et à une activation de l'apoptose (Holladay *et al.*, 1998). Cette mort cellulaire programmée pourrait être elle-même reliée à une augmentation du calcium extracellulaire et à un mauvais développement et maintien cellulaire des lignées de lymphocytes comme cela a été observé chez des souris exposées *in vitro* au fluoranthène (Yamaguchi *et al.*, 1996). Concernant les monocytes/macrophages, une inhibition de leur différenciation et de leur maturation a été démontrée chez l'Homme à la suite d'une exposition *in vitro* à du benzo[*a*]pyrène (Laupeze *et al.*, 2002). La perte de leur différenciation pourrait être due à une forte altération de la protéine inductrice CD1, à l'inhibition de la protéine kinase C et/ou à une interaction avec les récepteurs aryl hydrocarbure. Le benzo[*a*]pyrène provoquerait également une perte des marqueurs phénotypiques cellulaires, diminuant ainsi la maturation cellulaire (Laupeze *et al.*, 2002). Cette modulation du nombre de leucocytes est capable d'induire des déstabilisations dans l'activité immunitaire. Ainsi, comme pour les Invertébrés, une réduction du nombre de cellules phagocytaires suggère une altération de la phagocytose chez les organismes suite à une exposition aux hydrocarbures. En effet une inhibition de la capacité phagocytaire est constatée au niveau des macrophages circulants, rénaux et spléniques de nombreux poissons après injection intrapéritonéale de 7,12-diméthylbenz[*a*]anthracène (Seeley & Weeks-Perkins, 1997), de benzo[*a*]anthracène ou de benzo[*a*]pyrène (Carlson *et al.*, 2002; Lemaire-Gony *et al.*, 1995; Walczak *et al.*, 1987). En plus de l'absence de marqueurs de surface et d'une réduction du nombre des phagocytes, la perte de la reconnaissance par chimiotactisme lors d'une exposition *in situ* de tambour croca, *Leiostomus xanthurus*, et de la sole, *Trinectes maculatus*, à des cocktails d'hydrocarbures (Weeks *et al.*, 1986) pourrait

également expliquer la diminution de cette activité immunitaire. Néanmoins, cette réduction ne semble pas être systématique. En effet, chez des truites arc-en-ciel exposées à des effluents urbains, Hoeger *et al.* (2004) n'observent aucun effet sur la capacité phagocytaire des leucocytes sanguins même après 27 jours de contamination *in vivo*. De la même façon, Holladay *et al.* (1998) montrent qu'une injection intrapéritonéale de benzo[*a*]pyrène n'a aucune action sur la phagocytose des leucocytes du rein antérieur chez le tilapia du Nil.

La principale activité humorale non spécifique étudiée lors d'une pollution environnementale des eaux est la concentration en lysozyme. Une exposition longue chez la limande par du sédiment contaminé au pétrole (Tahir *et al.*, 1993) ou l'injection intrapéritonéale d'hydrocarbures chez la truite arc-en-ciel (Tahir & Secombes, 1995) provoquent sa diminution. A l'inverse, Jee *et al.* (2004) observent une augmentation significative de l'activité en lysozyme plasmatique et rénale chez le flet, *Paralichthys olivaceus*, suite à une exposition *in vivo* de quatre semaines à du phénanthrène. Les effluents urbains quant à eux ne semblent pas modifier *in vivo* l'activité plasmatique chez la truite arc-en-ciel (Hoeger *et al.*, 2004).

Bien que Kanemitsu *et al.* (1998) constatent une sur-activation de l'activité hémolytique du complément chez l'Homme suite au clivage sérique du composé C3 en C3b par des extraits de diesel l'action des polluants sur cette activité n'a été que très peu étudiée chez le poisson. Seuls Tahir & Secombes (1995) montrent l'absence d'effet, chez la truite arc-en-ciel, à la suite d'une injection intrapéritonéale d'hydrocarbures.

Lors d'une pollution par déversement accidentel d'hydrocarbures, les intervenants engagés dans les opérations de lutte tentent de mettre en place des moyens techniques endommageant le moins possible l'écosystème aquatique. Il apparaît donc essentiel de développer des outils de diagnostic pertinents qui permettent aux opérateurs de définir au mieux le moyen de lutte approprié à la pollution observée et qui fournissent des données en vue de l'établissement de réglementations. De plus, l'utilisation de deux espèces ayant des niches écologiques variables ouvre la discussion vers une intervention plus ciblée. Au cours de ce travail de recherche, des biomarqueurs reconnus, c'est-à-dire des paramètres biologiques susceptibles de refléter l'interaction entre un système biologique et un agent environnemental (Lagadic *et al.*, 1997), et de nouveaux indicateurs immunitaires sont testés afin de définir des séries d'analyses simples et favorisant l'obtention rapide de résultats sur l'état de santé des animaux aquatiques. Malgré une divergence de réponses provenant probablement de la grande diversité de polluants et d'espèces animales testés, ainsi que la variabilité des modes de contamination, il semble donc incontournable de développer la phagocytose, la stabilité membranaire des lysosomes et la concentration en lysozyme. Plus spécifiquement, l'activité PO chez les Mollusques Bivalves et l'activité du complément chez les poissons sont des indicateurs émergeants d'une pollution environnementale des eaux. De ce fait, l'action des hydrocarbures sur ces deux activités enzymatiques est recherchée. De plus, malgré une forte variabilité dans les réponses cellulaires, une modification dans la composition des différentes sous-populations cellulaires du milieu extracellulaire correspond à une modification physiologique largement décrite en tant que stress environnemental. Ainsi, l'étude des différentes sous-populations cellulaires semble être importante à la fois pour comprendre la variation des autres paramètres immunitaires, mais également pour avoir un aperçu de l'état de santé général de l'organisme. Enfin, à cette batterie d'outils immunotoxicologiques s'intéressant à l'immunité non spécifique propre aux deux espèces étudiées grâce au développement de tests fonctionnels, le suivi de l'expression de plusieurs gènes codants pour des effecteurs impliqués dans ces mécanismes est effectué.

CHAPITRE 3
EXPOSITIONS AUX HYDROCARBURES

La serre expérimentale du Cedre
Source : A. Bado-Nilles

Ce travail de recherche, cofinancé par le Conseil régional Poitou-Charentes et le *Cedre* dans le cadre du programme européen INTERREG IIIB – EROCIPS ("Emergency Response to coastal Oil, Chemical and Inert Pollution from Shipping"), a été réalisé en partenariat ou collaboration avec plusieurs laboratoires (Figure 3 - 1) :

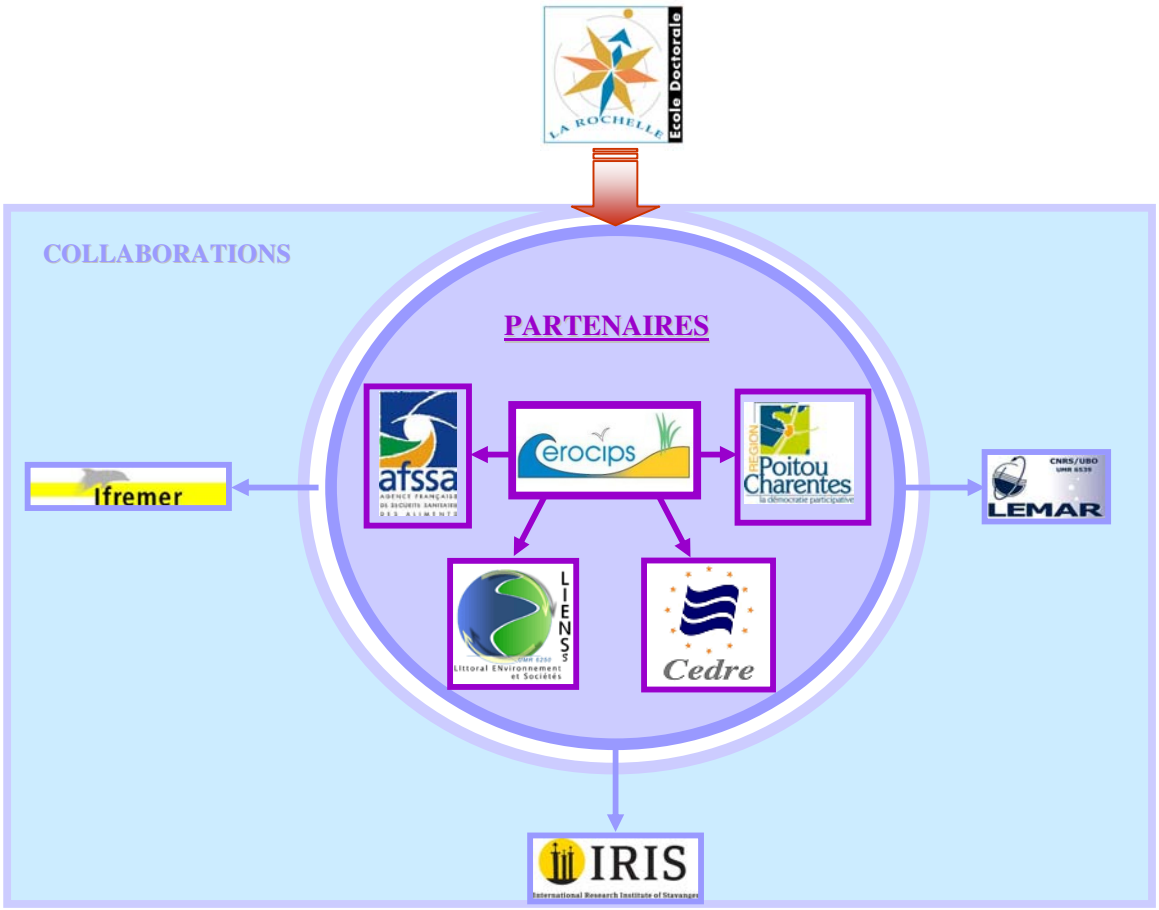


Figure 3 - 1 : Partenaires : Université de La Rochelle - LIENSs UMR CNRS 6250 - Equipe Réponse des Animaux MARins à la variabilité Environnementale ; *Cedre* - Service Recherche & Développement, Brest ; Afssa Ploufragan-Plouzané - Unité de Pathologie Virale des Poissons. Collaborations : IUEM Brest - LEMAR UMR CNRS 6539 - Biologie des interactions et de l'adaptation chez les organismes marins ; Ifremer La Tremblade - Laboratoire de Génétique et Pathologie ; Ifremer Brest - Département de Physiologie Fonctionnelle des Organismes Marins ; IRIS Norvège – Biomiljø. Ce travail de thèse, s'inscrivant dans le programme européen INTERREG IIIB Erocips "Emergency Response to coastal Oil, Chemical and Inert Pollution from Shipping", a été réalisé au sein de l'Ecole Doctorale de l'Université de la Rochelle.

Suite à une évaluation globale de la qualité du milieu par quantification des contaminants biodisponibles (concentration en polluants dissous…), la bioaccumulation dans la chair, la recherche des métabolites biliaires et l'impact des hydrocarbures sur l'activité immunitaire non spécifique de deux espèces marines des Pertuis Charentais sont recherchés. Ce travail de

thèse a pour but de définir l'importance du système immunitaire chez un Invertébré, l'huître creuse, *Crassostrea gigas*, et un Vertébré, le bar commun, *Dicentrarchus labrax*, en tant que cible de polluants de type hydrocarbure. Le second objectif de cette étude est de montrer l'intérêt d'utiliser des indicateurs et des descripteurs de type immunologique dans le cadre du suivi et du contrôle de l'état de santé de l'environnement. Ainsi, deux approches expérimentales seront successivement développées comme présenté en Figure 3 - 2 :

- Partie 1 : Evaluation *in vitro* de l'impact de polluants de type hydrocarbure et choix de descripteurs d'intérêt de l'immunité non spécifique chez les deux espèces étudiées.
- Partie 2 : Expositions *in vivo* des organismes marins de la zone littorale à une pollution occasionnelle :
    - De type fioul lourd (accident de l'*Erika*) ; pollution largement décrite comme ayant eu des effets délétères sur la faune marine.
    - Par le light cycle oil (LCO) ; produit léger issu du raffinage du pétrole et entrant dans la composition du fioul lourd précédemment testé.

Ces deux approches ont pour objectif de mettre en place des outils moléculaires et cellulaires dans la perspective d'utiliser ces marqueurs d'effets (intérêt mécanistique) lors d'une transposition à l'environnement (intérêt écologique) telle qu'une pollution *in situ* en cellules flottantes ou encore en mésocosme.

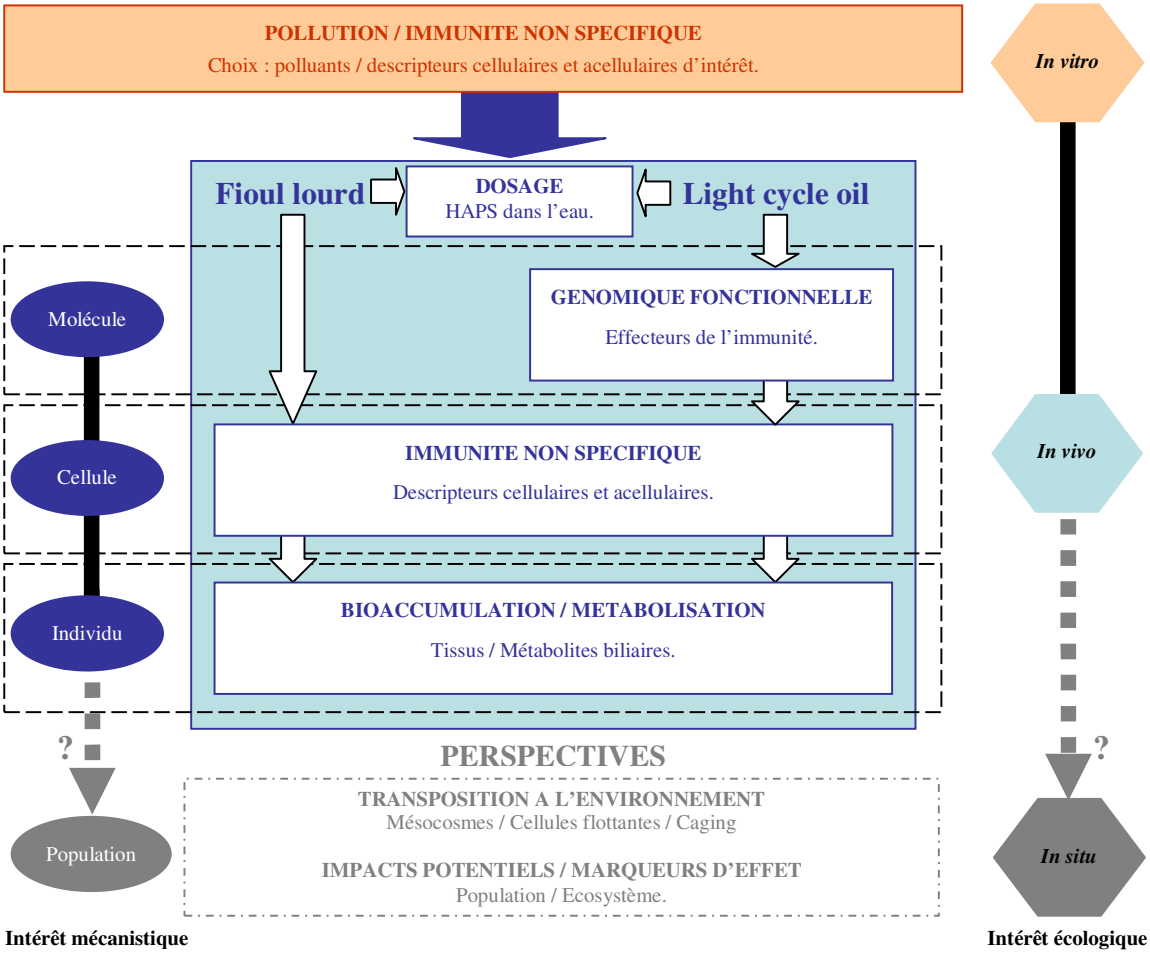
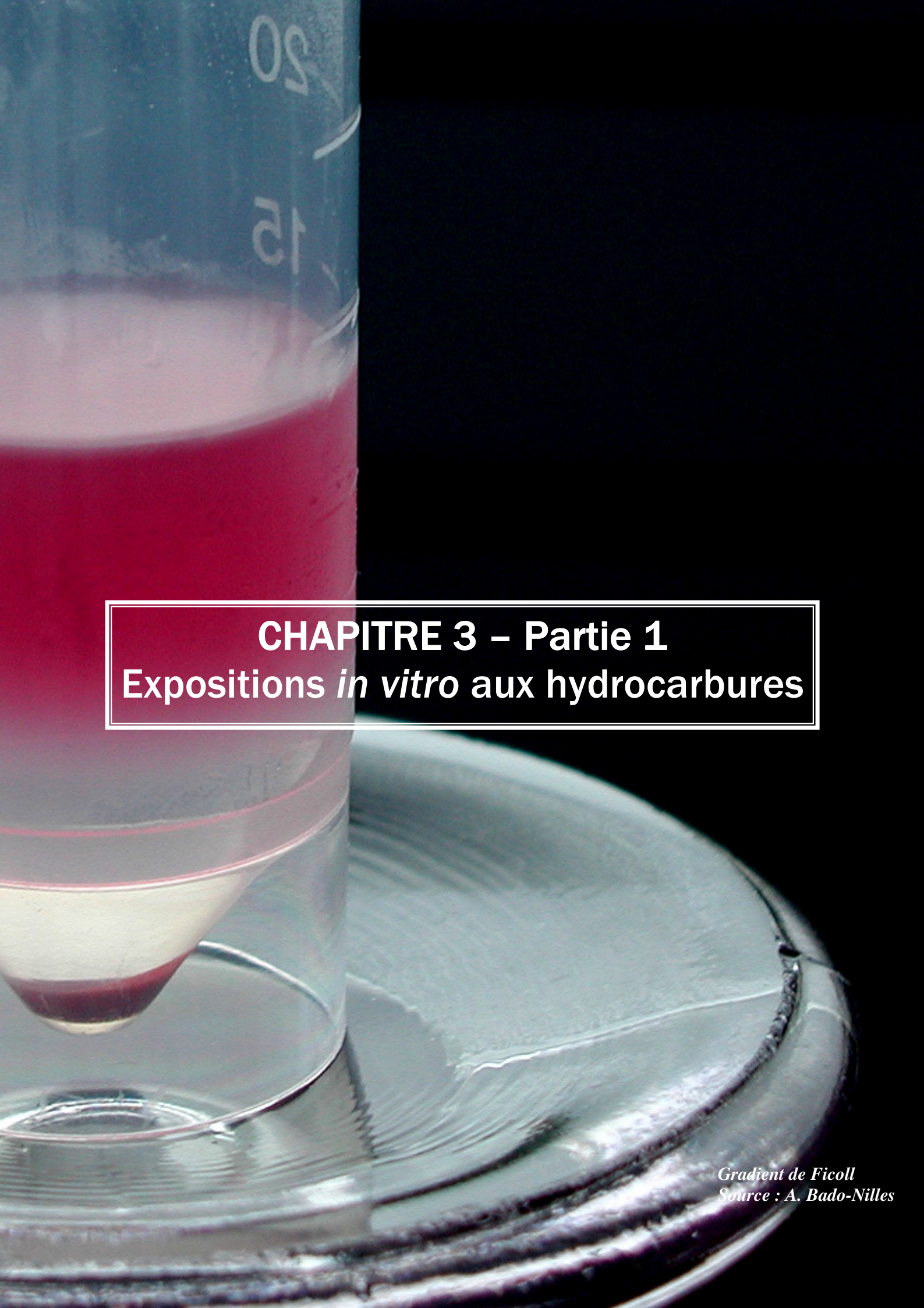


Figure 3 - 2 **:** Approches expérimentales réalisées à différents niveaux d'organisation biologique selon le *continuum* molécule – cellule - individu et portant sur l'étude des perturbations structurales et fonctionnelles engendrées par des variables forçantes (pollution) sur les mécanismes de défense des organismes marins de la zone littorale.

CHAPITRE 3 – Partie 1
Expositions *in vitro* aux hydrocarbures



Gradient de Ficoll
Source : A. Bado-Nilles

Des études récentes ont cherché à comprendre les effets de polluants organiques de type HAPs sur le système immunitaire des organismes vivants (Grundy *et al.*, 1996a; Kennedy & Farrell, 2008). Leur impact sur les activités immunitaires est issu soit d'un effet direct induit par l'action du xénobiotique sur les composants du système de défense (Kanemitsu *et al.*, 1998), soit d'un effet indirect lié à des modifications d'ordre physiologiques (Skouras *et al.*, 2003). Ainsi, des essais *in vitro* sont couramment entrepris pour vérifier si les effets observés sont identiques à ceux obtenus *in vivo* (Willett *et al.*, 2001). Cette approche expérimentale permet aussi de donner une première indication quant à l'action directe d'un composé toxique (Bai *et al.*, 2001; Behnisch *et al.*, 2003; Cavret & Feidt, 2005) et favorise l'évaluation et la comparaison de l'effet des xénobiotiques (Gagnaire *et al.*, 2006). Ainsi, ces derniers examinent l'effet de différents HAPs sur les capacités de défense de l'huître creuse suite à une exposition *in vitro* des hémocytes et constatent une similarité d'impact sur la présence en lysosome et le pourcentage de cellules estérases non-spécifiques.

La première partie de ce travail s'attache donc à étudier le potentiel immunotoxique des produits pétroliers, lors d'une pollution chronique et occasionnelle, par le biais d'une approche purement méthodologique. Au cours de ces expérimentations *in vitro*, l'action de trois produits pétroliers issus du raffinage (fioul lourd, gasoil et light cycle oil) sur les activités cellulaires et acellulaire de l'huître et le plasma du bar est déterminée. De plus, les 16 HAPs US-EPA contenus dans ces produits pétroliers sont retenus sur la base de leur immunotoxicité reconnue (Grundy *et al.*, 1996a; Reynaud & Deschaux, 2006) et sont testés seuls. Les concentrations utilisées sont établies à partir des concentrations chroniques mesurées dans le Bassin de Marennes-Oléron (Charente-Maritime, France) (Article 1, p.41) et celles trouvées *in situ* suite à une pollution occasionnelle (Boehm *et al.*, 2007 ; Law, 1978). Le choix des descripteurs immunologiques à tester, au sujet de l'huître creuse, se porte sur une large gamme d'activités immunitaires non spécifiques cellulaires (mortalité hémocytaire, phagocytose, pourcentage de cellules à activité estérase non-spécifique et présence de lysosome) et acellulaires (activité PO) (Article 1, p.41). En revanche, pour le bar commun, seules les activités acellulaires (activité du complément et lysozyme) sont étudiées du fait de la difficulté à maintenir fonctionnels, pendant 24h, des leucocytes après extraction

sur gradient de Ficoll (Article 2, p.61).

Sur les 16 HAPs testés séparément, seuls le fluoranthène et le benzo[*g,h,i*]périlène n'entraînent aucune réponse au niveau de l'hémolymphe ou du plasma chez les deux espèces étudiées. De plus, chacune des activités cellulaires et acellulaires considérées est immunomodulée par au moins l'une des 14 molécules restantes.

Concernant le fioul lourd, quelle que soit l'espèce analysée, aucun effet n'est observé sur les activités acellulaires. Par contre, il agit sur les paramètres cellulaires de l'huître creuse, notamment en induisant une diminution significative de la mortalité cellulaire et de la phagocytose.

Pour les produits légers issus du raffinage, le gasoil ne semble pas moduler l'activité immunitaire chez l'huître alors que le LCO induit chez le bar une augmentation de l'activité hémolytique de la voie alterne du complément (ACH_{50}) (Figure 3 - 3).

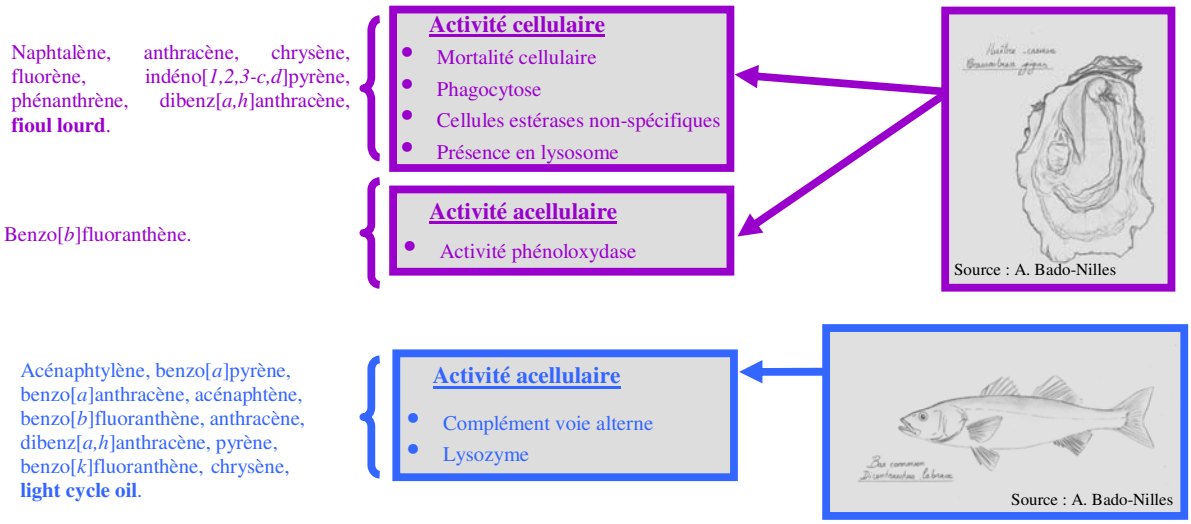


Figure 3 - 3 : Effets *in vitro* des 16 HAPS US-EPA et de trois produits pétroliers sur des descripteurs cellulaires et acellulaires chez l'huître creuse et le bar commun.

Cette première partie avait pour objectifs de :

1. Tester le potentiel immunotoxique chez l'huître creuse et le bar commun de trois produits pétroliers différents (fioul lourd, gasoil et LCO) et de 16 HAPs entrant dans leur composition.
2. Valider le choix des descripteurs immunologiques et des polluants pour réaliser l'approche expérimentale *in vivo*.

Chacun des éléments étudiés du système immunitaire sont impactés par au moins l'un des produits testés, à l'exception du gasoil, du fluoranthène et du benzo[*g,h,i*]périylène. Cependant, ces expériences *in vitro* ne peuvent pas reproduire l'ensemble des évènements susceptibles d'intervenir *in vivo* du fait de l'absence des conditions physiologiques générales de l'organisme. Ainsi, des expérimentations sur le vivant seront entreprises (Chapitre 3 - Partie 2). Du fait de leur action avérée *in vitro*, seuls deux produits pétroliers issus du raffinage, le fioul lourd et le LCO, seront étudiés *in vivo*. Par contre, leur éventuel impact sera recherché sur l'ensemble des descripteurs immunologiques décrits dans cette première partie.

Cette seconde partie permettra donc de caractériser l'effet d'une pollution occasionnelle au fioul lourd et au LCO sur les capacités de défense des organismes étudiés. Les résultats acquis rendront possible l'identificationr de descripteurs immunologiques susceptibles d'être utilisés lors d'une évaluation d'un risque environnemental.

ARTICLE 1: Effects of 16 pure hydrocarbons and two oils on haemocyte and haemolymphatic parameters in the Pacific oyster, *Crassostrea gigas* (Thunberg).

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Authors

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Abstract

The *in vitro* effects of polycyclic aromatic hydrocarbons (PAHs) on haemocyte and haemolymphatic parameters of the Pacific oyster, *Crassostrea gigas*, were tested using field concentrations (10^{-7} and 10^{-9} mg.mL^{-1}) observed in the Marennes-Oleron Basin (Charente-Maritime, France) and high concentrations (10^{-3} and 10^{-5} mg.mL^{-1}) observed during oil spills. As a first step, the effects of pollutants, after a 24 hour contamination period, were monitored on individual and pooled haemolymph samples and similar results were observed for both sample types. In a second step, haemolymphs from 45 oysters were withdrawn and pooled. Eighteen pollutants were tested *in vitro* after a 4 and 24 hour contamination period and ten of them showed significant modulations of the five haemocyte parameters tested. Seven pollutants (fluorene, pyrene, anthracene, phenanthrene, chrysene, indeno[*1,2,3-c,d*]pyrene and heavy fuel oil (HFO)) induced a decrease in haemocyte mortality. Fluorene, pyrene and HFO significantly decreased phagocytosis activity. Percentage of non-specific esterase positive cells, phenoloxidase activity and lysosome presence were increased by naphthalene, benzo[*b*]fluoranthene and dibenz[*a,h*]anthracene, respectively. Modulation of immune parameters in the Pacific oyster by PAHs suggested that PAH pollution may be related to enhanced susceptibility to diseases.

Keywords: Haemocyte activity; Flow cytometry; Spectrophotometry; Pacific oyster; PAHs; Toxicity.

1. Introduction

Among various chemical contaminants, the pollution caused by polycyclic aromatic hydrocarbons (PAHs) has led over recent years to numerous studies on the origin, distribution and fate of PAHs in the environment. Two main sources are identified: petrochemical (natural seepages, discharges of urban and industrial effluents, offshore oil production and oil spill) and pyrogenical (combustion processes due to human or natural fate). Concerning oil spill inputs, many authors have measured the concentrations of PAHs in the sediment and their effects on aquatic organisms before and after the event (Franco et al., 2006; Law, 1978; Morales-Caselles et al., 2006). These types of concerns were also studied in the case of chronic pollution (Budzinski et al., 1997; Halldorsson et al., 2004; Stehr et al., 2004; Van der Oost et al., 1991). Although many studies deal with PAH contamination of freshwater (Acheson et al., 1976; Tronczynski et al., 2004; WHO, 1997), only few authors have measured concentrations of the dissolved fraction of hydrocarbons in seawater (Boehm et al., 2007; Law, 1978; Maldonado et al., 1999). PAH concentrations following different oil spills ranged from 6.10^{-4} mg.mL^{-1} after the *Exxon Valdez* oil spill (Boehm et al., 2007) to 17.10^{-4} mg.mL^{-1} after the *Ekofisk* blowout (Law, 1978).

Anthropogenic contaminants, including PAHs, may affect the immunity of aquatic vertebrates (Reynaud et al., 2004; Yamaguchi et al., 1996) and invertebrates (Gagnaire et al., 2006a; Grundy et al., 1996a). Innate immunity in bivalve molluscs relies on haemocytes, the circulating cells present in extrapallial fluids, and haemolymph (Cheng, 1981; Johansson et al., 2000). Haemolymph contains also soluble effectors including phenoloxidase (PO) and antimicrobial peptides. Defence mechanisms, which include wound repair, coagulation, nodule formation, encapsulation, phagocytosis and cytotoxicity (Cheng, 1981), could be impaired by hydrocarbons (Coles et al., 1994; McVeigh et al., 2006; Wootton et al., 2003). Some authors described phagocytosis and other haemocyte parameters including non-specific esterase activity and lysosomal presence, as suitable biomarkers of pollution in different bivalve species (Auffret, 2005; Gagnaire et al., 2006a). PO was reported as been modified by xenobiotics in the Pacific oyster, *C. gigas* (Bouilly et al., 2006; Gagnaire et al., 2004).

Field concentrations of different PAHs were first determined in the Marennes-Oleron Basin (Charente-Maritime, France), where oyster production is an important economic

activity. *In vitro* effects of selected hydrocarbons at high and field concentrations were then monitored on haemocytes of Pacific oysters, *C. gigas*. Sixteen PAHs were selected from the United States Environmental Protection Agency (US-EPA) list and two oils (heavy fuel oil (HFO) and diesel oil) were also tested. Cell mortality, phagocytosis, percentage of non-specific esterase positive cells and lysosome presence were monitored using flow cytometry. Phenoloxidase (PO) activity was studied in the acellular fraction by spectrophotometry.

2. Material and methods

2.1. Concentrations of PAHs in seawater samples

Three samples were collected in October 2005 from each site (Fig. 1). The harbour area corresponded to an oyster-farming harbour. One litre of subsurface seawater was collected at a depth of 3 m with polyethylene Nyskin's bottles and directly transferred to Duran glass bottles (Bioblock) which were heated to 500 °C before use. Samples were kept at 4 °C in the dark until analysis. Analyses of the aromatic compounds were carried out two days later.

Samples were extracted with 30 mL of dichloromethane pestipur quality (SDS). After separation of the organic and aqueous phases, water was extracted two additional times by the same volume of dichloromethane (2 X 30 mL). The organic extracts were purified and treated using gas chromatography coupled with mass spectrometry (GC-MS, Hewlett Packard HP5890 coupled with an HP5972 mass selective detector) following procedures previously described (Douglas et al., 1992).

2.2. Oysters

Two hundred Pacific oysters, *Crassostrea gigas*, 8-10 cm in shell length, were purchased from a shellfish farm (La Tremblade, Charente-Maritimes, France) in October 2005. Oysters were maintained, at Ifremer's laboratory (La Tremblade, France), for one month in tanks receiving a constant flow of external seawater (temperature 15.3-16 °C, pH 7.6, salinity 33.9-34.5 ‰, free of nitrate and nitrite).

Samples N°	Type of localisation	Latitudes Longitudes
1	Open sea	46° 05' 09.53" N 1° 16' 00.53" O
2	Open sea	46° 03' 00.10" N 1° 13' 09.80" O
3	Open sea	46° 03' 09.90" N 1° 14' 00.20" O
4	Coastal	46° 00' 09.70" N 1° 16' 00.53" O
5	Coastal	45° 00' 07.47" N 1° 09' 02.64" O
6	Coastal	45° 58' 00.00" N 1° 06' 06.50" O
7	Open sea	45° 56' 04.80" N 1° 08' 04.75" O
8	Oyster-farming	45° 52' 04.03" N 1° 10' 00.60" O
9	Oyster-farming	45° 51' 02.30" N 1° 10' 06.67" O
10	Harbour	45° 46' 09.17" N 1° 07' 02.78" O

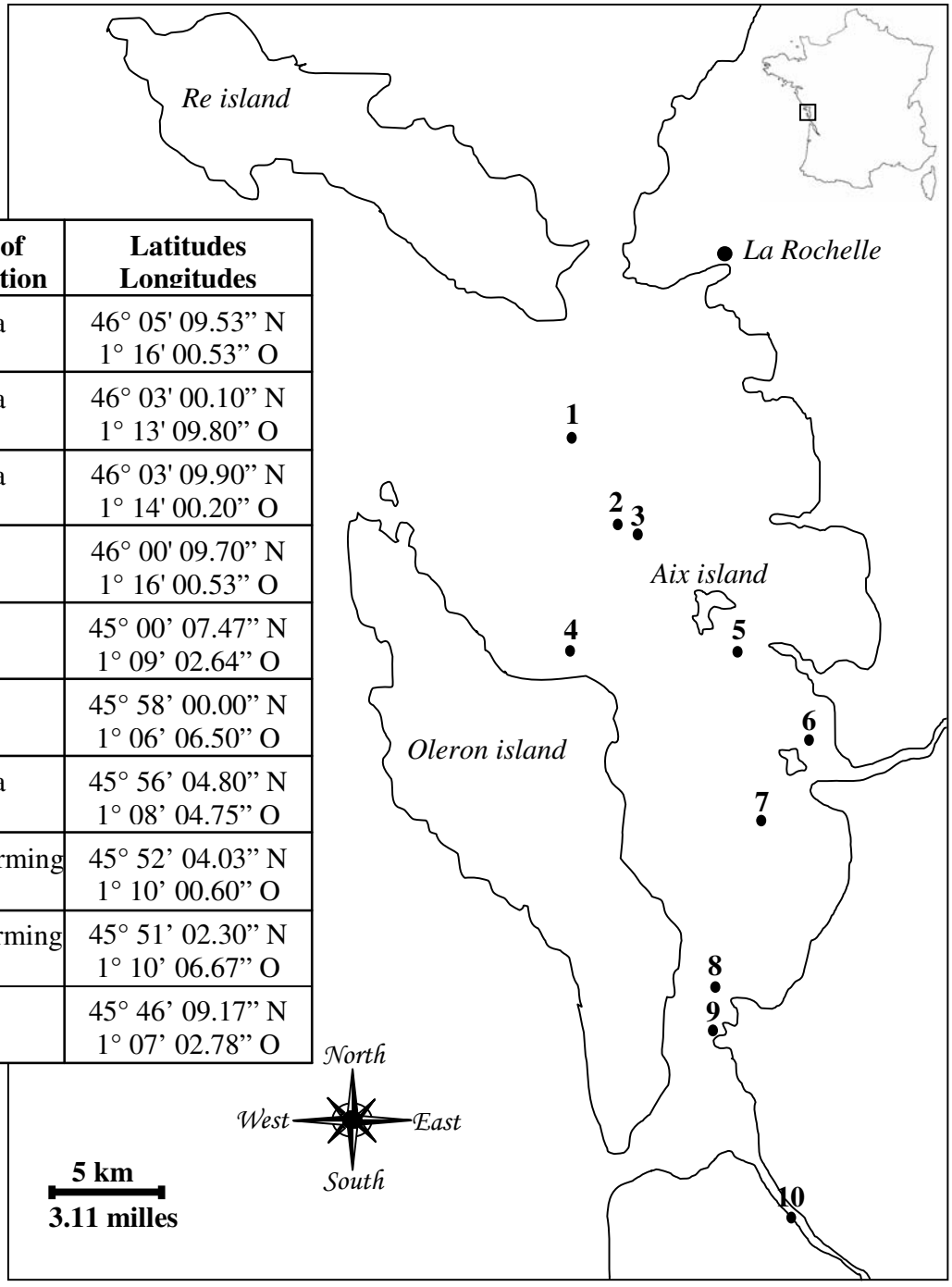

Fig. 1: Locations of the sampling sites in the Bay of Marennes-Oleron, background card issue from the National Geographic Institute.

Name of 16 PAHs US-EPA	Molecular weight ($g.mol^{-1}$)	Concentration ($\mu g.g^{-1} \pm SE$) in HFO	Concentration ($\mu g.g^{-1} \pm SE$) in diesel oil
Naphthalene	128,2	686.5 ± 51.5	1244.2 ± 49.9
Acenaphthylene	152,2	51.1 ± 1.8	72.4 ± 3.5
Acenaphthene	154,2	272.5 ± 8.4	90.7 ± 3.6
Fluorene	166,2	396.4 ± 11.5	187.2 ± 8.9
Phenanthrene	178,2	1936.9 ± 67.3	186.3 ± 15.2
Anthracene	178,2	213.6 ± 16.6	13.7 ± 0.8
Fluoranthene	202,3	125.2 ± 8.6	17.9 ± 1.1
Pyrene	202,3	516.2 ± 33.1	56.9 ± 3.5
Benz[*a*]anthracene	228,3	213.4 ± 8.0	1.4 ± 0.1
Chrysene	228,3	464.9 ± 8.2	6.5 ± 0.5
Benzo[*b+k*]fluoranthene	252,3	81.4 ± 6.8	n.d.
Benzo[*a*]pyrene	252,3	167.9 ± 4.5	n.d.
Benzo[*g,h,i*]perylene	276,3	48.1 ± 2.1	n.d.
Indeno [*1,2,3-c,d*] pyrene	276,3	16.6 ± 2.0	n.d.
Dibenz[*a,h*]anthracene	278,4	27.6 ± 3.3	n.d.

Table 1: Concentration of 16 priority PAHs of the US-EPA list in heavy fuel oil (HFO) and diesel oil. PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in $\mu g.g^{-1}$ (n = 3, mean ± standard error; n.d. = not detected).

2.3. Haemolymph collection

After breaching the oyster shell using pincers, approximately 2 mL of haemolymph were withdrawn from the posterior adductor muscle sinus using a 2 mL syringe equipped with a needle (0.6 x 25 mm). For each oyster, the haemolymph was filtered through a 60 µm mesh to eliminate debris and kept on ice until it was processed to reduce haemocyte aggregation (Auffret and Oubella, 1997).

For pre-experiments, which compared individual and pooled haemolymphs, 150 µL of ten haemolymph samples were pooled together. The remaining ten haemolymphs were kept for individual analysis. All samples were adjusted at 10^6 cells.mL^{-1} with artificial seawater (for 1 L: 234 g NaCl, 15 g KCl, 12 g $MgSO_4$ $4H_2O$, 1.5 g $CaCl_2$ $2H_2O$, 1.5 g $CaCl_2$ anhydrous; used at 1/10 dilution; all products come from Sigma) (Gagnaire et al., 2006a).

For the *in vitro* exposure, the haemolymph of 45 oysters was pooled, kept on ice to prevent haemocyte aggregation (Auffret and Oubella, 1997) and adjusted at 10^6 cells.mL^{-1} with artificial seawater (Gagnaire et al., 2006a).

Three pool samples were making for pre-experiments and for the *in vitro* exposure.

2.4. Experimental protocol

2.4.1. In vitro exposure protocol

For pre-experiments the difference between pool and individual contaminated samples was researched. Based on previously published results (Gagnaire et al., 2006a), two xenobiotics were selected. The effects of fluorene and pyrene were tested on cell mortality, phagocytosis percentage, percentage of non-specific esterase positive cells and lysosome presence. Both PAH were tested at 10^{-9} mg mL^{-1} at 15 °C during 24 h.

For *in vitro* exposure experiments, impact of chemicals on haemocyte parameters was monitored using 18 xenobiotics selected for their immunotoxic potential: 16 PAHs of the United States Environmental Protection Agency list (US-EPA) (WHO, 1997), and two oils (heavy fuel oil (HFO) and diesel oil). Concentrations of PAHs were determined in heavy fuel oil (HFO) and diesel oil (Table 1). High (10^{-3} and 10^{-5} mg.mL^{-1}) and field (10^{-7} and 10^{-9} mg.mL^{-1}) concentrations were tested for each PAH. The two oils were tested in their pure form and diluted to 1/1 000 and 1/10 000 in artificial seawater after a three-day contact period without mixing at ambient temperature (20°C) as previously described (Anderson et al., 1974).

Pollutants (PAHs and oils) were added individually at 5 µL per mL of haemocyte suspension. Before addition, PAHs were dissolved in cyclohexane (Sigma) and the ratio cyclohexane:haemocyte suspension did not exceed 0.5 % as recommended by manufacturers in order to avoid disturbance of cell parameters. In all experiments, the same volume of cyclohexane was used as solvent control and haemolymph alone was used as cell control. Samples were incubated at 15°C during 4 and 24 h as previously described by Gagnaire et al. (2006a). Experiments including controls were carried out three times.

2.4.2. Cell analysis by flow cytometry

Haemocyte mortality, phagocytosis percentage, percentage of non-specific esterase positive cells and lysosome presence were analysed with an EPICS XL 4 (Beckman Coulter). For each haemocyte sample, 10 000 events were counted using protocols previously described (Gagnaire et al., 2006a). Analyses were carried out on whole haemocytes without distinguishing cell subpopulation and results were expressed as percentage of positive cells.

Cell mortality was measured using FL3 (red fluorescence). Propidium iodide (PI, 1.0 $g.L^{-1}$, Molecular Probes) is membrane impermeant and is excluded from viable cells. Mortality was determined using 200 µL of haemocyte suspension and 10 µL of PI. Cell suspensions were incubated for 30 minutes at 4°C.

The phagocytosis percentage was determined using FL1 (green fluorescence). Fluorescent microspheres ($2.7x10^{10}$ $particles.mL^{-1}$, Fluorospheres® carboxylate-modified microspheres, diameter 1 µm, Molecular Probes) were used and the fluorescence setting was established using a suspension of fluorescent beads in distilled water. Only the events showing a fluorescence of at least three beads were considered positive for phagocytic activity. Phagocytic activity of haemocyte suspensions was analysed on 200 µL of haemolymph samples and 10 µL of a 1/10 dilution of fluorescent beads. Cell suspensions were incubated for one hour at room temperature.

Percentage of non-specific esterase positive cells was analysed using the non-specific liposoluble substrate fluoresceine diacetate (FDA, Molecular Probes). One µL of a FDA solution (400 µM) was added to 200 µL of haemocyte suspension. Cells were incubated for 30 minutes in the dark at room temperature and then the reaction was stopped on ice (5 min).

Lysosome presence was analysed with a commercial kit (LysoTracker ® Green DND-26, 1mM in DMSO, Molecular Probes) which consists of a fluorophore linked to a weak base that is partially protonated at neutral pH. The LysoTracker® is freely permeant to cell membranes and typically concentrated in lysosomes. One µL of a LysoTracker marker was added to 200 µL of haemocyte suspension. Samples were incubated for two hours in the dark at room temperature and then the reaction was stopped on ice (5 min).

2.4.3. PO activity analysis

Haemolymph samples were centrifuged (260 g, 10 min, 4°C) and supernatants recovered. Detection of phenoloxidase (PO) activity in acellular fraction samples was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-Dopa, Sigma) transformation in dopachromes as previously described by Gagnaire et al. (2004). Samples were distributed in 96-well microplates (Nunc, France). PO modulators were used to confirm the specificity of the detection. The purified trypsin TPCK (*N*-Tosyl-L-phenylalanine chloromethyl ketone, 1 $g.L^{-1}$, Sigma) was used as an activator and the β-2-mercaptoethanol (10 mM, Sigma) was used as an inhibitor. To determine the PO activity, 80 µL of cacodylate buffer (CAC buffer: sodium cacodylate (10 mM), trisodium citrate (100 mM), NaCl (0.45 M), $CaCl_2$ (10 mM), $MgCl_2$ (26 mM), pH 7.0), 20 µL of L-Dopa (3 $mg.mL^{-1}$) and 20 µL of samples were added in each well. To measure the PO activity modulation, 60 µL of CAC buffer, 20 µL of PO modulators, 20 µL of L-Dopa and 20 µL of samples were added to each well. Control (120 µL of CAC buffer) and negative control (100 µL of CAC buffer, 20 µL of L-Dopa) wells were used to determine respectively the purity of the buffer and the autooxidation capacities of L-Dopa. Each sample was tested in nine replicates and absorbance was measured at 490 nm after a 21 h incubation period at room temperature.

2.5. Statistical analysis

Statistical tests were carried out using XLStat Pro 7.5.3. A Kruskal-Wallis test, for non normal values, was used to analyse pollutant effects and a Mann-Whitney test was applied to compare the protocol effects. In the case of the rejection of H_0, a Dunn test for non normal values was used. P values lower than 0.05 were used to identify significant differences.

3. Results

3.1. PAH concentrations in the Marennes-Oleron Basin (Charente-Maritime, France)

The concentrations of 16 PAHs in seawater samples are given in Table 2. The values ranged from 0 to 53.8 $ng.L^{-1}$ with a total concentration of 70.5 ± 10.8 $ng.L^{-1}$.

Indeno[*1,2,3-c,d*]pyrene, benzo[*b*]fluoranthene and benzo[*g,h,i*]perylene were not detected at all sampling sites. Acenaphthene, chrysene, fluoranthene, fluorene, naphthalene and phenanthrene were detected at all sampling sites. Three types of sampling sites, "open sea", "coastal and harbour" and "oyster-farming", were differentiated in terms of total amounts of seawater PAH concentrations. The "open sea" sites showed the lowest concentrations with values ranging from 12.3 ± 1.4 ng.L^{-1} for naphthalene to 0.1 ng.L^{-1} for chrysene and with a total concentration of 39.0 ± 3.7 ng.L^{-1}.Twelve PAHs among the 16 analysed were detected. The "coastal and harbour" sites showed concentrations ranging from 33.8 ± 3.4 ng.L^{-1} for acenaphthene to 0.8 ± 0.5 ng.L^{-1} for anthracene and with a total concentration of 75.9 ± 3.3 ng.L^{-1}.Eight PAHs among the 16 analysed were detected. The highest total concentration (123.1 ± 18.1 ng.L^{-1}) was observed in the "oyster-farming" sites with values ranging from 48.7 ± 5.2 ng.L^{-1} for naphthalene to 0.2 ± 0.2 ng.L^{-1} for acenaphthylene. Nine PAHs among the 16 analysed were detected.

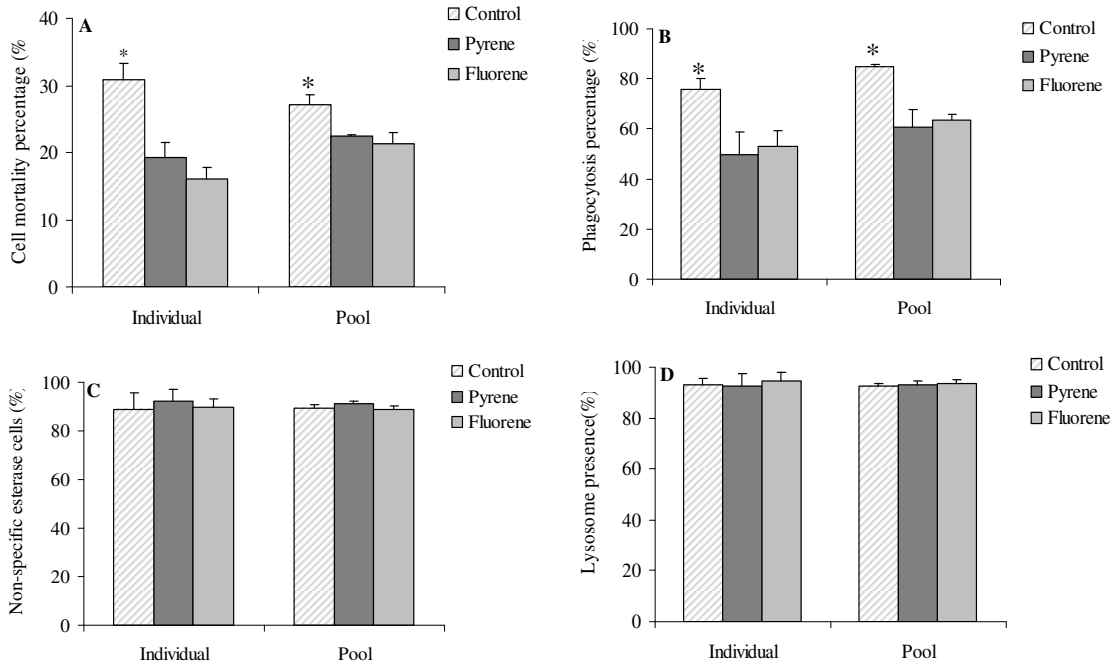

Fig. 2: Cellular mortality percentage (A), phagocytic percentage (B), percentage of non-specific positive esterase cells (C) and lysosome presence (D) of haemocytes measured by flow cytometry following an *in vitro* exposure of 24 h at 15°C to pyrene, fluorene at 10^{-9} mg.mL^{-1} for both samples (individual and pooled samples). Values are the mean of three replicates. The bars represent the standard error. * = statistical difference for $p \leq 0.05$.

Samples N° / 16 PAHs US-EPA	Open sea					Coastal and harbour					Oyster-farming			Mean (± SE)
	1	**2**	**3**	**7**	***Mean***	**4**	**5**	**6**	**10**	***Mean***	**8**	**9**	***Mean***	
Naphthalene	15.0 ± 0.6	14.4 ± 0.9	9.2 ± 0.6	10.4 ± 0.3	***12.3 ± 1.4***	25.8 ± 0.2	25.8 ± 0.4	25.7 ± 0.5	18.5 ± 0.5	***24.0 ± 1.8***	53.8 ± 0.2	43.5 ± 0.5	***48.7 ± 5.2***	**25.2 ± 4.6**
Acenaphthylene	2.6 ± 0.1	2.6 ± 0.0	2.4 ± 0.0	2.7 ± 0.1	***2.6 ± 0.1***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	0.4 ± 0.0	***0.2 ± 0.2***	**0.9 ± 0.4**
Acenaphthene	8.0 ± 0.1	8.0 ± 0.0	8.2 ± 0.2	8.1 ± 0.1	***8.1 ± 0.0***	33.6 ± 0.5	29.8 ± 0.3	28.3 ± 0.2	43.4 ± 0.5	***33.8 ± 3.4***	43.5 ± 0.3	33.6 ± 0.2	***38.6 ± 5.0***	**26.3 ± 4.7**
Fluorene	3.4 ± 0.1	4.9 ± 0.1	3.3 ± 0.1	4.9 ± 0.2	***4.1 ± 0.4***	1.8 ± 0.1	2.1 ± 0.0	2.0 ± 0.0	1.5 ± 0.0	***1.9 ± 0.1***	1.2 ± 0.0	1.4 ± 0.0	***1.3 ± 0.1***	**2.6 ± 0.4**
Phenanthrene	1.4 ± 0.1	2.0 ± 0.1	0.4 ± 0.0	1.5 ± 0.1	***1.3 ± 0.3***	8.4 ± 0.1	13.1 ± 0.2	5.1 ± 0.1	6.3 ± 0.2	***8.2 ± 1.8***	6.0 ± 0.1	13.2 ± 0.2	***9.6 ± 3.6***	**6.2 ± 1.5**
Anthracene	n.d.	n.d.	n.d.	n.d.	***n.d.***	1.3 ± 0.0	n.d.	n.d.	1.8 ± 0.0	***0.8 ± 0.5***	n.d.	2.1 ± 0.0	***1.1 ± 1.1***	**0.6 ± 0.3**
Fluoranthene	3.6 ± 0.1	3.5 ± 0.0	3.3 ± 0.0	3.5 ± 0.1	***3.5 ± 0.1***	2.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.1	1.0 ± 0.0	***1.4 ± 0.5***	1.2 ± 0.0	2.4 ± 0.0	***1.8 ± 0.6***	**2.2 ± 0.4**
Pyrene	3.1 ± 0.1	3.0 ± 0.1	4.0 ± 0.1	2.8 ± 0.1	***3.2 ± 0.3***	n.d.	2.7 ± 0.0	2.0 ± 0.0	3.9 ± 0.1	***2.2 ± 0.8***	2.6 ± 0.0	3.7 ± 0.0	***3.2 ± 0.5***	**2.7 ± 0.4**
Benz[*a*]anthracene	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	***0.4 ± 0.0***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.1 ± 0.1**
Chrysene	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	***0.1 ± 0.0***	2.0 ± 0.0	8.0 ± 0.1	3.0 ± 0.1	1.9 ± 0.0	***3.7 ± 1.4***	32.8 ± 0.3	4.7 ± 0.1	***18.8 ± 14.1***	**5.9 ± 3.2**
Benzo[*b*]fluoranthene	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**n.d.**
Benzo[*k*]fluoranthene	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	***0.8 ± 0.0***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.3 ± 0.1**
Benzo[*a*]pyrene	1.5 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	***1.4 ± 0.0***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.5 ± 0.2**
Benzo[*g,h,i*]perylene	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**n.d.**
Indeno [*1,2,3-c,d*] pyrene	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**n.d.**
Dibenz[*a,h*]anthracene	2.3 ± 0.0	n.d.	n.d.	2.4 ± 0.0	***1.2 ± 0.7***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.3 ± 0.3**
Mean (± SE)	**42.2 (± 5.2)**	**40.9 (± 2.6)**	**33.7 (± 0.9)**	**39.0 (± 0.4)**	***39.0 (± 3.7)***	**75.7 (± 2.9)**	**82.4 (± 3.4)**	**66.9 (± 2.6)**	**78.4 (± 3.2)**	***75.9 (± 3.3)***	**141.1 (± 6.1)**	**105.0 (± 5.8)**	***123.1 (± 18.1)***	**70.5 (± 10.8)**

Table 2: Concentration of 16 priority PAHs of the US-EPA list in seawater samples as function of sample localisations. PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in $ng.L^{-1}$ (n = 3, mean ± standard error; n.d. = not detected which corresponded to a detection limit of 0.1 $ng.L^{-1}$). With no statistical difference at $p \leq 0.05$ between each localisation type.

3.2. Pre-experiments: comparing individual and pooled haemocytes after incubation with pyrene and fluorene

Similar results were obtained between cell (haemolymph alone) and solvent controls (haemolymph plus cyclohexane) for each parameter studied (data not shown).

Cell mortality was not significantly different between individual (19.3 ± 2.2 %, 16.0 ± 1.7 % and 30.9 ± 2.3 % for pyrene, fluorene and control, respectively) and pooled haemocytes (22.5 ± 0.1 %, 21.3 ± 1.6 % and 27.1 ± 1.4 % for pyrene, fluorene and control, respectively (Fig. 2). Pyrene and fluorene at 10^{-9} mg.mL^{-1} induced a significant decrease in haemocyte mortality for both sample types.

Phagocytosis percentages were similar for individual (49.8 ± 9.1 %, 52.9 ± 6.1 % and 76.1 ± 4.0 % for pyrene, fluorene and control, respectively) and pooled haemocytes (60.7 ± 6.9 %, 63.4 ± 2.6 % and 85.0 ± 0.6 % for pyrene, fluorene and control, respectively). An equal decrease in phagocytosis percentage was shown with pyrene and fluorene at 10^{-9} mg.mL^{-1} compared to control samples.

Percentages of non-specific esterase positive cells were similar for individual (92.4 ± 4.7 %, 89.8 ± 3.6 % and 88.9 ± 6.5 % for pyrene, fluorene and control, respectively) and pooled haemocytes (91.0 ± 1.2 %, 88.9 ± 1.6 % and 89.2 ± 1.7 % for pyrene, fluorene and control, respectively). Percentages of cells presenting lysosomes were similar for individual (92.4 ± 5.3 %, 94.8 ± 3.1 % and 89.9 ± 2.3 % for pyrene, fluorene and control, respectively) and pooled haemocytes (93.2 ± 1.2 %, 93.6 ± 1.6 % and 92.4 ± 1.2 % for pyrene, fluorene and control, respectively). No significant difference in percentages of non-specific esterase positive cells and lysosome presence was observed in the presence of pyrene and fluorene compared to control samples.

3.3. *In vitro* exposure (Table 3)

Compared to control samples (28.3 ± 3.5 %), fluorene (21.0 ± 2.7 %), pyrene (20.7 ± 2.3 %), anthracene (23.6 ± 1.7 %), phenanthrene (23.0 ± 2.7 %), chrysene (23.0 ± 2.5 %), indeno[*1,2,3-c,d*]pyrene (20.1 ± 3.1 %) and HFO (21.8 ± 1.2 %) significantly decreased haemocyte mortality after a 24 h incubation. Phagocytosis activity was decreased by fluorene (28.0 ± 2.0 %), pyrene (27.6 ± 2.6 %) and pure HFO (33.5 ± 9.3 %) after a 24 h

18 pollutants used	Cell mortality (%)	Phagocytosis (%)	Non-specific esterases cells (%)	Lysosome presence (%)	PO activity ($A_{490\ nm}$)
Naphthalene			△ EC (**, 24h)		
Acenaphthylene					
Acenaphthene					
Fluorene	▼ EC (*, 24h)	▼ EC (*, 24h)			
Phenanthrene	▼ EC (*, 24h)				
Anthracene	▼ EC (*, 24h)				
Fluoranthene					
Pyrene	▼ EC (*, 24h)	▼ EC (*, 24h)			
Benz[*a*]anthracene					
Chrysene	▼ EC (*, 24h)				
Benzo[*b*]fluoranthene					△ 10^{-7}, 10^{-9} $g.L^{-1}$ (*, 24h)
Benzo[*k*]fluoranthene					
Benzo[*a*]pyrene					
Benzo[*g,h,i*]perylene					
Indeno[*1,2,3-c,d*]pyrene	▼ EC (**, 24h)				
Dibenz[*a,h*]anthracene				△ EC (**, 24h)	
HFO	▼ EC (**, 24h)	▼ pure (*, 24h)			
Diesel oil					

Table 3: Cellular mortality, phagocytosis, non-specific esterase positive cells percentages and lysosome presence of oysters measured by flow cytometry and phenoloxidase (PO) activity performed by spectrophotometry following an *in vitro* exposure of 4 and 24 h at 15°C to 18 hydrocarbons at 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} $mg.mL^{-1}$. Values are means of three replicates of 45 oysters (± standard error). * = statistical difference for $p \leq 0.05$ and ** for $p \leq 0.01$; △ = significant increase; ▼= significant decrease; EC = each concentration.

incubation compared to control samples (51.7 ± 12.0 %). On the contrary, percentage of non-specific esterase positive cells (93.2 ± 0.4 % and 89.1 ± 0.3 % for naphthalene and control, respectively) and lysosome presence (90.9 ± 2.3 % and 88.1 ± 3.0 % for dibenz[*a,h*]anthracene and control, respectively) were increased by naphthalene and dibenz[*a,h*]anthracene.

Phenoloxidase activity was increased by purified trypsin TPCK and inhibited by β-2-mercaptoethanol (data not shown). Phenoloxidase activity was significantly increased with benzo[*b*]fluoranthene at 10^{-7} (0.65 ± 0.07) and 10^{-9} mg.mL^{-1} (0.63 ± 0.08) after a 24 h incubation period compared to control samples (0.47 ± 0.10).

4. Discussion

The aim of the present work was to determine the *in vitro* effects of high and field concentrations of hydrocarbons on haemocyte parameters of Pacific oysters, *Crassostrea gigas*. The tested concentrations of hydrocarbons were determined based on the literature and experimental values (determining PAH concentrations in seawater in the Marennes-Oleron Basin). The high concentrations tested in the present study (10^{-3} and 10^{-5} mg.mL^{-1}) could be compared to those observed after an oil spill. After the *Prestige* oil spill total aromatic hydrocarbons were detected at an average concentration of 3.10^{-5} mg.mL^{-1} on the Northern Spanish coast (Gonzalez et al., 2006). The concentrations detected after the *Prestige* oil spill were in a lower range than those quoted in other accidentally polluted areas. Values up to 10^{-5} mg.mL^{-1} were reported in the coastal waters of Brittany after the *Amoco Cadiz* oil spill (Marchand, 1980). For field concentrations (10^{-7} and 10^{-9} mg.mL^{-1}), information is available for rivers, including German rivers, Dutch rivers, the Yellow River in China (WHO, 1997) and the Thames in Great Britain (Acheson et al., 1976). Field concentrations of PAHs around 10^{-9} mg.mL^{-1} were reported in the marine environment (Hellou et al., 2005; WHO, 1997). In the Marennes-Oleron Basin, no information on concentrations of hydrocarbons was available. In this study, PAH concentrations ranged from 10^{-7} to 10^{-9} mg.mL^{-1}. In concordance with results obtained in seawater on the German coast (WHO, 1997), the composition and the concentration of PAHs in the seawater of the Marennes-Oleron Basin

depend of the sampling sites. Our results showed that more PAHs were detected in "open sea" sites compared to "oyster-farming" and "coastal and harbour" sites. Nevertheless, lower total PAH concentration was observed in the open sea. In all sites, no detectable concentration of indeno[*1,2,3-c,d*]pyrene, benzo[*b*]fluoranthene and benzo[*g,h,i*]perylene was found. The absence of detection in seawater may be explained by their high molecular weight which makes them less soluble and increases their affinity with particles (Meador et al., 1995). Thus, for toxicology studies, field seawater concentrations of soluble PAHs ranged from 10^{-7} and 10^{-9} mg.mL^{-1}, without emulsion or microlayers, seems to be acceptable.

Studies on Pacific oyster, *C. gigas*, haemocytes have used either individual (Auffret and Oubella, 1997; Duchemin et al., 2007; Jeong and Cho, 2005) or pooled samples (Aton et al., 2006; Gagnaire et al., 2006b; Luna-González et al., 2003). The use of individual samples allows few successive tests in similar conditions and with the same specimens but with a greater range of variation (Auffret and Oubella, 1997). On the contrary, the use of pooled samples provided a sufficient quantity of cells to carry out multiple analyses with the same group of animals and minimize effects related to inter-individual variations (Gagnaire et al., 2004). Results show that values for haemocyte parameters assessed using a pool of ten oyster haemolymphs were similar to the mean of ten individual values for both pollutants. For each immunotoxicity studies, pooled samples may be used to test a large number of pollutants and/or of cell parameters. Concerning difference between pool and individual contaminated samples for PO activity, previous results shown that no significant difference between both sample types was observed (Thomas-Guyon et al., personal communication).

Among the 16 pure hydrocarbons and the two oils tested, ten chemicals (nine pure hydrocarbons plus HFO) were able to modify at least one haemocyte parameter after a 24 h incubation period. A decrease in cell mortality was reported for four PAHs (anthracene, chrysene, indeno[*1,2,3-c,d*]pyrene and phenanthrene) and a decrease in both cell mortality and phagocytosis percentage for three hydrocarbons (fluorene, pyrene and HFO). A phagocytosis decrease could therefore not be induced by an increase in cell destruction, but rather by a decrease in haemocyte immune capacities. Gagnaire et al. (2006a) observed that benzo[*a*]pyrene, phenanthrene and anthracene tested on Pacific oyster haemocytes induced a similar decrease in cell mortality after a 24 h incubation. The impact of hydrocarbons on

haemocytes was controversial. Sami et al. (1992) and Jeong and Cho (2005) reported a decrease in the number of haemocytes in the oysters, *C. virginica* and *C. gigas*, without affecting cell mortality, when Coles et al. (1994) observed that fluoranthene increased the total cell number without modifying subpopulation percentages. Furthermore, Grundy et al. (1996b) demonstrated an *in vitro* inhibitor effect on the common mussel, *Mytilus edulis*, with anthracene, fluoranthene and phenanthrene. In marine environment, microorganisms, especially bacteria, play an important role in the aerobic biodegradation of PAHs essentially for phenanthrene, fluorene and anthracene (Kasai et al., 2002). Moreover, microorganisms were naturally present in haemolymph related to the open circulatory system in bivalves (Cheng, 1981). Then, the diminution of cell mortality in contaminated haemocytes compared to control haemocytes could be explained by two hypothesis: i) for high concentrations, bacteria are more impaired by hydrocarbons than haemocytes; ii) for field concentrations, it means lower level of hydrocarbons, PAHs are more accessible to bacteria as carbon and energy sources than haemocytes.

Naphthalene has a low molecular weight and a toxic potential due to its polycyclic nature. In our experimental conditions, each concentration of naphthalene increased percentage of non-specific esterase positive cells, after a 24 h incubation. In the opposite, decrease of percentage of non-specific esterase positive cells were shown after 4 and 24 h of oyster haemocyte exposure to benzo[*a*]pyrene, phenanthrene, anthracene and fluoranthene (Gagnaire et al., 2006a). This discrepancy could be due to the concentrations used in each study.

In this study, dibenz[*a,h*]anthracene is associated with an increase in lysosome presence after *in vitro* contact at high and field concentrations. Lysosomes are usually decreased by PAHs which induced destabilisation of lysosomal membranes (Gagnaire et al., 2006a; Grundy et al., 1996b; Grundy et al., 1996a; McVeigh et al., 2006). However, Braunbeck and Appelbaum (1999) also observed a proliferation of lysosomes on intestinal epithelium in *Cyprinus carpio* after oral contamination with an ultra-low dose of cyclodiene insecticide endosulfan. This lysosomal proliferation has to be classed as an unspecific reaction of the intestinal mucosa to stress due to xenobiotics (Braunbeck and Appelbaum, 1999). In this study, the increase in lysosomal percentage might also represent unspecific signs of stress.

The PO system is an important immune defence mechanism in invertebrates which is found in the Pacific oyster, *Crassotrea gigas* (Hellio et al., 2007). In this work, PO activity increased after 24 h of *in vitro* contact between haemolymph free of cells and benzo[*b*]fluoranthene at high concentrations. Some authors have previously described an increase in PO activity after *in vivo* pollutant exposures, e.g. fluoranthene in mussels (Coles et al., 1994), tributyltin and copper in tunicates (Tujula et al., 2001) and cadmium in Pacific oysters (Bouilly et al., 2006). On the contrary, some pollutants decreased PO activity *in vitro* such as mercury in the Pacific oysters (Gagnaire et al., 2004) and *in vivo* such as trichlorfon in prawns (Chang et al., 2006). In these *in vitro* experimentations, benzo[*b*]fluoranthene does not modulate other haemocyte parameters, thus the increase in PO activity in Pacific oysters could be due to direct action of the pollutant on this activity.

5. Conclusions

This work analysed the *in vitro* effects of 16 pure hydrocarbons and two oils on immune haemocyte capacities. Each parameter was modulated by at least one pollutant. In the case of fluorene and pyrene, which are very representative in HFO, they have effects on both cellular mortality and phagocytic activity. Moreover, the PAHs which had immune effects are more accurately represented in HFO than in diesel oil, which had no effect on immune parameters. It could be interesting to develop the synergic or antagonist effects of these potential immunomodulators and to research their effects in *in vivo* experimentations.

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ARTICLE 2: Effects of two oils and 16 pure polycyclic aromatic hydrocarbons on plasmatic immune parameters in the European sea bass, *Dicentrarchus labrax* (Linné).

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Abstract

The in vitro effects of polycyclic aromatic hydrocarbons (PAHs) on two plasmatic immune parameters, lysozyme concentration and haemolytic alternative complement activity, of the European sea bass, *Dicentrarchus labrax*, were tested using field concentrations (10^{-7} and 10^{-9} mg.mL^{-1}) observed in the coastal area and high concentrations (10^{-3} and 10^{-5} mg.mL^{-1}) observed during oil spills. Peripheral blood from 105 fish was collected, centrifuged at 1 200 g, 10 min, 4°C and three plasma pools each of 35 fish were constituted. Effects on the two plasmatic immune parameters of two oils (heavy fuel oil and light cycle oil) and 16 pure PAHs, selected on the basis of the United States Environmental Protection Agency (US-EPA) list, were tested in vitro. Only three pure PAHs (anthracene, chrysene and dibenz[*a,h*]anthracene) modulated lysozyme concentration. Acenaphthene, acenaphthylene, anthracene, benzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, pyrene and light cycle oil modified the haemolytic alternative complement activity after 4h of incubation. This study is one of the first which investigates the direct effects of several PAHs on fish plasmatic immune functions and which describes the haemolytic complement activity of fish as suitable biomarkers of hydrocarbon pollution.

Keywords: Plasmatic immune activities; Spectrophotometry; European sea bass; Direct PAH toxicity; Field concentrations; High concentrations.

1. Introduction

Coastal ecosystems are subjected to increasing contamination due to compounds including polycyclic aromatic hydrocarbons (PAHs), pesticides and pharmaceutical products. Among various chemical contaminants, the pollution caused by PAHs has led over recent years to numerous studies on the origin, distribution and fate of PAHs in the aquatic environment. Two main sources are identified: petrochemical sources (natural seepages, discharges of urban and industrial effluents, offshore oil production and oil spill) and pyrogenical sources (combustion processes due to human activity or natural occurrence). The high dissolved concentration (from about 10^{-3} to 10^{-5} $mg.mL^{-1}$) observed in the marine environment concerned mostly oil spill inputs ranging from 6.10^{-4} $mg.mL^{-1}$after the *Exxon Valdez* oil spill (Boehm et al., 2007) to 3.10^{-5} $mg.mL^{-1}$ after the *Prestige* oil spill (Gonzalez et al., 2006). Concerning field pollution, concentrations ranging from 10^{-7} to 10^{-9} $mg.mL^{-1}$ in marine dissolved fraction were observed in situ (Bado-Nilles et al., 2008; Hellou et al., 2005; WHO, 1997). However, few works have studied in situ or in vivo the action of the natural water-soluble fraction of PAHs on aquatic organisms (Duesterloh et al., 2002; Malan, 1990).

Though PAHs are known to cause detrimental effects on animals, some studies have addressed the impact of these pollutants on aquatic organisms. In this context, many studies have focused on in situ effects of sediment contaminated by PAHs on aquatic organisms (Law, 1978; Morales-Caselles et al., 2006; Stehr et al., 2004; Van der Oost et al., 1991). Nevertheless, due to their mode of life, pelagic fish, as European sea bass, *Dicentrarchus labrax*, were mostly in contact with dissolved fraction of pollutant which enters fish predominantly via gills, skin and intestinal mucous membrane. These in situ contacts with field and high concentrations of PAHs could induce external abnormalities (Pollino and Holdway, 2002), somatic mutations (Cronin et al., 2002; Roy et al., 1999) and immunodepression (Holladay et al., 1998).

Like in mammals, the immune system of fish is constituted of innate and specific immune responses. The innate immune system is the first line of defence and it is principal in fish due to the relative inefficiency of the acquired immune system, its evolutionary status, and poikilotherm nature (Magnadóttir, 2006). It is also able to stimulate the specific immune system and maintain homeostasis. The innate immune system is independent of previous exposure to foreign antigens; therefore these components are often used to determine xenobiotic effects. Xenobiotics could act either

indirectly due to modulation of other physiological processes which induce an immunomodulation (Skouras et al., 2003a) or directly on components of the immune system (Kanemitsu et al., 1998).

To clarify xenobiotic effects, in vitro assays are usually used to investigate a mechanism by which pollutants can induce an effect in vivo (Bai et al., 2001; Cavret and Feidt, 2005) or to determine if an in vivo situation could cause the same effects as those reported in an in vitro study (Willett et al., 2001). This approach should more closely define how pollutants act on the function studied and give a first indication of the adverse effects caused directly by a toxic compound (Behnisch et al., 2003) allowing an evaluation and comparison of chemical effects (Gagnaire et al., 2006).

The aim of the present work was to define the in vitro effects of selected hydrocarbons at high and field concentrations on innate humoral immune characteristics in the European sea bass. Two oils were tested due to their impact during oil spill: heavy fuel oil (HFO) and light cycle oil (LCO). The composition of each oil was elucidated to permit identification of the sixteen PAHs listed in the United States Environmental Protection Agency (US-EPA, 1998) list. These 16 PAHs US-EPA, are also studied individually at field and high concentrations. Two parameters of the innate humoral activity were studied by spectrophotometry in the plasmatic peripheral blood fraction: the lysozyme concentration and the haemolytic activity of the alternative complement pathway (ACH_{50}).

2. Material and methods

2.1. Pollutants

Two oils were selected: a heavy fuel oil (HFO) and a light cycle oil (LCO). These two oils contained some or all of the 16 polycyclic aromatic hydrocarbons (PAHs) listed in the United States Environmental Protection Agency (US-EPA) list: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*g,h,i*]perylene, indeno[*1,2,3-c,d*]pyrene and dibenz[*a,h*]anthracene.

The same 16 PAHs US-EPA in solution at 100 $ng.\mu L^{-1}$ in cyclohexane (OEKANAL®, Sigma-Aldrich), used as standard, were also individually tested.

2.2. Sea bass

One hundred and five European sea bass, *Dicentrarchus labrax*, from one spawn, were maintained in 200 L tanks supplied with seawater at ambient temperature in an experimental facility at Agence Française de Sécurité Sanitaire des Aliments (Afssa) located in Plouzané (France). The sea bass were fed daily with dry commercial pellets at 1.5 % body weight (Grower Extrude Natura 4mm, Le Gouessant Aquaculture). At sample time, they weighed 190 ± 48 g for a length of 25 ± 2 cm.

2.3. Plasma collection

Fish were anaesthetised with phenoxy-2-ethanol (Merck). From each fish, 2 mL of peripheral blood were withdrawn from the caudal vein with a lithium heparinized vacutainer (BD Vacutainer^{TM} LH 85 U.I.). Blood samples were immediately centrifuged (1 200 g, 10 min, 4 °C). After centrifugation, three pools of plasma were gained, each constituted by mixing the plasma of 35 fish. Then, each pool of plasma was split into 146 aliquots of 300 µL in Micronics (Dutscher). The aliquots were kept on ice until immediate exposure to the test material.

2.4. PAHs concentration in pure oil

To 150 g of oil (HFO or LCO) were added 200 µL of predeuterated internal standards (SRM 2269, NIST, Gaithersburg, Md). This solution was completely dissolved with dichloromethane pestipur quality (SDS). The organic phase was filtered on GF/A filter (Whatman®) and concentrated. The purification of this concentrated extract was performed by transfer to a silica column (5 g of silica). Hydrocarbons were eluted with 50 mL of pentane/dichloromethane (80:20, v:v, SDS) and concentrated to 200 µL by means of a Turbo Vap 500 concentrator (Zyman, 880 mbar, 50 °C). Aromatic compounds were analysed by gas chromatography coupled with mass spectrophotometry (GC-MS) and PAHs were quantified relative to the predeuterated internal standards introduced at the beginning of the sample preparation procedure. Concentrations of the 16 PAHs US-EPA were quantified.

2.5. In vitro plasma exposure protocol

2.5.1. Pollutant dilutions

HFO and LCO were tested in their pure form and diluted to 1/1 000 and 1/10 000 in seawater after a three-day contact period without mixing at ambient temperature (20 °C) as previously described (Anderson et al., 1974).

High (10^{-3} and 10^{-5} mg.mL^{-1}) and field (10^{-7} and 10^{-9} mg.mL^{-1}) concentrations of the 16 PAHs US-EPA were tested. Each concentration was prepared daily by dilution in cyclohexane (Sigma). The ratio cyclohexane:plasma did not exceed 0.5 % (v:v), as recommended by manufacturers in order to avoid disturbance of parameter studies.

2.5.2. Pollutant and plasma mixture

Immediately after the toxicant dilution process, for each plasma pool and each pollutant, two aliquots of 300 µL of plasma (one by analysis) were mixed in Micronics (Dutscher) with 1.5 µL of pollutant for each concentration tested.

In each pool, three controls were used to check the quality of the plasma for each immune activity: 1.5 µL of cyclohexane were mixed with 300 µL of plasma to obtain solvent control, 1.5 µL of seawater were mixed with 300 µL of plasma for seawater control and 300 µL of plasma was used alone as a cell control.

All samples (controls or plasma mixed with pollutant) were incubated at 4 °C for 4h and 24h, until analyses were carried out.

2.6. Immune parameters

2.6.1. Lysozyme concentration

Plasma lysozyme activity was determined using a turbidimetric assay (Grinde et al., 1988), adapted to microtitration plates. Briefly, a bacterial suspension of *Micrococcus lysodeikticus* (Sigma) was prepared at a concentration of 1.25 g.L^{-1} in a 0.05 M sodium-phosphate buffer pH 6.2. Fifty µL of the samples were plated in 96 well microtitration plates. The reaction was initiated in a Labsystems'iEMS analyser, by addition of 160 µL.well^{-1} of *M. lysodeikticus* suspension using an automatic dispenser. Reading of optic density (O.D.) at a wavelength of 450 nm was performed every 15 s for 3 min, the plate being shaken before each reading. Using a standard hen egg white lysozyme (Sigma) in sodium-phosphate buffer, the concentration of lysozyme in sea bass plasma was expressed in mg.L^{-1}.

2.6.2. Haemolytic activity of the alternative complement pathway

Determination of the alternative pathway of plasma complement activity was carried out by haemolytic assay with rabbit red blood cells (RRC, Biomérieux) as described by Yano (1992) and adapted to microtitration plates. Sea bass samples, diluted at 1/64 in EGTA-Mg-GVB buffer to avoid natural haemolytic activity, was added in increasing amounts, from 10 to 100 µL.wells^{-1}, and was filled with EGTA-Mg-GVB buffer to a final volume of 100 µL. Fifty µL of 2 % RRC (Biomérieux) suspension were finally added in all wells. Control values of 0 and 100 % haemolysis were obtained using: 100 µL of EGTA-Mg-GVB buffer and 100 µL of non-decomplemented trout haemolytic serum at 1/50 in ultrapure water, respectively. Samples were incubated for one hour at 20 °C. The microplates were centrifuged (400 g, 5 min, 4 °C, Jouan). Then, 75 µL of supernatant from each well were transferred with 75 µL of phosphate buffer saline (PBS, Biomérieux) into an other 96-well microplate. The absorbance (A_{540}) was read in a Labsystems'iEMS analyser and the number of ACH_{50} units per mL of plasma was determined by reference to the 50 % haemolysis.

2.7. Statistical analysis

Statistical tests were carried out using XLStat 2008. Verification of normality was conducted using the Anderson-Darling test. Since the values were not normally distributed, non-parametric tests were performed. First the Kruskal-Wallis test was used to compare the different controls themselves. Then, a Kruskal-Wallis test was used to evaluate the effect of each pollutant at each concentration and incubation time in relation to mean control values. Significantly different groups were finally identified using a Dunn test. P values lower than 0.05 were used to identify significant differences.

3. Results

3.1. PAH concentration in oil tested

In the heavy fuel oil (HFO), the 16 polycyclic aromatic hydrocarbons (PAHs) were detected with concentrations ranging from 17 ± 2 µg.g^{-1} for indeno[*1,2,3-c,d*]pyrene to 1 937 ± 67 µg.g^{-1} for phenanthrene. The four most detected molecules were fluorene (396 ± 12 µg.g^{-1}), pyrene (516 ± 33 µg.g^{-1}), naphthalene (687 ± 52 µg.g^{-1}) and phenanthrene (1 937 ± 67 µg.g^{-1}) (Table 1).

Name of 16 PAHs US-EPA	Molecular weight (g.mol^{-1})	Concentration (µg.g^{-1} ± SE) in HFO	Concentration (µg.g^{-1} ± SE) in LCO
Naphthalene	128.2	687 ± 52	3 761 ± 49
Acenaphthylene	152.2	51 ± 2	241 ± 4
Acenaphthene	154.2	273 ± 8	1 017 ± 12
Fluorene	166.2	396 ± 12	1 732 ± 9
Phenanthrene	178.2	1 937 ± 67	9 204 ± 22
Anthracene	178.2	214 ± 17	524 ± 8
Fluoranthene	202.3	125 ± 9	843 ± 11
Pyrene	202.3	516 ± 33	801 ± 35
Benz[*a*]anthracene	228.3	213 ± 8	33 ± 5
Chrysene	228.3	465 ± 8	100 ± 10
Benzo[*b*+*k*]fluoranthene	252.3	81 ± 7	n.d.
Benzo[*a*]pyrene	252.3	168 ± 5	n.d.
Benzo[*g,h,i*]perylene	276.3	48 ± 2	n.d.
Indeno [*1,2,3-c,d*] pyrene	276.3	17 ± 2	n.d.
Dibenz[*a,h*]anthracene	278.4	28 ± 3	n.d.

Table 1: Concentration of 16 priority PAHs US-EPA in heavy fuel oil (HFO) and light cycle oil (LCO). PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in µg.g^{-1} (n = 3, mean ± standard error; n.d. = not detected).

In the light cycle oil (LCO), 10 of the 16 PAHs were detected with concentrations ranging from 33 ± 5 µg.g^{-1} for benzo[*a*]anthracene to 9 204 ± 22 µg.g^{-1} for phenanthrene. The four most detected molecules were acenaphthene (1 017 ± 12 µg.g^{-1}), fluorene (1 732 ± 9 µg.g^{-1}), naphthalene (3 761 ± 49 µg.g^{-1}) and phenanthrene (9 204 ± 22 µg.g^{-1}). The higher molecular weight molecules benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*g,h,i*]perylene, indeno[*1,2,3-c,d*]pyrene and dibenz[*a,h*]anthracene, were not found in LCO (Table 1).

Enzymatic activities	Control types	4h	24h
Lysozyme concentration (mg.L^{-1} ± S.E.)	Plasma	5.82 ± 0.32	5.63 ± 0.27
	Seawater	5.92 ± 0.15	5.72 ± 0.36
	Cyclohexane	6.22 ± 0.69	5.60 ± 0.30
Statistical analysis		*NS*	*NS*
ACH_{50} (Units.mL^{-1} ± S.E.)	Plasma	43.16 ± 2.10	40.20 ± 3.90
	Seawater	40.67 ± 3.21	38.79 ± 0.13
	Cyclohexane	43.38 ± 1.35	41.40 ± 2.97
Statistical analysis		*NS*	*NS*

Table 2: Different control types for lysozyme concentration and for haemolytic activity of alternative plasma complement pathway (ACH_{50}) measured by spectrophotometry following an in vitro incubation of 4h and 24h at 4 °C. Values are the mean of three pools of 35 fish. * = statistical difference for $p \leq 0.05$ and *NS* = no significant.

3.2. Effects of cyclohexane and seawater on plasmatic immune parameters tested

After 4h and 24h incubation between plasma, seawater and solvent controls, similar values were obtained for lysozyme concentration (from 5.60 ± 0.30 mg.L^{-1} to 6.22 ± 0.69 mg.L^{-1}) and haemolytic alternative complement activity (from 38.79 ± 0.13 Units.mL^{-1} to 43.38 ± 1.35 Units.mL^{-1}) (Table 2).

Enzymatic activities	Refined products	Incubation times	Concentrations			
			Control	pure	1/1 000	1/10 000
Lysozyme concentration (mg.L^{-1} ± S.E.)	HFO	4h	5.92 ± 0.47	6.12 ± 0.32	5.49 ± 0.33	6.97 ± 1.36
		24h	5.65 ± 0.26	6.34 ± 0.38	5.84 ± 0.57	5.30 ± 0.64
	LCO	4h	5.92 ± 0.47	5.26 ± 0.18	6.03 ± 0.79	5.99 ± 0.57
		24h	5.65 ± 0.26	5.61 ± 0.29	5.40 ± 0.27	5.30 ± 0.46
Statistical analysis			*NS*			
ACH_{50} (Units.mL^{-1} ± S.E.)	HFO	4h	42.7 ± 0.9	36.2 ± 6.2	40.4 ± 0.8	41.5 ± 3.8
		24h	40.4 ± 1.4	50.0 ± 11.1	53.5 ± 12.0	54.9 ± 10.5
	LCO	4h	42.7 ± 0.9	37.6 ± 4.9	35.3 ± 7.5	**50.8 ± 4.5 ***
		24h	40.4 ± 1.4	50.7 ± 9.7	53.9 ± 10.9	56.4 ± 12.9
Statistical analysis			*S: * p < 0.05 for 1/10 000 at 4h*			

Table 3 : Lysozyme concentration and haemolytic activity of alternative plasma complement pathway (ACH_{50}) of sea bass measured by spectrophotometry following an in vitro exposure of 4h and 24h at 4 °C to two oils (HFO and LCO) at three concentrations (pure form, 1/1 000 and 1/10 000). Values are means of three pools of 35 fish. * = statistical difference for $p \leq 0.05$ and *NS* = no significant.

16 PAHs US-EPA	Incubation times	Lysozyme concentration (mg.L^{-1}) Concentrations (mg.mL^{-1}) 10^{-9}	10^{-7}	10^{-5}	10^{-3}	Statistical analysis
Naphthalene	4h	6.03 ± 0.79	4.72 ± 0.56	5.49 ± 0.29	5.16 ± 0.28	*NS*
	24h	5.41 ± 0.55	5.49 ± 0.29	5.16 ± 0.28	5.07 ± 0.34	*NS*
Acenaphthylene	4h	6.01 ± 0.22	6.14 ± 0.19	5.07 ± 0.34	6.12 ± 0.18	*NS*
	24h	5.82 ± 0.26	5.64 ± 0.15	6.12 ± 0.18	5.93 ± 0.30	*NS*
Acenaphthene	4h	6.12 ± 0.40	5.61 ± 0.29	5.93 ± 0.30	5.65 ± 0.80	*NS*
	24h	5.60 ± 0.37	6.27 ± 0.08	5.65 ± 0.80	6.04 ± 0.19	*NS*
Fluorene	4h	7.51 ± 1.18	6.14 ± 0.39	6.03 ± 0.19	5.55 ± 0.12	*NS*
	24h	5.20 ± 0.37	5.35 ± 0.47	5.55 ± 0.12	5.09 ± 0.63	*NS*
Phenanthrene	4h	5.05 ± 0.63	7.07 ± 1.18	5.09 ± 0.63	6.26 ± 0.91	*NS*
	24h	4.87 ± 0.66	5.01 ± 0.27	6.26 ± 0.91	5.12 ± 1.32	*NS*
Anthracene	4h	6.16 ± 0.95	5.52 ± 0.63	5.11 ± 1.32	5.42 ± 0.55	*NS*
	24h	5.19 ± 0.88	4.82 ± 0.16	5.42 ± 0.55	**7.00 ± 0.24 ***	*S: * p < 0.05*
Fluoranthene	4h	6.09 ± 0.50	6.15 ± 0.50	7.00 ± 0.24	5.64 ± 0.39	*NS*
	24h	6.96 ± 0.80	5.91 ± 0.81	5.64 ± 0.39	6.99 ± 0.48	*NS*
Pyrene	4h	5.75 ± 0.30	4.49 ± 0.92	6.99 ± 0.48	8.28 ± 2.68	*NS*
	24h	6.59 ± 0.16	6.60 ± 1.33	8.28 ± 2.68	8.22 ± 2.16	*NS*
Benzo[*a*]anthacene	4h	7.79 ± 0.30	5.30 ± 0.54	8.22 ± 2.16	5.64 ± 0.22	*NS*
	24h	5.62 ± 0.31	5.52 ± 0.11	5.64 ± 0.22	5.08 ± 0.49	*NS*
Chrysene	4h	5.04 ± 1.00	5.72 ± 0.53	5.08 ± 0.49	**4.93 ± 0.06 ***	*S: * p < 0.05*
	24h	5.08 ± 0.36	5.67 ± 0.27	4.93 ± 0.06	7.19 ± 1.03	*NS*
Benzo[*b*]fluoranthene	4h	5.41 ± 0.63	5.81 ± 0.41	7.19 ± 1.03	5.98 ± 0.32	*NS*
	24h	5.30 ± 0.26	5.19 ± 0.36	6.00 ± 0.32	5.41 ± 0.66	*NS*
Benzo[*k*]fluoranthene	4h	5.71 ± 0.74	5.62 ± 0.49	5.41 ± 0.66	5.16 ± 0.47	*NS*
	24h	5.73 ± 0.29	5.30 ± 0.26	5.16 ± 0.47	5.72 ± 0.28	*NS*
Benzo[*a*]pyrene	4h	5.88 ± 0.56	5.71 ± 0.24	5.72 ± 0.28	5.16 ± 0.28	*NS*
	24h	5.41 ± 0.07	5.41 ± 0.16	5.16 ± 0.28	4.98 ± 0.60	*NS*
Benzo[*g,h,i*]perylene	4h	4.58 ± 0.63	5.20 ± 0.63	4.98 ± 0.60	5.75 ± 0.33	*NS*
	24h	4.87 ± 0.61	5.30 ± 0.26	5.75 ± 0.33	5.74 ± 0.73	*NS*
Indeno [*1,2,3-c,d*] pyrene	4h	6.11 ± 0.36	5.83 ± 0.39	5.74 ± 0.73	5.86 ± 0.31	*NS*
	24h	4.78 ± 0.48	5.05 ± 0.55	5.86 ± 0.31	6.16 ± 0.46	*NS*
Dibenz[*a,h*]anthracene	4h	**4.73 ± 0.24 ***	5.41 ± 0.60	6.16 ± 0.46	5.33 ± 0.26	*S: * p < 0.05*
	24h	5.09 ± 0.78	5.56 ± 0.20	5.33 ± 0.26	4.86 ± 0.25	*NS*
Control values correspond to: 4h = 5.92 ± 0.47 mg.L^{-1} ; 24h = 5.65 ± 0.26 mg.L^{-1}						

Table 4: Lysozyme concentration of sea bass measured by spectrophotometry following an in vitro exposure of 4h and 24h at 4 °C to 16 PAHs US-EPA (10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} mg.mL^{-1}). Values are means of three pools of 35 fish. * = statistical difference for $p \leq 0.05$ and *NS* = no significant.

16 PAHs US-EPA	Incubation times	ACH_{50} (Units.mL^{-1}) Concentrations (mg.mL^{-1})				*Statistical analysis*
		10^{-9}	10^{-7}	10^{-5}	10^{-3}	
Naphthalene	4h	54.0 ± 11.3	52.4 ± 8.2	46.7 ± 11.8	40.3 ± 10.2	*NS*
	24h	50.2 ± 7.5	53.2 ± 4.2	52.0 ± 6.1	56.6 ± 11.4	*NS*
Acenaphthylene	4h	35.9 ± 0.8	31.3 ± 6.2	29.5 ± 5.1	**20.6 ± 4.2 ***	*S: * p < 0.05*
	24h	44.4 ± 6.6	42.1 ± 1.2	42.1 ± 5.4	40.8 ± 1.4	*NS*
Acenaphthene	4h	**26.7 ± 3.5 ***	**25.5 ± 3.4 ***	**25.3 ± 6.4 ***	34.7 ± 1.3	*S: * p < 0.05*
	24h	50.9 ± 14.8	39.3 ± 2.2	38.8 ± 2.4	37.5 ± 5.4	*NS*
Fluorene	4h	42.5 ± 6.5	43.3 ± 9.0	40.6 ± 9.3	38.3 ± 7.4	*NS*
	24h	51.4 ± 5.2	52.9 ± 4.5	54.3 ± 13.7	51.5 ± 3.9	*NS*
Phenanthrene	4h	40.6 ± 4.3	42.6 ± 3.5	41.8 ± 5.0	39.4 ± 4.5	*NS*
	24h	46.8 ± 4.8	51.2 ± 6.4	48.1 ± 6.1	49.1 ± 5.0	*NS*
Anthracene	4h	**57.1 ± 4.6 ****	**55.6 ± 6.5 ***	**49.2 ± 3.1 ***	**46.5 ± 3.5 ***	*S: * p < 0.05 and ** p < 0.01*
	24h	66.7 ± 11.1	56.8 ± 8.5	57.6 ± 8.8	54.1 ± 8.9	*NS*
Fluoranthene	4h	38.8 ± 6.0	35.9 ± 5.1	36.0 ± 1.5	46.4 ± 4.3	*NS*
	24h	51.2 ± 4.5	60.4 ± 8.0	53.3 ± 6.0	50.2 ± 10.2	*NS*
Pyrene	4h	39.4 ± 2.1	**48.0 ± 1.3 ***	**50.7 ± 4.9 ***	43.4 ± 3.0	*S: * p < 0.05*
	24h	40.6 ± 7.2	46.4 ± 5.2	50.3 ± 8.8	44.2 ± 7.3	*NS*
Benzo[*a*]anthacene	4h	**55.1 ± 1.6 ****	**53.0 ± 2.0 ***	**58.6 ± 3.7 ****	**53.4 ± 4.5 ***	*S: * p < 0.05 and ** p < 0.01*
	24h	53.5 ± 9.9	58.3 ± 8.7	66.1 ± 9.1	56.4 ± 6.8	*NS*
Chrysene	4h	39.0 ± 3.2	38.3 ± 5.2	42.5 ± 3.8	41.4 ± 2.7	*NS*
	24h	54.7 ± 6.5	56.5 ± 5.5	51.3 ± 2.7	48.1 ± 2.6	*NS*
Benzo[*b*]fluoranthene	4h	51.1 ± 7.3	**52.8 ± 6.9 ***	48.1 ± 2.7	**64.6 ± 8.3 ****	*S: * p < 0.05 and ** p < 0.01*
	24h	52.9 ± 9.9	55.2 ± 8.4	49.8 ± 9.4	51.4 ± 7.0	*NS*
Benzo[*k*]fluoranthene	4h	**49.9 ± 2.7 ***	**61.3 ± 1.7 ****	46.1 ± 4.2	48.0 ± 4.6	*S: * p < 0.05 and ** p < 0.01*
	24h	44.6 ± 6.3	49.4 ± 4.9	54.7 ± 11.0	49.8 ± 12.3	*NS*
Benzo[*a*]pyrene	4h	45.8 ± 0.8	**54.6 ± 7.3 ***	**49.8 ± 1.6 ***	47.3 ± 5.3	*S: * p < 0.05*
	24h	42.7 ± 11.0	50.9 ± 11.8	48.0 ± 7.8	43.8 ± 9.8	*NS*
Benzo[*g,h,i*]perylene	4h	45.3 ± 2.5	44.7 ± 1.7	46.9 ± 5.7	43.7 ± 1.8	*NS*
	24h	44.7 ± 10.6	50.1 ± 9.1	53.4 ± 11.3	49.4 ± 9.2	*NS*
Indeno [*1,2,3-c,d*] pyrene	4h	39.7 ± 6.9	44.0 ± 8.6	33.4 ± 9.6	42.4 ± 4.4	*NS*
	24h	52.2 ± 6.5	51.9 ± 4.2	51.1 ± 4.6	45.1 ± 6.3	*NS*
Dibenz[*a,h*]anthracene	4h	44.4 ± 2.4	44.2 ± 4.5	45.9 ± 8.7	39.5 ± 6.5	*NS*
	24h	50.8 ± 1.0	52.3 ± 4.3	56.2 ± 5.1	58.2 ± 7.4	*NS*
Control values correspond to: 4h = 42.7 ± 0.9 mg.L^{-1} ; 24h = 40.4 ± 1.4 mg.L^{-1}						

Table 5: Haemolytic activity of alternative plasma complement pathway (ACH_{50}) of sea bass measured by spectrophotometry following an in vitro exposure of 4h and 24h at 4 °C to 16 PAHs US-EPA (10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} mg.mL^{-1}). Values are means of three pools of 35 fish. * = statistical difference for $p \leq 0.05$, ** for $p \leq 0.01$ and *NS* = no significant.

3.2. Effects of pollutants on plasmatic lysozyme concentration

There was no difference between control and oil exposure (HFO and LCO) at each concentration and incubation time (Table 3).

Three of the 16 PAHs US-EPA individually tested showed significant modulations of lysozyme concentration (Table 4).A significant increase of lysozyme concentration was observed after 24h incubation with anthracene at 10^{-3} mg.mL^{-1} (7.00 ± 0.24 mg.L^{-1}) compared to control samples (5.71 ± 0.17 mg.L^{-1}).

On the other hand, after 4h incubation, chrysene at 10^{-3} mg.mL^{-1} (4.93 ± 0.06 mg.L^{-1}) and dibenz[*a,h*]anthracene at 10^{-9} mg.mL^{-1} (4.73 ± 0.31 mg.L^{-1}) significantly decreased lysozyme concentration compared to control samples (5.92 ± 0.30 mg.L^{-1}).

3.3. Effects of pollutants on haemolytic alternative complement activity

Only field concentration (1/10 000) of LCO (50.8 ± 4.5 Units.mL^{-1}) induced a significant elevation of haemolytic activity of complement compared to control samples (42.7 ± 0.9 Units.mL^{-1}). The HFO had none effect on this activity (Table 3).

Among the 16 PAHs US-EPA individually tested, nine produced significant modulation of ACH_{50} after 4h incubation with pollutants. After 24h incubation, no effect was observed (Table 5).

Compared to control samples, anthracene and benzo[*a*]anthracene significantly increased ACH_{50} at all concentrations tested.

Similarly, an elevation of haemolytic activity of complement was observed at field concentrations with benzo[*k*]fluoranthene (61.3 ± 1.7 Units.mL^{-1} at 10^{-7} mg.mL^{-1} and 49.9 ± 2.7 Units.mL^{-1} at 10^{-9} mg.mL^{-1}) compared to control samples (42.7 ± 0.9 Units.mL^{-1}).

Conversely, a significant diminution of this activity was shown after incubation of plasma at high concentration of acenaphthylene (20.6 ± 4.2 Units.mL^{-1} at 10^{-3} mg.mL^{-1}) compared to control samples (42.7 ± 0.9 Units.mL^{-1}), while acenaphthene induced a significant decrease of ACH_{50} at both high (20.6 ± 4.2 Units.mL^{-1} at 10^{-5} mg.mL^{-1}) and field (25.5 ± 3.4 Units.mL^{-1} at 10^{-7} mg.mL^{-1} and 26.7 ± 3.5 Units.mL^{-1} at 10^{-9} mg.mL^{-1}) concentrations compared to control samples (42.7 ± 0.9 Units.mL^{-1}).

The other pollutants, benzo[*a*]pyrene, benzo[*b*]fluoranthene and pyrene, significantly induced an increase of ACH_{50} compared to control samples at some high and field concentrations.

4. Discussion

The petroleum products which pollute the coasts are called heavy fuel oil (HFO). This product is a mixture of catalytic cracking product and light cycle oil (LCO) used by some refiners in order to facilitate transportation by tankers of these residues (Ding et al., 2007). After oil spillage, these products, containing polycyclic aromatic hydrocarbons (PAHs), could induce modification in the marine biological environment. In fact, fish obtained from PAH-polluted areas, present lesions thought to be related to immunosuppression (Stentiford et al., 2003). The innate immune system of fish is often used as a biomarker of xenobiotic effects. Indeed, for toxicological studies, lysozyme levels have been most frequently examined in fish plasma or serum (Reynaud and Deschaux, 2006). Nevertheless, little is known about the direct action of pollutants. Another physiological component of innate humoral immunity is the complement, of which a key role in fish in innate immune response is recognized. Moreover, due to their down-regulation in many situations of stress (Tort et al., 1996) and their activation in the absence of pathogens, these physiological components appear to be possible biomarkers for pollution. Nevertheless, at the present time only few authors have investigated effects on this phenomenon following environmental pollution (Tahir and Secombes, 1995; Wu et al., 2007). Thus, the main aim of the present work, after a characterisation of the tested oils, was to determine the in vitro effects of high and field concentrations of hydrocarbons on these two plasmatic parameters of the European sea bass.

The two oils used, HFO and LCO, contained PAHs listed in the United States Environmental Protection Agency (US-EPA) list. HFO contains all 16 PAHs US-EPA, whilst LCO contains only the 11 PAHs US-EPA with low and medium molecular weight (≤ 228.3 g.mol^{-1}). Concerning HFO, naphthalene (128.2 g.mol^{-1}), fluorene (166.2 g.mol^{-1}), phenanthrene (178.2 g.mol^{-1}), pyrene (202.3 g.mol^{-1}) and chrysene (228.3 g.mol^{-1}) were

largely detected, whilst for LCO, the acenaphthene (154.2 $g.mol^{-1}$), a lower molecular weight compound, replaced chrysene. The PAH composition of each oil induces dissimilar viscosity. In fact, HFO presented a viscosity of 38 cSt at 100 °C, whilst LCO had a viscosity of 1.24 cSt at 100 °C (TotalFina, personal communication). During the three-day contact between oil and seawater, as described by Anderson et al. (1974) in order to obtain a dissolved fraction of PAHs, the kinetic rate of PAHs into the seawater was dependant on the composition and the viscosity of oil. Indeed, in contrast to in vivo exposure to LCO (Bado-Nilles et al., submitted-a), after HFO exposure (Bado-Nilles et al., submitted-b), high molecular weight compounds ($\geq$ 252.3 $g.mol^{-1}$) were detected in the seawater dissolved fraction. In this way, it became apparent that the two oils tested could have dissimilar effects in function of their composition and of the dilution used. Thus, a first idea of the impact of two different cocktails of PAHs could be given after LCO and HFO exposure.

Concerning the lysozyme concentration in sea bass plasma mixed in vitro with HFO and LCO, no modification was observed. So it appears that direct contact between this enzyme and PAH mixture did not affect the active mechanism of the lysozyme activity, which attacked the 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell walls of gram-positive bacteria (Holloway et al., 1993). In the same way, no variation on plasmatic lysozyme concentrations was shown in in vivo exposed sea bass to HFO or to municipal sewage effluent, and these, in spite of some PAHs (e.g. naphthalene, acenaphthene, pyrene and benzo[*a*]pyrene) that were bioaccumulated in fish tissues (Bado-Nilles et al., submitted-b; Hoeger et al., 2004). These bioavailable PAHs, which bioaccumulated in the organism, seemed to have had no direct effect on the lysozyme concentration. In the present work, several proposals can be put forward to explain this absence of effect: 1) synergic/antagonist action of the PAH cocktail found in oils, 2) the minor presence of compounds which could induce an in vitro modification of this immune parameter, 3) protein destabilisation due to a poor incubation process which abrogated the PAH effect, 4) evaporation of PAH compound, which produce a diminution of contamination, or of plasma, which could produce a concentration of the protein tested. Nevertheless, no significant difference for control values was obtained in the presence of seawater, which was used to dissolve pollutants for in vitro exposure. Thus, seawater did not produce a variation on the plasmatic immune parameter tested. Moreover, an absence of

difference between the two incubation times tested (4h and 24h) was observed for plasmatic and seawater controls and these values were roughly similar to other sea bass lysozyme concentrations (Caruso et al., 2005). These results showed that the incubation temperature used (4 °C) do not induce important protein destabilisation and that the incubation process prevents evaporation of PAHs and plasma. It is concluded that the exposure protocol used give only information about direct action of pollutants. Thus in the present work, contamination of plasma by HFO and LCO have no direct effect on lysozyme concentration.

In the same way, for ACH_{50}, no significant difference was detected at each incubation time for control values (plasmatic and seawater controls), these values were roughly similar to other sea bass ACH_{50} (Caruso et al., 2005). So, the difference observed in ACH_{50} may be due to a direct action of oils. Whereas HFO direct contact with plasma does not appear to induce an effect on ACH_{50}, an increase of this activity was observed in the presence of LCO after 4h incubation at the lower concentration tested. In vivo exposure to dissolved fraction of HFO induces bioaccumulation of molecules with low molecular weights, largely detected in the LCO fraction (Bado-Nilles et al., submitted-b). Thus, the presence of some PAHs with low molecular weight seems to influence the increase of ACH_{50} in vitro as in vivo. In spite of no significant effect was found in vitro after 24h incubation, the trend of ACH_{50} increase was maintained.

In order to try to define the exact function of each PAH present in the refined products, the effects of each PAH US-EPA on the two immune parameters tested were performed in vitro. Three pure PAHs induced modulation of lysozyme: anthracene produced an increase of enzymatic activity at high concentration after 24h incubation, while a decrease of this same activity was noted after 4h incubation with chrysene and dibenz[*a,h*]anthracene. These results were hard to explain in function of the number of benzene rings (two for anthracene, four for chrysene and five for dibenz[*a,h*]anthracene), or molecular weight of these PAHs (low for anthracene: 178.2 $g.mol^{-1}$, medium for chrysene: 228.3 $g.mol^{-1}$ and high for dibenz[*a,h*]anthracene: 278.4 $g.mol^{-1}$). Moreover, it was difficult to explain that after in vitro incubation between plasma and phenanthrene, from 10^{-9} $mg.mL^{-1}$ (5.61 pM) to 10^{-3} $mg.mL^{-1}$ (5.61 µM), no effect was demonstrated when in vivo exposure to phenanthrene dissolved fraction (> 1 µM) increased the plasmatic lysozyme activity in the olive flounder,

Paralichthys olivaceus (Jee et al., 2004). The nature of effect, direct or indirect, could explain a part of these differences. Indeed, at the time of in vivo pollution, contaminants may cause indirect effects on lysozyme modulation via endocrine disruption (e.g. elevating levels of cortisol), immune (e.g. impairment of lysosome stability) and physiological (e.g. damaging cells or modification of cytochrome P450 1A induction) processes (Bakirel et al., 2005; Skouras et al., 2003b). These indirect actions, due to cellular or metabolic damage, could not be involved in lysozyme modification by PAHs in vitro. Thus, alteration of lysozyme concentration was due here only to action of anthracene, chrysene and dibenz[*a,h*]anthracene. In fact, as for seawater control, the incubation period does not induce protein destabilisation and the evaporation of PAHs and plasma was prevented. Indeed, no significant difference for control values was obtained in the presence of cyclohexane certainly due to the manufacturers recommended dilution used (0.5 %) and, as with seawater, values obtained after 4h and 24h were similar to other sea bass lysozyme concentrations (Caruso et al., 2005). In the presence of high concentration of anthracene the lysozyme was increased significantly. During this in vitro experiment with only plasma, the lysozyme quantity could not be increased. However, the liposoluble properties of the anthracene could induce damages on cellular membranes when they transported through the bacterial membrane of *Micrococcus lysodeikticus*. In this way, the destabilisation of bacterial suspension by anthracene could facilitate the attack of the bacterial cell wall by the lysozyme. Conversely, after chrysene high contamination and dibenz[*a,h*]anthracene field exposure, a possible destruction of lysozyme protein and/or a decrease of their enzymatic activity might be supposed after 4h incubation. Nevertheless, no effect was found after 24h incubation, perhaps due to the destruction or destabilisation of PAH molecules in plasma. Therefore, after 4h incubation, pollutants might not induce either a real destruction of lysozyme protein or irremediable alteration of this enzymatic protein but they might induce a reversible decrease of lysozyme activity.

As for lysozyme control values, for ACH_{50}, no significant difference was detected at each incubation time for control values (plasmatic and cyclohexane controls), and these values were roughly similar to other sea bass ACH_{50} at each incubation time (4h and 24h) (Caruso et al., 2005). Thus, whatever concentration tested, opposite effects on ACH_{50} modulation, occured according to the molecule tested. The in vitro decrease of ACH_{50}

induced by acenaphthene (154.2 $g.mol^{-1}$) and acenaphthylene (152.2 $g.mol^{-1}$), two low molecular weight PAHs, could be related to either inhibition of pathway activation by modification of structural conformation of some plasmatic components (e.g. component C3) or molecular toxicity as described after in vivo exposure to cadmium (Wu et al., 2007). Moreover, a possible first destabilisation of red blood cell membrane by PAH could be proposed too, as for LCO exposure. Moreover, for each modulation of the ACH_{50}, no effect was found after 24h which would explain that these PAHs might not induce either a real destruction of ACH_{50} or irremediable alteration of this enzymatic cascade but they might induce a reversible decrease of their activity. On the contrary, anthracene, benzo[*a*]pyrene, benzo[*a*]anthracene, benzo[*k*]fluoranthene, benzo[*b*]fluoranthene and pyrene induced an increase of the haemolytic activity of the complement, which suggests a stimulation of some components of the enzymatic cascade. In fact, in human serum, diesel exhaust particle extracts induced in vitro the cleavage of the third component of complement (C3) in serum to C3b (Kanemitsu et al., 1998).

This work, to our knowledge, is the first to analyse the direct in vitro effects of two oils and 16 pure hydrocarbons on humoral non specific immune capacities of the European sea bass. Lysozyme was little affected by oils and PAHs, thus, it must be combined with other type of biomarkers to analyse suitably hydrocarbon exposures. By contrast, haemolytic alternative complement activity was affected by LCO and six pure PAHs. Thus, although few authors have investigated the effects of environmental pollutant on this parameter, this enzymatic activity appears to be suitable as a biomarker for in vitro hydrocarbon contamination at high as field concentrations. Moreover, the PAHs which had effects on immunological parameters were generally more representative in LCO than in HFO. Finally, it could be interesting to investigate further the synergistic or antagonistic effects of these pure PAHs, both in vitro and in vivo, to better understand the effects of oils on immunological parameters in marine organisms.

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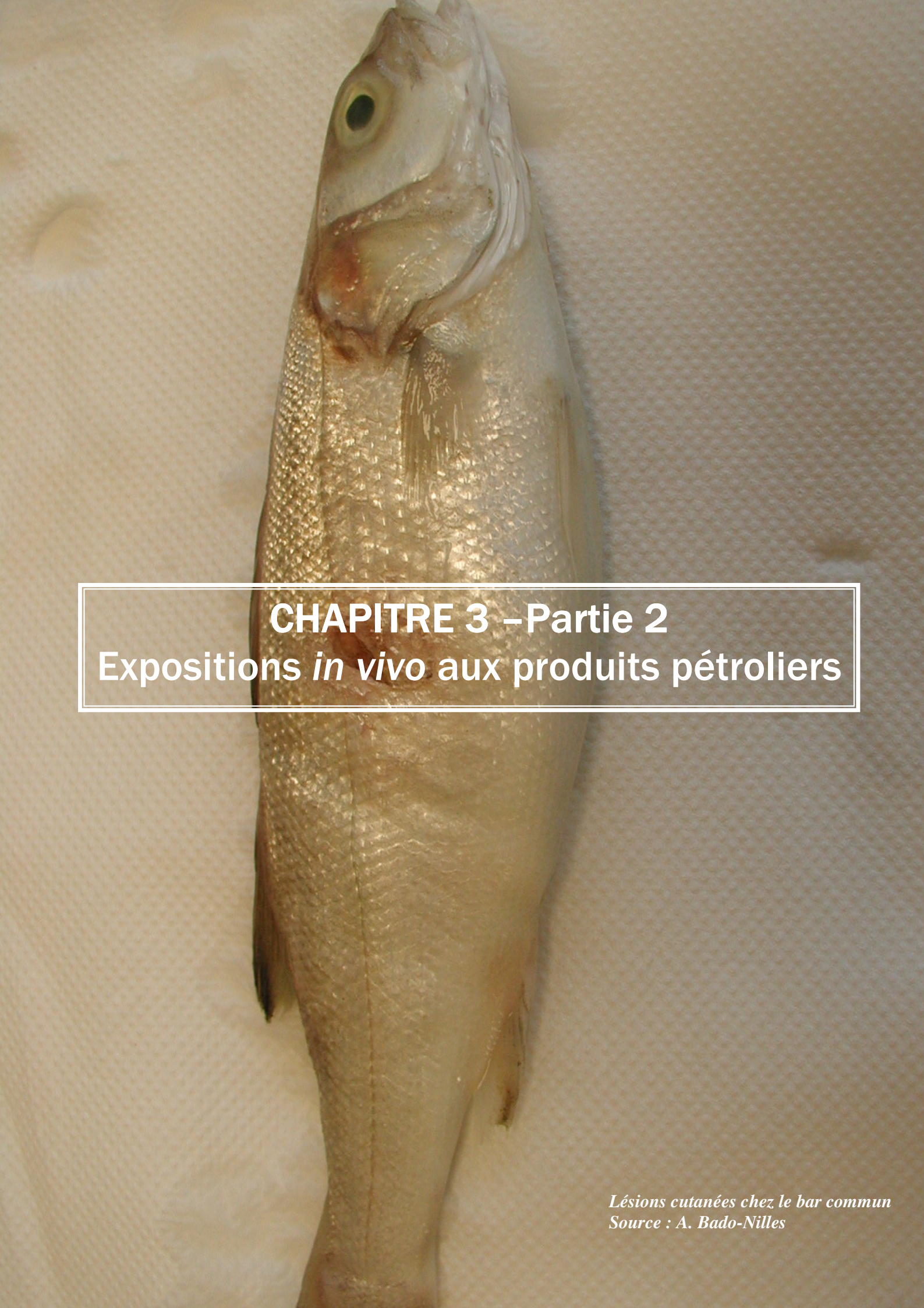

CHAPITRE 3 –Partie 2
Expositions *in vivo* aux produits pétroliers

Lésions cutanées chez le bar commun
Source : A. Bado-Nilles

Après le choix des polluants et des descripteurs d'intérêt, cette seconde partie du travail s'attache à évaluer l'impact *in vivo* d'un fioul lourd issu de l'*Erika* (Articles 3, p.88, et Article 5, p.124) et d'une fraction pétrolière légère le constituant, le LCO (Articles 6, p.152, et Article 7, p.177), sur le système immunitaire de l'huître creuse et du bar commun soumis à une exposition de type accidentel. Le LCO est issu des distillations atmosphériques et sous vide réalisées par les industriels lors du raffinage des produits pétroliers en vue d'optimiser la production des composés légers à forte valeur économique, tels que les diesels, les gasoils et les kérosènes. Les produits nommés fluxants, tel que le LCO, correspondent aux résidus de ce craquage catalytique (Poveda Jaramillo *et al.*, 2004). Ce fluxant est régulièrement utilisé pour réduire la viscosité des fiouls lourds et favoriser ainsi leur transport (Ding *et al.*, 2007). Comme décrit précédemment (Chapitre 2), le transport par mer des fiouls lourds induit des risques pour l'environnement liés notamment aux déversements accidentels. En effet, ces dernières années, d'importants accidents maritimes sont venus souiller le littoral Européen, induisant une forte médiatisation due à des pertes économiques et écologiques conséquentes (e.g. l'*Erika* et le *Prestige*).

Pour réaliser ces études *in vivo* un nouveau dispositif expérimental adapté de la méthode d'Anderson *et al.* (1974) a été conçu afin d'exposer les animaux uniquement à la fraction soluble de la substance polluante (Article 5, p.124). Ce dispositif favorise l'obtention d'une exposition homogène dans le temps et assimilable à celles obtenues *in situ* suite à une marée noire, de 733 $ng.L^{-1}$ pour le fioul lourd à 1 600 $ng.L^{-1}$ avec le LCO. En effet, les concentrations en HAPs dissous recensées au cours des différents accidents maritimes vont de 600 $ng.L^{-1}$ après la marée noire de l'*Exxon Valdez* (Boehm et al., 2007) à 1 700 $ng.L^{-1}$ après l'accident de l'*Ekofisk* (Law, 1978). De plus, il répond à différents critères de maintien en vie des organismes, telle que la stabilité des paramètres physico-chimiques de l'eau. L'ensemble des ces paramètres valide le dispositif expérimental utilisé. Comme expliqué dans le Chapitre 2, à la suite de ces déversements occasionnels, les hydrocarbures subissent une étape d'oxydation ou de biotransformation ce qui augmente leur biodisponibilité vis-à-vis de la faune et la flore marines. La quantification de ces hydrocarbures bioaccumulés dans les organismes a nécessité la mise au point du protocole d'extraction de ces derniers au

sein du *Cedre*. Il a été notamment constaté que les poissons vont bioaccumuler préférentiellement des composés de faible poids moléculaire (e.g. naphtalène et acénaphtène ; Article 3, p.88 Article 5, p.124 et Article 7, p.177), alors que les Bivalves bioaccumulent majoritairement les composés de poids moléculaire élevé (e.g. phénanthrène; Article 3, p.88 Article 5, p.124 et Article 6, p.152), et ceci en raison de leur faible capacité de métabolisation. De plus, l'élimination des HAPs bioaccumulés est très différente : elle se fait selon des processus de diffusion passive chez les Bivalves, tandis que les poissons vont excréter activement par la bile ou l'urine (Meador et al, 1995, Budzinski *et al.*, 2004). Ainsi, la quantification des métabolites biliaires chez les Vertébrés est une technique de détection des composés rapidement biotransformés, à savoir les composés à 4-cycles benzéniques comme les métabolites du pyrène. De fait, ces métabolites sont détectés dans la bile suite à l'exposition de sept jours aux hydrocarbures alors qu'ils ne sont pas retrouvés dans les tissus du poisson (Article 7, p.177). De nombreux facteurs sont donc à l'origine de l'accumulation différentielle entre les Invertébrés et les Vertébrés malgré une voie d'entrée similaire (épithéliums branchiaux et cutanés). Comme les HAPs, constituants des produits pétroliers et de leurs fluxants, et leurs métabolites possèdent une action connue sur la reproduction (Cajaraville *et al.*, 1993) et les capacités de défense (Reynaud & Deschaux, 2006), l'impact sur les réserves halieutiques doit être déterminé. Cependant, l'analyse des résultats soulève le problème de l'effet de l'échantillonnage sur les capacités de défense des organismes (Article 4, p.113) et la question de la répétitivité de ces résultats se pose (Article 5, p.121). Suite aux expérimentations, il apparaît souhaitable d'effectuer des prélèvements hebdomadaires des organismes afin d'éviter tout effet de stress dû aux manipulations et donc de maintenir des paramètres immunitaires stables dans le temps tout en favorisant la répétitivité des analyses biologiques pour chaque condition d'essais (Figure 3 - 4).

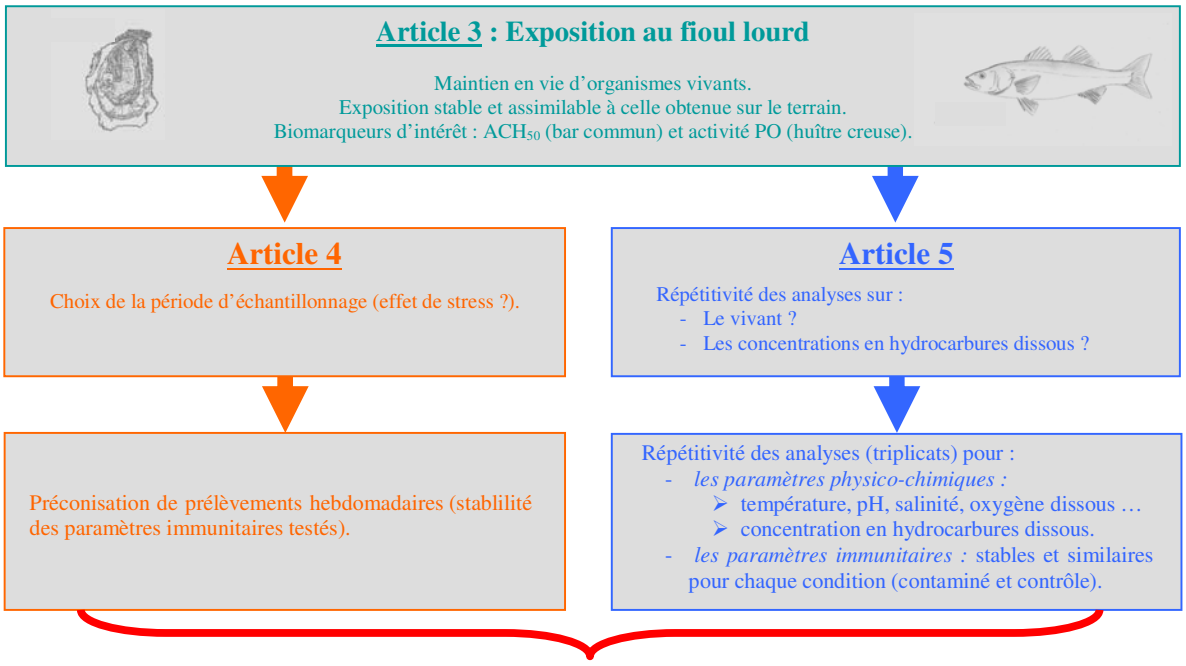

Figure 3 - 4 : Représentation de la validation des protocoles et du dispositif expérimental.

A la suite de ces séries d'expérimentations avec du fioul lourd (Articles 3, p.88, et Article 5, p.124) et du LCO (Articles 6, p.152, et Article 7, p.177), les paramètres cellulaires sont peu modifiés, alors que les deux cascades enzymatiques acellulaires analysées s'affichent comme des biomarqueurs d'intérêt. En effet, jusqu'à 14 jours après l'arrêt de la contamination, l'activité PO de l'huître est diminuée de manière significative et l'ACH_{50} est clairement augmentée chez le bar. Ainsi, cette modulation des paramètres immunitaires témoigne d'un impact significatif mais, néanmoins, toujours réversible car elle n'est est plus retrouvée en fin de décontamination.

Actuellement, très peu de données sont disponibles quant à l'action des polluants à l'encontre de ces deux descripteurs immunologiques (PO et ACH_{50}). Aussi, l'analyse des tests fonctionnels a été complétée par une étude du suivi de l'expression de certains gènes d'intérêt impliqués dans la régulation de ces deux biomarqueurs :

- Pour les Bivalves, les gènes codant pour la *laccase*, une oxydase cuivre-dépendante comme la PO, et le *molluscan defence precursor*, un précurseur du système proPO-PO, sont étudiés.
- Chez les poissons, le suivi de l'expression des gènes codant pour le *TNF-alpha*, qui est un facteur sérique jouant un rôle clef dans les réponses inflammatoires, est entrepris. Or, suite à l'exposition à la fraction soluble en fioul lourd et en LCO, les réponses inflammatoires sont proposées en tant que précurseur de l'augmentation de l'activité du complément (Article 3, p.88 et Article 7, p.177). De plus, suite aux observations de Kanemitsu *et al.* (1998), qui lient la suractivation du complément voie alterne au clivage du composé C3 par les particules de diesel, l'expression des gènes codant pour cette protéine plasmatique (*C3*) est également quantifiée.

Le suivi des gènes codant pour les effecteurs impliqués dans la cascade enzymatique montre une modulation du *molluscan defence precursor* et de la *laccase* après contamination à la fraction soluble en LCO. Bien que le *molluscan defence precursor*, qui induirait le clivage de la proPO en PO, semble augmenter, l'activité PO est significativement diminuée après l'exposition des Bivalves au LCO, et ceci, même après 14 jours de décontamination. Ainsi, la diminution de cette activité enzymatique ne semble pas être reliée à la perte du clivage de la proPO en PO. Elle pourrait donc être consécutive à une action directe ou synergique d'un et/ou plusieurs HAP(s) présent(s) dans la fraction soluble ou bioaccumulé(s) dans les tissus (naphtalène, acénaphtylène, acénaphtène, fluorène ou phénanthrène). De plus, en supposant que la PO corresponde bien à la *laccase* séquencée (Renault *et al.*, communication personnelle), l'hypothèse d'une sur-régulation dans l'optique d'un effet compensatoire est à émettre (Figure 3 - 5).

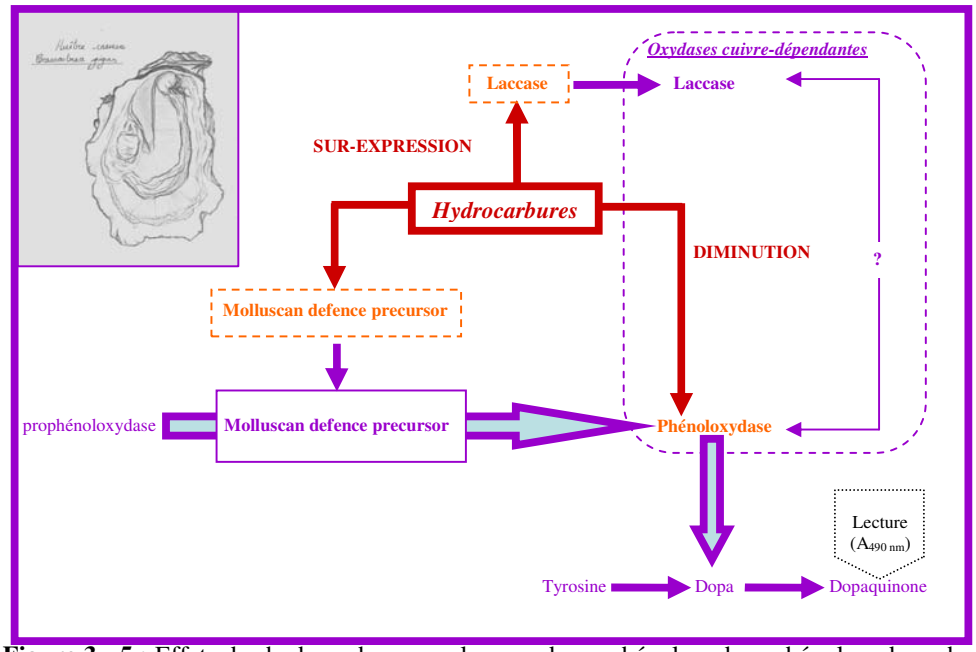

Figure 3 - 5 : Effets des hydrocarbures sur la cascade prophénoloxydase-phénoloxydase chez l'huître creuse, *Crassostrea gigas*, avec : gènes , **cibles**.

L'étude en génomique fonctionnelle entreprise chez le bar commun montre une sur-expression du gène codant pour le *TNF-alpha*, molécule clef dans la réponse inflammatoire, illustrant probablement, à l'échelle moléculaire, les importantes lésions cutanées observées chez les bars soumis à une exposition au LCO. Cette forte réaction inflammatoire pourrait être à l'origine de l'activation de la voie alterne du complément observée. De plus, cette activation de l'ACH_{50} est, elle même, liée au clivage du composé C3 (Kanemitsu *et al.*, 1998). Or une sous-expression, non significative, du gène codant pour ce composé est constatée. Un effet compensatoire, à cette forte réaction inflammatoire dans un souci de régulation est à envisager (Figure 3 - 6).

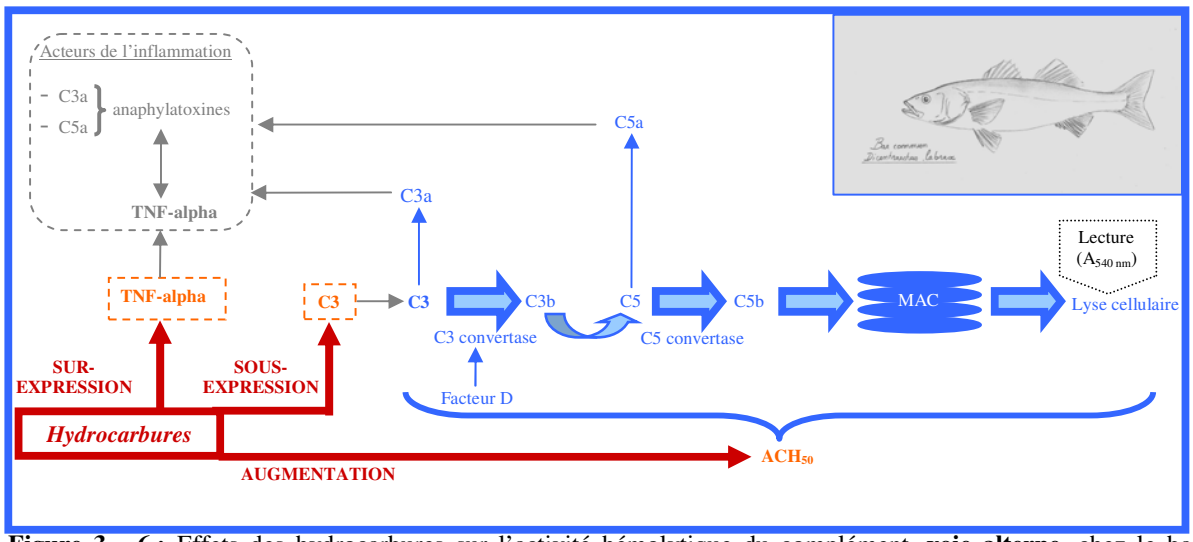

Figure 3 - 6 : Effets des hydrocarbures sur l'activité hémolytique du complément, **voie alterne**, chez le bar commun, *Dicentrarchus labrax*, avec : gènes , **cibles**.

La seconde partie de ce travail avait pour objectif de :

1. Valider un dispositif expérimental permettant le maintien en vie des organismes et l'exposition à des concentrations en hydrocarbures solubles stables et réalistes.
2. Mettre au point les techniques d'extraction au *Cedre* afin de quantifier la bioaccumulation des hydrocarbures dans les tissus des organismes exposés et les métabolites biliaires chez le bar.
3. Définir des descripteurs immonologiques pertinents utilisables en cas de déversement accidentel.

Le dispositif expérimental utilisé a permis une contamination des animaux démontrée, *premièrement* par la mise en évidence d'hydrocabures bioaccumulés, et *deuxièmement* au niveau immunitaire, par une modulation réversible de deux paramètres acellulaires : la PO chez l'huître et l'ACH_{50} chez le bar. Ainsi, l'évaluation du risque chimique en expositions contrôlées de laboratoire a révélé l'intérêt de ces deux outils de diagnostic, robustes, peu coûteux et fiables, pouvant être transposés dans des conditions réelles de terrain.

Les modes d'action des hydrocarbures sur ces deux paramètres acellulaires apparaissent complexes et doivent être approfondis. Concernant l'activité PO chez les Bivalves, la recherche de l'action des hydrocarbures sur les autres systèmes endogènes d'activation (protéases, récepteurs membranaires des hémocytes…) devrait être entreprise. Pour l'ACH_{50} chez les poissons, l'impact des polluants sur les anaphylatoxines (C3a et C5a) qui interviennent dans la réponse inflammatoire et sur les convertases (C3 et C5 convertase) devrait être précisé.

ARTICLE 3: Effects of HFO on immune parameters of European sea bass, *Dicentrarchus labrax*, and Pacific oyster, *Crassostrea gigas*.

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Abstract

The European sea bass, *Dicentrarchus labrax*, and the Pacific oyster, *Crassostrea gigas*, were respectively exposed to a soluble fraction of heavy fuel oil during five and nine days. Then, organisms were transferred for one month to non contaminated seawater. The bioaccumulation and elimination of PAHs in contaminated tissues were dissimilar between species. In fish, acenaphthene and naphthalene were detected and naphthalene was always quantified 30 days after the beginning of the recovery period. Whereas in oysters, pyrene and phenanthrene were bioaccumulated and 14 days after the exposure, no more PAHs were detected. Concerning innate immune parameters, the increase of haemolytic activity of alternative complement pathway in fish and the reduction of the phenoloxidase activity in oysters endured respectively one and two weeks in contaminated organisms. This indicates that these two enzymatic cascades could be quite attractive for monitoring pollution by oil.

Keywords: *Dicentrarchus labrax*; *Crassostrea gigas*; Heavy fuel oil; Immune parameters; Complement activity, Phenoloxidase activity, Bioaccumulation.

1. Introduction

In last years, many studies have been directed toward elucidating the relationship between environmental pollutants and the occurrence of stress-related and disease conditions in aquatic animals. In fact, the estuarine environment is a major source of potential chemical pollutants emitted from human activities including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). The PAHs may dissolved throughout the water column, become adsorbed onto particles and enter into the sediments where they may persist, only undergoing very slow degradation (Meador et al., 1995). These pollutants are well-known as environmental pollutants at low concentrations and appear in the United States Environmental Protection Agency (US-EPA) priority pollutant list due to their mutagenic and carcinogenic properties. PAHs are phototoxic (Shiaris, 1989), reprotoxic (Diamond et al., 1995) and immunotoxic (Yamaguchi et al., 1996). In mammals and fish, the immunotoxic effects of PAHs have been widely demonstrated (Krieger et al., 1994; Vos et al., 1989; Weeks and Warinner, 1984) but few studies reported immunological effects in marine Bivalves (Coles et al., 1994).

Marine organisms may be continually exposed to fluctuations of the environment including xenobiotics (Gagnaire et al., 2003). The vulnerability of aquatic species to chemical pollution depends on pollutant properties, pollutant concentrations entering ecosystems and the capacity of ecosystems to resist pollutants and especially biodegradation (Fochtman, 2000). However bioaccumulation constitutes a natural response and may be the consequence of direct contamination by water or indirect contamination through the food chain (Amiard-Triquet, 1989). When bioaccumulation occurs, pollutants can have direct interactions with tissues and cells such as immune cells.

It seems that xenobiotic-mediated suppression of innate immune responses, which impact on resistance to pathogen, would have more impact than suppression of an acquired immune response. Moreover, little is known about mechanisms by which PAHs induce immunotoxicity in vertebrates and more especially in fish (Reynaud and Deschaux, 2006). The internal defence mechanisms may prevent infections from pathogenic microorganisms and parasites by cellular reactions such as phagocytosis which eliminated invaders (Glinski and Jarosz, 1997). Intruders may also be eliminated by humoral components formed by

complement system or prophenoloxidase/phenoloxidase system, lysozyme activity and lectins (Glinski and Jarosz, 1997). It seems that xenobiotic-mediated suppression of these innate immune responses, impacts resistance to pathogens. Nevertheless, little is known about mechanisms by which PAHs induce immunotoxicity in organisms (Reynaud and Deschaux, 2006).

The aims of the present work were (i) to develop an *in vivo* system of experimental contamination by heavy fuel oil (HFO) representative of concentrations experienced after an oil spill and (ii) to carry out a preliminary validation of the experimental system by studying pollutant effects on immune parameters simultaneously in two marine species, the European sea bass, *Dicentrarchus labrax*, and the Pacific oyster, *Crassostrea gigas*. The HFO used was similar to the oil which was released into the Atlantic coastal waters after the Erika oil spill which occurred in December 1999. The capacity of organisms to recover their initial status after exposure to a soluble fraction of HFO was analysed during one month based on monitoring of cell mortality, cell subpopulations and phagocytosis activity by flow cytometry. Humoral innate immune parameters were performed too: for fish haemolytic activity of alternative complement pathway (ACH_{50}) were studied and for oyster phenoloxidase (PO) activity was quantified.

2. Materials and methods

2.1. Organisms

One hundred and twenty European sea bass, *Dicentrarchus labrax*, 144 ± 32 g, came from one pond and were maintained in an experimental facility at Afssa site de Plouzané (France).

Pacific oysters, *Crassostrea gigas*, 8-10 cm in shell length, were purchased from a shellfish farm located in the Brest bay (Brittany, France).

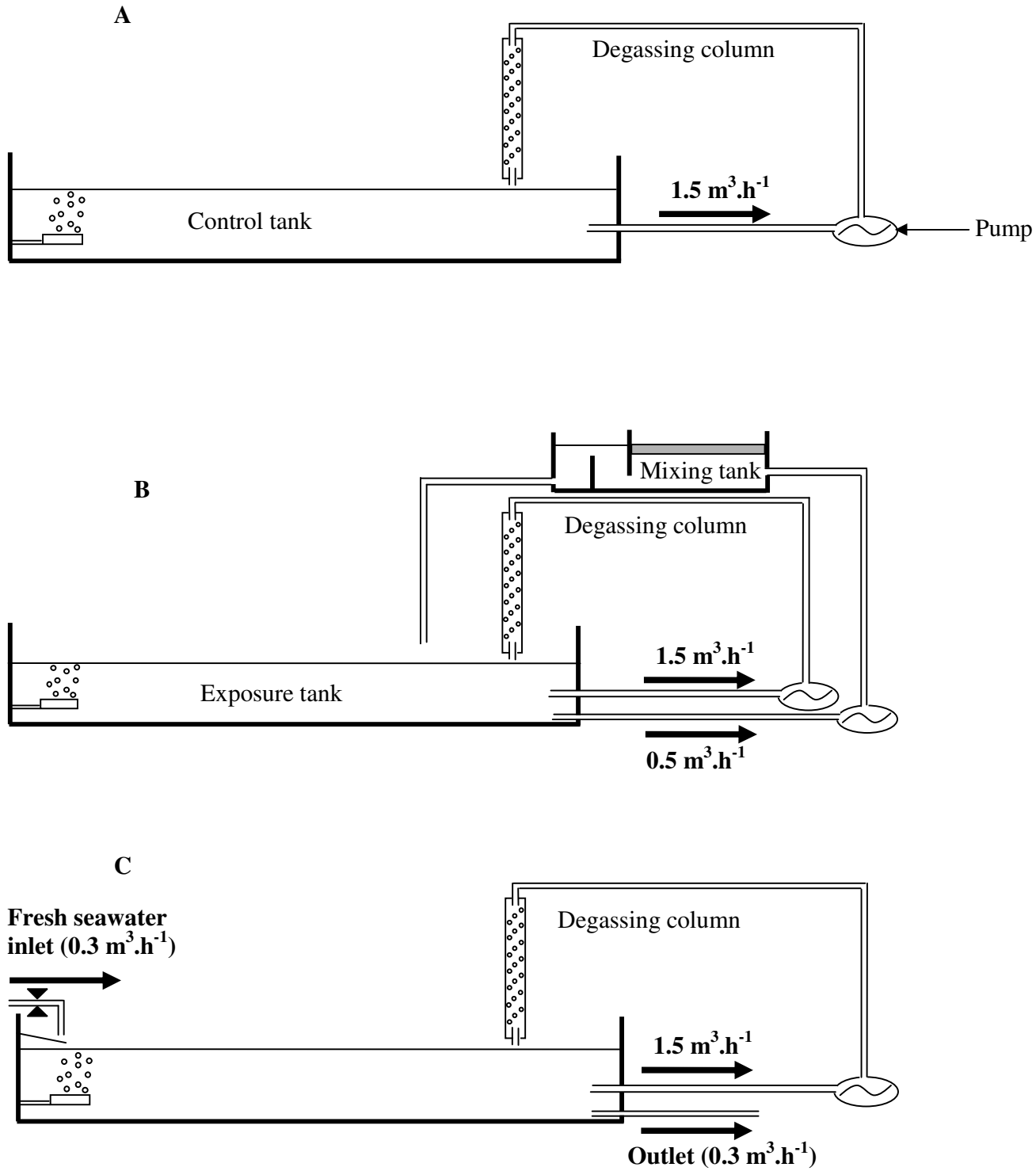

Fig. 1: Experimental tanks for control oysters (A) and contaminated oysters (B) as a closed circuit and during the recovery period (C) as an open circuit.

European sea bass and Pacific oysters were acclimated together in 1 200 L tanks with a flow of 0.3 $m^3.h^{-1}$ at 12 ± 1°C (dissolved oxygen 96 ± 4 %, pH 7.6 ± 0.4, salinity 36 ± 2 ‰, free of nitrate and nitrite). Sea bass were fed every day with 150 g of granulates (Grower Extrude Natura 4mm, Le Gouessant Aquaculture) and oysters every three days with 15 L of *Isochrysis galbana* at 4.10^6 cell.mL^{-1}. This rate allows maintaining per day a minima concentration of 15.10^4 cell.mL^{-1} which is enough to ensure a continuous feeding of oyster (Rico-Villa et al., 2006).

At the end of the acclimatisation period, a small notch was carved in the dorsal shell of the oysters, to favour direct contact between oyster tissues and pollutants.

Name of PAH compounds	**Molecular weight ($g.mol^{-1}$)**	**Concentration ($\mu g.g^{-1}$ ± SE)**
Naphthalene	128.2	687 ± 89
Acenaphthylene	152.2	51 ± 3
Acenaphthene	154.2	273 ± 14
Fluorene	166.2	396 ± 20
Phenanthrene	178.2	1937 ± 116
Anthracene	178.2	214 ± 28
Fluoranthene	202.3	125 ± 14
Pyrene	202.3	516 ± 57
Benz[*a*]anthacene	228.3	213 ± 13
Chrysene	228.3	465 ± 14
Benzo[*b*+*k*]fluoranthene	252.3	81 ± 11
Benzo[*a*]pyrene	252.3	168 ± 7
Benzo[*g,h,i*]perylene	276.3	48 ± 3
Indeno [*1,2,3-c,d*] pyrene	276.3	17 ± 3
Dibenz[*a,h*]anthracene	278.4	28 ± 5

Table 1: Concentration of 16 priority PAHs of the US-EPA list in the heavy fuel oil used during the exposure period. PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in $\mu g.g^{-1}$ (n = 3, mean ± standard error).

2.2. Pollutants

A heavy fuel oil (HFO), similar to that of the Erika, which contained 24-25% of saturate hydrocarbons, 54-55% of aromatic hydrocarbons and 20-21% of polar compounds, was selected to perform the exposure. The HFO contained among others the 16 PAHs of US-EPA list which are benzo[*a*]anthracene, naphtalene, acenaphtylene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[*1,2,3-c,d*]pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene at concentrations ranging from 1 936.9 ± 116.6 $\mu g.g^{-1}$ for phenanthrene to 27.6 ± 5.7 $\mu g.g^{-1}$ for dibenz[*a,h*]anthracene (Table 1).

2.3. Experimental arrangement

2.3.1. Experimental system

The experimental system take place in the *Cedre* (Brest, France). It was constituted of four tanks, one control tank (Fig. 1A), one exposed tank (Fig. 1B) and two tanks for recovery and acclimatisation period (Fig. 1C). Each tank had a volume of 1 200 L and was 120 cm deep. The tanks were placed in a greenhouse, which is thermoregulated (T = 14 ± 1 °C) and has one total renewal of the air in 6 h.

For the four different tanks, an air stone and a degassing column were used to maintain a level of dissolved oxygen around 96 %. The degassing column is constituted by a 2 m high pipe, with a diameter of 20 cm, which is filled with 40 kg of glass beads (diameter of 6 mm). In addition, the flow of seawater (1.5 $m^3.h^{-1}$) which goes through this column allows the elimination of the dissolved carbon dioxide (Fig. 1).

For the exposure period, a mixing tank was connected to the exposure tank in order to generate a closed circulation of seawater with a flow of 0.5 $m^3.h^{-1}$ (Fig. 1B). At the water surface of the mixing tank, three litres of HFO were released. This equipment allows seawater contaminated by only the soluble fraction of the HFO in the exposure tank to be obtained. This system was adapted from Anderson et al. (1974) and modified by *Cedre* to obtain a stable oil concentration in the exposure tank throughout the experimentation (733 ± 111 $ng.L^{-1}$). After two weeks of mixture of HFO and seawater, organisms were placed on exposure tanks.

2.3.2. Exposure conditions

After the acclimatisation period, 60 sea bass were placed with 60 oysters in the control tank (Fig. 1A) and 60 other sea bass were exposed with 60 oysters (Fig. 1B) with the natural light / dark cycle. Fish were exposed during five days and oyster during nine days. Control groups received clean seawater during the same period. During this exposure period, control and exposed organisms were not fed. The seawater quality was monitored in both tanks (oxygen level, temperature, pH, salinity, nitrate and nitrite) and, also, the concentration of the HFO soluble fraction.

2.3.3. Recovery period

After the exposure period, organisms of each tank were transfered into two other tanks, which are similar to acclimatized tank (Fig. 1C), with an inlet of clean seawater with a flow rate of 0.3 $m^3.h^{-1}$. Organisms were put back in the same condition as during the acclimatisation period. During the 30 day recovery period, organisms were fed again in the same condition as during acclimatized period.

2.4. Sampling and sample preparation

2.4.1. Seawater

One litre of seawater was collected in triplicates in heated (500 °C) Duran glass bottles (Bioblock) on Days (-8), (-4) and (-2) after the beginning of the exposure period to characterise the seawater quality.

2.4.2. Sea bass

Fish were anaesthetised with phenoxy-2-ethanol. Peripheral blood was collected on the first day of the recovery period, immediately after the disconnection of the mixing tank (Day 0), and at Days 1, 3, 14 and 30 of the recovery period. For each fish, 2 mL of blood were withdrawn from the caudal vein with a lithium heparinized vacutainer (BD Vacutainer™ LH 85 U.I.). Then, fish were stunned and weighed. The blood and fish were kept on ice until they were processed.

For flow cytometry analysis, 0.5 mL of blood collected from 10 contaminated and from 10 control fish (n = 10) were immediately diluted with 10 mL of Leibovitz 15 medium

(L15, Eurobio) containing 10 U. heparin lithium (Sigma). Then, samples were loaded onto Ficoll gradient (Histopaque®1077, Eurobio) to density of 1.07-1.08 $g.cm^{-3}$. After centrifugation (400 g, 30 min, 15 °C), mononuclear cells at the interface were collected and were washed twice (400 g, 5 min, 4 °C) with L15. Finally, cells were resuspended in 1 mL of L15 medium.

For spectrophotometry analysis, remaining blood samples were centrifuged (1 200 g, 10 min, 4 °C). After centrifugation, the plasma of each fish were split into two aliquots of 100 µL and stored at -80°C for further analysis.

After blood collection, about 20 g of muscle of 10 control and 10 contaminated fish were collected and frozen at -80°C until analysis.

2.4.3. Oyster

Haemolymph was collected on the first day of the recovery period, immediately after the disconnection of the mixing tank (Day 0), and at Day 3, 14 and 30 post exposure.

After opening the oyster shell by cutting off the adductor muscle, approximately 2 mL of haemolymph was withdrawn from the pericardial cavity using a 2 mL syringe equipped with a 0.7 X 30 mm needle. The haemolymph samples were kept on ice until they were processed to reduce haemocyte aggregation (Auffret and Oubella, 1997).

As for cytometry, 1 mL of haemolymph from five oysters was collected individually from five contaminated oysters and from five control oysters (n = 5). All samples were treated immediately and separately to study cellular activity.

As for spectrophotometry, three pooled samples, each composed of haemolymph from five oysters were formed to obtain a sufficient volume of haemolymph. The three pooled haemolymph samples were centrifuged (260 g, 10 min, 4 °C). The acellular fraction (supernatant) was frozen at - 80 °C for further analysis.

2.5. Analytical methods

2.5.1. Seawater PAH concentrations

For the seawater PAH content, samples were extracted with 30 mL of dichloromethane pestipur quality (SDS). After separation of the organic and aqueous phases, water was extracted two additional times by the same volume of dichloromethane

(2 X 30 mL). The combined extracts were purified and treated using gas chromatography coupled with mass spectrometry (GC-MS, Hewlett Packard HP5890 coupled with an HP5972 mass selective detector) following published procedures (Douglas et al., 1992). Finally, the 16 PAHs US-EPA was quantified.

2.5.2. PAH concentrations

PAH levels in fish muscles were determined by GC-MS using the procedure of Baumard et al. (1997) with some modifications. Prior to extraction, each muscle sample was homogenized using an Ultraturax (Janke & Kunkel, IKA®-Labortechnik). One hundred and fifty µL of predeuterated internal standards (CUS-7249, Ultra Scientific, Analytical solutions) were added to 16 g of homogenized muscle and samples were digested for 4 h under reflux in 50 mL of an ethanolic solution of potassium hydroxide (2 $mol.L^{-1}$, Fisher Chemicals). After cooling, decantation and addition of 20 mL of demineralised water, the digest was extracted in a 250 mL funnel 2 times with 20 mL of pentane (SDS). The extract was evaporated with a Turbo Vap 500 concentrator (Zyman, Hopkinton, MA, USA, at 880 mbar and 50 °C) to obtain 1 mL of concentrated extract. The purification of the extract was performed by transfer to a silica column (5 g of silica). Hydrocarbons were eluted with 50 mL of pentane:dichloromethane (80:20, v:v, SDS) and concentrated to 200 µL by means of a Turbo Vap 500 concentrator (Zyman, 880 mbar, 50°C). Aromatic compounds were analyzed by GC-MS and PAHs were quantified relative to the predeuterated internal standards introduced at the beginning of the sample preparation procedure.

Whole tissues of the 15 control and 15 contaminated oysters were collected, pooled and frozen at – 80 °C until further analysis. The PAHs levels were determined by GC-MS (Laboratoire Municipal de Rouen, ETSA, Rouen, France) (Munschy et al., 2005).

2.5.3. Blood leucocytes and haemocytes analysis by flow cytometry

Cell viability was assessed using the trypan blue exclusion method. Cell enumeration was performed with a Thoma's cell haemocytometer and adjusted at 10^6 $cells.mL^{-1}$ with L15 medium for leucocytes and with Tris Buffer Saline (TBS, 1 000 $mOsm.L^{-1}$) for haemocytes. Morphological characteristics, cell mortality and phagocytosis percentage were analysed with a Facscalibur flow cytometer (Becton Dickinson) using protocols previously described

(Gagnaire et al., 2003). For each cell sample, 10 000 events were counted. Analyses were carried out on whole immune cells without distinguishing subpopulation and results were expressed as percentage of positive cells.

Cellular subpopulation percentages and structure (size and complexity) were analyzed using FSC and SSC photodetectors, respectively, which enable differentiation between cell types and to establish the proportion in fish of lymphocytes and monocytes-granulocytes and in oysters of hyalinocytes and granulocytes. These parameters were monitored using 200 µL of cell suspension without previous treatment.

Cell mortality was measured using FL3 (red fluorescence). Propidium iodide (PI, 1.0 $g.L^{-1}$, Molecular Probes) is membrane impermeant and is excluded from viable cells. Mortality was determined using 200 µL of haemocyte suspension and 10 µL of PI. Cell suspensions were incubated for 30 minutes at 4 °C.

The phagocytosis percentage was determined using FL1 (green fluorescence). Fluorescent microspheres ($2.7x10^{10}$ $particles.mL^{-1}$, Fluorospheres® carboxylate-modified microspheres, diameter 1 µm, Molecular Probes) were used and the fluorescence setting was established using a suspension of fluorescent beads in distilled water. Only the events showing a fluorescence of at least three beads were considered positive for phagocytic activity. Phagocytic activity of haemocyte suspensions was analysed on 200 µL of haemolymph samples and 10 µL of a 1/10 dilution of fluorescent beads. Cell suspensions were incubated for one hour at room temperature.

2.5.4. Plasma and haemolymph analysis by spectrophotometry

2.5.4.1. Fish analysis

Determination of the alternative pathway of plasma complement activity was carried out by haemolytic assay with rabbit red blood cells (RRC, Biomérieux) as described by Yano (1992) and adapted to microtitration plates. Sea bass samples, diluted at 1/64 in EGTA-Mg-GVB buffer to avoid natural haemolytic activity, was added in increasing amounts, from 10 to 100 $\mu L.wells^{-1}$, and was filled with EGTA-Mg-GVB buffer to a final volume of 100 µL. Fifty µL of 2 % RRC (Biomérieux) suspension were finally added in all wells. Control values of 0 and 100 % haemolysis were obtained using: 100 µL of EGTA-Mg-GVB buffer and 100 µL of non-decomplemented trout haemolytic serum at 1/50

in ultrapure water, respectively. Samples were incubated for one hour at 20 °C. The microplates were centrifuged (400 g, 5 min, 4 °C, Jouan). Then, 75 µL of supernatant from each well were transferred with 75 µL of phosphate buffer saline (PBS, Biomérieux) into an other 96-well microplate. The absorbance (A_{540}) was read in a Labsystems'iEMS analyser and the number of ACH_{50} units per mL of plasma was determined by reference to the 50 % haemolysis.

2.5.4.2. Oyster analysis

Haemolymph samples were centrifuged (260 g, 10 min, 4°C) and supernatants recovered. Detection of phenoloxidase (PO) activity in acellular fraction samples was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-Dopa, Sigma) transformation in dopachromes as previously described by Gagnaire et al. (2004). Samples were distributed in 96-well microplates (Nunc, France). PO modulators were used to confirm the specificity of the detection. The purified trypsin TPCK (*N*-Tosyl-L-phenylalanine chloromethyl ketone, 1 $g.L^{-1}$, Sigma) was used as an activator and the β-2-mercaptoethanol (10 mM, Sigma) was used as an inhibitor. To determine the PO activity, 80 µL of cacodylate buffer (CAC buffer: sodium cacodylate (10 mM), trisodium citrate (100 mM), NaCl (0.45 M), $CaCl_2$ (10 mM), $MgCl_2$ (26 mM), pH 7.0), 20 µL of L-Dopa (3 $mg.mL^{-1}$) and 20 µL of samples were added in each well. To measure the PO activity modulation, 60 µL of CAC buffer, 20 µL of PO modulators, 20 µL of L-Dopa and 20 µL of samples were added to each well. Control (120 µL of CAC buffer) and negative control (100 µL of CAC buffer, 20 µL of L-Dopa) wells were used to determine respectively the purity of the buffer and the autooxidation capacities of L-Dopa. Each sample was tested in nine replicates and absorbance was measured at 490 nm after a 21 h incubation period at room temperature.

Total protein concentration was measured using the Bradford method (Micro BCA Protein Assay Kit, Pierce, Rockford, IL). The bovine albumin serum (BSA, from 0.0 to 1.0 $g.L^{-1}$, Pierce, Rockford, IL) was used as a standard. Ten µL of samples or of the standard were distributed in 96-well microplates with 90 µL of milliQ water and 100 µL of reagent kit. The microplates were then incubated for 2 h at 37 °C and the absorbance was read at 570 nm (A_{570}).

2.5. Statistical analysis

Statistical tests were carried out using XLStat Pro 7.5.3. Verification of normality and of homogeneity of covariance matrices (homocedasticity) were conducted using respectively the Anderson-Darling test and the Bartlett test. For normal values and homogeneous variance, an F-test was applied to analyse HFO effects. P values lower than 0.05 were used to identify significant differences.

3. Results

Name of PAH compounds	**Concentration at Day (-8)**	**Concentration at Day (-4)**	**Concentration at Day (-2)**	**Mean concentration ($ng.L^{-1}$ ± SE)**
Naphthalene	88	60	48	66 ± 12
Acenaphthylene	4	18	2	8 ± 5
Acenaphthene	15	22	18	19 ± 2
Fluorene	7	7	7	7 ± 0
Phenanthrene	482	300	614	466 ± 91
Anthracene	37	33	31	34 ± 2
Fluoranthene	37	48	64	50 ± 8
Pyrene	4	5	7	5 ± 1
Chrysene	7	9	10	9 ± 1
Benzo[a]anthracene	65	32	13	37 ± 15
Benzo[b]fluoranthene	7	15	32	18 ± 7
Benzo[k]fluoranthene	5	11	23	13 ± 5
Benzo[a]pyrene	4	4	5	5 ± 0
Indeno[1,2,3-c,d]pyrene	n.d.	n.d.	n.d.	n.d.
Benzo[g,h,i]perylene	n.d.	n.d.	n.d.	n.d.
Dibenz[a,h]anthracene	n.d.	n.d.	n.d.	n.d.
Sum of the 16 PAHs	**762**	**564**	**874**	**733 ± 157**

Table 2: Concentration of 16 US-EPA PAHs in contaminated tanks throughout the *in vvivo* experiment. The results are expressed in $ng.L^{-1}$ in seawater (n = mean values establish from three analysis at Day (-8), Day (-4) and Day (-2) after the beginning of the exposure period, mean ± standard error, n.d. = not detected).

No sea bass and oyster mortality was observed during the course of the experiment. The physico-chemical parameters of the seawater was the same as that of the acclimatisation period during all the experimentation (dissolved oxygen 96 ± 4 %, pH 7.6 ± 0.4, salinity 36 ± 2 ‰, temperature 12 ± 1 C, free of nitrate and nitrite).

3.1. PAH concentrations in seawater

In the control tank, PAHs were not detected. In the exposure tank, 13 out of 16 PAHs were detected with a total mean concentration of 733 ± 111 $ng.L^{-1}$ and with mean concentrations ranging from 5 ± 0 $ng.L^{-1}$ for benzo[*a*]pyrene to 466 ± 91 $ng.L^{-1}$ for phenanthrene. The concentrations of diaromatic compounds decreased during the exposure period (e.g. naphthalene, from 88 to 48 $ng.L^{-1}$). For compounds with more benzenic cycles, kinetics of dissolution were inverted: at Day (-8) concentrations are globally lower than at Day (-2) (e.g. benzo[b]fluoranthene, from 7 to 32 $ng.L^{-1}$). Right from the beginning, the three heaviest (indeno[*1,2,3-c,d*]pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene) were undetected (Table 2).

Sampling dates	Fish muscles (µg.kg^{-1} of dry weight)		Oyster tissues (µg.kg^{-1} of dry weight)	
	Control	**Contaminated**	**Control**	**Contaminated**
D0	n.d.	56.8 (naphthalene: 45.2, acenaphthene: 11.6)	n.d.	22.8 (phenanthrene: 5.8, pyrene: 17.00)
D1	n.d.	52.3 (naphthalene: 43.2, acenaphthene: 9.1)	-	-
D3	n.d.	37.9 (naphthalene: 29.7, acenaphthene: 8.2)	n.d.	7.6 (pyrene)
D9	n.d.	32.4 (naphthalene: 27.2, acenaphthene: 5.2)	-	-
D14	n.d.	6.4 (naphthalene: 6.4)	n.d.	n.d.
D30	n.d.	5.7 (naphthalene: 5.7)	n.d.	n.d.

Table 3: Concentration of the 16 US-EPA PAHs in sea bass muscles and oyster tissues. The results are expressed in µg.kg^{-1} of dry weight (n = 10 for fish and n = 15 for oysters, n.d. = not detected). The experiment was performed by gas chromatography coupled with mass spectroscopy (GC-MS).

	Experimental conditions	Recovery period											
		Day 0		Day 1		Day 3		Day 9		Day 14		Day 30	
	Values ± SE	Control	*HFO*	Control	*HFO*	Control	*HFO*	Control	*HFO*	Control	*HFO*	Control	*HFO*
European sea bass	Lymphocytes (%)	72.2 ± 9.1	*74.9 ± 3.1*	72.8 ± 4.2	*65.2 ± 2.7*	71.9 ± 6.1	*64.4 ± 8.2*	75.1 ± 7.4	*66.9 ± 5.6*	77.7 ± 6.1	*78.5 ± 5.3*	71.5 ± 9.3	*57.1 ± 9.9*
	Monocytes / Granulocytes (%)	15.4 ± 4.9	*14.0 ± 1.2*	19.6 ± 3.7	*23.8 ± 2.9*	18.2 ± 4.8	*20.9 ± 5.0*	16.2 ± 5.6	*20.3 ± 3.1*	12.8 ± 3.6	*13.4 ± 3.9*	16.7 ± 3.9	*22.3 ± 4.4*
	Mortality (%)	**0.8 ± 0.2**	***1.7 ± 0.3****	1.1 ± 0.3	*0.5 ± 0.1*	0.7 ± 0.3	*0.7 ± 0.2*	1.6 ± 0.6	*1.2 ± 0.3*	0.7 ± 0.1	*1.1 ± 0.2*	0.7 ± 0.2	*0.7 ± 0.2*
	Phagocytosis (%)	35.3 ± 7.9	*26.9 ± 6.5*	44.1 ± 9.8	*36.9 ± 15.4*	27.3 ± 14.0	*26.4 ± 9.9*	16.9 ± 3.9	*12.7 ± 2.0*	3.5 ± 0.5	*3.3 ± 1.0*	20.6 ± 8.7	*11.3 ± 5.9*
Pacific oyster	Granulocytes (%)	47.2 ± 6.0	*37.8 ± 2.1*	-	-	38.3 ± 4.9	*31.2 ± 4.3*	-	-	32.2 ± 2.8	*26.5 ± 1.4*	26.6 ± 2.7	*25.4 ± 4.0*
	Hyalinocytes (%)	52.8 ± 6.0	*62.2 ± 2.1*	-	-	61.7 ± 4.9	*68.8 ± 4.3*	-	-	67.8 ± 2.8	*73.5 ± 1.4*	73.4 ± 2.7	*74.6 ± 4.0*
	Mortality (%)	10.9 ± 1.2	*11.7 ± 1.6*	-	-	4.9 ± 0.9	*7.8 ± 0.9*	-	-	6.6 ± 1.6	*6.1 ± 1.1*	5.7 ± 1.0	*6.5 ± 2.3*
	Phagocytosis (%)	**35.6 ±4.2**	***24.5 ±0.5****	-	-	27.5 ±2.9	*23.7 ±2.5*	-	-	24.8 ±4.0	*23.1±5.7*	23.6 ±4.4	*28.5 ±3.0*

Table 4: cellular subpopulation, cell mortality and phagocytosis percentage monitored by flow cytometry after *in vivo* exposure of sea bass and oyster to heavy fuel oil (HFO). Day 0, Day 1, Day 3, Day 9, Day 14 and Day 30 concerned the recovery period. No effect was detected during the entire recovery period and for all values (n = 10 for fish and n = 15 for oysters, mean ± standard error). * = statistical difference for $p \leq 0.05$.

3.2. PAH concentrations in fish muscles and oyster tissues

PAHs were neither detected in the muscles of control fish nor in the tissues of the control oyster (lower than 5 $\mu g.kg^{-1}$of dry weight with correspond to limit detection of GC-MS, Table 3).

For the fish, after five days of exposure, two PAHs were detected in muscle of contaminated sea bass, with 56.8 $\mu g.kg^{-1}$ of dry weight composed of about 79.6 % of naphthalene and 20.4 % of acenaphthene. After one day of recovery period, fish had eliminated 4.4 % of naphthalene and 21.6 % of acenaphthene. Then, 34.3 % of naphthalene and 29.3 % of acenaphthene had been eliminated on Day 3 and 39.8 % of naphthalene and 55.2 % of acenaphthene had been eliminated at Day 9. From the 14^{th} day of the recovery period, only naphthalene was detected in fish muscle with 6.4 $\mu g.kg^{-1}$ of dry weight (85.5 % of elimination) on Day 14 and 5.7 $\mu g.kg^{-1}$ of dry weight (87.4 % of elimination) on Day 30.

Concerning oysters, after nine days of exposure, the contaminated oyster tissues had bioaccumulated only two PAHs, with 22.8 $\mu g.kg^{-1}$ of wet weight composed of about 25.5 % of phenanthrene and 74.5 % of pyrene. At Day 3 post-contamination, the oysters had eliminated 100 % of the phenanthrene and 55.3 % of the pyrene bioaccumulated in their tissues. From the 14^{th} day of the recovery period, PAHs were no more detected in oyster tissues.

3.3. PAH effects on cellular parameters

Concerning fish results, leucocyte subpopulations were represented by mean values of 14.6 ± 3.9 % for granulocytes-monocytes and 70.7 ± 6.4 % for lymphocytes. Leucocyte subpopulation percentages and phagocytic activity were not significantly different in control and contaminated fish at the end of the contamination period and during the recovery period. Nevertheless, the cell mortality had significantly increased in the contaminated fish (1.7 %) in comparison to the control fish (0.8 %) before transfer into recovery tanks (Table 4).

In oysters, haemocyte subpopulations were represented by mean values of 33.2 ± 7.5 % of granulocytes and 66.8 ± 7.5 % of hyalinocytes. Haemocyte subpopulation percentages and cell mortality were not significantly different in the control and contaminated oysters during the recovery period. At the end of the contamination period, the

phagocytosis activity had significantly decreased in the contaminated oysters (24 %) compared to controls (36 %) (Table 4).

3.4. PAH effects on extracellular parameters

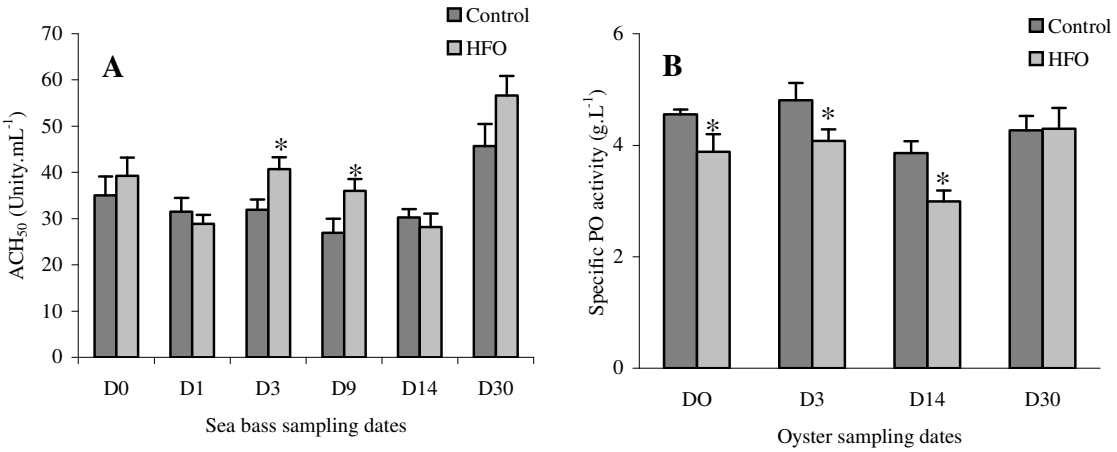

Fig. 2: Haemolytic activity of alternative complement pathway (ACH_{50}, **A**) in fish and specific phenoloxidase activity (PO, **B**) in oysters measured by spectrophotometry during the recovery period following exposure to heavy fuel oil (HFO). N = 10 for fish and n = 15 for oysters. The bars represent the standard error. * = statistical difference for $p \leq 0.05$.

In fish, the ACH_{50} was significantly increased in contaminated fish compared to controls with 40.7 ± 2.5 ACH_{50} units.mL^{-1} for contaminated fish and 31.9 ± 2.2 ACH_{50} units.mL^{-1} for control fish at Day 3 and with 36.1 ± 2.9 ACH_{50} units.mL^{-1} for contaminated fish and 27.0 ± 3.0 ACH_{50} units.mL^{-1} for control fish at Day 9. At each other sampling dates of the recovery period, no significant difference was observed between the contaminated fish and the control fish (Fig. 2).

Concerning specific PO activity in oysters, it was significantly decreased in contaminated oysters compared to controls from Day 0 to Day 14 of the recovery period. This difference decreased, they past to 0.9 at Day 0, to 0.8 at Day 3 and to 0.7 at Day 14, and this difference was no longer significant by Day 30 (< 0.1) (Fig. 2).

4. Discussion

4.1. Chemical analysis: validation of the experimental system

Pollutant detection and exposure in soluble fractions depend on the dissolution technique used. In this study, the Anderson method (Anderson et al., 1974) has been chosen and modified by the *Cedre* to expose organisms only to the soluble fraction of heavy fuel oil (HFO). During the entire exposure period of the experiment, the global concentration of the soluble fraction of HFO (733 $ng.L^{-1}$) remained constant, validating the experimental system, which moreover enables a homogenate exposure in the whole tank. This concentration was within the classic range often observed after an oil spill. In fact, much wider ranges of concentrations are described in the literature covering the concentrations of PAHs in seawater after different slick. After the *Exxon Valdez* wreckage, Boehm et al. (2007) reported seawater PAH concentrations ranging up to 600 $ng.L^{-1}$. Law (1978) also reported high concentrations of PAHs in seawater ranging up to 1 700 $ng.L^{-1}$ after the *Ekofisk* blowouts. Thirteen out of 16 PAHs present in the HFO were detected in the soluble fraction, obtained after the HFO has been in contact with seawater for two weeks. No detectable concentration of indeno[*1,2,3-c,d*]pyrene, benzo[*g,h,i*]perylene and dibenz[*a,h*]anthracene was found. The absence of these three compounds may be explained by their low content in HFO and their high molecular weight which make them less soluble. The low solubility of pyrene (202.25 $g.mol^{-1}$), related to its high molecular weight, probably explains the small quantity of pyrene (5.0 $ng.L^{-1}$) in seawater, in spite of its high concentration in HFO (516 $\mu g.g^{-1}$). On the contrary, the high concentration of phenanthrene in the HFO (1 936 $\mu g.g^{-1}$), and its low molecular weight (178.23 $g.mol^{-1}$), explain its high concentration in seawater. So, the composition in PAHs of the soluble fraction depends on the initial concentration of the different elements in heavy fuel oil, of their molecular weight and of their number of benzenic cycles which determine also their solubilisation kinetic (Meador et al., 1995). Moreover, the seawater contamination by HFO is an equilibrium between all compounds and their kinetics of dissolution which explain why the total mean concentration of PAHs stay stable during the exposure period. These results, close to those observed during the behaviour of an oil slick at sea, validate the experimental system. Thus, organisms contaminated by the soluble fraction of HFO were exposed to the 13 PAHs during different labs of time, five days

for fish and nine for oysters. This discrepancy could explain several kinetics of bioaccumulation.

4.2. Chemical analysis: PAH bioaccumulation

In the present study, the contamination period induced PAH bioaccumulation in fish muscles (naphthalene; acenaphthene) and in oyster tissues (pyrene; phenanthrene) confirming the efficacy of the experimental system. Several factors may be at the origin of this difference between the uptake and accumulation of PAHs in fish and oysters: (i) physical and chemical properties of PAHs (i.e. molecular weight, solubility, half-life period, bioavailability); (ii) time of exposure and environmental parameters such as water oxygenation and temperature; (iii) intraspecific factors such as living conditions, pelagic and carnivorous for certain species, sessile and filter-feeding for the others, or physiological status of the animals (e.g. ventilatory rate, reproductive condition, age, capacities to uptake and metabolize PAHs, rate of excretion) (Meador et al., 1995; Varanasi et al., 1985). In fact, the fish possess very rapid rates of uptake, which may be related in part to high ventilatory rates, they can metabolize PAHs much more efficiently and hence acquire faster elimination rates (Meador et al., 1995). In this work, comparable conditions for fish and oysters were supplied by the different experimental procedures: similar exposure to soluble fraction of hydrocarbons leads to an analogous route of contaminant uptake by diffusion across gills and intestinal or cutaneous teguments and homogenate old and length for each species. Thus, the principal differences between the two species was the exposure time, five days for fish and nine days for oysters, and the differential accumulations of PAHs in function of molecular weight and number of rings. Van der Oost et al. (1994) have shown that the eel, *Anguilla anguilla*, presents a higher proportion of 2- and 3-ring PAHs and less of the 4- and 5-ring compounds. The elimination of higher lipophilic PAHs (3-, 4- and 5-rings) was due to fish metabolism (Varanasi et al., 1985) for which the typical effect is the formation of hydrophobic metabolites which are rapidly excreted (Van der Oost et al., 1994). These differential accumulations of PAHs shown here were in concordance with the accumulation of naphthalene and acenaphthene, both 2-ring molecules, in fish muscle. The oysters, on the other hand, have the highest mean weight percentage of PAHs accumulated which was produced by 3- and 4-ring compounds (Wade et al., 1988), like the pyrene and the

phenanthrene bioaccumulated in this study. In contrast with fish, the accumulation of higher molecular weight compounds in oyster tissues seem to be due to the low capacity of bivalves to metabolize PAHs (Varanasi et al., 1985), whereas the 2- and 3-ring molecules apparently were released directly through the skin and gills without being metabolised (Varanasi et al., 1985). This metabolism discrepancy explains why fish have bioaccumulated naphthalene and acenaphthene, 2-ring compounds, whereas oyster bioaccumulated preferentially phenanthrene and pyrene, respectively 3- and 4-rings. Currently, fish which metabolize efficiently higher PAHs, may have excreted PAHs metabolites at a similar rate to the rate of uptake of parent compounds. Nevertheless, since accumulation of PAHs in fish was directly proportional to the ability of the organisms to metabolize PAHs, the absence of metabolization of lower molecular weight compounds seems to induce an increase of accumulation.

Currently, metabolism, excretion and diffusive loss are processes that can decrease tissue concentrations of parent PAHs. The principal discrepancy between bivalves and fish was the type of elimination. In fact, bivalves favour diffusive loss, the decrease in tissue burden caused by simple diffusion, whereas fish prefer an active excretion, a physiological process that eliminates parent compounds and metabolites through bile or urine (Meador et al., 1995). In addition to excretion type, molecular weight and PAH hydrophobicity induced different rate of elimination (Neff, 1979). All these factors could explain the difference between the time taken to eliminate the molecules in oyster and fish tissues. Indeed, lower molecular weight PAHs can diffuse easily out of organisms through its gills, whereas a PAH with higher molecular weight is not appreciably eliminated through this route (Meador et al., 1995). This indicates that the sea bass could diffuse easily naphthalene and acenaphthene through its gills; nevertheless, a part of these PAHs could be metabolized and excreted later. In the same way, since oysters present an intensive filtration by their gills, phenanthrene and pyrene could be easily eliminated by important diffusive loss. Therefore, a relationship between the type of excretion and the bioaccumulated PAH decline could explain the difference observed between oysters and fish elimination duration.

4.3. PAHs and immunological analysis

In this study, no organism mortality was recorded. Neither internal nor external lesions were observed in exposed animals, probably due to the short exposure time and the controlled external conditions (e.g. filtered water, constant temperature and oxygenation…) which prevent or at least delay secondary infections often observed after immunotoxicity phenomena. The experiment has been designed in order to detect effects of pollutants on innate immune parameters. All experimental conditions were equal in both control and contaminated tanks except for the presence of pollutants. If pollutants were capable to induce some modification of cellular parameters, significant differences between both conditions (control versus contaminated) were expected. Similar temporal trends detected in both control and contaminated organism were thus out of the scope of the study. However, such results indicated that the rearing conditions may be improved.

Variations in cellular composition of blood and haemolymph, as total and differential cell counts, are reported among the first physiological disturbance described in organisms exposed to environmental stressors (Fisher, 1988; Reynaud and Deschaux, 2006). In the two species tested, exposure to 733 $ng.L^{-1}$ of the soluble fraction of HFO did not significantly modulate cellular subpopulation percentages. In fact, in sea bass, no modifications to the classic proportions of granulocytes-monocytes (20 %) and lymphocytes (80 %) (Scapigliati et al., 2003) were observed. And approximately 40 % of oyster haemocytes were granulocytes and 60 % were hyalinocytes, which correspond to classical values reported for Pacific oysters (Hégaret et al., 2003). The principal discrepancy between fish and oyster cellular composition concern the cellular mortality: a significant increase in sea bass leucocyte mortality was observed after the five day exposure to HFO (Day 0), whereas no haemocyte mortality was noted in the invertebrates. This difference was probably due to the PAHs bioaccumulated in organisms. Indeed, the naphthalene, quantified in fish muscle and absent in oyster tissues, is known to cause deleterious membrane-damaging, which induces a decrease in the number of immune cells (Ahmad et al., 2003). In the present work, the liposoluble properties of naphthalene and acenaphthene can put several proposals to explain this increase of leucocyte mortality: 1) the naphthalene could induce damages on cellular membranes, as glycoproteins and proteins alteration, when they are transported through the leucocyte membrane; 2) the penetration of naphthalene in lysosome induced membrane

rupture which induced pH modification in leucocyte and definitely cell destruction (Grundy et al., 1996a).

Moreover, this lysosomal damage could also interfere with phagocytic percentage. In the present work, fish does not present modification of this immune parameter, thus naphthalene impact seems to induce cellular mortality by direct damage on leucocyte membrane. In the opposite, phagocytic percentage was significantly decreased by short-term exposure to 733 $ng.L^{-1}$ of soluble fraction of HFO. This might be correlated with the bioaccumulation of PAHs in oyster tissues at this sampling date, and also particularly of phenanthrene which is known to modify phagocytic activity (Grundy et al., 1996a). These authors suggested that PAHs, or their metabolites to a lesser degree, disrupt lysosomal integrity by altering membrane fluidity which prevents deformation of the membrane essential in the process of phagocytosis (Grundy et al., 1996a). Moreover, the disruption of lysosomal membrane integrity induces an acidification of haemocytes and then an alteration of internal pH of haemocytes. This acidosis affects proteolytic enzymes which may also contribute to the decrease in phagocytic activity (Grundy et al., 1996b). Thus for oysters, as described by Ayffret et al. (2006), among functional parameters phagocytic capacity of haemocytes appears as a possible tool for monitoring pollution.

Concerning innate and humoral immune parameters, the complement of fish is an essential part of the innate immune system which shares some similarities with the ProPO system in invertebrates. Concerning sea bass, a significant increase in haemolytic activity of the alternative pathway was observed in contaminated fish from Day 3 to Day 9 of the recovery period. This result suggests that high amounts of complement components were secreted probably by mononucleated phagocytes which are known to be an important source of these components in mammals (McPhade and Whaley, 1993). This activation of the metabolism of these cells might reflect an inflammatory response probably due to the bioaccumulation of naphthalene and/or acenaphthene whose toxic potential due to their polycyclic aromatic nature was known. This inflammatory phenomenon, already described following PAH contamination (Myers et al., 1998; Stentiford et al., 2003), was associated, when exposure time was extended, by cellular damage favouring an increase in pathologies due to opportunist pathogens. An activation of the alternative pathway to fight these aggressors could be considered (Sakai, 1992). Now, for Pacific oyster, PO activity decreased

after a short exposure to the soluble fraction of HFO and the recovery of this activity was obtained after two weeks. Some other pollutants caused a similar decrease in PO activity *in vitro* such as mercury in the Pacific oyster (Gagnaire et al., 2004) and *in vivo* such as trichlorfon in prawns (Chang et al., 2006).

An *in vivo* system of experimental contamination by HFO was developed and a preliminary validation was carried out. PAHs were detected both in seawater and organisms tissues confirming the efficacy of the developed system. In a second step, effects of HFO were reported on some immune parameters in the European sea bass, *D. labrax*, and in the Pacific oyster, *C. gigas*. *In vivo* contamination with the soluble fraction of HFO cause leucocyte death in fish and affect oyster's phagocytosis. Moreover, the two enzymatic cascades (phenoloxidase activity in bivalves and ACH_{50} in fish) seem to be quite attractive for monitoring pollution by oil. Nevertheless, a recovery period could rapidly recondition immune parameters after this short-time exposure.

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ARTICLE 4: Plasmatic non-specific immune response in European sea bass, *Dicentrarchus labrax* (Linné), after handling stress.

Article en préparation

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Abstract

For experimental condition, the effects of sampling, an acute handling stress condition, should not be neglected because they could mask impacts of other types of factor like contamination, pathogen activities or diet condition. One hundred and sixty sea bass, *Dicentrarchus labrax*, were exposed to two different types of sampling date during 30 days, in order to search effects of sampling on modulation of two innate immune parameters: haemolytic activity based on the alternative complement pathway (ACH_{50}) and the lysozyme concentration. The first treatment corresponds to a nearer sampling and the second treatment to a weekly sampling. The two plasmatic innate responses of fish, which were known to be modulated by stress condition, were studied in the plasmatic peripheral blood fraction by spectrophotometry. Results shown an increase of the ACH_{50} with the nearer sampling when no effect was observed for lysozyme concentration. Thus, for further *in vivo* experimentations with sea bass, a weekly sampling must be used to annihilate handling stress and to analyse effects of other types of factors.

Keywords: Haemolytic activity of alternative complement pathway; Lysozyme concentration; European sea bass; Handling stress.

1. Introduction

Welfare associated in aquaculture was linked with stress response due to prolonged, repeated or unavoidable conditions (Ashley, 2007). Two types of common source of stress were currently observed in aquaculture management: acute (handling, capture, tank cleaning, anaesthesia, air exposure, physical disturbance, transport) (Demers and Bayne, 1997; Möck and Peters, 1990; Ortuño et al., 2002a; Varsamos et al., 2006) or chronic (crowding, confinement, diet, environmental variations) (Caruso et al., 2005; Pulsford et al., 1995; Rotllant et al., 1997; Snieszko, 1974; Tort et al., 1996a; Vazzana et al., 2002) stress procedures. These stress conditions could modified some biological function at long-term period such as growth, reproductive function, hydromineral and energy balance (Wendelaar Bonga, 1997), disease resistance (Snieszko, 1974; Varsamos et al., 2006) and immune function (Rotllant et al., 1997; Varsamos et al., 2006).

Like in mammals, the immune system of fish is constituted of innate and specific immune responses. The innate immune system is the first line of defence and it is primordial in fish due to the relative deficiency of specific immune system, its evolutionary status and poikilotherme nature. It is also able to stimulate the specific immune system and maintain homeostasis. The defence mechanisms of fish, specific and innate immune responses, were modified by profound and diverse effects due to tertiary responses of stressors (Wendelaar Bonga, 1997). These tertiary responses occur after immediate and long-term actions of endocrine hormones (catecholamine and corticosteroids). Many authors search effects of stress condition on cellular parameters such as circulating leucocyte and erythrocyte numbers (Tort et al., 1996b), haematocrit content and haemagglutinating of immune cell (Caruso et al., 2005), phagocytosis activity and respiratory burst of macrophages and granulocytes (Ortuño et al., 2001). The humoral innate activity was quite investigated too, such as haemolytic activity of alternative complement pathway (ACH_{50}), lysozyme concentration, total immunoglobuline concentrations (Rotllant et al., 1997) or total protein concentrations (Vazzana et al., 2002; Yin et al., 1995). A general consensus considers the lysozyme concentration and the ACH_{50}, two plasmatic innate immune parameters, as sensitive indicators of stress conditions (Caruso et al., 2005; Tort et al., 1996b). In fact, these two enzymatic activities were described to be modulated by many types of acute and chronic

stressor like crowding (Ortuño et al., 2002a; Yin et al., 1995), confinement (Ruane et al., 1999), handling (Demers and Bayne, 1997; Rotllant et al., 1997), regime modification (Holland and Lambris, 2002) and anaesthesia treatment (Ortuño et al., 2002b; Ortuño et al., 2002c).

Due to the action of stressor conditions, as handling stress linked to sampling dates, innate immune parameters could be severely modified. Moreover, variation in immune responses due to stressful situations could mask impacts of other types of experimental factors like contamination, pathogen activities or diet condition. Thus, it seems to be important to determine the effects of sampling on immune parameters before beginning experimentations. The aim of the present work was to define the *in vivo* impacts of selected sampling dates on European sea bass, *Dicentrarchus labrax*, plasmatic immune activities. Two types of treatment were used: a nearer sampling and a weekly sampling. The lysozyme concentration and the ACH_{50} were studied in the plasmatic peripheral blood fraction by spectrophotometry.

2. Materials and methods

Fig. 1: Schematic representation of the experimental setup for the two types of treatment used. Treatment 1 corresponds to a nearly sampling date and treatment 2 to a weekly sampling date.

2.1. Sea bass

One hundred and sixty European sea bass, *Dicentrarchus labrax*, 41.6 ± 0.6 g and 14.9 ± 0.5 cm, came from one pond and were divided into four groups of 40 fish in 300 L tanks in May 2006. Then, they were acclimatized for two weeks in these tanks with a flow of 0.1 $m^3.h^{-1}$at 17.6 ± 0.2°C (dissolved oxygen 95 ± 1 %, pH 7.9 ± 0.1, salinity 36 ± 1 ‰, free of nitrate and nitrite). Sea bass were fed daily with dry commercial pellets at 2 % body weight (Grower Extrude Natura 3.5mm, Le Gouessant Aquaculture).

2.2. Experimental treatment and sampling

In order to account for possible tank effects, experiments were conducted in duplicate tanks. The experimental system was constituted of four tanks, two tanks for near sampling (D0, D1, D3, D9, D14 and D30) and two tanks for weekly sampling (D0, D7, D14 and D30) (Fig. 1). At each sampling date, five fish by tanks were randomly captured and sampled.

To obtain blood samples, fish were anaesthetised with phenoxy-2-ethanol (Merck). For each fish, 1 mL of peripheral blood was withdrawn from the caudal vein with a lithium heparinized vacutainer (BD Vacutainer™ LH 85 U.I.). Blood samples were centrifuged (1 200g, 10 min, 4 °C). Then, plasma of each fish was split into two aliquots of 200 µL. These aliquots were kept on ice until further analysis.

2.3. Analytical procedures

Plasma lysozyme activity was determined using a turbidimetric assay (Grinde et al., 1988), adapted to microtitration plates. Briefly, a bacterial suspension of *Micrococcus lysodeikticus* (Sigma) was prepared at a concentration of 1.25 $g.L^{-1}$ in a 0.05 M sodium-phosphate buffer pH 6.2. Fifty µL of the individual plasma samples were plated in 96 well microtitration plates. The reaction was initiated in a multiscan spectrophotometer, by addition of 160 $\mu L.well^{-1}$ of *M. lysodeikticus* suspension using an automatic dispenser. Reading of D.O. at a wavelength of 450 nm were performed every 15 s for 3 min, the plate being shaken before each reading. Using a standard hen egg white lysozyme (Sigma) in sodium-phosphate buffer, the concentration of lysozyme in sea bass plasma was expressed in $mg.L^{-1}$.

The alternative pathway of plasma complement activity was carried out by a haemolytic assays with rabbit red blood cells (RRC, Biomérieux) as described by Yano (1992) and adapted to microtitration plates. Sea bass samples, diluted at 1/64 in EGTA-Mg-GVB buffer to avoid natural haemolytic activity, was added in increasing amounts, from 10 to 90 µL.wells^{-1}, and was filled with EGTA-Mg-GVB buffer to get a final volume of 100 µL. Fifty µL of 2% RRC (Biomérieux) suspension were finally added in all wells. Control values of 0 and 100 % haemolysis were obtained using: 100 µL of EGTA-Mg-GVB buffer and 100 µL of non-decomplemented trout haemolytic serum at 1/50 in ultrapure water, respectively. Samples were incubated during one hour at 20°C. The microplates were centrifuged (400g, 5min, 4°C, Jouan). Then, 75 µL of supernatant of each well were deposed with 75 µL of phosphate buffer saline (PBS, Sigma) into an other 96-well microplate. The absorbance (A_{540}) was read and the number of ACH_{50} units per mL of plasma was determined in reference to the 50 % haemolysis.

2.4. Statistical analysis

Statistical tests were carried out using XLStat 2008. Verification of normality was conducted using the Anderson-Darling test. Since the values were normal, a one factor ANOVA test was used to observe modulation of each plasmatic parameter at each sampling date. Significantly different groups were finally identified using a Student-Newman-Keuls test for normal values. P values lower than 0.05 were used to identify significant differences.

3. Results

No sea bass mortality was observed and the physico-chemical parameters of the seawater was the same during the course of the experiment (dissolved oxygen 95 ± 1 %, pH 7.9 ± 0.1, salinity 36 ± 1 ‰, temperature 17.6 ± 0.2 C, free of nitrate and nitrite).

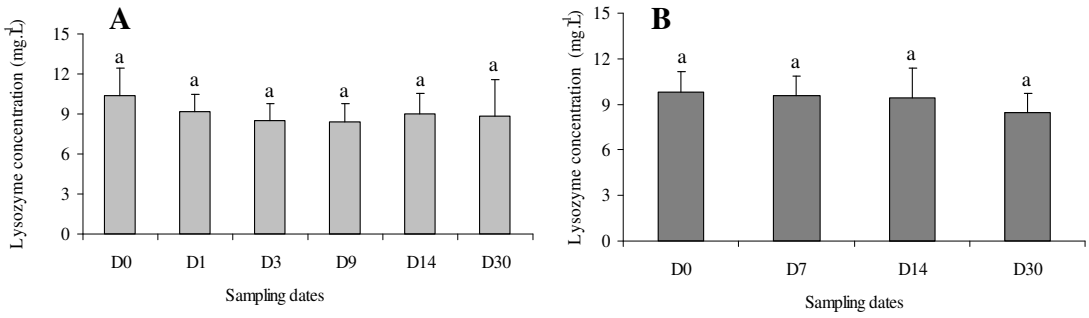

Fig. 2: Plasmatic lysozyme concentration ($mg.L^{-1}$) evaluated by spectrophotometry following a handling stress due to two types of sampling used: treatment 1 (nearly sampling, A) and treatment 2 (weekly sampling, B). Values are the mean of ten fish. The bars represent the standard error. Different letters denote statistically significant differences between the groups (explained in the text).

3.1. Effects of handling stress on plasmatic lysozyme concentration

At the first day of sampling (Day 0), similar results were shown between the two types of treatment with a mean lysozyme concentration of 10.0 ± 0.9 $mg.L^{-1}$. Then, letter (a) was assigned at the Day 0 of each treatment (Fig. 2).

Moreover, no significant difference was shown for lysozyme concentration after the handling stress due to near sampling (treatment 1) and weekly sampling (treatment 2), then similar letter (a) was assigned at each sampling dates.

The mean lysozyme concentration obtained for each treatment and date was 9.2 ± 2.7 $mg.L^{-1}$.

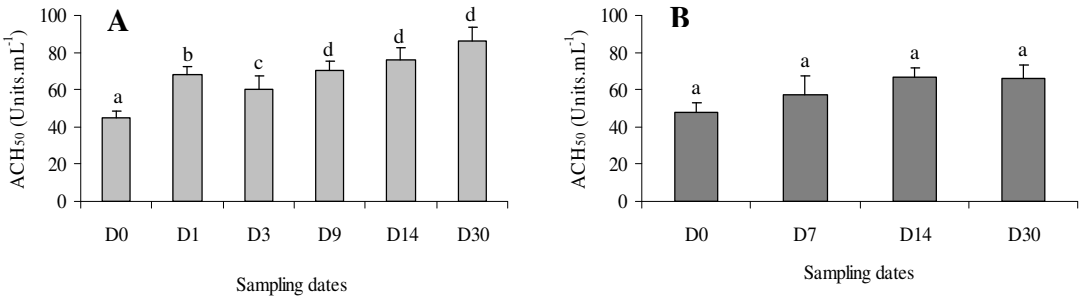

Fig. 3: Haemolytic alternative complement activity (ACH_{50}, $Unit.mL^{-1}$) evaluated by spectrophotometry following a handling stress due to two types of sampling used: treatment 1 (nearly sampling, **A**) and treatment 2 (weekly sampling, **B**). Values are the mean of ten fish. The bars represent the standard error. Different letters denote statistically significant differences between the groups (explained in the text).

3.2. Effects of handling stress on haemolytic alternative complement activity (ACH_{50})

The letter (a) was assigned at the Day 0 of the two treatments due to obtainment of similar results between the two types of treatment with a mean ACH_{50} of 48.8 ± 7.3 Units.mL^{-1} (Fig. 3).

Concerning the nearly treatment (treatment 1), for each sampling date a significant increase of ACH_{50} was shown compared to the first day of sampling (Day 0). The ACH_{50} was higher at Day 1 (67.9 ± 4.3 Units.mL^{-1}) compared to Day 3 (60.3 ± 7.1 Units.mL^{-1}), these results were present by dissimilar letters (b for D1 and c for D3). Then, higher values were observed from Day 9 to Day 30 with no significant difference between these dates, and then letter (d) was given at these sampling dates. The mean value obtain from Day 9 to Day 30 was 77.5 ± 4.7 Units.mL^{-1}.

In the opposite, for the weekly treatment (treatment 2), no significant difference was shown for ACH_{50} after the handling stress with a mean ACH_{50} of 66.9 ± 4.3 Units.mL^{-1}, then similar letter (a) was assigned at each sampling date of treatment 2.

4. Discussion

In the present study, the absence of fish mortality suggest that the experimental condition used underwent a normal and not harmful stress condition which could be overcome by physiological mechanisms or which were nor critical enough to induce negative consequence on fish metabolism. Nevertheless, stressful conditions induce primary response, such as the release of corticosteroid and catecholamines, which induce changes in the immune system (Wendelaar Bonga, 1997). In the present work, lysozyme concentration and haemolytic activity of alternative complement pathway (ACH_{50}) were used as indicators of immunomodulation induced by sampling date. Since marked individual variability is commonly noted for immune parameters as lysozyme concentration (Demers and Bayne, 1997) and ACH_{50} (Ortuño et al., 2002a), repeat analysis by replicates and important numbers of animals were taken. By doing so, results obtain show significant changes in the immune indicators measured.

4.1. Plasmatic lysozyme concentration

Both the strength of the stressor and the condition of the fish determine whether the lysozyme component of the innate immune system will be activated or suppressed under stress (Möck and Peters, 1990). Regarding lysozyme concentration, no significant difference was observed between each tank, treatment and sampling date, with a mean level (9.13 ± 2.73 mg.L^{-1}) which correspond roughly to concentrations obtained in unstressed European sea bass, *Dicentrarchus labrax* (Bado-Nilles et al., submitted; Caruso et al., 2005). Therefore, lysozyme concentration do not seem to be impacted by handling stress. Usually, the decrease in plasma of lysozyme concentration observed during chronic stress conditions (Caruso et al., 2005; Ruane et al., 1999; Yin et al., 1995) was the result of two processes: i) an increase of other immune defence mechanisms due to stress period (Möck and Peters, 1990; Rotllant et al., 1997); ii) some differences in the responses of the pituitary-interrenal axis as the higher cortisol response (Ruane et al., 1999).

4.2. Haemolytic activity of alternative complement pathway

The response of complement system is a rapid and sensitive indicator of immune parameters which is interesting to use for chronic and repeated acute stress conditions (Tort et al., 1996a; Tort et al., 1996b). In the present work, at the first day of the experiment (Day 0), similar results were obtained between each tank and treatment with a mean value for ACH_{50} of 48.8 ± 7.3 Units.mL^{-1}. This mean value corresponds roughly to concentrations obtained in unstressed sea bass (Bado-Nilles et al., submitted; Caruso et al., 2005). In the opposite, concerning the nearly treatment, a significant increase was observed from Day 1 to Day 30 due to repeated stressful conditions. Ruane et al. (1999) observed, after confinement condition, that the ACH_{50} activation was due to differences in the responses of the pituitary-interrenal axis. Moreover, this increase corresponds to a part of the adaptive effect of the immune system preparing the animal to react to infection that could occur during the stressful period (Demers and Bayne, 1997; Ruane et al., 1999). Furthermore, a kind of oscillation was observed from Day 1 to Day 9, which seems to be explained by down-regulation mechanisms and its bi-directional features between the endocrine and immune systems (Ortuño et al., 2001; Tort et al., 1996a). In fact, an overcompensation response was often found in biological process until re-equilibrium (Ortuño et al., 2001; Tort

et al., 1996a). From Day 9 to Day 30 no significant difference was detected, probably explainable by the space between each sampling (more than five days). In fact some authors observed that control values were reached after four days post-stress (Ortuño et al., 2001; Ortuño et al., 2002a). Likewise, weekly sampling induced no significant difference between each sampling date and tank; certainly explain by the same hypothesis.

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ARTICLE 5: The health of aquatic organisms after a spill: a new experimental system.

Article en préparation

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Abstract

Oil and chemical spills at sea are considered as major factor of stress for marine organisms. In order to study the biological effects of such events in controlled conditions, an experimental system was implemented in the *Cedre*. Two types of organism were exposed to the soluble fraction of oil during one week: an invertebrate, the Pacific oyster, *Crassostrea gigas*, and a vertebrate, the Sea bass, *Dicentrarchus labrax*. All physico-chemical parameters were stable and the contaminant concentration was constant in the exposure tanks. Then, a recovery period of two weeks was launched. To validate this experimental equipment, bioaccumulation of PAHs in the tissues and effect biomarkers were measured. For oysters, three PAHs out of twelve detected in the seawater were bioaccumulated at the end of the contamination period and, for fish, only two out of twelve. Although bioaccumulation of PAHs in contaminated organisms decreased during the recovery period, they were always detected at the end of the recovery period (14 days). Biliary metabolites, a physiological parameter, and the alternative complement pathway, an immunological parameter, were significantly increased in exposed sea bass after one week of exposure, and they were always higher after the recovery period. The phenoloxidase activity, a haemolymphatic parameter, was significantly decreased in the exposed oysters, both after the exposure period and the recovery period. This new experimental system can reproduce a realistic and reproducible pollution after a spill and allows to perform impact studies on organisms.

Key words: Spill; Experimental system; *Crassostrea gigas*; *Dicentrarchus labrax*; Phenoloxidase activity; Complement activity; Biliary metabolites; Bioaccumulation.

1. Introduction

During the last decades, many oil spills were observed in the Atlantic coast. The 20th century was noted by the *Erika* tanker accident which spread out 20 000 tonnes of its Heavy Fuel Oil (HFO) into the sea. The last major pollution in the Atlantic coast was the *Prestige* accident in November 2002 with a freight of 63 000 tonnes of HFO. In the immediate aftermath of the spill, damages attributed to oil were measured in several species of bivalves (Bocquené et al., 2004; Cajaraville et al., 2006) and fish (Budzinski et al., 2004; Morales-Caselles et al., 2006). In case of oil spills, hydrocarbons are considered as a major factor of stress for marine organisms via their mutagenic, carcinogenic, phototoxic (Shiaris, 1989), reprotoxic (Diamond et al., 1995) and immunotoxic (Yamaguchi et al., 1996) properties. For these reasons many institutes perform research which deals with the effects of oil on physiological, immunological, histological and genetical parameters of some aquatic organisms. In addition, some studies were performed after a chemical spill to evaluate the impact of released compounds, like those made after the sinking of the *Ievoli Sun* (2000).

Nevertheless, the evaluation of effects of crude oil, refined petroleum products and other hazardous noxious substances (HNS) represent a significant challenge, first, due to their behaviour in marine environment (evaporation and dissolution processes, emulsification…), and secondly, due to their chemical composition (the oil is a mixture of potentially several thousands of chemical molecules). These molecules cover a wide range of physico-chemical behaviour which creates a dynamic system multiphase. Some authors proposed experimental systems for testing chronic and / or accidental toxicities of complex mixtures of substances poorly soluble in water. Cohen et al (2001a) and (2001b) used a chemically dispersed crude oil which was mixed with seawater before being delivered to the exposure tanks by a peristaltic pump. This type of protocol allows getting a homogenised contamination of the seawater in each place of the exposure tank. Nevertheless, the use of dispersant is not representative of what occurs naturally after an accidental spill. Duesterloh et al. (2002) used a protocol which allows exposing marine organisms to the water-soluble fraction (WSF) of oil only. The system used is made of a column filled with glass beads beforehand covered in oil. The WSF is obtained by a flow of seawater which goes through the column before being supplied to the exposure tank. If with this system organisms are not

exposed directly to the slick, the chemical composition of the WSF is not typical of the experience obtained during the behavioural of an oil spill at sea. In fact, the WSF contains compounds that normally should not be present in the water column due to the evaporation process. In addition, they took out the monocyclic aromatic compounds of the oil before they soaked the glass beads. However, in the environment, these compounds are able to solubilize in water and then to impact marine organisms. Finally, Baussant et al. (2001a) designed a continuous flow system (CFS) where oil is added mechanically to sea water in a dark cylinder before being supplied to the exposure tank. In this cylinder, a propeller, able to rotate at a speed of approximately 250 rpm, allows to obtain oil droplets in suspension (average 10 µm in diameter). In that case, all compounds of the oil are solubilised, including the light ones which normally should be evaporated. In addition, emulsification process can occur due to the mechanical energy applied in the chamber, involving a decrease of the dissolution rate for some compounds. Moreover, few oil droplets are observed in the marine environment after a spill without the use of dispersants, except where a strong storm took place.

Therefore, this paper presents an experimental system enabling the exposure of aquatic organisms to a stable dissolved fraction of hydrophobic xenobiotics without droplets and in controlled conditions (pH, temperature, dissolved oxygen, photoperiod …). This tool, present in the *Cedre* at Brest (France) can reproduce in a realistic way the behaviour of oil released at the sea surface. The dissolved fraction obtained is compared to the water accommodation fraction (WAF) made with the same oil in order to characterise chemically the pollutant which will be used for the organisms exposure, notably the impact of the evaporation process on the chemical composition of the soluble fraction of the oil. The validation of this system is achieved by performing a short term exposure of marine invertebrates and vertebrates followed by a recovery period. The level of the contamination is determined by bioaccumulation and biomarkers analysis.

2. Materials and methods

An equipment dedicated for the evaluation of environmental impact of pollutant, was built in a greenhouse thermoregulated (T = 14 ± 1 °C) and with one total renewal of the air in six hours.

2.1. Exposure system

The system was designed in order to perform a controlled pollution by chemical or organic pollutants and this independently of the product solubility. This exposure system was adapted from the Anderson method (Anderson et al., 1974) and modified by the *Cedre* to obtain, during all the experimentation, a constant concentration in the exposure tanks. This experimental system consists of six similar and independent units themselves composed of (Fig. 1):

- One rectangular mixing tank in stainless steel,
- One cylindrical exposure tank in height density polyethylene (HDPE),
- One degassing column.

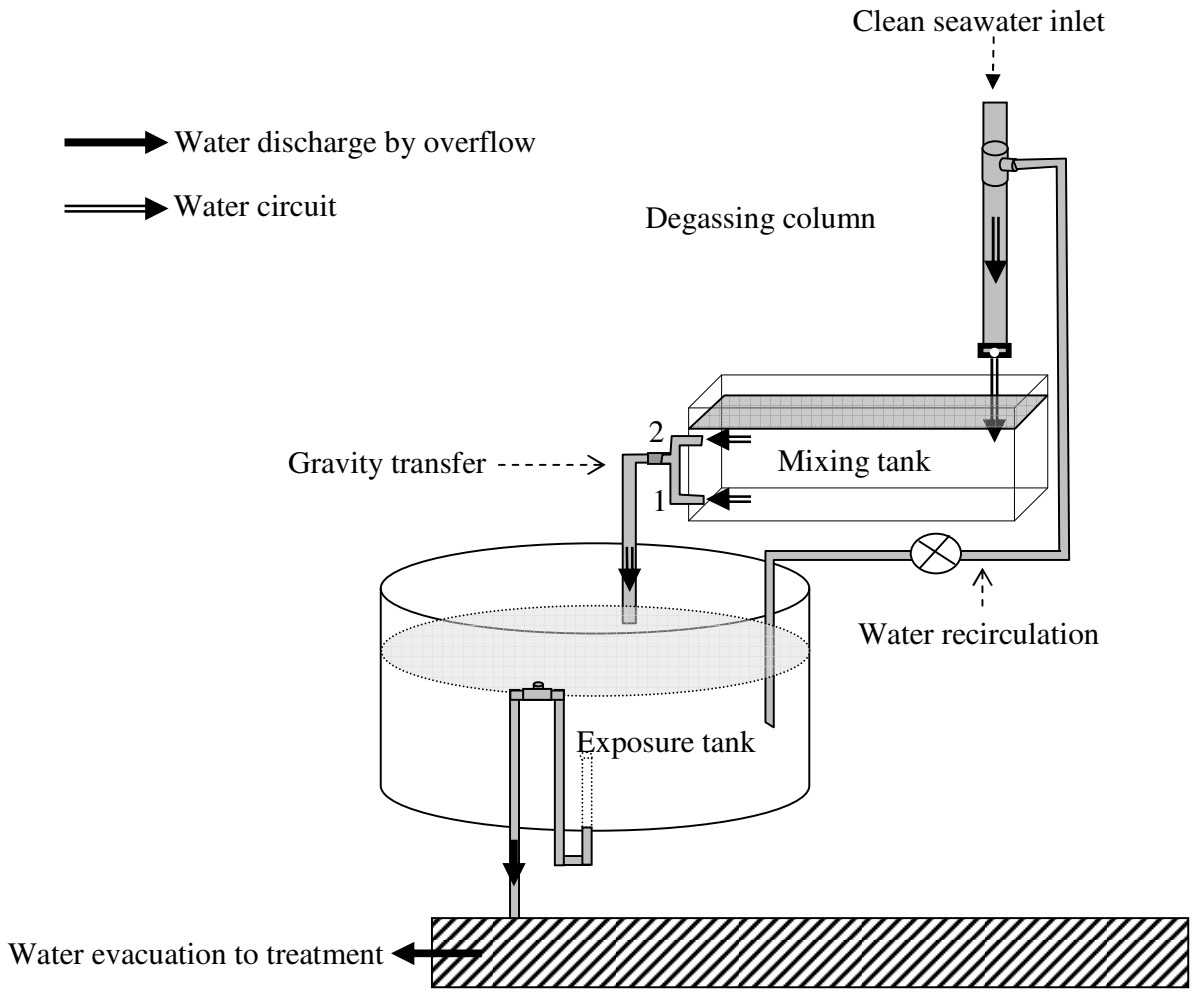

Fig. 1: Descriptive scheme of one unit coming from the experimental system present at the *Cedre* and which was composed about six units.

2.2.1. The mixing tanks

In the mixing tank of 316 L, the pollutant is in contact with the aqueous phase (Fig. 2). Xenobiotics are placed into the first compartment. The partitions of this tank prevent small oil slicks and suspended droplets to be flushed by the seawater flow.

The density of the pollutants used in this mixing tank can be either higher or lower than the seawater (respectively floating and sinking products). When floating products are used, only the orifice (1) is opened, when sinking products are used the orifice (2) is opened. In both cases the water flows out to the exposure tanks by gravity through strengthen ringed pipe, which resist to dissolved pollutants.

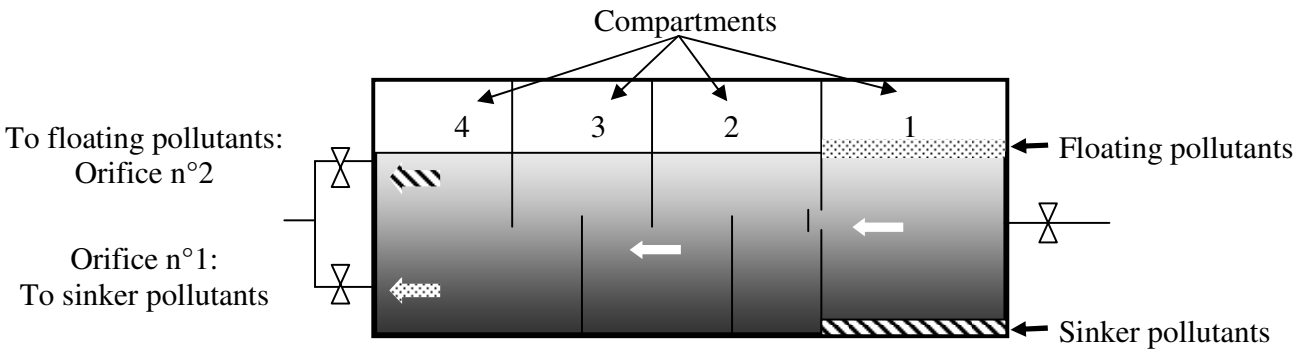

Fig. 2: Descriptive scheme of one mixing tank.

2.2.2. The exposure tanks

The exposure tanks (300 L), which received organisms, are cylindrical and they are connected to a system of polluted water recuperation by overflow. During the exposure period, a second circulation of sea water is added in order to prevent any decreasing in the concentration of dissolved pollutants. Water is pumped at flow rate of 0.5 $m^3.h^{-1}$ into the exposure tanks and goes through a degassing column before being sent into the mixing tanks. The oxygen saturation of water in each tank is maintained by a compressor, which injects air *via* an air stone (Fig. 2).

2.2.3. The degassing columns

A degassing column is installed between both tanks (exposure and mixing) in order to favour the gaseous exchange by percolation of sea water. During the percolation process, the water is oxygenated (oxygen transfer from air to water) and the level of dissolved carbon dioxide produced by organisms is reduced (carbon dioxide transfer from water to air). The degassing column is constituted by a 1 m high pipe, with a diameter of 20 cm, which is filled with 20 kg of glass beads (diameter of 6 mm). A gas extractor equipped with an activated carbon filter is connected to this column in order to prevent any releases of chemical compounds in the atmosphere (Fig. 2).

2.2. Physico-chemical validation of the system

A study on the chemical behaviour of the oil in the system was performed in order to characterise the level and the composition of the dissolved fraction in the exposure tanks.

Three polluted and three control tanks were prepared to carry out this test. This validation was performed only with the system in the configuration 1, it means for a floating pollutant.

2.2.1. Seawater quality

In all tanks, seawater was sampled daily to measure physico-chemical (temperature, dissolved oxygen, pH, salinity, suspension particles, ammonium, nitrates, nitrites, ortho-phosphates) and bacteriological (Coliforms, *Escherichia coli*, Salmonelles, *Vibrio parahaemolyticus*) parameters.

2.2.2. Pollutant

A heavy fuel oil (HFO), similar to that of the Erika, was selected to perform the validation of the system.

2.2.2.1.Chemical characterisation of heavy fuel oil (HFO)

The HFO used consisted of 24-25% of saturate hydrocarbons, 54-55% of aromatic hydrocarbons and 20-21% of polar compounds. The GC-MS characterisation has shown that this oil contained among others the 16 polycyclic aromatic hydrocarbons (PAHs) of US-EPA list which are benzo[*a*]anthracene, naphtalene, acenaphtylene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[*1,2,3-c,d*]pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene at concentrations ranging from 1 936.9 ± 116.6 $\mu g.g^{-1}$ for phenanthrene to 27.6 ± 5.7 $\mu g.g^{-1}$ for dibenz[*a,h*]anthracene (Table 1).

Name of PAH compounds	Number of aromatic cycles	Molecular weight ($g.mol^{-1}$)	Concentration in HFO ($\mu g.g^{-1} \pm$ SE)	Solubility ($mg.L^{-1}$ at 25 °C)	Vapour pressure (Pa at 25 °C)
Naphthalene	2	128,2	687 ± 89	31.8	10.5
Acenaphthene	2	154,2	273 ± 14	3.7	0.36
Acenaphthylene	2	152,2	51 ± 3	-	-
Fluorene	2	166,2	396 ± 20	1.98	0.09
Phenanthrene	3	178,2	1937 ± 116	1.2	0.091
Anthracene	3	178,2	214 ± 28	1.29	0.055
Fluoranthene	3	202,3	125 ± 14	0.26	0.0012
Pyrene	4	202,3	516 ± 57	0.13	3.0
Benz[*a*]anthracene	4	228,3	213 ± 13	0.01	-
Chrysene	4	228,3	465 ± 14	2.10^{-3}	$8.4 . 10^{-5}$
Benzo[*b*+*k*]fluoranthene	4	252,3	81 ± 11	-	-
Benzo[*a*]pyrene	5	252,3	168 ± 7	$3 . 10^{-3}$	$7.3 . 10^{-7}$
Indeno[*1,2,3-c,d*]pyrene	5	276,3	17 ± 3	$6.2 . 10^{-2}$	$1.3 . 10^{-8}$
Dibenz[*a,h*]anthracene	5	278,4	28 ± 5	$0.5 . 10^{-3}$	$1.3 . 10^{-8}$
Benzo[*g,h,i*]perylene	6	276,3	48 ± 3	$2.6 . 10^{-4}$	$1.4 . 10^{-8}$

Table 1: Concentration, solubility and vapour pressure of 16 priority PAHs of the US-EPA list in the heavy fuel oil used during the exposure period. PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in $\mu g.g^{-1}$ (n = 3, mean ± standard error).

2.2.2.2.Characterisation of the dissolved fraction of the HFO

In order to accurately characterize the soluble fraction of the heavy fuel oil, a water accommodated fraction (WAF) was prepared by stirring HFO (3.2 g) with seawater (1.6 L) in a Schott Glas bottle (Bioblock) with minimum headspace (20%) for 24 h, 72 h, 120 h and 168 h at room temperature (20°C). The vortex speed was set to adjusting at 200 turn.min^{-1} to increase the total petroleum hydrocarbon (TPH) content of the water column. The mixture was then allowed to settle down for one hour to separate water and oil phases. The water phase, which constituted the WAF, was isolated *via* a tap located at the bottom of the mixing bottle and transferred to a clean glass bottle for analysis by using the SBSE technique (Stir Bar Sorptive Extraction) and thermal desorption GC-MS according to the protocol described by Roy et al. (2005). For the whole target molecules, the limits of detection of the SBSE technique are, at least, below or close to 1 ng.L^{-1}. This technique was used to identify the compounds contained in the oil that can dissolve without taking into account others processes which characterise the behaviour of a product at sea like the evaporation process.

2.2.3. Determination of the level of dissolved PAH in the experimental system

After the two first weeks of contact between HFO and seawater in the mixing tanks of the contamination system, 1 L of seawater was taken in heated (500 °C) Duran glass bottles (Bioblock) at different dates (Day -7, Day -5, Day -3 and Day 0) to determine the PAH contents in each exposure tanks (3, 4 and 5) (Fig. 3). Samples were extracted with 30 mL of dichloromethane pestipur quality. After separation of the organic and aqueous phases, water was extracted two additional times by the same volume of dichloromethane (2 X 30 mL). The combined extracts were purified and treated using gas chromatography coupled with mass spectrometry (GC-MS, Hewlett Packard HP5890 coupled with an HP5972 mass selective detector) following published procedures (Douglas et al., 1992).

2.3. Biological validation of the system

Two types of organisms were used to validate the exposure system: an invertebrate, the Pacific oyster, *Crassostrea gigas*, and a vertebrate, the European sea bass, *Dicentrarchus labrax*. In order to evaluate the effects of the dissolved fraction of the HFO, the animals were

divided into six groups: three control groups and three exposed groups. The seawater quality and the PAH concentrations were measured as described in the case of the physico-chemical validation.

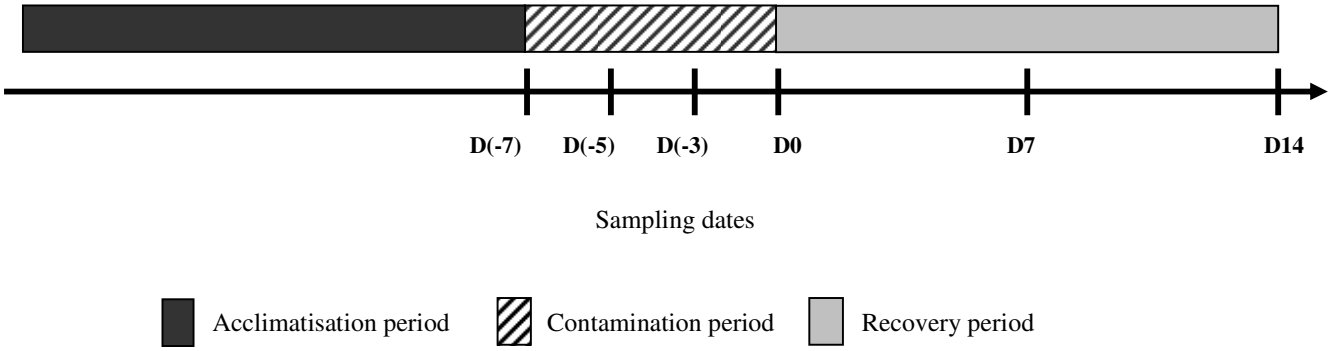

Fig. 3: Schematic representation of experiment design and of each sampling date for the two types of experimentation (without and with organisms).

2.3.1. Organisms collection

Pacific oysters, *Crassostrea gigas*, 8-10 cm in shell length, were purchased from a shellfish farm located in the Brest bay (Brittany, France) in May 2005. European sea bass, *Dicentrarchus labrax*, 80 ± 23 g and 20 ± 2 cm, were purchased from Afssa site de Ploufragan-Plouzané located in Plouzané (France) in May 2005. All fish are originated from the same source. Organisms sustain a natural light / dark cycle during all the experimentation.

2.3.2. Experimental design and viability of organisms

During the three weeks of experimentation, dead organisms and macroscopically lesions were counted.

2.3.2.1. Acclimatisation period

Oysters were acclimatised during three days and fish during two weeks in 1 200 L tanks with a flow of 0.3 $m^3.h^{-1}$ at 18.6 ± 0.1°C (dissolved oxygen 91.6 ± 0.4 %, pH 8.2 ± 0.1, salinity 36.1 ± 2.0 ‰, free of nitrate and nitrite).

The oysters were fed every three days with 15 L of *Isochrysis galbana* at 4.10^6 cell.mL^{-1} provided by the international aquarium Océanopolis (Brest, France) in order to maintain a minimum concentration of 15.10^4 cell.mL^{-1} which is enough to ensure a continuous feeding (Rico-Villa et al., 2006). Sea bass were fed every day with 150 g of granulates (Grower Extrude Natura 4mm, Le Gouessant Aquaculture).

2.3.2.2. Contamination period

After the acclimatisation period, 40 oysters and 20 fish were placed together in each control tank while 40 oysters were exposed with 20 fish in each contaminated tank. During the seven days of contamination period, control and exposed animals were not fed.

2.3.2.3. Recovery period

After the contamination period, the mixing tank was disconnected and fresh seawater was flowed at a rate of 0.3 m^3.h^{-1}. Oysters and sea bass were put back in the same condition as during the contamination exposure period but without exposure. During the two weeks recovery period, the organisms were fed again.

2.3.2.4. Samples and sampling dates

Five oysters and five sea bass were collected, in each exposure tanks, on the first (Day (-7)) and on the last (Day 0) days of the contamination period and after seven and 14 days of recovery (respectively Day 7 and Day 14) (Fig. 3).

2.3.3. PAH bioaccumulations in organisms tissues

2.3.3.1. Oysters

Whole tissues of the 15 control and 15 contaminated oysters were collected and frozen at – 80 °C until analysis. The PAHs concentrations were determined by GC-MS using the procedure of Baumard et al. (1997) with some modifications. Prior to extraction, the whole tissue was homogenized using an Ultraturax (Janke & Kunkel, IKA®-Labortechnik). The 150 µL of predeuterated internal standards (CUS-7249, Ultra Scientific, Analytical solutions) were added to 3 g of homogenized whole tissue and the sample was digested for

4 h under reflux in 50 mL of an ethanolic solution of potassium hydroxide (2 mol.L^{-1}, Fisher Chemicals). After cooling, decantation and addition of 20 mL of demineralised water, the digest was extracted in a 250 mL funnel two times with 20 mL of pentane (SDS). The extract was evaporated with a Turbo Vap 500 concentrator (Zyman, Hopkinton, MA, USA, at 880 mbar and 50 °C) to obtain 1 mL of concentrated extract. The purification of the extract was performed by transferred to a silica column (5 g of silica). The hydrocarbons were eluted with 50 mL of pentane/dichloromethane (80/20, v/v, SDS) and concentrated to 200 µL by means of a Turbo Vap 500 concentrator (Zyman, 880 mbar, 50°C). Aromatic compounds were analyzed by GC-MS and PAHs were quantified relative to the predeuterated internal standards introduced at the beginning of the sample preparation procedure.

2.3.3.2. Fish

Sixteen g of muscle of the 15 control and contaminated fish were collected and frozen at - 80°C until analyses. The PAHs concentrations were determined by GC-MS using the procedure of Baumard et al. (1997) as described for oysters.

2.3.4. Use of biomarkers to validated the exposure of organisms to pollutant

2.3.4.1. Oysters

After opening the oyster shell by cutting off the adductor muscle, approximately 2 mL of haemolymph was withdrawn from the pericardial cavity using a 2 mL syringe equipped with a 0.7 X 30 mm needle. The haemolymph samples were kept on ice until they were processed to reduce haemocyte aggregation (Auffret and Oubella, 1997) and three pooled samples, each composed of haemolymph from five oysters, were formed to obtain a sufficient volume of haemolymph. The three pooled haemolymph samples were centrifuged (260 g, 10 min, 4 °C). The acellular fraction (supernatant) was frozen at - 80 °C for further analysis.

Detection of phenoloxidase (PO) activity in acellular fraction samples was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-Dopa, Sigma) transformation in dopachromes as described previously by Gagnaire et al. (2004). The products used to determine the PO activity where purchased from Sigma. Samples were distributed in 96-well

microplates (Nunc, France). Modulators of this enzymatic activity were used to confirm the specificity of the detected activity. The purified trypsin TPCK (*N*-Tosyl-L-phenylalanine chloromethyl ketone, 1 $g.L^{-1}$) was used as an activator of PO activity and the β-2-mercaptoethanol (10 mM) was tested as an inhibitor. To determine the PO activity of all samples, 80 µL of cacodylate buffer (CAC buffer: sodium cacodylate (10 mM), trisodium citrate (100 mM), NaCl (0.45 M), $CaCl_2$ (10 mM), $MgCl_2$ (26 mM), pH 7.0), 20 µL of L-Dopa (3 $mg.mL^{-1}$) and 20 µL of samples were added in each well. To measure the PO activity modulation, 60 µL of CAC buffer, 20 µL of PO activity modulators, 20 µL of L-Dopa and 20 µL of samples were added in each well. Control (120 µL of CAC buffer) and negative control (100 µL of CAC buffer, 20 µL of L-Dopa) wells were used to determine respectively purity of buffer and autooxidation capacities of L-Dopa. Each sample was tested in triplicate wells and A_{490} was measured after 21 h incubation period at room temperature.

2.3.4.2. Fish

Fish were anaesthetised with phenoxy-2-ethanol. For each fish, 1-2 mL of peripheral blood were withdrawn from the caudal vein with a lithium heparinized vacutainer (BD Vacutainer™ LH 85 U.I.). Blood samples were centrifuged (1 200 g, 10 min, 4 °C, Jouan) and the plasma was frozen at - 80 °C to analyse the alternative pathway of plasma complement activities (ACH_{50}).

After blood collection, the gall bladder was excised, weighted and stored at –80°C for later analyses. One microlitre of bile samples was diluted in 4 mL of absolute ethanol (VWR International). Fluorescence analyses of bile samples were performed with a spectropfluorimeter (Fluorimeter 10-AU, Turner Designs Sunyvale, California). The excitation and emission wavelength used to determine the fluorescence intensity of bile samples by Fixed Fluorescence (FF) was 341/383 nm. This couple of wavelength corresponds to four-rings PAH compounds, pyrene metabolites (Aas et al., 1998).

The alternative pathway of plasma complement activity was carried out by a haemolytic assays with rabbit red blood cells (RRC, Biomérieux) as described by Yano (1992) and adapted to microtitration plates. Sea bass plasma, diluted at 1/64 in EGTA-Mg-GVB buffer to avoid natural haemolytic activity, was added in increasing amounts, from 10 to 90 $\mu L.wells^{-1}$, and was filled with EGTA-Mg-GVB buffer to get a final volume of 100 µL.

Fifty µL of 2 % RRC (Biomérieux) suspension were finally added in all wells. Control values of 0 and 100 % haemolysis were obtained using: 100 µL of EGTA-Mg-GVB buffer and 100 µL of non-decomplemented trout haemolytic serum at 1/50 in ultrapure water, respectively. Samples were incubated during one hour at 20 °C. The microplates were centrifuged (400 g, 5 min, 4 °C, Jouan). Then, 75 µL of supernatant of each well were deposed with 75 µL of phosphate buffer saline (PBS, Sigma) into an other 96-well microplate. The absorbance (A_{540}) was read and the number of ACH_{50} units per mL of plasma was determined in reference to the 50 % haemolysis.

2.5. Statistical analysis

Statistical tests were carried out using XLStat Pro 2007. P values lower than 0.05 were used to identify significant differences.

To determine the homogeneity of the different parameters tested, a Kruskall-Wallis test, for no normal values, was used. In the case of rejection of *Ho*, an *a posteriori* Dunn test was used. To analyse HFO effects on organism's biomarkers, a Mann-Whitney test, for no normal values, was applied.

3. Results

3.1. Physico-chemical properties

3.1.1. Seawater quality

During all the experimental period, temperature (18.5 ± 0.1 °C), pH (8.2 ± 0.1), dissolved oxygen (91.6 ± 0.1 %) and salinity (36°± 0.0 PSU, data not shown) stayed constant. No significant difference was detected between each exposure tank. Seawater into all experimental tanks contained neither search bacteria nor suspended particles. Nitrates, nitrites, ammonium and ortho-phosphates are absent into seawater used for each tank (Table 2).

	UNITY	QUANTITY
Bacteriological parameters		
Coliformes	/ 100 ml	< 15
Escherichia coli	/ 100 ml	< 15
Salmonella	For 2 L	Absence
Vibrio parahaemolyticus	/ 10 ml	Absence
Physico-chemical parameters		
Temperature	° C	18.5 ± 0.1
pH	-	8.2 ± 0.1
Dissolved oxygen	%	91.6 ± 0.1
Suspended particles	mg/L	< 2
DBO 5	mg/L	1.7
Ammonium	mg/L	0.02
Nitrites (NO_2)	mg/L	< 0.01
Nitrates (NO_3)	mg/L	0.2
Ortho-phosphates (PO_4)	mg/L	0.03

Table 2: Main characteristics of the seawater quality for bacteriological and physico-chemical parameters. For each parameter, three samples were taken at all exposure tanks.

3.1.2. Chemical composition of the HFO Water Accommodated Fraction (WAF)

The chemical composition of the WAF has been studied at various sample dates in order to characterise the kinetics of solubilisation of the 16 PAHs. All data are reported in the Table 3. Results are in accordance with the solubility limits of the polycyclic aromatic hydrocarbons: the most soluble, naphtalene (only two rings or benzenic cycles), is present at the highest concentration and the less soluble, benzo[*g,h,i*]perylene (six rings), is present at the lowest concentration in the WAF. The hydrophobic characteristic of these molecules increases proportionally with the number of rings. Concerning solubilisation kinetics, it appears that the concentration in the aqueous phase of PAHs with 4, 5 and 6 rings increases with time: the highest concentrations are obtained after 7 days of contact between oil and seawater (indeno[*1,2,3-c,d*]pyrene ≈ 7.5 ng.L^{-1}, benzo[*a*]pyrene ≈ 40 ng.L^{-1}, dibenz[*a,h*]anthracene ≈ 4.5 ng.L^{-1} and benzo[*g,h,i*]perylene ≈ 11 ng.L^{-1}). For molecules with two and three rings, the kinetic of solubilisation is slightly different: during the 5 first days of contact between oil and seawater, concentrations increase but after, it is possible to observe a small decrease, except for naphthalene for which concentrations vary widely.

Name of PAH compounds	Concentration of dissolved PAH in WAF (ng.L^{-1})			
	Day (-7)	**Day (-5)**	**Day (-3)**	**Day 0**
Naphthalene	37110,79	47167,06	23463,30	4480,37
Acenaphthene	4187,94	4256,44	4816,14	4245,76
Acenaphthylene	3643,63	3733,67	4180,88	3817,27
Fluorene	5232,08	5828,24	6051,24	4997,62
Phenanthrene	7888,10	9008,50	8735,84	7723,06
Anthracene	613,45	688,33	694,52	571,29
Fluoranthene	114,97	177,69	116,33	106,21
Pyrene	435,91	450,47	461,64	298,95
Benz[*a*]anthracene	99,90	91,86	106,28	146,40
Chrysene	35,73	31,02	38,40	60,87
Benzo[*b+k*]fluoranthene	10,97	27,43	40,22	54,48
Benzo[*a*]pyrene	7,76	16,01	24,25	39,61
Indeno[*1,2,3-c,d*]pyrene	4,15	4,49	4,86	7,44
Dibenz[*a,h*]anthracene	2,77	2,82	2,70	4,32
Benzo[*g,h,i*]perylene	5,30	6,27	7,41	11,00

Table 3: Concentration of 16 US-EPA PAHs in WAF. The results are expressed in ng.L^{-1} in seawater at Day (-7), Day (-5), Day (-3) and Day 0 after the beginning of the exposure period, mean ± standard error, n.d. = not detected).

3.1.3. Dissolved fraction of HFO in the experimental system

In the control tanks, PAHs were not detected. No PAH difference was detected between each polluted tank for all sampling dates. In polluted tanks, 12 out of 16 PAHs were detected with a total mean concentration of 1 270 ± 86 ng.L^{-1} and with mean concentrations ranging from 9 ± 2 ng.L^{-1} for chrysene to 371 ± 27 ng.L^{-1} for phenanthrene. The concentrations of diaromatic compounds decreased during the exposure period (e.g. naphthalene, from 88 to 48 ng.L^{-1}). For compounds with more benzenic cycles (from three to four benzenic cycles), kinetics of dissolution were stable: at Day (-7) concentrations are globally similar than at Day 0 (e.g. anthracene, from 56 to 55 ng.L^{-1}). At the end, the four heaviest compounds with five benzenic cycles (benzo[*a*]pyrene, indeno[*1,2,3-c,d*]pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene) were undetected (Table 4).

Name of PAH compounds	Concentration at Day (-7) ($ng.L^{-1}$ ± SE)	Concentration at Day (-5) ($ng.L^{-1}$ ± SE)	Concentration at Day (-3) ($ng.L^{-1}$ ± SE)	Concentration at Day (0) ($ng.L^{-1}$ ± SE)	Mean concentration ($ng.L^{-1}$ ± SE)
Naphthalene	88 ± 6	68 ± 6	60 ± 9	48 ± 7	66 ± 8
Acenaphthene	308 ± 26	244 ± 12	212 ± 13	179 ± 7	236 ± 27
Acenaphthylene	224 ± 22	141 ± 2	76 ± 16	59 ± 13	125 ± 37
Fluorene	319 ± 12	222 ± 13	251 ± 58	125 ± 24	229 ± 40
Phenanthrene	308 ± 13	373 ± 28	365 ± 25	438 ± 43	371 ± 27
Anthracene	56 ± 3	54 ± 1	51 ± 4	55 ± 1	54 ± 1
Fluoranthene	16 ± 0	16 ± 1	18 ± 1	12 ± 1	15 ± 1
Pyrene	51 ± 4	35 ± 2	51 ± 13	66 ± 3	51 ± 6
Benzo[*a*]anthracene	19 ± 3	35 ± 2	19 ± 7	4 ± 1	19 ± 6
Chrysene	9 ± 5	15 ± 2	8 ± 2	4 ± 0	9 ± 2
Benzo[*b*]fluoranthene	76 ± 24	77 ± 3	72 ± 2	87 ± 6	76 ± 4
Benzo[*k*]fluoranthene	19 ± 4	29 ± 10	16 ± 6	11 ± 4	19 ± 4
Benzo[*a*]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.
Indeno[*1,2,3-c,d*]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenz[*a,h*]anthracene	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[*g,h,i*]perylene	n.d.	n.d.	n.d.	n.d.	n.d.
Sum of the 16 PAHs	**1493 ± 51**	**1309 ± 30**	**1199 ± 82**	**1088 ± 59**	**1270 ± 86**

Table 4: Concentration of 16 US-EPA PAHs in contaminated tanks throughout the *in vivo* experiment. The results are expressed in $ng.L^{-1}$ in seawater (n = mean values establish from 3 analysis at Day (-7), Day (-5), Day (-3) and Day 0 after the beginning of the exposure period, mean ± standard error, n.d. = not detected).

3.2. Biological analysis

3.2.1. Viability of organisms

During contamination and recovery periods neither mortality nor macroscopically lesions were detected in sea bass and oysters (data not shown).

3.2.2. PAHs bioaccumulation in organisms tissues

3.2.2.1.Oysters

PAHs were not detected in each oyster group before the contamination period (Day (-7)) and during all the experimental period for the control oysters (lower than 5 $\mu g.kg^{-1}$ of wet weight, Table 5). After seven days of exposure (Day 0), the contaminated oyster tissues had bioaccumulated only three PAHs, with 136.8 ± 13.2 $\mu g.kg^{-1}$ of dry weight composed of about 12.6 % of fluorene (17.3 ± 3.2 $\mu g.kg^{-1}$ of dry weight), 60.3 % of phenanthrene (82.5 ± 11.2 $\mu g.kg^{-1}$ of dry weight) and 27.0 % of anthracene (37.0 ± 5.7 $\mu g.kg^{-1}$ of dry weight). After seven days of recovery (Day 7), the oysters had

eliminated 26.0 % of the fluorene (12.8 ± 3.5 µg.kg^{-1} of dry weight), 17.1 % of the phenanthrene (68.4 ± 22.1 µg.kg^{-1} of dry weight) and 50.8 % of the anthracene (18.2 ± 7.7 µg.kg^{-1} of dry weight) bioaccumulated in their tissues. At Day 7, the PAHs still bioaccumulated (99.5 ± 25.9 µg.kg^{-1} of dry weight) correspond to 12.9 % of fluorene, 68.7 % of phenanthrene and 18.3 % of anthracene. On Day 14, the oyster tissues still contained 80.7 ± 12.4 µg.kg^{-1} of dry weight of each three PAHs detected with 11.4 % of the fluorene (9.2 ± 1.1 µg.kg^{-1} of dry weight), 73.7 % of the phenanthrene (59.5 ± 8.0 µg.kg^{-1} of dry weight) and 14.7 % of the anthracene (11.9 ± 1.2 µg.kg^{-1} of dry weight). On Day 14, oysters have eliminated 46.8 % of the fluorene, 27.9 % of the phenanthrene and 67.8 % of the anthracene bioaccumulated in their tissues. Finally, after 14 days of recovery period, oysters have eliminated 41 % of the total PAHs bioaccumulated.

Sampling dates	**Fish muscles (µg.kg^{-1} of dry weight)**		**Oyster tissues (µg.kg^{-1} of dry weight)**	
	Control	**Contaminated**	**Control**	**Contaminated**
Day (-7)	n.d.	n.d.	n.d.	n.d.
Day 0	n.d.	31.8 ± 7.1 (naphthalene: 10.9 ± 4.3, acenaphthene: 20.9 ± 5.7)	n.d.	136.8 ± 13.2 (fluorene: 17.3 ± 3.2; phenanthrene: 82.5 ± 11.2; anthracene: 37.0 ± 5.7)
Day 7	n.d.	10.5 ± 1.6 (acenaphthene: 10.5 ± 1.6)	n.d.	99.5 ± 25.9 (fluorene: 12.8 ± 3.5; phenanthrene: 68.4 ± 22.1; anthracene: 18.2 ± 7.7)
Day 14	n.d.	8.6 ± 1.5 (acenaphthene: 8.6 ± 1.5)	n.d.	80.7 ± 12.4 (fluorene: 9.2 ± 1.1; phenanthrene: 59.5 ± 8.0; anthracene: 11.9 ± 1.2)

Table 5: Concentration of the 16 US-EPA PAHs in sea bass muscles and oyster tissues. The results are expressed in µg.kg^{-1} of dry weight (n = 15 for fish and n = 15 for oysters, n.d. = not detected). The experiment was performed by gas chromatography coupled with mass spectroscopy (GC-MS).

3.2.2.2.Fish

PAHs were not detected in fish groups before the contamination period (Day (-7)) and during all the experimental period for the control fish (lower than 5 µg.kg^{-1} of wet weight, Table 5). After seven days of exposure (Day 0), the contaminated fish muscles had bioaccumulated only two PAHs, with 31.8 ± 7.1 µg.kg^{-1} of dry weight composed of about

34.3 % of naphthalene (10.9 ± 4.3 µg.kg^{-1} of dry weight) and 65.7 % of acenaphthene (20.9 ± 5.7 µg.kg^{-1} of dry weight). After seven days of recovery period (Day 7), the fish had eliminated 100 % of the naphthalene and 49.8 % of the acenaphthene (10.5 ± 1.6 µg.kg^{-1} of dry weight) bioaccumulated in their muscles. On Day 14, the fish muscles still contained only 8.6 ± 1.5 µg.kg^{-1} of dry weight of acenaphthene, with a depuration of 58.9 %. Finally, after 14 days of recovery period, fish have eliminated 83 % of the total PAHs bioaccumulated.

3.2.3. Biomarkers analysis

3.2.3.1.Phenoloxidase (PO) activity in oysters

The PO activity was not significantly different between exposed and control oysters before contamination period (Day (-7)) with a mean PO activity of 0.362 ± 0.007. A significant decrease in exposed oysters compared to control oysters from Day 0 to Day 14 of the recovery period was observed. The mean PO activity for exposed and control oysters was respectively 0.303 ± 0.008 and 0.405 ± 0.027 (Fig. 4).

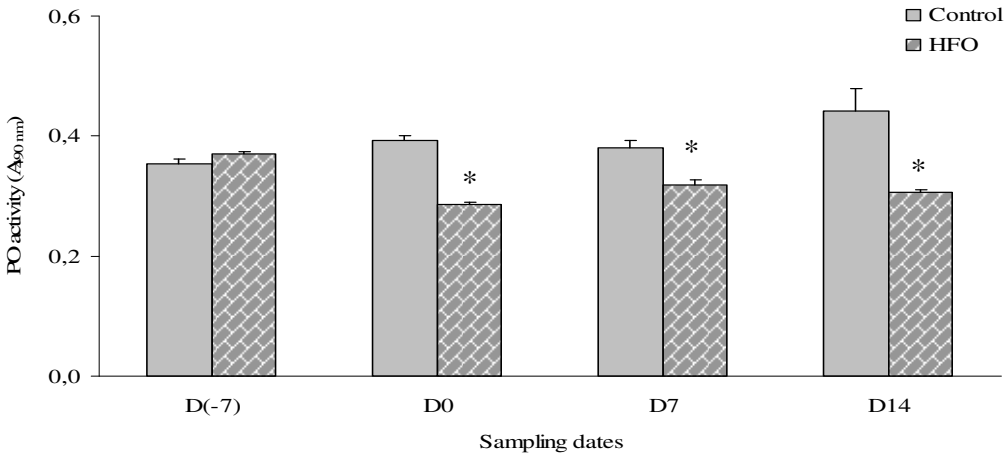

Fig. 4: Phenoloxidase activity measured in oysters by spectrophotometry ($A_{490\ nm}$) during the experimental period: before exposure to heavy fuel oil (HFO, Days (-7)) and following the contamination period (Day 0 to Day 14). Values are the mean of three pools of five oysters. The bars represent the standard error. * = statistical difference for $p \leq 0.05$.

3.2.3.2.Biliary metabolite in fish

The RFU at 343/383 nm was significantly different between exposed and control fish from Day 0 to Day 7 of the recovery period with respectively 13.2 ± 0.4 and 1.7 ± 0.0 for

exposed fish and 0.23 ± 0.0 and 0.33 ± 0.0 for control fish. No significant difference was observed between exposed and control fish before exposure period (Day (-7)) and after 14 days of recovery period (Day 14, Fig 5).

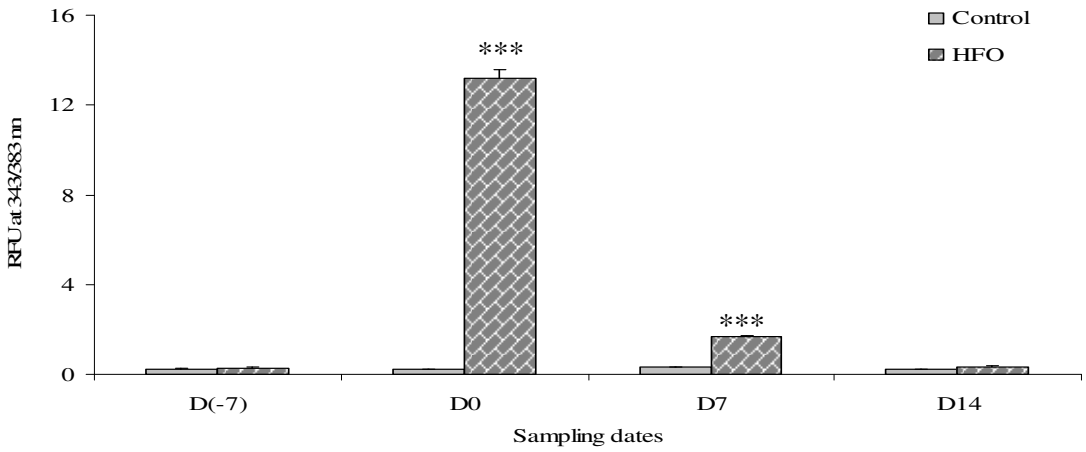

Fig. 5: Quantification of Relative Fluorescence Unit (RFU) at 343/383 nm in sea bass during the experimental period: before exposure to heavy fuel oil (HFO, Days (-7)) and following the contamination period (Day 0 to Day 14). Values are the mean of 15 sea bass. The bars represent the standard error. *** = statistical difference for p≤0.001.

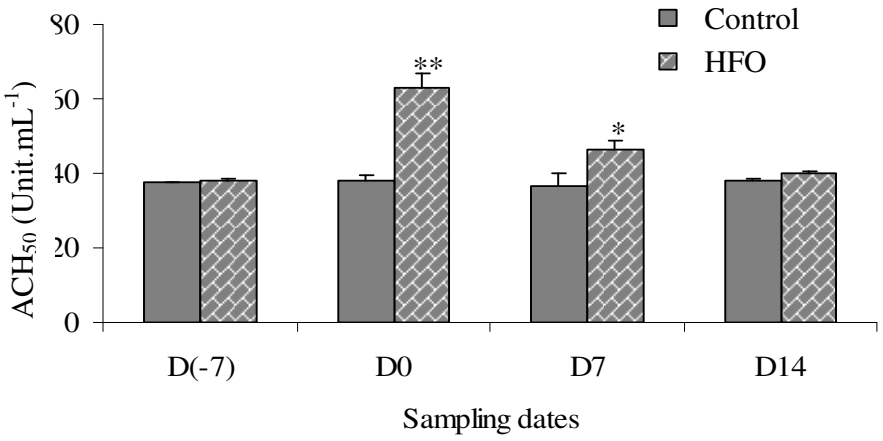

Fig. 6: Quantification of the haemolytic activity of the alternative complement pathway (ACH_{50}) in sea bass during the experimental period: before exposure to heavy fuel oil (HFO, Days (-7)) and following the contamination period (Day 0 to Day 14). Values are the mean of 15 sea bass. The bars represent the standard error. * = statistical difference for p≤0.05 and ** for p≤0.01.

3.2.3.3.Alternative complement pathway (ACH_{50}) in fish

The alternative activity of complement was not significantly different between exposed and control fish before exposure period (Day (-7)) with a mean of 37.8 ± 0.3 ACH_{50} units.mL^{-1}. The haemolytic activity of the ACH_{50} was significantly increased after seven days of exposure (63.1 ± 3.9 ACH_{50} units.mL^{-1} at Day 0). This difference was observed also after seven days of recovery period (46.3 ± 2.2 ACH_{50} units.mL^{-1} at Day 7). After 14 days of recovery period (Day 14), the values of ACH_{50} come back to control threshold baseline (40.1 ± 0.6 ACH_{50} units.mL^{-1}, Fig.6).

4. Discussion

Despite the fact that some authors have demonstrated that the complexity of oil types and environmental fate make difficult to perform realistic laboratory experiments that stimulate natural conditions (Baussant et al., 2001b; Duesterloh et al., 2002), the goal of this study was to present a new experimental system enabling to investigate effects of dissolved xenobiotic on marine and freshwater organisms. During the experimentation presented here, two types of marine organisms were exposed to the dissolved fraction of a heavy fuel oil (HFO): an invertebrate, the Pacific oyster, *Crassostrea gigas*, and a vertebrate, the European sea bass, *Dicentrarchus labrax*. The dissolved fraction of HFO was obtained according to a new designed system, in order to reproduce, after two weeks of contact between HFO and seawater, a homogenised polluted water column similar to what can exist after an oil spill and without emulsion interference.

4.1. Physico-chemical properties

Biological equilibrium of marine species is known to be strongly dependent upon environmental conditions (Claireaux and Lagardere, 1999; Saroglia et al., 2000). Consequently any experimental set-up involving biological analysis on fish and on bivalves must allow a precise control of its physico-chemical parameters. In this experiment, the results obtained show that temperature, salinity, pH and dissolved oxygen can be maintained

several weeks at satisfying levels (18 °C, 36 PSU, 8 and 92 % respectively). Furthermore, ammonium, ortho-phosphate, nitrate and nitrite were not found in the seawater.

Concerning the protocol used for the organisms exposed, it was possible to detect in each dissolved fraction of all contaminated tanks, twelve out of the 16 US-EPA polycyclic aromatic hydrocarbons (PAHs) present in the original HFO. The low solubility limit of the four heaviest aromatic hydrocarbons explains their absence in the water (table 1). It is also the case for the chrysene which has a solubility limit of 0.002 $mg.L^{-1}$. The concentration of the PAHs with three and four rings increases slightly during the entire contamination period which is in accordance with the results obtained during the WAF test. It is important to underline that during a WAF test, most hydrocarbons dissolve in the water, which explains why the final concentration (Day 0) are globally higher than at the beginning (Day -7). The behaviour of compounds with two rings is more complex: a small decrease is observed along the exposure period. For the WAF test, this observation is true only at the end, it means after five days of the oil-water contact (between Day -3 and Day 0). The main reason is that these compounds have the highest vapour pressure: they evaporate easily, even during the WAF test because of the presence of a small air space (saturation of the gaseous phase). With naphthalene, this phenomenon is even more important due to the fact that this compound has a vapour pressure (10.5 Pa) vastly superior to the other PAHs (< 1 Pa). It is the reason why little naphthalene is detected in the exposure tanks even if its proportion in the original HFO is high. With respect to the proportion, the concentrations of HAPs in the exposure tanks are in accordance with their abundance in the original oil, except for naphthalene due to the evaporation process and for chrysene due to its low solubility limit. In addition, during the chemical validation of the equipment, concentrations of HAPs in contaminated tanks were very close (standard error lowers than 26) which shows the accuracy of the experimental set-up and the good reproducibility of the water contamination method. These points are absolutely necessary for the statistical treatment of the data obtained in experiments dedicated to characterising the impact of a pollutant on organisms.

The experimental equipment developed at Cedre reproduces the behaviour of oil at sea in a realistic way; it takes into account important phenomena which influence significantly the chemical ageing process of a pollutant. It is not the case when the water

contamination is obtained by adding dispersants: firstly, the natural tendency of compounds to evaporate is not taken into account; secondly, fish are exposed to oil droplets and not only to the WAF which causes specific impacts, for example the alteration of the gills from the oil droplets physically attached. Furthermore, even if dispersants are biodegradable and not toxic, it appears that the toxicity of dispersant combined with the oil is greater than the toxicity of oil alone or dispersant alone. It is also the case with the Continuous Flow System which allows water contamination by creating oils droplets: this method of contamination of the water underestimates the evaporation rate of the lightest PAHs. This increases in an arbitrary manner the oil toxicity because organisms are very sensitive to an exposure of two ring PAHs like naphthalene. In addition, a mechanical dispersion of oil could induce an inverse emulsion (water trapped within the oil droplets) which could slightly reduce the dissolution kinetics of several compounds. The last contamination technique, glass beads covered in oil, all compounds are forced to dissolve even those with low solubility limit like chrysene. Here again, it is not possible to consider the evaporation rate of the oil which normally reduces the concentration of the lightest PAHs.

The physico-chemical and PAHs analysis of the sea water carried out during the validation test shows that this new experimental system creates reproducible data allowing the experiments to be compared (statistical treatment of the results). The range of concentrations is within the concentrations reported following the accident of the *Exxon Valdez* oil spill (Boehm et al., 2007) and also the *Ekofisk blowout* (Law, 1978). This experimental system could also be used for chronic pollution at a background level for example pesticide or hydrophobic xenobiotics.

4.2. Biological analysis

During this experimentation, no individual mortality was recorded. No macroscopic external and internal lesions were observed in both sea bass and oysters, probably due to the short exposure time and the controlled external conditions (i.e. filtered water, constant temperature and oxygenation…) which prevent or at least delay secondary infections often observed after immunotoxicity phenomena.

The seven days exposure to the dissolved fraction of HFO induced a bioaccumulation of some PAHs on oyster tissues (fluorene, phenanthrene and anthracene) and sea bass

muscles (naphthalene and acenaphthene). These results are accordance with previous studies that showed that for the oyster bioaccumulation, four and nine days of contamination with dissolved fraction of HFO induced a bioaccumulation of naphthalene, fluorene and phenanthrene (Neff et al., 1976), and pyrene and phenanthrene (Bado-Nilles et al., submitted). Moreover, in these two cases, fluorene and anthracene concentrations were low and phenanthrene concentration was high when compared with this study. For fish, in accordance to Bado-Nilles et al. (submitted), naphthalene and acenaphthene were bioaccumulated into fish muscles. The difference between PAHs bioaccumulated in oysters and fish are not surprising when we know that uptake and accumulation of PAHs depends on several factors: physical and chemical properties (i.e. molecular weight, solubility, half-life period, bioavailability), time of exposure and environmental parameters such as water oxygenation and temperature and biological factors (i.e. way of life, physiological status) (Meador et al., 1995). Nevertheless, due to the characteristics of the experimental system, implying stable environmental condition and homogeneous assessment of HFO, it would seem that this is mainly physical factors, way of life of organisms (pelagic and carnivorous for fish; sessile and filter feeding for oysters) and metabolisation types that explain the bioaccumulation observed in the two species.

The two types of depuration pathway was used for PAHs: i) metabolism wich biotransform parent PAHs to metabolites that are rendered more water soluble to facilitate excretion through bile or urine; ii) simple diffusion out of the organism that is controlled by thermodynamic partitioning between compartments (e.g. tissue and water) (Meador et al., 1995). In this study, for oysters, the half-life of anthracene was seven days and approximately 14 days for fluorene. After the 14 days of the recovery period, only 28 % of phenanthrene was depurated. Bado-Nilles et al. (submitted) observed that a dissolved fraction of HFO induced a quite short half-life for phenanthrene (less than three days) and the oyster tissues were cleaned up after two weeks of recovery period. Nevertheless, in this work, phenanthrene was more bioaccumulated in oyster tissues. For fish, they had totally eliminated naphthalene after seven days of recovery period, when the half-life of acenaphthene was just reached. In the same way, the half-life for acenaphthene was approximately seven days after a contamination to dissolved HFO (Bado-Nilles et al., submitted). In contradiction to Bado-Nilles et al. (submitted), the sea bass seems to depurate

more quickly their muscles (87 % eliminated after 14 days) that oysters, which have only eliminate 41 % after 14 days of recovery period. The time of depuration was influenced principally by the molecular weight of bioaccumulated compounds (Neff et al., 1976) and the depuration pathway used (Meador et al., 1995). In fact, higher PAHs, as present in oyster tissues, will slowly diffuse out of rich lipid storage tissue, which limits the amount of PAH available for metabolism. Additionally, lower PAHs, as detected in fish muscle, can diffuse easily out of organism through its gills. Moreover, fish are known to have very short half-lives of PAHs due to their capacities to reduce tissue concentrations of parent hydrocarbon by the quick conversion of PAH compounds to metabolites via the mixed function oxidase system (MFO), which requires cytochrome P1A and NADPH (reduced nicotinamide adenine dinucleotide phosphate). In the opposite, invertebrates, which possess low metabolic activities and which used essentially simple diffusion, will generally have high persistence of parent PAHs in their tissues and will more closely reflect environmental exposure (Meador et al., 1995).

The last part of these experimentations was to develop immunological and physiological (Gagnon and Holdway, 2000) effect biomarkers in order to validate the contamination by this experimental system. In this analysis, phenoloxidase activity decreased after a short exposure to the dissolved fraction of HFO and the recovery of this activity was obtained after more than two weeks. A significant increase of haemolytic activity of alternative pathway was observed in contaminated fish from Day 0 to Day 7 of the recovery period. In accordance to Bado-Nilles et al. (submitted), naphthalene and acenaphthene, which were bioaccumulated in fish muscles, could be at the origin of the beginning of an inflammatory response which increases haemolytic activity of alternative pathway. About fluorescent intensity of pyrene, it was increase after the seven days of exposure to dissolved fraction of HFO (Day 0) and after seven of the recovery period (Day 7). The pyrene metabolite quantities come back, after 14 days of the recovery period (Day 14), to control threshold baseline. Similar results were shown with Australian bass, *Macquaria novemaculeata* (Cohen et al., 2003), and with marine flatfish dab, *Limanda limanda* (Van Schanke et al., 2001), after 12 days of recovery period. Finally, these two enzymatic cascades (phenoloxidase activity in bivalve and haemolytic activity of alternative pathway in fish) and

the biliary metabolite quantification validate, as bioaccumulation of PAHs, this experimental system.

To conclude, this experimental equipment favour the obtainment of stable physico-chemical parameters to allows organism life and permit the reproduction of realistic pollutions of homogenate seawater column by hydrophobic xenobiotics. Furthermore, this experimental system exposes vertebrate and invertebrate at dissolved fraction of pollutants, as validate by bioaccumulation and effect biomarkers. Moreover, the same system permit the passage to recovery period without organisms handling and then to stimulate a real spill.

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ARTICLE 6: In vivo effects of light cycle oil soluble fraction on immune function and gene expression in marine species: Part I. in an Invertebrate, the Pacific oyster, *Crassostrea gigas* (Thunberg).

Article en préparation

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Abstract

The effects of a dissolved fraction of light cycle oil (LCO, 1 600 $ng.L^{-1}$) on haemocyte parameters, phenoloxidase (PO) activity and selected gene expression, in the Pacific oyster, *Crassostrea gigas*, were tested after seven days of exposure. At the end of the contamination, oysters were transferred to non contaminated seawater and a recovery period was conducted for two weeks. Five PAHs out of ten detected in the seawater were bioaccumulated at the end of the contamination period. Bioaccumulation of PAHs in contaminated tissues decreased during the recovery period and 14 days after the seven day exposure, 69 % of bioaccumulated PAHs were detected in the tissues of contaminated oysters. This exposure condition induces 21 % of oyster mortality and green coloration of their shell. A significant decrease of PO activity was observed after seven days of seawater contamination and before 14 days of recovery period. Modification of the expression of tested genes was noted. To conclude, modulation of immune-related parameters was demonstrated using three different methods (flow cytometry, spectrophotometry and gene expression) in Pacific oysters after contact with dissolved fraction of LCO. Each compound which constitutes the LCO could therefore induce modulation of immune-related gene and thus decrease the capacity of oyster to respond to an infectious agent.

Keywords: Crassostrea gigas; Haemocytes; Polycyclic aromatic hydrocarbons; Immune parameters; Bioaccumulation; Gene expression.

1. Introduction

Refining industry has look for new alternatives to convert the denser streams and in addition maximize the production of lighter and more value-added products such as gasoline and diesel (Nylen et al., 2004). In order to reduce the heavy oil production and increase value-added products, petroleum refiners selected a severe cracking of crude feedstock which raise the middle distillate products (Xu et al., 2003). All of these operations increase the supply of light cycle oil (LCO). Actually, LCO is a fraction of products obtained by the catalytic cracking of residues, either obtained by atmospheric or vacuum distillation (Poveda Jaramillo et al., 2004). LCO corresponds to middle distillates at atmospheric pressure (144-404 °C) of these cracked molecules, the lightest products being used to form gasolines. This LCO refined product is rich in polycyclic aromatic hydrocarbons (PAHs), aromatic sulphurs and nitrogen compounds, and possesses lower cetane number and higher density (Nylen et al., 2006; Xu et al., 2003). This composition define a LCO with a poor quality comparing to other products derived from distillation process of crude oils (Xu et al., 2003). Nevertheless, after distillation product, LCO accounts for approximately 10-20 weight percent of the fluid catalytic cracking products (Nylen et al., 2006). These products are commonly used to produce higher home fuel or to increase or maintain fluid catalytic cracking federate (Ding et al., 2007). Also, some refiners use at the end of the fluid catalytic cracking process the LCO to fluidify residues in order to facilitate its transportation by tankers (Ding et al., 2007). These mixtures, call heavy fuel oil (HFO), are largely detected after the wreckage of tankers.

In marine organisms, the biological toxic effects of PAHs was well studied in particularly for HFO (Bocquené et al., 2004; Claireaux et al., 2004), dispersed oil (Cohen et al., 2003; Cohen et al., 2001; Gagnon and Holdway, 2000), crude oil (Cohen et al., 2003; Crocker et al., 1974; Gagnon and Holdway, 2000) and municipal sewage effluent exposures (Hoeger et al., 2004; Salo et al., 2007). Moreover, many authors research the effects of persistent PAHs in sediment (Baumard et al., 1999; Beyer et al., 1996; Camus et al., 2003) or of one or more PAHs (Grundy et al., 1996; Nott et al., 1985; Walczak et al., 1987). Nevertheless, the LCO fraction, which is composed essentially of the priority PAHs of the United States Environmental Protection Agency (US-EPA) pollutant lists, was not studied for

the moment. The vulnerability of aquatic species to pollutions depends on pollutant properties, pollutant concentrations and the capacity of ecosystems to resist to pollutants and especially biodegradation (Fochtman, 2000). However bioaccumulation constitutes a natural response and may be the consequence of direct contamination by water or indirect contamination through the food chain (Amiard-Triquet, 1989). When bioaccumulation occurs, pollutants can have direct interactions with tissues and cells such as immune cells.

The internal defence mechanisms may prevent infections from pathogenic microorganisms and parasites by cellular reactions such as phagocytosis which eliminated invaders (Glinski and Jarosz, 1997). Intruders may also be destroyed by humoral components formed by prophenoloxidase/phenoloxidase system, or lysozyme activity (Glinski and Jarosz, 1997). Xenobiotics may affect such systems and activities which could enhance the susceptibility of animals to infectious diseases.

The aims of the present work were to carry out a preliminary experimentation concerning effect of refined petroleum, the LCO, on immune parameters and gene expression simultaneously in two marine species, an invertebrate, the Pacific oyster, *Crassostrea gigas*, and a lower vertebrate, the European sea bass, *Dicentrarchus labrax*. The results obtained for the Pacific oysters were reported in the present article. This organism, as many bivalve molluscs, is considered as ideal indicator for the assessment of environmental pollution (Lopez-Barea and Pueyo, 1998). They are used in several programs monitoring pollution in estuarine ecosystems due to their ubiquitous, benthic and sedentary way of life and their suspensivore mode of nutrition which leads them to bioaccumulate contaminants within their tissues by intensive filtration (Domart-Coulon et al., 2000). In this study, the capacity of adult Pacific oysters to recover their initial status after a seven day exposure to a dissolved fraction of LCO was analysed based on monitoring of cell mortality, haemocyte subpopulations, phagocytosis activity, percentage of non-specific esterase positive cells and lysosome presence by flow cytometry. Phenoloxidase (PO) activity was studied in the acellular fraction of haemolymph by spectrophotometry. The expression of four immune related genes (laccase, macrophage express protein, myeloïd differentiation factor and molluscan defence precursor) and one tumor suppressor gene (p53) was monitored by real time RT PCR. All parameters were performed during a recovery period of two weeks. The

results obtained for European sea bass were noticed in an other article (Bado-Nilles et al., submitted-a).

2. Materials and methods

Name of 16 PAHs US-EPA	Molecular weight ($g.mol^{-1}$)	Concentration ($\mu g.g^{-1} \pm$ SE) in LCO
Naphthalene	128,2	3 761 ± 49
Acenaphthylene	152,2	241 ± 4
Acenaphthene	154,2	1 017 ± 12
Fluorene	166,2	1 732 ± 9
Phenanthrene	178,2	9 204 ± 22
Anthracene	178,2	524 ± 8
Fluoranthene	202,3	843 ± 11
Pyrene	202,3	801 ± 35
Chrysene	228,3	100 ± 10
Benzo[*a*]anthracene	228,3	33 ± 5
Benzo[*b*+*k*]fluoranthene	252,3	n.d.
Benzo[*a*]pyrene	252,3	n.d.
Benzo[*g,h,i*]perylene	276,3	n.d.
Indeno [*1,2,3-c,d*] pyrene	276,3	n.d.
Dibenz[*a,h*]anthracene	278,4	n.d.

Table 1: Concentration of 16 priority PAHs US-EPA in light cycle oil (LCO). PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in $\mu g.g^{-1}$ (n = 3, mean ± standard error; n.d. = not detected, < 0.1 $\mu g.g^{-1}$).

2.1. Oysters

Pacific oysters, *Crassostrea gigas*, 9-10 cm in shell length, were purchased from a shellfish farm located in the Brest bay (Brittany, France) in October 2006. Oysters were acclimated with European sea bass, *Dicentrarchus labrax* (Bado-Nilles et al., submitted-a) for one week in 1 200 L tanks with a flow of 0.3 $m^3.h^{-1}$ at 16 ± 1°C (dissolved oxygen 96 ± 4 %, pH 7.8 ± 0.2, salinity 36 ± 2 ‰, free of nitrate and nitrite). The oysters were fed every three days with 15 L of *Isochrysis galbana* at 4.10^6 $cell.mL^{-1}$ provided by the

Océanopolis aquarium (Brest, France). This rate allows maintaining a minima concentration of 15.10^4 cell.mL^{-1} per day which is enough to ensure a continuous feeding (Rico-Villa et al., 2006).

2.2. Pollutants

Light cycle oil (LCO), which contained 85-86 % of aromatic hydrocarbons, 1-2 % of sulphurs and 4-2 % of polar compounds, was selected to perform the exposure. The LCO contain 10 of the 16 PAHs of US-EPA list which are naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene and benzo[*a*]anthracene at concentrations ranging from 9 204 ± 22 µg.g^{-1} for phenanthrene to 33 ± 5 µg.g^{-1} for benzo[*a*]anthracene (Table 1).

2.3. Experimental design

The experiment was carried out twice.

2.3.1. Experimental system

The experimental system used was constituted about six similar and independent units, three control units and three contaminated units. Each unit (616 L) was composed about one rectangular mixing tank (316 L) to generate soluble fraction of LCO, one cylindrical exposure tank (300 L) to expose organisms and one degassing column to maintain the level of oxygen around 96 %. The experimental system was placed in a greenhouse, which is thermoregulated (T = 14 ± 1 °C) and has one total renewal of the air in 6 h. For the exposure period, mixing tanks were connected to exposure tanks with a release of three litres of LCO at the water surface. After the contamination period, the mixing tank was disconnected and fresh seawater was flowed at a rate of 0.3 m^3.h^{-1}.

This system was adapted from Anderson et al. (1974) and modified by *Cedre* in order to obtain a stable oil concentration in the exposure tank throughout the experiment (1 600 ± 315 ng.L^{-1}). The stable concentration of dissolved fraction of LCO was obtained after two weeks of mixture of LCO and seawater.

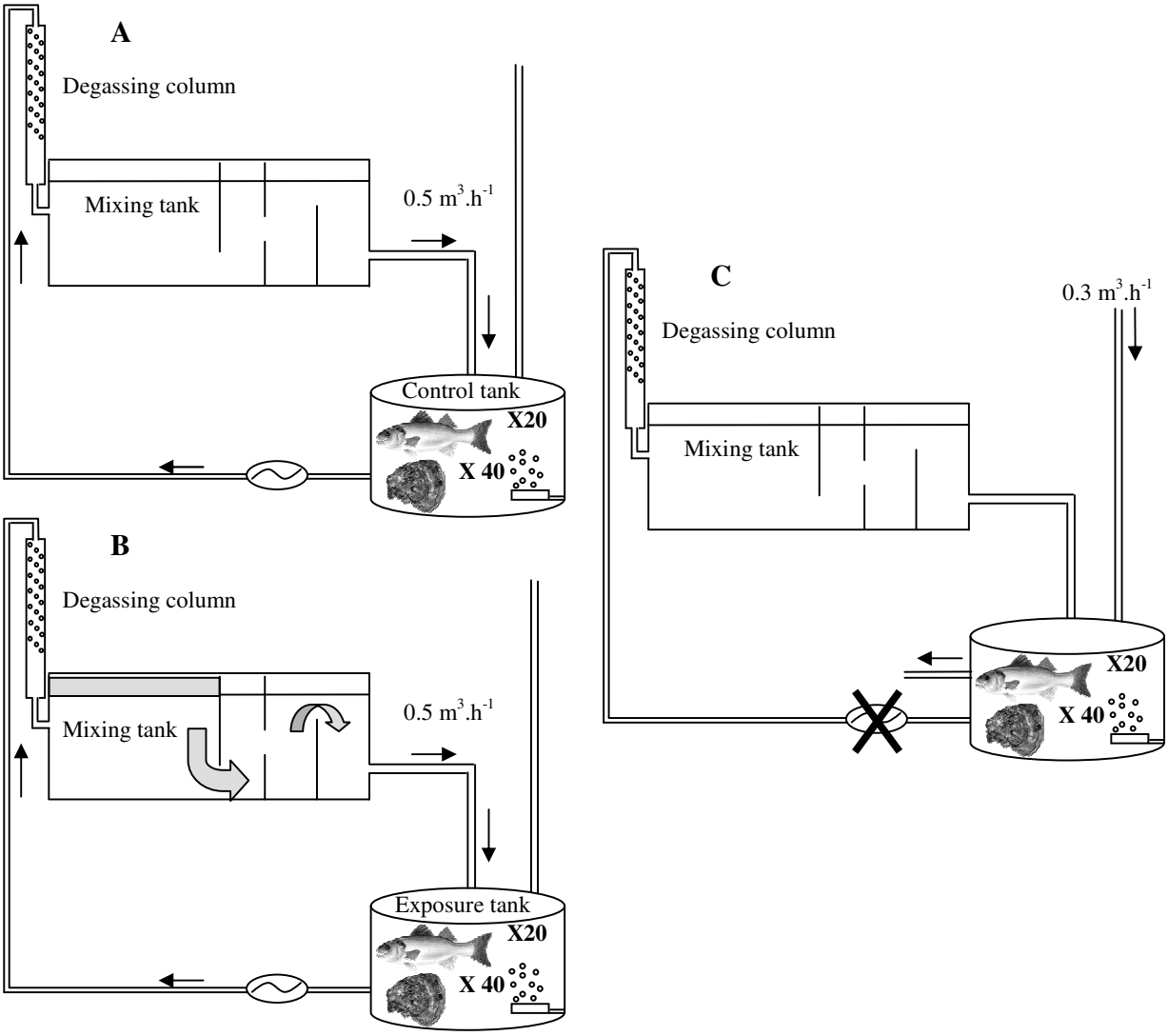

Fig. 1: Experimental units for control organisms (A) and contaminated organisms (B) as a closed circuit during the contamination period and as an open circuit (C) during the recovery period.

2.3.2. Exposure conditions and recovery period

After the acclimatization period, 150 oysters were placed with sea bass in each control unit while 150 oysters were exposed with sea bass in each contaminated unit (Fig. 1) with the natural light / dark cycle. During this exposure period, control and exposed organisms were not fed.

After the contamination period, the mixing tank was disconnected and fresh seawater was flowed at a rate of 0.3 $m^3.h^{-1}$ in the exposure tanks (Fig. 1). During the two weeks recovery period, the organisms were fed again.

During all the experiment, the seawater quality was monitored in each tank (oxygen level, temperature, pH, salinity, nitrate and nitrite) and, also, the concentration of the LCO dissolved fraction.

2.3.4 Samples and sampling date

Seawater sampling

One litre of seawater was collected daily in each expose tank in heated (500 °C) Duran glass bottles (Bioblock) to determine pollutant concentrations during the exposure period.

Oyster sampling

Oyster mortality was daily recorded. Fifteen oysters were collected in each exposure tanks: five for flow cytometry, spectrophotometry and PAH tissue analysis and ten for RNA extraction and also PAH tissue analysis. Oyster samples were collected on the first (Day (-7)) and on the last (Day 0) days of the contamination period and after seven and 14 days of recovery period (respectively Day 7 and Day 14). For each sampling date, external macroscopically lesions and shell abnormalities were recorded.

Haemolymph collection

After carving a small notch in the dorsal shell of the oyster, approximately 2 mL of haemolymph were withdrawn from the posterior adductor muscle sinus by using a 2 mL syringe equipped with a 0.7 X 30 mm needle. Haemolymph samples were filtered through a 60 µm mesh to eliminate aggregates and external particles. Then, they were kept on ice until they were processed.

2.4. Analytical methods

2.4.1. Seawater PAH concentrations

Seawater samples were extracted with 30 mL of dichloromethane pestipur quality (SDS). After separation of the organic and aqueous phases, water was extracted two additional times by the same volume of dichloromethane (2 X 30 mL). The combined extracts were purified and treated using gas chromatography coupled with mass spectrometry

(GC-MS, Hewlett Packard HP5890 coupled with an HP5972 mass selective detector) following previously published procedures (Douglas et al., 1992).

2.4.2. PAH concentrations in o*yster tissue*

Whole tissues of the 15 control and 15 contaminated oysters were collected and frozen at – 80 °C until analysis. The PAH concentrations were determined by GC-MS using the procedure of Baumard et al. (1997) with some modifications. Prior to extraction, the whole tissue was homogenized using an Ultraturax (Janke & Kunkel, IKA®-Labortechnik). The 150 µL of predeuterated internal standards (CUS-7249, Ultra Scientific, Analytical solutions) were added to 3 g of homogenized whole tissue and the sample was digested for 4 h under reflux in 50 mL of an ethanolic solution of potassium hydroxide (2 $mol.L^{-1}$, Fisher Chemicals). After cooling, decantation and addition of 20 mL of demineralised water, the digest was extracted in a 250 mL funnel two times with 20 mL of pentane (SDS). The extract was evaporated with a Turbo Vap 500 concentrator (Zyman, Hopkinton, MA, USA, at 880 mbar and 50 °C) to obtain 1 mL of concentrated extract. The purification of the extract was performed by transferring to a silica column (5 g of silica). The hydrocarbons were eluted with 50 mL of pentane:dichloromethane (80:20, v:v, SDS) and concentrated to 200 µL by means using a Turbo Vap 500 concentrator (880 mbar, 50°C). Aromatic compounds were analyzed by GC-MS and PAHs were quantified relative to the predeuterated internal standards introduced at the beginning of the sample preparation procedure.

2.4.3. Haemocyte parameters

A pool of five oysters by exposure tank was prepared to obtain a sufficient volume of haemolymph. Haemocytes were counted with a Thoma's haemocytometer and adjusted at 10^6 $cells.mL^{-1}$ with Tris Buffer Saline (TBS, 1 000 $mOsm.L^{-1}$). One mL of haemolymph from each pool was collected immediately and split into five aliquots of 200 µL to study cellular activities by flow cytometry. Resting haemolymph pool samples were centrifuged (260 g, 10 min, 4 °C) and the acellular fraction (supernatant) was frozen at - 80 °C for further analysis by spectrophotometry.

Morphological characteristics, haemocyte mortality, phagocytosis percentage, percentage of non-specific esterase positive cells and lysosome presence were analysed with

an EPICS XL 4 (Beckman Coulter) flow cytometer. For each haemocyte sample, 10 000 events were counted using protocols previously described (Gagnaire et al., 2006).

Haemocyte subpopulation percentages and cell characteristics (size and complexity) were analyzed using FSC (Forward Light Scatter) and SSC (Side Light Scatter) photodetectors, respectively. The proportion of hyalinocytes (lower SSC/FSC) and granulocytes (high SSC/FSC) were thus established using 200 µL of one aliquot by pool without previous treatment.

Cell mortality was measured using FL3 (red fluorescence). Mortality was determined using 200 µL of haemocyte suspension and 10 µL of propidium iodide (PI, 1.0 $g.L^{-1}$, Molecular Probes). Cell suspensions were incubated for 30 minutes at 4°C. Propidium iodide is membrane impermeant and is excluded from viable cells.

The phagocytosis percentage was determined using FL1 (green fluorescence). Fluorescent microspheres ($2.7x10^{10}$ $particles.mL^{-1}$, Fluorospheres® carboxylate-modified microspheres, diameter 1 µm, Molecular Probes) were used and the fluorescence setting was established using a suspension of fluorescent beads in distilled water. Only the events showing a fluorescence of at least three beads were considered positive for phagocytic activity. Phagocytic activity of haemocyte suspensions was analysed on 200 µL of haemolymph samples and 10 µL of a 1/10 dilution of fluorescent beads. Cell suspensions were incubated for one hour at room temperature.

Percentage of non-specific esterase positive cells was defined using the non-specific liposoluble substrate fluoresceine diacetate (FDA, Molecular Probes). One µL of a FDA solution (400 µM) was added to 200 µL of haemocyte suspension. Cells were incubated for 30 minutes in the dark at room temperature and then the reaction was stopped on ice (5 min).

Lysosome presence was analysed with a commercial kit (LysoTracker ® Green DND-26, 1mM in DMSO, Molecular Probes) which consists in a fluorophore linked to a weak base that is partially protonated at neutral pH. The LysoTracker® is freely permeant to cell membranes and typically concentrated in lysosomes. One µL of a LysoTracker marker was added to 200 µL of haemocyte suspension. Samples were incubated for two hours in the dark at room temperature and then the reaction was stopped on ice (5 min).

2.4.4. Haemolymph analysis by spectrophotometry

Detection of phenoloxidase (PO) activity in acellular fraction samples of each pool was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-Dopa, Sigma) transformation in dopachromes as described previously by Gagnaire et al. 2004. The products used to determine the PO activity where purchased from Sigma. Samples were distributed in 96-well microplates (Nunc, France). Modulators of this enzymatic activity were used to confirm the specificity of the detected activity. The purified trypsin TPCK (*N*-Tosyl-L-phenylalanine chloromethyl ketone, 1 $g.L^{-1}$) was used as an activator of PO activity and the β-2-mercaptoethanol (10 mM) was tested as an inhibitor. To determine the PO activity of all samples, 80 µL of cacodylate buffer (CAC buffer: sodium cacodylate (10 mM), trisodium citrate (100 mM), NaCl (0.45 M), $CaCl_2$ (10 mM), $MgCl_2$ (26 mM), pH 7.0), 20 µL of L-Dopa (3 $mg.mL^{-1}$) and 20 µL of samples were added in each well. To measure the PO activity modulation, 60 µL of CAC buffer, 20 µL of PO activity modulators, 20 µL of L-Dopa and 20 µL of samples were added in each well. Control (120 µL of CAC buffer) and negative control (100 µL of CAC buffer, 20 µL of L-Dopa) wells were used to determine respectively purity of buffer and autooxidation capacities of L-Dopa. Each sample was tested in triplicate wells and A_{490} was measured after 21 h incubation period at room temperature.

2.4.4. Oyster gene expression

Gene selection (Table 2)

Four immune related genes (laccase, macrophage express protein, myeloïd differentiation factor and molluscan defence precursor) previously identified (Renault and Faury, personal communication) and a gene encoding expression of tumor suppressor gene (p53) from *C. gigas* cDNA database were selected. The Elongation factor I (EF I) gene was used as reference.

Genes (abbreviations)	Encoding gene types	5' – 3' primer sequence		GeneBank number
Laccase (LAC)	Immune gene	Forward Reverse	Gga tgg gtg gga ggc gaa ac Tgc gtc aca aag gga aca cca	EF999948
Macrophage express protein (MAC)	Immune gene	Forward Reverse	Acc gag acc gag ttt cag ggg gta g Gcc acc gaa agc cgg aga aga tgt c	None
Myeloïd differentiation factor (MYEL)	Immune gene	Forward Reverse	Cgt gcc atg gac gga taa caa cg Ggc cca gca gta cct ctg tgg aat c	None
Molluscan defence precursor (DEF)	Immune gene	Forward Reverse	Tcc ctc cag cga tca cta gat cca c Cat tct gcc cgg aga caa tca gat g	None
p53-like protein (p53)	Tumor suppressor gene	Forward Reverse	Cat gtg tct ggg atc ctg tg Ctg cct ttt cgt ctg ctt tc	AM236465
Elongation factor I (EFI)	Reference gene	Forward Reverse	Acc acc ctg gtg aga tca ag Acg acg atc gca ttt ctc tt	BQ426516

Table 2: Information concerning the four genes selected for real-time PCR analysis: gene names and their abbreviations, encoding gene type, primer sequences and GenBank Accession numbers.

Haemocyte preparation and total RNA extraction

Haemolymph from 30 oysters was pooled for each condition in order to obtain a sufficient quantity of haemocytes for RNA extraction. Each pool was centrifuged (1 200 g, 10 min, 4 °C) and the acellular fraction (supernatant) were eliminated.

Five mL of Trizol (Trizol® reagent, Invitrogen™; one mL Trizol for 0.5 - 1.10^{-6} cells) was mixed with haemocytes and incubated at ambient temperature (10 min, 15 °C) to extract total RNA. Two hundred µL of trizol dichloroform isoamylalcohol (TDI, MP Biomedical North America) were added. Samples (supernatant and TDI) were incubated at ambient temperature (3 min, 15 °C), they were then centrifuged (72 g, 15 min, 4 °C) and supernatants were collected. Five hundred µL of isopropanol trizol (IP, MP Biomedical North America) were added. Samples were incubated (10 min, 15 °C) and centrifuged (72 g, 10 min, 4 °C). Pellets were washed two times with one mL of 75 % ethanol (Sigma) and suspended in 22 µL of sterile water (MP Biomedical North America). Suspended samples were incubated (10 min, 55 °C), diluted at 1/100 and RNA concentrations was measured at $A_{260\ nm}$ using the conversion factor 1 OD = 40 µg RNA.

Samples were treated with the RQ1 RNase – free DNase (Promega) to remove DNA. The quality of extracted RNA was checked on 2 % agarose gel. RNA concentrations and purity were measured again by absorption spectrophotometry ($A_{260\ nm}$). Only high purity samples ($OD_{260/280} > 1.7$) were further processed.

Reverse transcription (RT)

Aliquots of 2 µg were reverse transcribed using SuperScript™ III Reverse Transcriptase kit (Invitrogen). Reverse transcriptase was carried out following manufacturer's recommendations using oligo$(dT)_{20}$ (Invitrogen), 10 mM of dNTP (Invitrogen) and RNaseOUT™ recombinant RNase Inhibitor (40 units.μL^{-1}, Invitrogen).

Real-time PCR analysis

The relative levels of gene transcripts in haemocytes from control and contaminated oysters were investigated by real time-PCR using an Icycler (Stratagène). Aliquots of the RT mixture were diluted at 1/40 with sterile water (MP Biomedical North America) before use.

Real-time PCR was performed in triplicates in a total volume of 25 µL in the presence of diluted cDNA (5 µL), Full Velocity® SYBR® Green QPCR Master Mix (12.5 µL, Stratagène®), sterile water (2.5 µL, MP Biomedical North America) and each specific primer pair (2.5 µL, Eurogentec S.A.) (Table 2). Amplification of a specific cDNA (Elongation factor I, coefficient of variation < 5 %) was performed to confirm the steady-state expression of a housekeeping gene, allowing an internal control for gene expression. Each microplate includes negative control (cDNA treated with DNase) and blank control (sterile water).

The cycling conditions consisted of Taq polymerase thermal initial activation (10 min, 95 °C) followed by 40 cycles of denaturation (30 s, 95 °C) and annealing/elongation (1 min, 60 °C). Finally, a melting curve of PCR product (60-95 °C) was performed to ensure the production of a single specific product.

PCR efficiency (E) was determined for each primer pair by constructing a standard curve from serial dilutions (10^{-1} to 5.10^{-4}). E value needs to range from 99 % to 100 %. Cycle threshold (Ct) values corresponded to the number of cycle at which the fluorescence emission monitored in real-time exceeded the threshold limit. Ct and E were obtained using the iCyclerTMiQ, Optical System Software, v. 3.0a (Biorad). The relative expression ratio for a considered gene is based on the PCR efficiency (E) and the Ct of the contaminated

Experimental conditions / Percentage (% ± SE)	Sampling dates							
	Day (-7)		Day (0)		Day (7)		Day (14)	
	Control	Contaminated	Control	Contaminated	Control	Contaminated	Control	Contaminated
Individual mortality	0	0	0	2	0	8	0	11
Granulocytes	28.9 (± 1.9)	26.9 (± 1.9)	22.2 (± 2.8)	23.7 (± 2.9)	35.5 (± 2.3)	38.5 (± 4.7)	21.6 (± 2.1)	20.3 (± 4.6)
Hyalinocytes	65.2 (± 1.8)	66.7 (± 2.0)	65.5 (± 2.6)	65.2 (± 3.1)	58.7 (± 2.6)	55.5 (± 4.5)	59.3 (± 3.6)	59.3 (± 3.6)
Mortality	4.9 (± 0.5)	4.2 (± 0.5)	2.7 (± 0.2)	2.2 (± 0.3)	3.8 (± 0.7)	4.9 (± 0.5)	4.4 (± 1.2)	4.6 (± 1.3)
Phagocytosis	37.3 (± 2.4)	40.7 (± 2.8)	30.8 (± 3.3)	28.2 (± 2.3)	33.7 (± 3.9)	33.0 (± 2.2)	30.5 (± 3.5)	32.1 (± 1.6)
Non-specific esterase positive cells	99.4 (± 0.1)	99.2 (± 0.2)	98.8 (± 0.2)	98.9 (± 0.2)	98.5 (± 0.2)	94.5 (± 3.7)	80.5 (± 8.0)	83.9 (± 6.0)
Lysosome presence	99.6 (± 0.1)	99.6 (± 0.2)	99.2 (± 0.2)	98.8 (± 0.5)	99.6 (± 0.4)	99.2 (± 0.3)	93.5 (± 6.1)	99.4 (± 0.2)

Table 3: Individual mortality was count and haemocytes (subpopulation, cell mortality, phagocytosis, non-specific esterase positive cell percentages and lysosome presence) were monitored by flow cytometry after two *in vivo* experimental exposures to light cycle oil (LCO). Day (-7) concerned the post-exposure and Day 0, Day 7 and Day 14 concerned the recovery period. No effect was detected for the two experiments for all values. Values are the mean of six pools of five oysters (three pools by experiment). * =statistical difference for $p \leq 0.05$.

samples versus the control samples expressed in comparison to the reference gene (EF I) as described previously (Pfaffl, 2001):

$$\text{Ratio} = \frac{(\text{E studied gene})^{\Delta \text{Ct studied gene (contaminated – control)}}}{(\text{E reference gene})^{\Delta \text{Ct reference gene (contaminated – control)}}}$$

2.5. Statistical analysis

Statistical tests were carried out using XLStat 2008. Verification of normality was conducted using the Anderson-Darling test. Since the values were non normal, the Mann-Whitney test was used to analyse LCO effects at each sampling date. P values lower than 0.05 were used to identify significant differences.

3. Results

Results were the mean of two experiments with LCO exposure at 1 600 ± 315 $ng.L^{-1}$. An oyster mortality of 21 % was counted in contaminated organisms during the recovery period, with 2 % at Day 0, 8 % at Day 7 and 11 % at Day 14 (Table 3), and shells were coloured on green but no lesion was macroscopically observed (data not shown). In control units, no mortality was shown and no lesion and shell abnormality were macroscopically detected. Seawater parameters were stable during the acclimatisation period and the experiment (dissolved oxygen 96 ± 4 %, pH 7.6 ± 0.4, salinity 36 ± 1 ‰, temperature 12 ± 1 °C, free of nitrate and nitrite) in each unit.

3.1. PAH concentrations in seawater

In control tanks, PAHs were not detected. Each PAH which composed the LCO was detected in contaminated units with a total mean concentration of 1 600 ± 315 $ng.L^{-1}$ and with mean concentrations ranging from 20 ± 4 $ng.L^{-1}$ for fluoranthene and 20 ± 8 $ng.L^{-1}$ for phenanthrene to 668 ± 224 $ng.L^{-1}$ for acenaphthene. Concerning the diaromatic (e.g. naphthalene, from 196 to 5 $ng.L^{-1}$) and triaromatic compounds (e.g. phenanthrene, from 30 to 5 $ng.L^{-1}$), concentrations decreased during the exposure period. For the compounds with four aromatic cycles, kinetics of dissolution were stable (e.g. pyrene, from 126 to 159 $ng.L^{-1}$) (Table 4).

Name of PAH compounds	Concentration ($ng.L^{-1} \pm SE$)					
	Day (-6)	**Day (-5)**	**Day (-4)**	**Day (-3)**	**Day (-2)**	***Mean***
Naphthalene	196	174	15	5	5	*79 ±43*
Acenaphthylene	96	208	80	27	21	*86 ±34*
Acenaphthene	1 368	307	996	504	167	*668 ±224*
Fluorene	437	10	174	124	122	*173 ±71*
Phenanthrene	30	48	6	9	5	*20 ±8*
Anthracene	86	79	53	46	48	*62 ±8*
Fluoranthene	32	19	21	11	15	*20 ±4*
Pyrene	126	110	96	48	159	*108 ±18*
Chrysene	287	99	176	202	324	*166 ±56*
Benzo[*a*]anthracene	111	69	96	378	175	*218 ±40*
Benzo[*b+k*]fluoranthene	n.d.	n.d.	n.d.	n.d.	n.d.	*n.d.*
Benzo[*a*]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	*n.d.*
Indeno[*1,2,3-c,d*]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	*n.d.*
Benzo[*g,h,i*]perylene	n.d.	n.d.	n.d.	n.d.	n.d.	*n.d.*
Dibenz[*a,h*]anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	*n.d.*
Sum of the 16 PAHs	**2 769**	**1 124**	**1 713**	**1 355**	**1 039**	***1 600 ±315***

Table 4: Concentration of 16 US-EPA PAHs in contaminated units throughout the two *in vivo* experimental exposures to light cycle oil (LCO). The results are expressed in $ng.L^{-1}$ in seawater (n = mean values establish from five analysis at Day (-6), Day (-5), Day (-4), Day (-3) and Day (-2) after the beginning of the exposure period, mean ± standard error, n.d. = not detected, < 5 $ng.L^{-1}$).

3.2. PAH concentrations in oyster tissues

PAHs were detected neither before the contamination period in each oyster group (Day (-7)) nor during all the experimental period for the control oysters (lower than 5 $\mu g.kg^{-1}$ of dry weight) (Table 5).

After seven days of exposure (Day 0), contaminated oyster had bioaccumulated five PAHs, with 702 ± 99 $\mu g.kg^{-1}$ of dry weight composed in largely about acenaphthene (57.9 %) and naphthalene (30.9 %). Fluorene (6.0 %), acenaphthylene (3.6 %) and phenanthrene (1.7 %) were less bioaccumulated.

After seven days of recovery (Day 7), contaminated oysters had particularly eliminated fluorene (34.5 %) bioaccumulated in their tissues. The other bioaccumulated molecules presented approximately the same elimination rate (phenanthrene: 18.2 %; acenaphthene: 17.1 %; acenaphthylene: 16.2 %; naphthalene: 14.7 %). At Day 7, the PAHs still bioaccumulated (580 ± 97 $\mu g.kg^{-1}$ of dry weight) corresponded to 58.1 % of acenaphthene, 31.9 % of naphthalene, 4.7 % of fluorene, 3.6 % of acenaphthylene and 1.7 % of phenanthrene.

On Day 14, the oyster tissues still contained 484 ± 45 $\mu g.kg^{-1}$ of dry weight of each five bioaccumulated PAH with 66.1 % of acenaphthene, 25.0 % of naphthalene, 3.9 % of fluorene and acenaphthylene and 1.1 % of phenanthrene. Oysters have mostly eliminated fluorene (55.2 %), phenanthrene (54.5 %) and naphthalene (44.1 %). Acenaphthylene (25.2 %) and acenaphthene (21.3 %) were the PAH with the bit elimination rate. Finally, after 14 days of recovery period, oysters have eliminated 31.1 % of the total PAHs bioaccumulated.

Sampling dates	**Control oysters ($\mu g.kg^{-1}$ of dry weight ± SE)**	**Contaminated oysters ($\mu g.kg^{-1}$ of dry weight ± SE)**
Day (-7)	n.d.	n.d.
Day (0)	n.d.	702 ± 99 *naphthalene: 217 ± 40; acenaphthylene: 25 ± 9; acenaphthene: 406 ± 72; fluorene: 42 ± 8; phenanthrene: 12 ± 4*
Day (7)	n.d.	580 ± 97 *naphthalene: 185 ± 29; acenaphthylene: 21 ± 8; acenaphthene: 337 ± 64; fluorene: 27 ± 10; phenanthrene: 10 ± 4*
Day (14)	n.d.	484 ± 45 *naphthalene: 121 ± 16; acenaphthylene: 19 ± 3; acenaphthene: 319 ± 33; fluorene: 19 ± 3; phenanthrene: 5 ± 2*

Table 5: Concentration of the 16 US-EPA PAHs in oyster tissues after two *in vivo* experimental exposures to light cycle oil (LCO). The results are expressed in $\mu g.kg^{-1}$ of dry weight (n.d. = not detected, < 5 $\mu g.kg^{-1}$). Values are the mean of 30 oysters (15 oysters by experiment). The experiment was performed by gas chromatography coupled with mass spectroscopy (GC-MS).

3.3. PAH effects on haemocyte parameters

All haemocyte parameters analysed by flow cytometry were not significantly different in the control and contaminated oysters at each sampling date. Haemocyte

subpopulations were represented by 26.9 ± 1.3 % of granulocytes and 61.7 ± 1.3 % of hyalinocytes. Haemocyte subpopulation and mortality (4.0 ± 0.3 %), phagocytosis percentage (33.3 ± 1.1 %), non-specific esterase positive cells (94.2 ± 1.6 %) and lysosome presence (98.6 ± 0.8 %) were maintained constant during both experiments (Table 3).

3.4. PAH effects on phenoloxidase (PO) activity

PO activity was significantly decreased in contaminated oysters compared to controls from Day 0 to Day 14 of the recovery period. This difference was similar for each sampling date which a mean value of 0.274 ± 0.012 for contaminated oysters and 0.349 ± 0.005 for control oysters (Fig. 2).

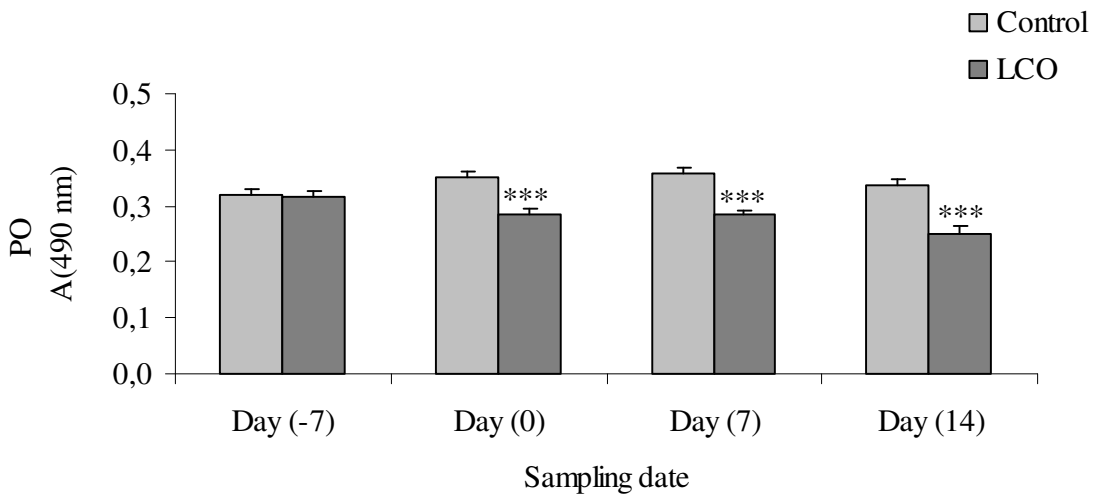

Fig. 2: Phenoloxidase (PO) activity measured by spectrophotometry following two *in vivo* experimental exposures to light cycle oil (LCO). Values are the mean of six pools of five oysters (three pools by experiment). The bars represent the standard error. *** = statistical difference for $p \leq 0.001$.

3.5. PAH effects on gene expression

After the seven day period of LCO exposure (Day 0), relative expression of LAC (1.8 ± 0.6) and MAC (3.3 ± 0.9) was higher compared to those from control oysters. A weak lower relative expression of transcripts was observed for p53 (0.8 ± 0.1) (Fig. 3).

Relative expression of MAC (0.7 ± 0.1), DEF (0.7 ± 0.2) and p53 (0.4 ± 0.3) was weakly lower compared to those from control organisms at Day 7 of the recovery period.

At the last day of the recovery period (Day 14), a lower relative expression of transcripts compared to those from control was observed for LAC (0.8 ± 0.0) and MYEL (0.3 ± 0.0). MAC (1.4 ± 0.0) and DEF (1.5 ± 0.1) presented higher expression of their transcript.

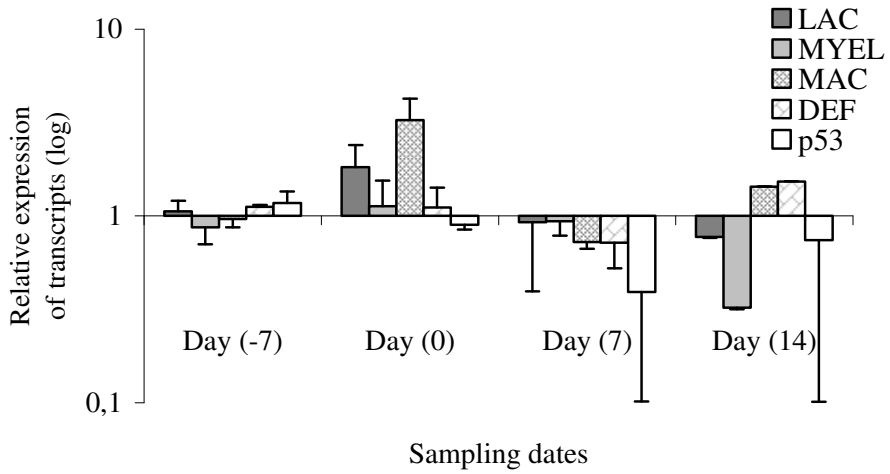

Fig. 3: Relative expression of the five gene transcripts (normalised to elongation factor I) in contaminated oysters compared to controls following two *in vivo* experimental exposures to light cycle oil (LCO). RNA was extracted from two pools of haemocytes (one pool of 30 oysters by experiment). Control values were represented by the axis located at 1 relative expression of transcripts. The bars represent the standard error. See Table 2 for the category of the genes.

4. Discussion

The Pacific oyster, *Crassostrea gigas*, has been selected in order to research the effects of LCO dissolved fraction on marine organisms.

4.1. Chemical analysis

Pollutant detection and exposure in dissolved fractions depend on the dissolution technique used. The Anderson method (Anderson et al., 1974) has been chosen and modified by the *Cedre* to expose organisms only to the dissolved fraction of LCO. The dissolved fraction, obtained after two weeks of contact with seawater (1 600 $ng.L^{-1}$), remained constant and can be compared to those observed after an HFO oil spill (Boehm et al., 2007; Law,

1978). The more soluble PAHs, due to their low and mean (≤ 228.3 g.mol^{-1}) molecular weights, were represented in pure LCO. All of these PAHs were detected in the dissolved fraction used. PAH concentration of four benzenic cycles stayed constant during the entire contamination period and two-ring molecules (naphthalene, acenaphthene, acenaphthylene and fluorene) decrease slowly. The stable concentration of three-ring molecules could be explained together by their evaporation processes and their fast dissolution kinetics rate. Concerning the level of each PAH in seawater dissolved fraction, they seems to be not connected to abundance of PAHs in the refined petrol but certainly to an equilibrium between all compounds and their kinetics of dissolution. In the present work, exposure to the dissolved fraction of LCO conducted to the bioaccumulation of some PAHs on oyster tissues (naphthalene, acenaphthene, acenaphthylene, fluorene and phenanthrene). Others authors observed that naphthalene, fluorene and phenanthrene could be bioaccumulated by the Eastern oyster, *Crassostrea virginica* (Neff et al., 1976) after exposure to the same dissolved PAHs. The bioaccumulation process was dependant about molecular weight and seawater concentration. PAHs with low molecular weight (≤ 178.2 g.mol^{-1}) were more bioaccumulated as the others due to their important water-soluble capacities and than their larger bioavailability for organisms (Neff et al., 1976). Moreover, the bioaccumulation capacity seems to be dependant about their concentrations in seawater.

Concerning depuration, after 14 days of recovery period, half-life for PAHs was reported in oyster tissues: fluorene (55.2 %), phenanthrene (54.5 %) and naphthalene (44.1 %). Acenaphthylene (25.2 %) and acenaphthene (21.3 %), which present very similar molecular conformation, have a slower elimination rate. Depuration rate is dependant to toxicant doses, exposure time, toxicity mixtures, species and other factors such as suspended organic matter, temperature, salinity changes and form of contaminants present in the water column (Meador et al., 1995). Since in invertebrates depuration mainly relies on simple diffusion, oysters could normally release preferentially water-soluble molecules (e.g. naphthalene). Nevertheless, the quantity and the conformation of each bioaccumulated PAH were also involved in elimination processes (Meador et al., 1995). Tissue concentrations, molecular weights and conformations of compound seem to act on these depuration rates.

4.2. LCO and biological parameters

In this study, 21 % of contaminated oysters died due to exposure to LCO dissolved fraction (1 600 ng.L^{-1}). This refined product was constituted about PAHs with low and mean molecular weights which are known to induce important physiologic, immunologic, mutagenic and carcinogenic effects (WHO, 1997). An important destabilisation of these processes by pollutant could explain the oyster mortality recorded. Nevertheless, few immune parameters tested were modulated, thus this individual mortality could be due to physiological disturbance. In fact, Mahoney and Noyes (1982) observed that chronic exposure to hydrocarbons could induce Eastern oyster mortality by reduction of food intake or utilization. Although, the shell of contaminated oysters showed a green colour, no lesions were macroscopically observed.

Although some oysters died, no modulation of haemocyte parameters was noted after seven days of exposure to dissolved fraction of LCO. In this study, no significant modulation of haemocyte subpopulation percentage was reported. Exposure of common mussel, *Mytilus edulis*, to fluoranthene did not induce modification of haemocyte subpopulation percentage (Coles et al., 1994). As shown by Jeong and Cho (2005), fluoranthene exposure had also no effect on *C. gigas* haemocyte mortality. Our results suggest that PAHs at the concentration used do not affect the oyster immune system by killing haemocytes. Moreover, in contradiction with previous studies (Auffret, 2005; Gagnaire et al., 2006; Lowe et al., 1995), phagocytosis percentage, non-specific esterase positive cell percentage and lysosome presence were not affected by tested PAHs. Lowe et al. (1995) observed *in vivo* a significant modulation of lysosomal membrane due to exposure to only one PAH, the fluoranthene, whereas in this experiment organisms were exposed to a cocktail of PAHs. Gagnaire et al. (2006) observed that fluoranthene, anthracene, pyrene, phenanthrene and benzo[*a*]pyrene have an impact on haemocyte parameters in function of concentration tested. Thus, the present results could be due either to antagonist effects of some dissolved PAHs on these haemocyte activities or to the dissolved concentration of each PAH.

Phenoloxidase (PO) activity was modified by LCO exposure and a significant decrease of this activity was reported during two weeks. Similar results were shown after a short exposure to the dissolved fraction of HFO and after two weeks of recovery period (Bado-Nilles et al., submitted-b). Other pollutants caused a similar decrease in PO activity *in*

vitro such as mercury in the Pacific oyster (Gagnaire et al., 2004) and *in vivo* such as trichlorfon in prawns (Chang et al., 2006). On the contrary, Coles et al. (1994) had previously shown that fluoranthene induced an *in vivo* increase in PO activity in the blue mussel, *Mytilus edulis*.

4.3. LCO and gene expression

The present study provides a first evidence of LCO dissolved fraction effects on gene expression in the Pacific oyster, *C. gigas.* Four immune-related genes (laccase, macrophage express protein, myeloïd differentiation factor and molluscan defence precursor) and a tumor suppressor gene (p53) were monitored by real time RT PCR. Nevertheless, only the expression of laccase and macrophage express protein was discussed here due to their important increase after seven days of exposure to LCO dissolved fraction.

Laccases (benzenediol:oxygen oxidoreductases; E.C. 1.10.3.2) are copper-containing oxidoreductive enzymes that catalyze a one-electron oxidation of broad range of polyphenols and aromatic substrates (Claus, 2004). In the present study, an up-regulation of laccase gene was observed after seven days of oyster exposure to LCO dissolved fraction (Day 0). Currently this tyrosinase was studied in *Trametes versicolor* for bioremediation of PAH because it can catalyze oxydation of aromatics (e.g. anthracene, benzo[*a*]pyrene) by an indirect mechanism involving the participation of an oxidative mediator (Dodor et al., 2004). Thus, it could be speculated that increase of laccase gene expression enables a protection against bioaccumulated PAHs. During the recovery period, mRNA expression was progressively decreased after 14 days of recovery (Day 14). This slow decrease might be due to regulation of enzymatic concentration as a function of quantity of bioaccumulated PAHs.

The macrophage express protein is a perforin-like protein and has been reported in marine molluscs (Mah et al., 2004). This perforin-like is up-regulated by lipopolysaccharide (LPS) in the sponge, *Suberites domuncula,* and displays a toxic effect on bacteria (Wiens et al., 2005). In the present work, an increased expression of macrophage express protein gene was observed after seven days of exposure to dissolved fraction of LCO (Day 0). After seven day of recovery period (Day 7), the expression of the macrophage express protein decreased. The product of macrophage express protein gene is an important actor of innate immunity in

different species. Thus, expression modulation of this gene by LCO exposure may indicate that PAHs are able to modulate immune functions in the Pacific oyster, *C. gigas*.

4. Conclusions

Effects of LCO were reported on some monitored parameters in the Pacific oyster, *C. gigas*. In vivo contamination with the soluble fraction of LCO does not cause haemocyte cell death, but has an effect on PO activity before and after recovery period. Moreover, some immune-related genes, including laccase and macrophage express protein, which are important components of the innate immune response against pathogens, were modulated. This study seems to indicate a possible existence of a relationship between PAHs and susceptibility of animals to infectious diseases as shown by Grundy et al. (1996) and Pipe and Coles (1995) and need further studies.

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ARTICLE 7: In vivo effects of light cycle oil soluble fraction on immune function and gene expression in marine species: Part II. in a Vertebrate, the European sea bass, *Dicentrarchus labrax* (Linné).

Article en préparation

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Abstract

The effects of a dissolved fraction of light cycle oil (LCO, 1 600 $ng.L^{-1}$) on leucocyte parameters, lysozyme concentration, alternative complement pathway (ACH_{50}) activity in plasma and candidate gene expressions in spleen of the European sea bass, *Dicentrarchus labrax* (L.), were tested after seven days of exposure. At the end of the contamination, fish were transferred to non contaminated seawater and a recovery period was conducted for two weeks. Five polycyclic aromatic hydrocarbons (PAHs) out of ten detected in the seawater were bioaccumulated in muscle at the end of the contamination. Bioaccumulation of PAHs in contaminated muscle decreased during the recovery period and 14 days after the exposure, 60 % of the PAHs bioaccumulated at Day 0 were detected in the muscle of contaminated fish. Some biliary metabolites were accumulated into gall bladder. Exposure condition to dissolved fraction of light cycle oil (1 600 $ng.L^{-1}$) induces external and internal macroscopically detectable lesions and 12 % of fish mortality. Each immune parameter, apart from non-specific esterase positive cell percentage, approached by flow cytometry and spectrophotometry were modulated by pollutant. Modification of immune-related gene (TNF and C3) expressions was noted but tumor suppressor gene (P53) expression was not affected. To conclude, modulation of immune-related parameters was demonstrated using three different methods (flow cytometry, spectrophotometry and gene expression) in European sea bass after contact with dissolved fraction of LCO. Each compound which constitutes the LCO could therefore induce modulation of immune system and thus modified the capacity of fish to respond to an infectious agent.

Keywords: *Dicentrarchus labrax*; Leucocytes; Polycyclic aromatic hydrocarbons; Immune parameters; Bioaccumulation; Gene expression.

1. Introduction

The LCO products are commonly used to produce higher home fuel or to increase or maintain fluid catalytic cracking federate (Ding et al., 2007). Moreover certain refiners use the LCO at the end of cracking catalytic process in order to facilitate its transport by tankers (Ding et al., 2007). These mixtures, called heavy fuel oil (HFO), are largely detected in the environment after the wreckage of tankers. During the last decades, oil spills were observed in the Atlantic coast in particular after the accident of the Erika tanker which spread out in the sea 20.000 tons of its HFO A major pollution occurred also in November 2002 after the *Prestige* tanker accident with a freight of 63 000 tons of HFO. In case of oil spills, polycyclic aromatic hydrocarbons (PAHs) are considered as a major factor of stress for marine organisms. Some PAHs appear in the priority pollutant list of the US environmental protection agency (US-EPA) due to their mutagenic, carcinogenic, phototoxic (Shiaris, 1989), reprotoxic (Diamond et al., 1995) and immunotoxic (Yamaguchi et al., 1996) properties. Thus, in the immediate aftermath of the spill, damages attributed to HFO were observed and measured in several species of bivalves (Bocquené et al., 2004; Cajaraville et al., 2006) and fish (Budzinski et al., 2004; Morales-Caselles et al., 2006). Nevertheless, the LCO fraction, which is composed mainly of low molecular weight PAHs, was not studied until now.

Physiological disorders could result from the natural accumulation of PAHs in fish tissues. This bioaccumulation process is due to different mechanisms: a direct uptake from water by gills or skin and a uptake of suspended particles due to ingestion and the consumption of contaminated food (Van der Oost et al., 2003). PAHs are generally bioaccumulated in specific organs, such as the liver where the biotransformation is most effective, and lipid-rich tissues (e.g. muscle) and their metabolites can be found in the gas bladder (Van der Oost et al., 2003). This bioaccumulation into muscle and bile of PAH metabolites induce physiological disturbances and particularly, on immune system (Ribeiro et al., 2005). In Vertebrates, contrary to oysters which have only an innate immune system, both specific and non-specific immunity may be altered by PAHs (Reynaud and Deschaux, 2006). It seems that xenobiotic-mediated suppression of the innate immune responses, involved in resistance to pathogens, would have more impact than the suppression of the

acquired immune response. However, little is known about mechanisms by which PAHs induced immunotoxicity in vertebrates and more especially in fish (Reynaud and Deschaux, 2006).

The aims of the present study were to investigate the effects of refined petroleum, the LCO, on immune parameters and candidate gene expressions simultaneously in two marine species, the Pacific oyster, *Crassostrea gigas*, and the European sea bass, *Dicentrarchus labrax*. The results obtained for Pacific oyster are reported in an other article (Bado-Nilles et al., submitted-a). The capacity of adult European sea bass to recover their initial status after a seven days exposure to a dissolved fraction of LCO was analysed based on monitoring of cell mortality, haemocyte subpopulations, phagocytosis activity, percentage of non-specific esterase positive cells and lysosome presence by flow cytometry. Lysozyme concentration and haemolytic alternative complement pathway (ACH_{50}) were studied in the plasma by spectrophotometry. Two immune related genes (TNF-alpha, component C3) and one tumor suppressor gene (P53) expression were monitored by real time RT PCR. All parameters were monitored during a recovery period of two weeks.

2. Materials and methods

2.1. Sea bass

One hundred and twenty European sea bass, *Dicentrarchus labrax*, 136 ± 32 g and 22 ± 2 cm, came from one pond and were maintained in an experimental facility at AGENCE FRANÇAISE DE SACURITÉ SANITAIRE DES ALIMENTS site de Ploufragan-Plouzané (Afssa, France). On October 2006, fish were transferred to the *Cedre* institute (Brest, France). Sea bass were acclimatized with Pacific oyster, *Crassostrea gigas* (Bado-Nilles et al., submitted-a), for two weeks in 1 200 L tanks with a flow of 0.3 $m^3.h^{-1}$ at 16 ± 1°C (dissolved oxygen 96 ± 4 %, pH 7.8 ± 0.2, salinity 36 ± 2 ‰, free of nitrate and nitrite). Sea bass were fed every day with 150 g of granulates (Grower Extrude Natura 4mm, Le Gouessant Aquaculture).

2.2. Pollutants

Light cycle oil (LCO), which contained 85-86 % of aromatic hydrocarbons, 1-2 % of sulphurs and 4-2 % of polar compounds, was selected to perform the exposure. The composition of LCO was described previously (Bado-Nilles et al., submitted-a). Due to the specificity of the exposure system, the sea bass were contaminated only by the dissolved fraction of this LCO.

2.3. Experimental arrangement

2.3.1. Exposure system

The experimental system was constituted of six units (616 L), three control units and three contaminated units. The tanks were placed in a greenhouse, which is thermoregulated (T = 14 ± 1 °C) and has one total renewal of the air in 6 hours. For the exposure period, the protocol used and the dissolved fraction of LCO were described previously (Bado-Nilles et al., submitted-a).

2.3.2. Exposure conditions

After the acclimatisation period, 20 sea bass were placed with oysters in each control units while 20 sea bass were exposed with oysters in each contaminated unit with the natural light / dark cycle. During the seven days of exposure period, control and exposed organisms were not fed. The seawater quality was monitored in both tanks (oxygen level, temperature, pH, salinity, nitrate and nitrite) and, also, the concentration of the LCO soluble fraction.

2.3.3. Recovery period

After the contamination period, the mixing tank was disconnected and fresh seawater was flowed at a rate of 0.3 $m^3.h^{-1}$. Oysters and sea bass were put back in the same condition as during the contamination exposure period but without exposure. During the two weeks recovery period, the organisms were fed again.

2.3.4 Samples and sampling date

Seawater sampling and PAH concentrations

During the exposure period, one litre of seawater was collected daily in each exposed tanks in heated (500 °C) Duran glass bottles (Bioblock) to characterise the seawater quality. Results were reported in previous publication (Bado-Nilles et al., submitted-a).

Sea bass sampling

Fish mortality was counted daily. Five sea bass were collected in each exposure tanks for flow cytometry, spectrophotometry, PAH tissue analysis and RNA extraction. Sea bass samplings were made on the first (Day (-7)) and on the last (Day 0) days of the contamination period and after seven and 14 days of recovery period (respectively Day 7 and Day 14). At each sampling date, external and internal macroscopically lesions were researched.

Blood, muscle and organ collections

Fish were anaesthetised with phenoxy-2-ethanol and then peripheral blood was collected. For each fish, 1-2 mL of blood were withdrawn from the caudal vein with a lithium heparinized vacutainer (BD Vacutainer^{TM} LH 85 U.I.). Blood was kept on ice until it was processed. Then, fish were stunned and weighed. After handling, the gall bladder and the spleen were excised, weighted and stored at – 80°C for later analyses. Sixteen g of muscle of the 15 control and contaminated fish were collected and frozen at - 80°C too until analyses.

2.4. Analytical methods

2.4.1. PAH concentrations on sea bass muscle

The PAHs concentrations in the 16 g of each muscle were determined by GC-MS using the procedure of Baumard et al. (1997) as described for oysters (Bado-Nilles et al., submitted-a).

2.4.2. Sea bass biliary metabolite concentrations

One microlitre of bile of each gall bladder was sampled. Each bile sample was diluted in 4 mL of absolute ethanol (VWR International). Fluorescence analyses of bile samples

were performed with a spectropfluorimeter (Fluorimeter 10-AU, Turner Designs Sunyvale, California). The excitation and emission wavelength used to determine the fluorescence intensity of bile samples by Fixed Fluorescence (FF) were 290/335 nm and 341/383 nm. The couple of wavelength 290/335 nm characterizes the naphthalene type metabolites (Lin et al., 1996) and 341/383 nm corresponds to four-rings PAH compounds, pyrene metabolites (Aas et al., 1998).

2.4.3. Sea bass immune parameters

Blood leucocyte preparation and analysis by flow cytometry

For flow cytometry analysis, 0.5 mL of the blood collected from 15 contaminated and from 15 control fish (n = 15) were immediately diluted with 10 mL of Leibovitz 15 medium (L15, Eurobio) containing 10 U. heparin lithium (Sigma). Then, samples were loaded onto Ficoll gradient (Histopaque®1077, Eurobio) to density of 1.07-1.08 $g.cm^{-3}$. After centrifugation (400 g, 30 min, 15 °C), mononuclear cells at the interface were collected and were washed twice (400 g, 5 min, 4 °C) with L15. Finally, cells were resuspended in 1 mL of L15 medium. Then, cell viability was assessed using the trypan blue exclusion method and enumeration was performed with a Thoma's cell haemocytometer and adjusted at 10^6 $cells.mL^{-1}$ with L15 medium.

Morphological characteristics, leucocyte mortality, phagocytosis percentage, percentage of non-specific esterase positive cells and lysosome presence were analysed with an EPICS XL 4 (Beckman Coulter) using protocols previously described (Bado-Nilles et al., submitted-a). For each leucocyte sample, 10 000 events were counted. Analyses were carried out on whole leucocytes without distinguishing cell subpopulation and results were expressed as percentage of positive cells.

Plasma preparation and analysis by spectrophotometry

For spectrophotometry analysis, resting blood samples were centrifuged (1 200 g, 10 min, 4 °C). After centrifugation, the plasma of each fish was split into 2 aliquots of 100 µL and stored at - 80°C for further analysis.

Plasma lysozyme activity was determined using a turbidimetric assay (Grinde et al., 1988), adapted to microtitration plates. Briefly, a bacterial suspension of *Micrococcus*

lysodeikticus (Sigma) was prepared at a concentration of 1.25 $g.L^{-1}$ in a 0.05 M sodium-phosphate buffer pH 6.2. Fifty µL of the individual plasma samples were plated in 96 well microtitration plates. The reaction was initiated in a multiscan spectrophotometer, by addition of 160 $\mu L.well^{-1}$ of *M. lysodeikticus* suspension using an automatic dispenser. Reading of D.O. at a wavelength of 450 nm were performed every 15 s for 3 min, the plate being shaken before each reading. Using a standard hen egg white lysozyme (Sigma) in sodium-phosphate buffer, the concentration of lysozyme in sea bass plasma was expressed in $mg.L^{-1}$.

The alternative pathway of plasma complement activity was carried out by a haemolytic assays with rabbit red blood cells (RRC, Biomérieux) as described by Yano (1992) and adapted to microtitration plates. Sea bass plasma, diluted at 1/64 in EGTA-Mg-GVB buffer to avoid natural haemolytic activity, was added in increasing amounts, from 10 to 90 $\mu L.wells^{-1}$, and was filled with EGTA-Mg-GVB buffer to get a final volume of 100 µL. Fifty µL of 2 % RRC (Biomérieux) suspension were finally added in all wells. Control values of 0 and 100 % haemolysis were obtained using: 100 µL of EGTA-Mg-GVB buffer and 100µL of non-decomplemented trout haemolytic serum at 1/50 in ultrapure water, respectively. Samples were incubated during one hour at 20 °C. The microplates were centrifuged (400 g, 5 min, 4 °C, Jouan). Then, 75 µL of supernatant of each well were deposed with 75 µL of phosphate buffer saline (PBS, Sigma) into an other 96-well microplate. The absorbance (A_{540}) was read and the number of ACH_{50} units per mL of plasma was determined in reference to the 50 % haemolysis.

Genes (abbreviations)	**Encoding gene types**	**5' – 3' primer sequence**		**GeneBank number**
Complement component C3 (C3)	Immune gene	Forward	tat gcc ctt ctt gct ctg gt	Marine Genomic Europe (Not published)
		Reverse	gcc tga gtt gat cca tag cc	
Tumor Necrosis Factor alpha (TNF-a)	Immune gene	Forward	tca cca cag agc act gga ag	DQ200910
		Reverse	aga ccg atc tcc aca tca cc	
p53-like protein (P53)	Tumor suppressor gene	Forward	tcc cgg tta cat ccg act ac	Marine Genomic Europe (Not published)
		Reverse	gct ccg aaa aag tcg aag tg	
Elongation factor I (EFI)	Reference gene	Forward	gct tcg agg aaa tca cca ag	AJ866727
		Reverse	ca acct tcc atc cct tga ac	

Table 1: Information concerning the three genes selected for real-time PCR analysis: gene names and their abbreviations, encoding gene type, primer sequences, GenBank Accession numbers and references**.**

2.4.4. Sea bass gene expression

Gene selection

The sequence of two genes encoding protein involved in immune system (complement component C3 and tumor necrosis factor alpha) and one gene encoding tumor suppressor actor (P53) were obtained from public and marine Genomic Europe databases (Table 1). The Elongation factor I (EF I) gene was used as reference.

Gene expression

Total RNA was extracted from spleen using Trizol Reagent (Invitrogen^{TM}) as described by the manufacturer's kit. The RNA concentrations were measured at $A_{260\ nm}$ using the Nanodrop® Spectrophotometer ND-1000. Only high purity samples ($OD_{260/280} > 1.8$) were further processed. The quality of the isolated RNA was checked on electrophoretic run on 1.2 % agarose gel. Aliquots of 1 µg were reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) as described by the manufacturer's kit. Expression of C3, TNF-alpha and P53 were investigated by real-time PCR using the MyIQ^{TM} single color real-time PCR detection system (Biorad). Reverse transcription product were diluted at 5.10^{-2} with sterile water (MP Biomedical North America), and 5 µL were used for each real-time PCR reaction. Triplicates were run for each RT product. Real-time PCR was performed using IQ^{TM} SYBR® Green Supermix (BioRad) according to the manufacturer's instruction. The level of EF I RNAs was monitored using the same sample set to allow normalization. Real time PCR data were treated using ΔΔCT method available in iCyclerMyIQ^{TM}5, Optical System Software, v. 2.0 (Biorad).

2.5. Statistical analysis

Statistical tests were carried out using XLStat 2008. Verification of normality was conducted using the Anderson-Darling test. Since the values were normal, the F-test was used to analyse LCO effects in at each sampling date. P values lower than 0.05 were used to identify significant differences.

3. Results

Data obtained resulted from the experiments with LCO exposure at 1 600 ± 315 ng.L^{-1} (Bado-Nilles et al., submitted-a). A sea bass mortality of 12 % was counted in contaminated animals during the recovery period and external and internal macroscopically lesions were observed (data not shown). Concerning control units, no mortality and no macroscopically lesion were detected. Seawater parameters were stable during the acclimatisation period and the experiment (dissolved oxygen 96 ± 4 %, pH 7.6 ± 0.4, salinity 36 ± 1 ‰, temperature 12 ± 1 °C, free of nitrate and nitrite) in each unit.

3.1. PAH concentrations in fish tissues

PAHs were detected neither before the contamination period (Day (-7)) in each fish group nor during all the experimental period for the control fish (lower than 5 µg.kg^{-1} of dry weight) (Table 2).

After seven days of exposure (Day 0), the contaminated fish muscles had bioaccumulated five PAHs, with 321 ± 14 µg.kg^{-1} of dry weight composed largely about fluorene (37.3 %, 120 ± 7 µg.kg^{-1} of dry weight), phenanthrene (31.8 %, 102 ± 6 µg.kg^{-1} of dry weight) and acenaphthene (19.6 %, 63 ± 9 µg.kg^{-1} of dry weight). Anthracene (6.1 %, 20 ± 3 µg.kg^{-1} of dry weight) and naphthalene (5.2 %, 17 ± 4 µg.kg^{-1} of dry weight) were less bioaccumulated after the seven days of exposure.

After seven days of recovery (Day 7), the fish muscle had particularly eliminated naphthalene (69.6 %) bioaccumulated in their muscles. The other bioaccumulated molecules present weakly elimination rate (phenanthrene: 27.2 %; fluorene: 18.1 %; acenaphthene: 4.7 %; anthracene: 1.6 %). At Day 7, the PAHs still bioaccumulated (257 ± 16 µg.kg^{-1} of dry weight) correspond to 38.2 % of fluorene (98 ± 7 µg.kg^{-1} of dry weight), 28.9 % of phenanthrene (74 ± 7 µg.kg^{-1} of dry weight), 23.4 % of acenaphthene (60 ± 9 µg.kg^{-1} of dry weight), 7.5 % of anthracene (19 ± 2 µg.kg^{-1} of dry weight) and 2.0 % of naphthalene (5 ± 1 µg.kg^{-1} of dry weight).

On Day 14, the fish muscle still contained 163 ± 22 µg.kg^{-1} of dry weight without naphthalene which was totally eliminated. Each four resting PAHs were detected with 42.7 %

of fluorene (70 ± 18 µg.kg^{-1} of dry weight), 28.7 % of acenaphthene (47 ± 10 µg.kg^{-1} of dry weight), 23.2 % of phenanthrene (38 ± 6 µg.kg^{-1} of dry weight) and 5.5 % of anthracene (9 ± 2 µg.kg^{-1} of dry weight). Fish have mostly eliminated phenanthrene (62.9 %), anthracene (54.6 %) and fluorene (41.7 %) than acenaphthene (25.5 %).

Finally, after 14 days of recovery period, fish have eliminated 49.1 % of the total PAHs bioaccumulated.

Sampling dates	**Control fish (µg.kg^{-1} of dry weight ± SE)**	**Contaminated fish (µg.kg^{-1} of dry weight ± SE)**
Day (-7)	n.d.	n.d.
Day (0)	n.d.	321 ± 14 *naphthalene: 17 ± 4; acenaphthene: 63 ± 9; anthracene: 20 ± 3; fluorene: 120 ± 7; phenanthrene: 102 ± 6*
Day (7)	n.d.	257 ± 16 *naphthalene: 5 ± 1; acenaphthene: 60 ± 9; anthracene: 20 ± 2; fluorene: 98 ± 7; phenanthrene: 74 ± 7*
Day (14)	n.d.	163 ± 22 *acenaphthene: 47 ± 10; anthracene: 9 ± 2; fluorene: 70 ± 18; phenanthrene: 38 ± 6*

Table 2: Concentration of the 16 US-EPA PAHs in fish tissues after two *in vivo* experimental exposures to light cycle oil (LCO). The results are expressed in µg.kg^{-1} of dry weight (n.d. = not detected, < 5 µg.kg^{-1}). Values are the mean of 30 fish (15 fish by experiment). The experiment was performed by gas chromatography coupled with mass spectroscopy (GC-MS).

3.2. Biliary metabolites

The Relative Fluorescence Unit (RFU) at 290/335 nm (naphthalene type metabolites) and 341/383 (pyrene type metabolites) was equal between exposed and control fish before exposure condition (Day (-7)) with a mean value of 0.26 ± 0.03 and 0.27 ± 0.02 respectively. After exposure period (Day 0), a significant increase was observed for contaminated fish at 290/335 nm (23.28 ± 2.27) and at 341/383 nm (31.19 ± 6.88). These values decrease highly in exposed fish after seven and 14 days of recovery period (Day 7 and 14) with a mean RFU of 2.53 ± 0.34 at 290/335 nm and 0.42 ± 0.06 at 341/383 nm (Fig. 1).

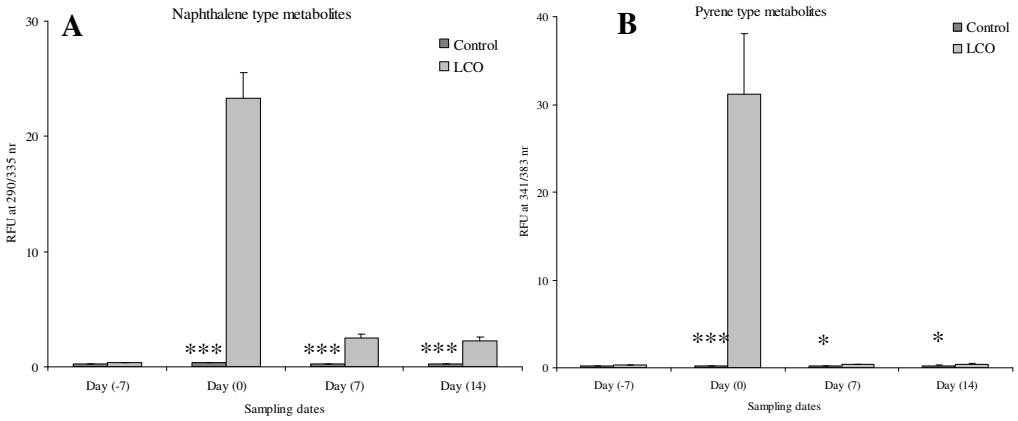

Fig. 1: Quantification of Relative Fluorescence Unit (RFU) at 290/335 nm (naphthalene type metabolites, **A)** and 341/383 nm (pyrene type metabolites, **B)** in sea bass during the experimental period: before exposure to light cycle oil (LCO, Days (-7)) and following the contamination period (Day 0 to Day 14). Values are the mean of 30 sea bass. The bars represent the standard error. * = statistical difference for $p \leq 0.05$ and *** for $p \leq 0.001$.

3.3. PAH effects on leucocyte subpopulation percentages, cell mortality, phagocytosis, non-specific esterase positive cells and lysosome presence

All leucocyte parameters analysed by flow cytometry were equal in control and contaminated fish before the exposure period (Day (-7)) with a mean value of: 85.2 ± 5.0 % of lymphocytes, 16.5 ± 2.2 % of granulocytes-monocytes, 1.2 ± 0.1 % of granulocytes-monocytes, 16.5 ± 2.2 % of leucocyte mortality, 36.5 ± 3.4 % of phagocytosis, 93.7 ± 1.9 % of non-specific esterase positive cells and 99.5 ± 4.6 % of lysosome presence (Fig. 2).

Leucocyte subpopulations were modified by exposure to LCO dissolved fraction with a decrease of lymphocyte (37.6 ± 3.9 %) and an increase of granulocytes-monocytes (47.1 ± 2.9 %). Moreover, leucocyte mortality was increase after exposure condition with a mean value of 3.7 ± 0.3 % from Day 0 to Day 14.

Phagocytosis percentages (14.9 ± 2.3 %) and lysosome presence (84.9 ± 3.8 %) were significantly decreased after the contamination period to dissolved fraction of LCO. On the other hand, non-specific esterase positive cell percentages were not modified by pollutant: 93.2 ± 0.8 %.

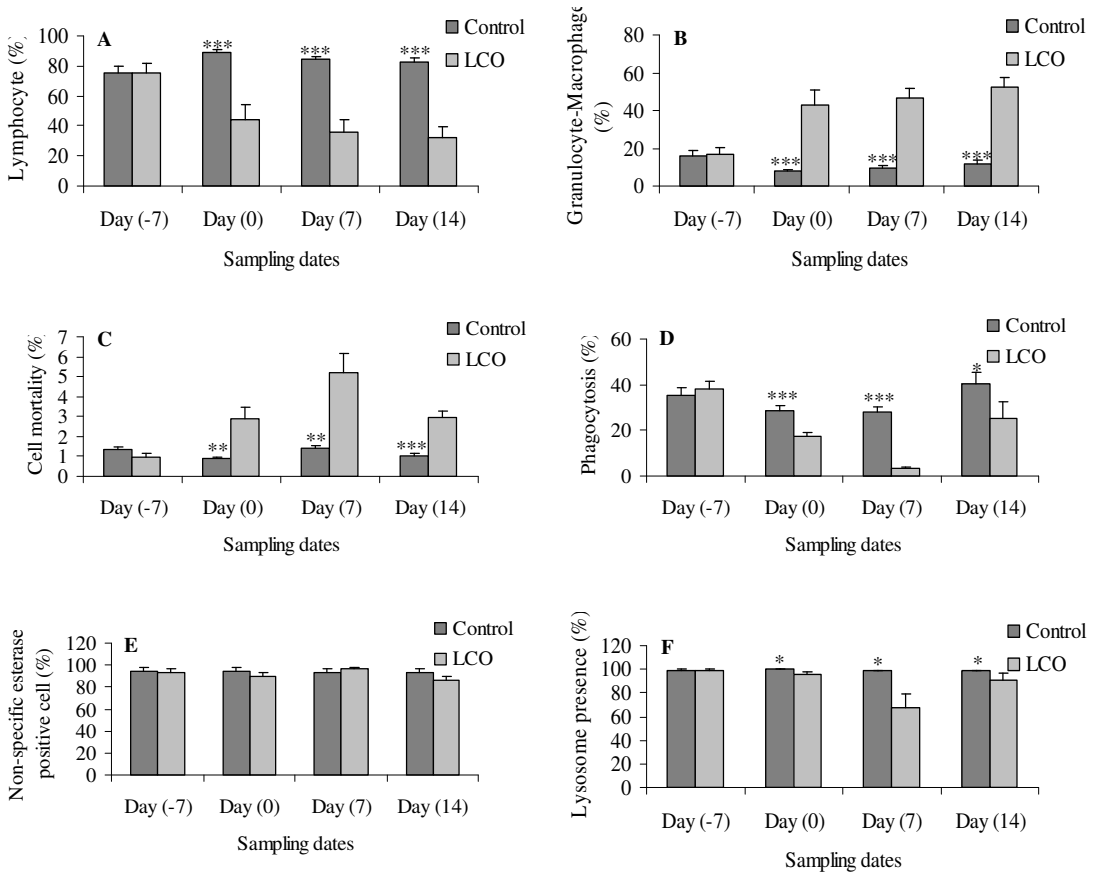

Fig 2: Leucocytes (lymphocyte (**A**), granulocyte-monocyte (**B**), cell mortality (**C**), phagocytosis (**D**), non-specific esterase positive cell (**E**) percentages and lysosome presence (**F**)) were monitored by flow cytometry after two *in vivo* experimental exposures to light cycle oil (LCO). Day (-7) concerned the post-exposure and Day 0, Day 7 and Day 14 concerned the recovery period. No effect was detected for the two experiments for all values. Values are the mean of 30 fish (15 fish by experiment). * = statistical difference for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

3.4. PAH effects on lysozyme concentration and haemolytic activity of the alternative complement pathway (ACH_{50})

Each plasmatic activity quantified by spectrophotometry was equal in control and contaminated fish before the exposure period (Day (-7)) with a mean value of: 5.6 ± 0.3 mg.mL^{-1} of lysozyme and 20.4 ± 1.1 U.ACH_{50} (Fig. 3).

Concerning lysozyme concentration, a seven days exposure to LCO dissolved fraction induced a significant decrease of this activity (3.7 ± 0.6 mg.mL^{-1}) compared to control (4.9 ± 0.3 mg.mL^{-1}). After the seven and 14 day's of recovery period, no significant difference was noted with a mean concentration of 5.7 ± 0.4 mg.mL^{-1}.

The haemolytic activity of the alternative of complement was significantly increased in contaminated fish compared to controls with 23.3 ± 1.0 $U.ACH_{50}$ for contaminated fish and 17.0 ± 2.1 $U.ACH_{50}$ for control fish at Day 0. At Day 7 and 14, the ACH_{50} was increased too in contaminated fish with respectively: 34.4 ± 2.2 $U.ACH_{50}$ and 50.4 ± 7.0 $U.ACH_{50}$.

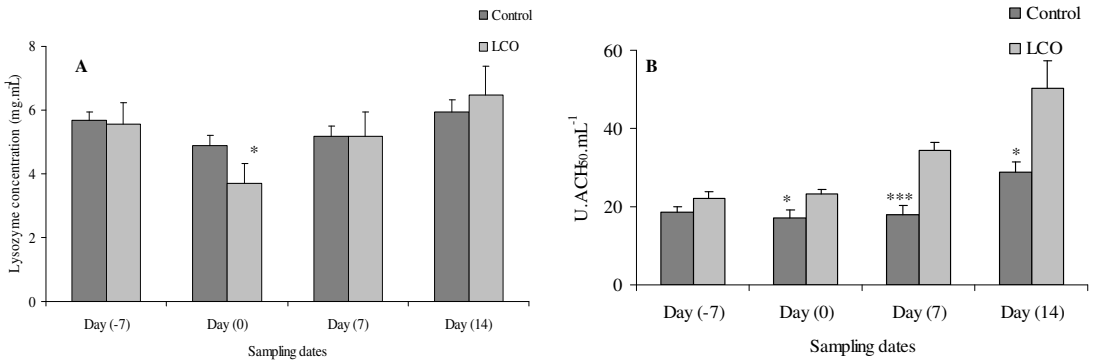

Fig 3: Plasmatic activities (lysozyme concentration (**A**), haemolytic activity of the alternative complement pathway (U.ACH50, **B**)) were monitored by spectrophotometry after two *in vivo* experimental exposures to light cycle oil (LCO). Day (-7) concerned the post-exposure and Day 0, Day 7 and Day 14 concerned the recovery period. No effect was detected for the two experiments for all values. Values are the mean of 30 fish (15 fish by experiment). * = statistical difference for $p \leq 0.05$ and *** for $p \leq 0.001$.

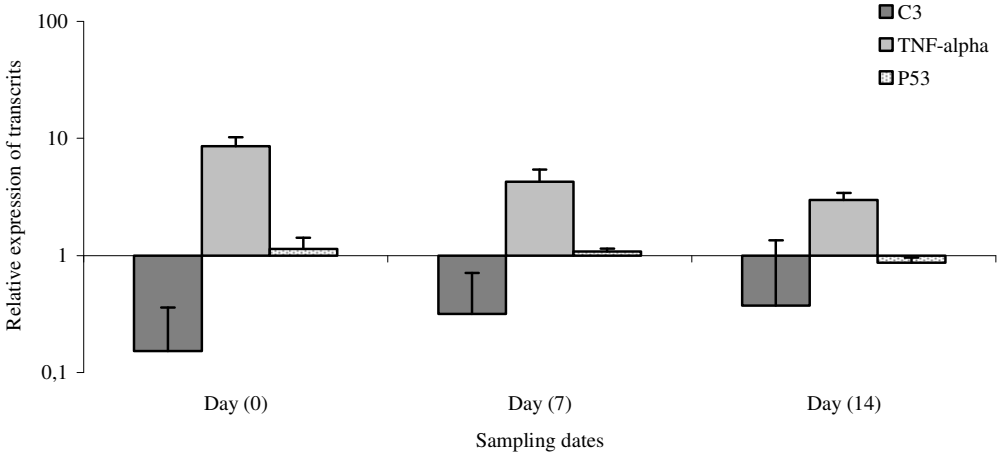

Fig. 4: Relative expression of the three gene transcripts (normalised to elongation factor I) in contaminated fish compared to controls after *in vivo* experimental exposure to light cycle oil (LCO). RNA was extracted from 15 fish spleens by condition. The X-axis corresponds to logarithm values. Control values were represented by one relative expression of transcripts. The bars represent the standard error. See Table 1 for the category of the genes.

3.5. PAH effects on gene expression

After the seven days of LCO exposure (Day 0), relative expression of TNF-alpha (8.6 ± 1.6) was higher compared to those from control fish. Likewise, a gradual time decrease of the relative expression of TNF- alpha was shown from Day 7 (4.2 ± 1.2) to Day 14 (3.0 ± 0.4) (Fig. 4).

Concerning relative expression of C3 and P53 genes, similar relative expression gene was shown compared to control samples during the entire recovery period from Day 0 to Day 14 with a mean value of 0.3 ± 0.1 and 1.0 ± 0.1 respectively.

4. Discussion

As described previously in oyster study, no study has investigated until now effects of light cycle oil (LCO) on organisms. The European sea bass, *Dicentrarchus labrax*, has been selected in order to research the effects of LCO dissolved fraction on marine organisms. This fish corresponds to a pelagic fish which bioaccumulated pollutants preferentially by gills. Thus, this work will study effects of dissolved fraction of LCO on immune system where it was a lack of knowledge.

4.1. Chemical analysis: PAH bioaccumulation

After tanker wreckage, some information was given about concentration of the 16 polycyclic aromatic hydrocarbons (PAHs) US-EPA without differentiation about origin of these compounds. In fact, in this case, it is very difficult to differentiate the origin of each compound when the single difference between heavy fuel oil (HFO) and LCO products is the presence or absence of some PAHs. In fact, HFO contains each 16 PAHs US-EPA when LCO contains only the PAHs with low and medium molecular weight ($\leq$ 228.3 g.mol^{-1}). Thus, the concentration chosen for this study was compared to a HFO oil spill. The experimental system used to dissolved oil permits the procurement of a LCO dissolved fraction (1 600 ng.L^{-1}) near of the *Amoco Cadiz* (Marchand, 1980) or of the *Ekofisk* blowouts (Law, 1978). The major conclusion of the oyster work has concerned the contamination kinetics in organisms. In fact, organisms contaminated by this dissolved fraction of LCO

were exposed to each 10 PAHs detected in petroleum products during different labs time which could explain several kinetics of bioaccumulation.

Just at the end of the seven days of contamination, fish muscles had bioaccumulated five out of the 10 compounds detected in seawater: naphthalene (128.2 $g.mol^{-1}$), acenaphthene (152.2 $g.mol^{-1}$), fluorene (166.2 $g.mol^{-1}$), phenanthrene and anthracene (178.2 $g.mol^{-1}$). These PAHs were largely bioaccumulated by Pacific oyster, *Crassostrea gigas*, too. A part of these compounds (naphthalene, acenaphthene) were also detected after a five day exposure to HFO dissolved fraction (Bado-Nilles et al., submitted-b). In comparison to the HFO exposure, fluorene and anthracene were more represented into seawater column which could explain larger bioavailability for organisms. At the opposite, phenanthrene seemed to be less biodisponible but it was all the same bioaccumulated. This discrepancy might be due to the longest period of exposure: seven days in this study against five days with HFO (Bado-Nilles et al., submitted-b). As for Pacific oyster, the absence of some PAHs seemed to be linked with their slow kinetics of accumulation due to mean molecular weight ($\leq$ 228.3 $g.mol^{-1}$). Indeed, bioaccumulation of PAHs was among other dependent on molecular weight, toxicant dose and exposure time (Meador et al., 1995).

During the recovery period, the fish had attained half-life of naphthalene (70 %) after seven days and had totally eliminated this PAH after the 14 days post-exposure. For fluorene (42 %), phenanthrene (63 %) and anthracene (55 %), the half-life was reached after 14 days of recovery period and acenaphthene (26 %) have a slow elimination rate. Difference between all these elimination kinetics proved that biodegradation rate was faster for lower molecular weights (Hellou et al., 1999) due to their poorer hydrophobicity (Neff et al., 1976). Nevertheless, concerning acenaphthene, this hypothesis was not right. In fact, many factors modified the depuration rate as PAH level in muscle and molecular conformation (Meador et al., 1995). As for oyster, acenaphthene, which are a molecule with two aromatic rings and one pentenic ring, was more slowly eliminate. In the present work, the two type of depuration rate between oyster and fish was roughly similar. Nevertheless, invertebrate used preferentially the passive diffusion to clean up their tissues, whereas fish metabolized PAHs and excreted metabolites into bile in order to reduce tissue concentrations of parent hydrocarbons (Meador et al., 1995).

The measure of naphthalene and pyrene biliary metabolite, by fluorescence intensity, confirmed that fish were exposed to substantially higher level of PAHs. The fluorescence intensity of each compound was increased after the seven days of exposure to dissolved fraction of LCO (Day 0) and decrease highly during recovery period. After the 14 days of recovery period, the two metabolite quantities were almost at control threshold baseline. Similar results were shown with Australian bass, *Macquaria novemaculeata* (Cohen et al., 2003), and with marine flatfish dab, *Limanda limanda* (Van Schanke et al., 2001), after 12 days of recovery period. This important naphthalene biliary metabolite could explain important elimination rate of this bioaccumulated PAHs during the recovery period. In the same way, the prompt metabolization of four-ring PAHs might explicate their no detection in fish muscle probably link to concentration near limit detection value (lower than 5 $\mu g.kg^{-1}$ of dry weight).

4.2. LCO and biological parameters

An important destabilisation of physiologic, immunologic, mutagenic and carcinogenic effects in relation to PAHs with low and mean molecular weight (WHO, 1997), could explain the fish mortality recorded (17 %) and the presence of external and internal macroscopically lesions. Furthermore, some authors observed that an oil spill, as the *Exxon valdez* for fish embryons (Cronin et al., 2002; Roy et al., 1999), induced fish mortality for several years. The inflammatory phenomenon described following PAH contamination (Myers et al., 1998; Stentiford et al., 2003) could be associated to cellular damage which favour an increase in pathologies. These pathologies could be due to opportunist pathogens which might be detected by some macroscopically lesions.

At the opposite of oyster study, many cellular damages were noted after exposure to LCO dissolved fraction in fish immune cells. A significant modulation of leucocyte subpopulation percentage was observed after the seven day exposure to LCO: lymphocyte percentages were decreased when granulocyte-monocyte percentages were increased. This destabilisation of subpopulation percentage could be explained by two hypotheses. The first hypothesis concerns the important increase in cellular mortality which could affect only lymphocyte population. This hypothesis is in accordance with Hoeger et al. (2004) which showed that only the lymphocyte number of rainbow trout, *Oncorhynchus mykiss*, was

decreased by municipal sewage effluent. In this first hypothesis, the increase of granulocyte-monocyte percentage was just an artefact of analysis due to the proportionality between the two leucocyte subpopulations. In the second hypothesis, the reverse could be shown: granulocyte-monocyte percentages were increased but without their entire immune capacity. In fact, as often shown by other authors (Cajaraville et al., 2000; Weeks et al., 1986), phagocytosis percentage and lysosomal presence were decreased by pollutant exposure. As shown for oyster study, non-specific esterase positive cell percentages were not modified by exposure condition. This second hypothesis could be sustained by Van Grevenynghe et al. (2003) which observed that PAHs strongly impair functional differentiation and maturation of human macrophage. In this second hypothesis, the decrease in lymphocyte percentages was an artefact of the analysis. Nevertheless, a mix of these two hypotheses must not be neglected.

Lysozyme concentration is described as a suitable biomarker for hydrocarbon exposure (Reynaud and Deschaux, 2006), however, in the present work it was little affected by LCO dissolved fraction after the seven days of exposure. Moreover, lysozyme concentration appeared to be quite variable in fish lysozyme responses following PAH contamination (Skouras et al., 2003). Thus lysozyme concentration should be used cautiously as a suitable biomarker for hydrocarbon exposure in function of types of PAH exposure and of concentrations found. At the opposite, the haemolytic activity of alternative plasma complement pathway (ACH_{50}), which shares some similarities with the ProPO/PO system in invertebrates, was to be developed recently as a suitable biomarker of hydrocarbon exposures (Bado-Nilles et al., submitted-b; Kanemitsu et al., 1998). As shown after HFO exposure (Bado-Nilles et al., submitted-b), in the oyster experimentation phenoloxidase activity decreased (Bado-Nilles et al., submitted-a) and ACH_{50} increased. This ACH_{50} increase could be due to a direct interaction between chemical molecules and immune components (Bado-Nilles et al., submitted-c) or to indirect effects linked with inflammatory response (Bado-Nilles et al., submitted-b). Concerning the first hypothesis (direct action), Bado-Nilles et al. (submitted-c) showed *in vitro* that some PAHs as anthracene, which was bioaccumulated in fish muscle in this study, induced an increase in the ACH_{50}. This *in vitro* increase would be related to the stimulation of some compounds of the enzymatic cascade. In fact, in human serum, the alternative pathway of the complement system was activated *in*

vitro by diesel exhaust particle extracts by the cleavage of the third component of the complement (C3) in serum to C3b (Kanemitsu et al., 1998). Moreover, the spleen relative expression of C3 tends to be decreased during experiment maybe to maintain a weak cleavage of C3 by pollutant. In the second hypothesis (indirect effect), an inflammatory response due to bioaccumulation of molecule with low molecular weights ($\leq$ 228.3 $g.mol^{-1}$), as in the present work, was capable to increase ACH_{50} (Bado-Nilles et al., submitted-b). This inflammatory phenomenon, already described following PAH contamination (Myers et al., 1998; Stentiford et al., 2003), was associated to cellular damages which could be detected by the macroscopically lesions observed in exposed fish. Moreover, as described previously for pollutant exposure as pesticides (Sato et al., 1998) or hydrocarbons (Ushio et al., 1999), a significant up-expression in spleen of TNF-alpha related gene, which plays a central role in mediating the response in inflammatory process, was described after *in vivo* exposure to dissolved fraction of LCO. Moreover, Moore et al. (1999) showed that a pro-inflammatory cytokine is required for *de novo* carcinogenesis and that TNF-alpha increased the rate of early stages of tumor promotion. Thus the contaminated fish, which had important TNF-alpha expression, seemed to be in early stages of tumor promotion. Nevertheless, the related gene expression of tumor suppressor gene (P53), which controlled the neoplasia formation (Barker et al., 1997), would not be modified in this fish experimentation on the contrary to oyster exposure (Bado-Nilles et al., submitted-a). Nevertheless, the inactivation of P53 and the up-regulation of TNF-alpha could largely increased risk of malignant disease sustained by the important lesions in skin and organs.

5. Conclusions

Effects of LCO were reported on some immune parameters in the European sea bass, *D. labrax*. *In vivo* contamination with the soluble fraction of LCO caused important immune modifications as demonstrated using three different methods (flow cytometry, spectrophotometry and gene expression). Thus the enzymatic cascade (ACH_{50}) and the pro-inflammatory cytokine (TNF-alpha) seemed to be quite attractive in fish for monitoring pollution by oil. These studies confirmed an existence of a relationship between PAHs and susceptibility of animals to infectious diseases and risk of tumor development.

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CHAPITRE 4
CONCLUSIONS GENERALES ET PERSPECTIVES

Haven (Avril 1991)
Source : Cedre

L'objectif de cette étude était de déterminer l'impact de substances anthropiques de type hydrocarbure sur l' "état de santé" et plus particulièrement sur le système immunitaire non spécifique de deux organismes marins, l'huître creuse et le bar commun. Ces deux animaux sont fortement représentés, entre autre, dans le Bassin de Marennes-Oléron (Charente-Maritime) où leur importance économique y est loin d'être négligeable. Pour mener à bien ce projet, une étude expérimentale et multidisciplinaire (chimie, biochimie et biologie) a été entreprise à différents niveaux d'intégration (du moléculaire à l'organisme). Ce travail souhaite tendre vers une application pratique grâce à l'utilisation d'un développement méthodologique prenant en compte à la fois une approche immunotoxique et chimique. D'un point de vue scientifique, un essai de la compréhension des mécanismes d'action des polluants sur le système immunitaire de ces deux espèces marines, a été réalisé. Sur le plan opérationnel, ce travail a permis de progresser dans la validation d'outils expérimentaux et de diagnostic pertinents capables d'aider les intervenants dans les opérations de lutte.

Lors de la première partie de cette thèse, le but a été d'une part de sélectionner parmi les produits pétroliers existants, des polluants susceptibles de contaminer les eaux superficielles, et d'autre part de mettre en évidence des biomarqueurs d'intérêt. Pour ce faire, une recherche *in vitro* du potentiel immunotoxique de trois produits pétroliers, lors d'une pollution chronique assimilable à celle du Bassin de Marennes-Oléron (10^{-9} à 10^{-7} mg.mL^{-1}) et occasionnelle quantifiée lors des accidents maritimes (10^{-5} à 10^{-3} mg.mL^{-1}), a été effectuée par le biais d'une approche purement méthodologique. L'objectif de cette démarche était de déterminer l'action directe de trois produits pétroliers, un fioul lourd de type *Erika*, l'un de ces fluxants, le LCO, et un pétrole raffiné à haute valeur ajoutée, le gasoil, sur l'hémolymphe de l'huître creuse et le plasma du bar commun. Du fait de leur présence dans chaque produit pétrolier testé, de leur persistance dans l'environnement, de leur forte capacité à être bioaccumulés et de leur impact avéré sur le système immunitaire, les 16 HAPs prioritaires de l'US-EPA ont été étudiés individuellement. A l'exception du gasoil, du fluoranthène et du benzo*[g,h,i]*pérylène, l'ensemble des molécules et des produits pétroliers expérimentés s'avèrent intéressants car ils ont modulé au moins l'un des paramètres immunitaires non

spécifiques étudiés qu'ils soient cellulaires ou acellulaires, à savoir chez le Bivalve : la mortalité cellulaire, la phagocytose, la présence en lysosome, le pourcentage de cellules estérases non-spécifique, l'activité PO ; et chez le poisson : la concentration en lysozyme et l'activité du complément voie alterne.

Afin de valider ces outils de diagnostic, des études *in vivo* ont donc été entreprises. En effet, les expérimentations *in vitro* ne peuvent en aucun cas reproduire l'ensemble des évènements susceptibles d'intervenir *in vivo* du fait, en autre, de l'absence des conditions physiologiques générales de l'organisme. En raison de leurs actions constatées *in vitro*, seuls deux produits pétroliers issus du raffinage, le fioul lourd et le LCO, ont été étudiés. Par contre, l'impact de cette pollution occasionnelle a été recherché sur l'ensemble des descripteurs immunologiques analysés lors de l'étude *in vitro*. En effet, tous ont subi des variations plus ou moins importantes et il paraît intéressant, en vue d'essayer de déterminer de bons descripteurs immunologiques d'une pollution par hydrocarbures, de tester leur éventuelle sensibilité *in vivo*. D'un point de vue expérimental, divers modes de contamination peuvent être utilisés : la pollution du sédiment, la contamination par voie orale, le "caging" et la contamination par l'eau. Ce dernier, qui correspond à la mise en présence directe des organismes avec une eau polluée par un ou plusieurs xénobiotiques, a été choisi dans le cadre de cette étude car il se rapproche des conditions "naturelles" tout en favorisant la maîtrise de l'ensemble des stress environnementaux tels que les variations des paramètres physico-chimiques de l'eau ou encore l'action d'un agent pathogène potentiel présent dans le milieu.

L'ensemble de ces expérimentations *in vivo* a pu être réalisé grâce à un outil expérimental défini dans le cadre de la programmation 2003 du *Cedre* (Cedre R.03.16.C/3043), adapté de la méthode d'Anderson *et al.* (1974), et validé au cours de cette thèse. Ce système permet d'exposer les organismes tests uniquement à la fraction soluble d'un polluant évitant ainsi qu'ils soient contaminés par contact direct avec la nappe de surface. Ainsi des expositions homogènes dans le temps, de 733 $ng.L^{-1}$ pour le fioul lourd à 1 600 $ng.L^{-1}$ avec le LCO, et assimilables à celles observées *in situ* suite à une marée noire ont été obtenues. Une répétitivité des analyses physico-chimiques par prélèvements journaliers a été constatée et un protocole analytique permettant de quantifier la bioaccumulation de la substance chimique

dans les tissus des organismes exposés a été mis en place au laboratoire du *Cedre*. La quantification des 16 HAPs US-EPA dans les tissus d'huîtres creuses et le muscle des bars communs a notamment démontré que les poissons vont bioaccumuler préférentiellement des composés de faible poids moléculaire, alors que les Bivalves bioaccumulent majoritairement les composés de poids moléculaire élevé, et ceci en raison de leur faible capacité de métabolisation. De plus, contrairement aux Mollusques filtreurs qui éliminent essentiellement les polluants par des processus de diffusion passive, les Téléostéens excrètent activement les métabolites par la bile ou l'urine. Ainsi, afin de détecter les composés rapidement biotransformés et donc souvent faiblement détectables dans les chairs (inférieur à 5 $\mu g.kg^{-1}$), une quantification des métabolites biliaires chez les Vertébrés a été effectuée notamment pour les composés à 4-cycles benzéniques comme les métabolites du pyrène. De fait, ces métabolites sont détectés dans la bile suite à l'exposition de sept jours aux hydrocarbures alors qu'ils ne sont pas retrouvés dans les muscles du poisson. D'autre part, aucun phénomène de mortalité relatif aux conditions expérimentales n'a été mis en évidence démontrant ainsi que le système utilisé répond bien aux différents critères de maintien en vie des organismes. Enfin, afin d'éviter tout effet de stress dû aux manipulations et donc de maintenir des paramètres immunitaires stables dans le temps pour chaque condition d'essais, il apparaît souhaitable de respecter une périodicité hebdomadaire pour la réalisation des prélèvements sur les animaux.

Concernant les capacités de défense, une exposition expérimentale à des concentrations de type accidentel à la fraction soluble de fioul lourd ou de LCO entraîne une modulation de certains de ses éléments. Bien que peu d'effets aient été observés sur les paramètres cellulaires, les paramètres acellulaires comme l'activité PO et l'ACH_{50}, sont significativement modifiés. Ainsi, une diminution de la PO est notée chez l'huître creuse alors qu'une augmentation de l'ACH_{50} est observée chez le bar commun. Ces perturbations apparaissent dès la fin de la période de contamination qui, dans le cadre de ce travail, a duré sept jours, et sont toujours présentes 7 à 14 jours après la fin de l'exposition témoignant d'un impact significatif. Néanmoins, celles-ci sont toujours réversibles chez les organismes survivants car elles ne sont plus retrouvées en fin de décontamination. Seuls les HAPs légers

sont présents à la fois dans le fioul lourd et le LCO, ils pourraient donc être responsables des variations observées.

Afin de mieux comprendre l'impact de ces hydrocarbures à l'encontre de ces deux descripteurs immunologiques (PO et ACH_{50}), l'analyse des tests fonctionnels a été complétée, lors de l'exposition *in vivo* des deux organismes à la fraction soluble du LCO, par une étude du suivi de l'expression de certains gènes d'intérêt impliqués dans la régulation de ces deux descripteurs. Chez l'huître creuse, un effet antagoniste est observé entre la modulation du gène codant pour le *molluscan defence precursor*, qui induit le clivage de la proPO en PO, et l'activité PO proprement dite. La diminution de cette activité enzymatique ne semble donc pas être reliée à la perte de l'activation du système proPO-PO, mais probablement à un effet simple ou synergique d'un et/ou plusieurs des HAP(s) présent(s) dans la fraction soluble ou bioaccumulé(s) dans les tissus (naphtalène, acénaphtylène, acénaphtène, fluorène ou phénanthrène) et ceci malgré leurs absences d'impact *in vitro* quand ils sont testés individuellement. De plus, la sur-régulation observée pour la *laccase*, qui est supposée correspondre à la PO, pourrait s'expliquer par un effet compensatoire. Concernant l'activité du complément voie alterne chez les bars, son augmentation, suite à une exposition au LCO, pourrait être en partie liée à l'inflammation tissulaire détectable par des lésions cutanées et illustrée probablement, à l'échelle moléculaire, par la sur-expression du gène codant pour le *TNF-alpha*, molécule clef de la réponse inflammatoire. Cependant, dans un souci de régulation interne à l'organisme, un effet compensatoire à cette forte réaction inflammatoire est envisageable en raison de la sous-expression, non significative, de l'expression du gène codant pour le *C3*. Il serait donc intéressant d'approfondir, au niveau moléculaire, la régulation de ces deux cascades enzymatiques sous l'action des hydrocarbures.

L'ensemble des analyses, *in vitro* et *in vivo* réalisées au cours de ce travail de thèse, souligne l'intérêt de l'étude de l'activité PO chez l'huître creuse et de l'ACH_{50} chez le bar commun comme outils de diagnostic lors d'une pollution. En effet, ces expérimentations confirment la sensibilité de ces deux activités humorales qui apparaissent donc susceptibles d'être de bons biomarqueurs d'intérêt et qui pourraient ainsi être candidats à la batterie de tests utilisables

en immunotoxicité du fait de leur robustesse, de leur faible coût et de leur rapidité de mise en oeuvre.

Dans la perspective de valider ces descripteurs immunologiques à l'ensemble des constituants du pétrole (les différentes molécules, les fluxants, les résidus de raffinage…), et suite aux observations de Barron *et al.* (1999), qui démontrent que la toxicité relative des alcanes et des isoprénoïdes, également présents dans les produits pétroliers, est importante, il serait intéressant d'étendre ce type d'analyses, *in vitro* et *in vivo,* à l'impact de ces autres composants, et par extension aux pollutions chroniques. De plus, un complément d'informations grâce à des études *in situ* semblent indispensables afin de confirmer ou d'infirmer ces résultats.

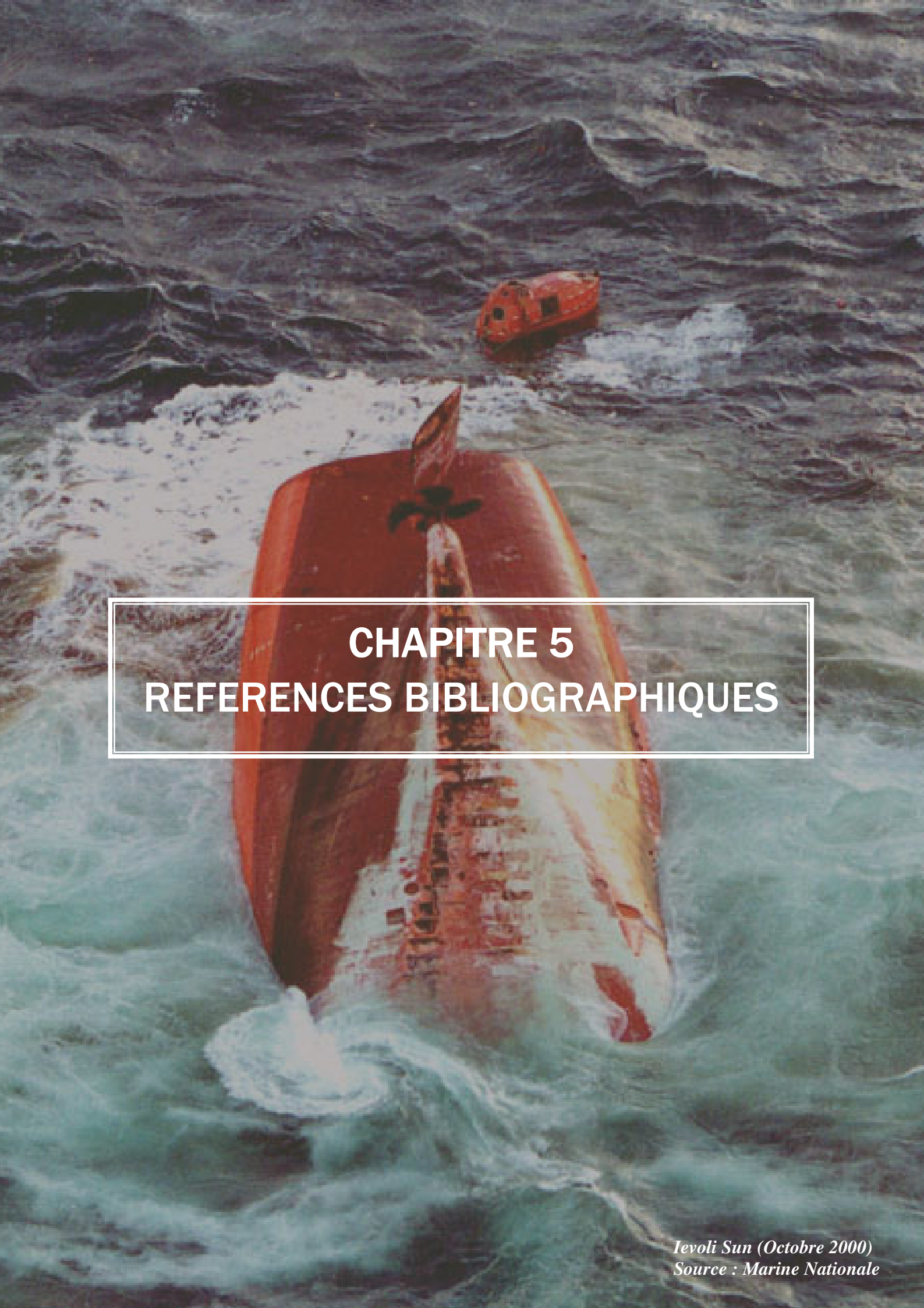
Les hydrocarbures provoquent donc une modulation du système immunitaire aussi bien chez l'huître creuse que chez le bar commun. Ceci n'est probablement pas sans conséquence sur l'homéostasie des animaux et plus particulièrement sur leurs capacités de défense entraînant probablement une sensibilité accrue des animaux face aux maladies induites par les nombreux agents pathogènes présents naturellement dans le milieu (Grundy *et al.*, 1996a; Pipe & Coles, 1995). Une approche plus spécifique sur l'état de santé de l'animal serait donc intéressante à développer notamment par le biais de l'étude de l'action d'une pollution occasionnelle avant ou après le contact avec un de ces agents. Elle permettrait d'une part d'aborder l'immunité spécifique, tout au moins chez les Vertébrés, et de tester grâce à des infections expérimentales, le potentiel global de défense des organismes. Cette étude supplémentaire, un instant envisagée dans le cadre de cette thèse, n'a pu être effectuée du fait de l'absence d'un dispositif expérimental approprié à la décontamination des eaux infectées par des agents infectieux. Elle devrait être réalisée dans le cadre d'une thèse, cofinancée par l'Afssa et la région Bretagne, qui débute actuellement et qui a pour objectif d'étudier l'impact de polluants (hydrocarbures et pesticides) sur les écosystèmes aquatiques avec une évaluation des effets d'une exposition sur l'état sanitaire des poissons et une mise en perspective avec les seuils de qualité environnementale. L'aspect sanitaire sera complété par un volet réglementaire visant à établir des seuils de contamination acceptable garantissant l'obtention de poissons sains et à faibles niveaux de résidus dans les chairs.

D'autre part, l'ensemble des projets européens auxquels participent les différents partenaires et collaborateurs de cette thèse souligne l'importance actuelle accordée par les Ministères et les décideurs sur la thématique de la pollution environnementale des eaux.

Ainsi, le projet PRAGMA ("A pragmatic and integrated approach for the evaluation of environmental impact of oil and chemicals spilled at sea: input to European Guidelines") accepté en 2005 démontre les effets liés à une exposition au long terme à la fraction soluble d'un pétrole et d'un produit chimique, le styrène, sur les paramètres physiologiques et immunologiques du turbot et de la moule commune (http://www.iris.no/pragma). Le projet RESPIL ("Response means to chemicals spilled at sea and environmental damage") accepté en 2006 met en évidence les différents effets liés à une exposition de quelques jours à la fraction soluble de l'éthylbenzène, du cumène et de l'aniline sur l'activité immunitaire de la moule commune, et ceci lors d'exposition *in situ* (http://www.iris.no/respill).

En complément à ces études concernant l'évaluation d'outils de diagnostic, un autre aspect de cette thématique sur la pollution des milieux aquatiques, à savoir les moyens de lutte contre les hydrocarbures, a été accepté en 2007 par l'ANR PRECODD ("Programme Ecotechnologies et Développement Durable"). Il s'agit du projet DISCOBIOL ("Dispersants et technique de lutte en milieux côtiers : effets biologiques et apports à la réglementation") qui a pour but d'établir un argumentaire scientifique quant à l'innocuité des tensioactifs associés aux hydrocarbures sur les écosystèmes littoraux.

Pour conclure, ce travail s'intègre parfaitement dans les préoccupations sociétales actuelles *via* des outils de diagnostic écologiques et sanitaires qui visent à l'excellence environnementale.



CHAPITRE 5
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Ievoli Sun (Octobre 2000)
Source : Marine Nationale

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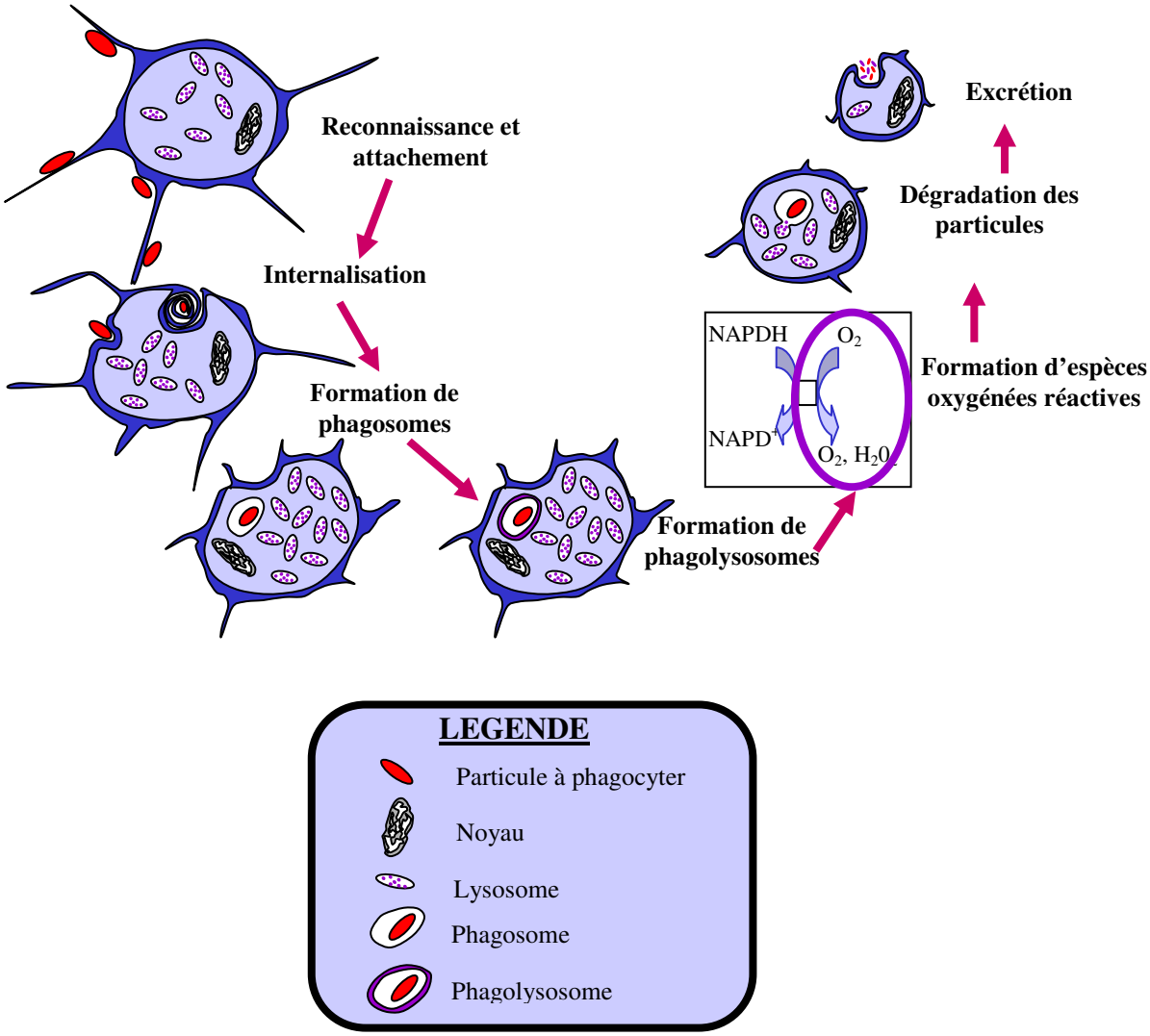
Annexes

Cavo Cambanos (Mars 1981)
Source : Thierry Frot

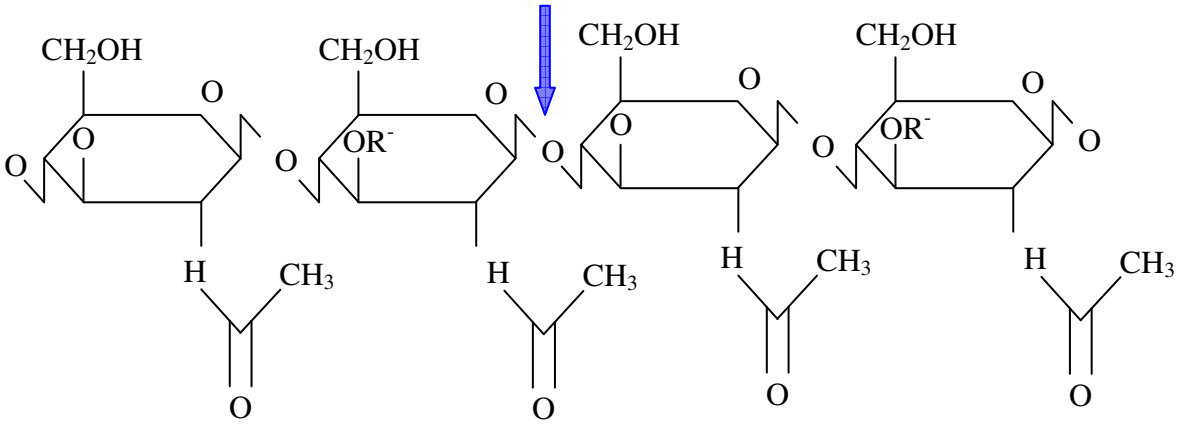
ANNEXE 1 : La phagocytose

Mécanisme général de la phagocytose, modifié d'après Cheng (1983), Cheng & Rodrick (1975) et Fisher (1986) :



ANNEXE 2 : Le lysozyme

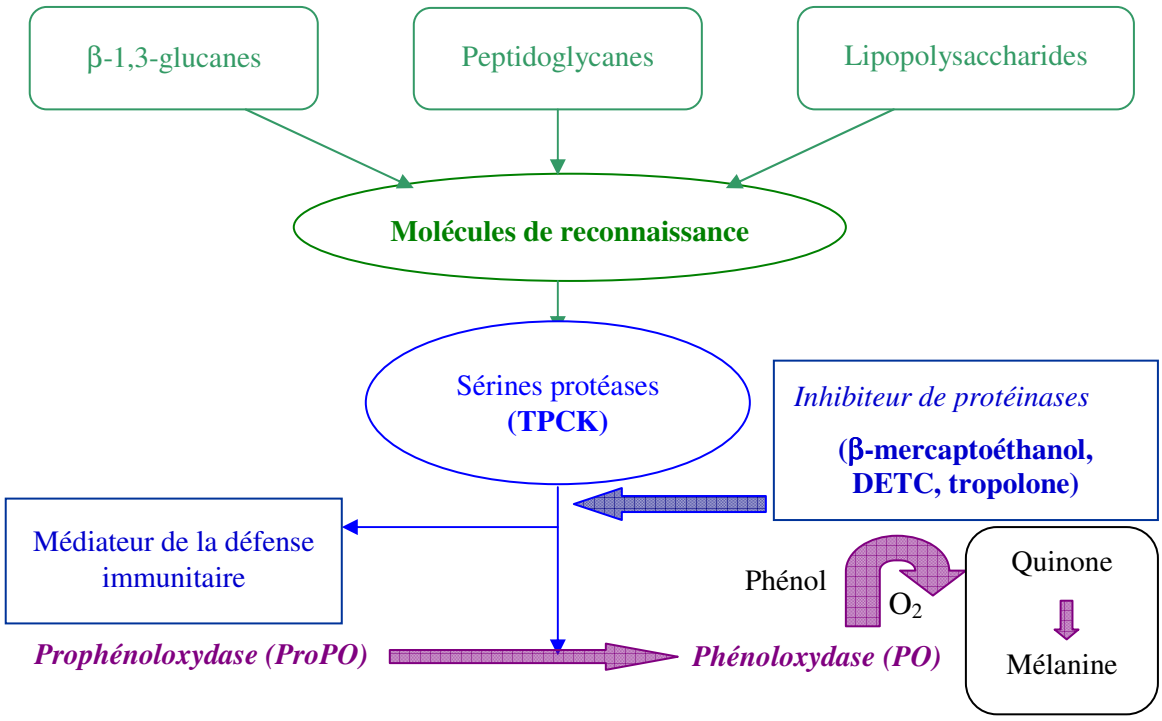
Mécanisme général du clivage de la membrane bactérienne par le lysozyme au niveau de la liaison glycosidique β(1,4), indiquée par la flèche bleue :



$OR^- = OCH_3CHCOO^-$

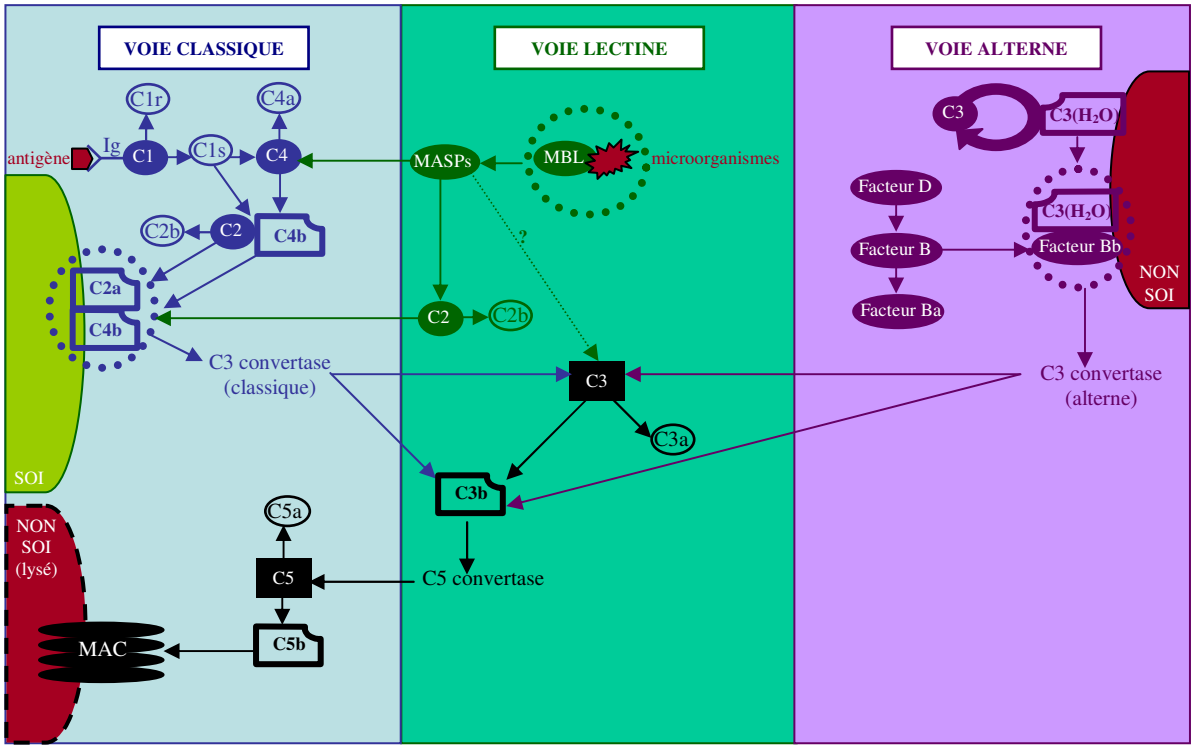
ANNEXE 3 : L'activité phénoloxydase

Représentation schématique simplifiée de l'activation du système prophénoloxydase-phénoloxydase, modifié d'après Söderhäll & Cerenius (1992) :

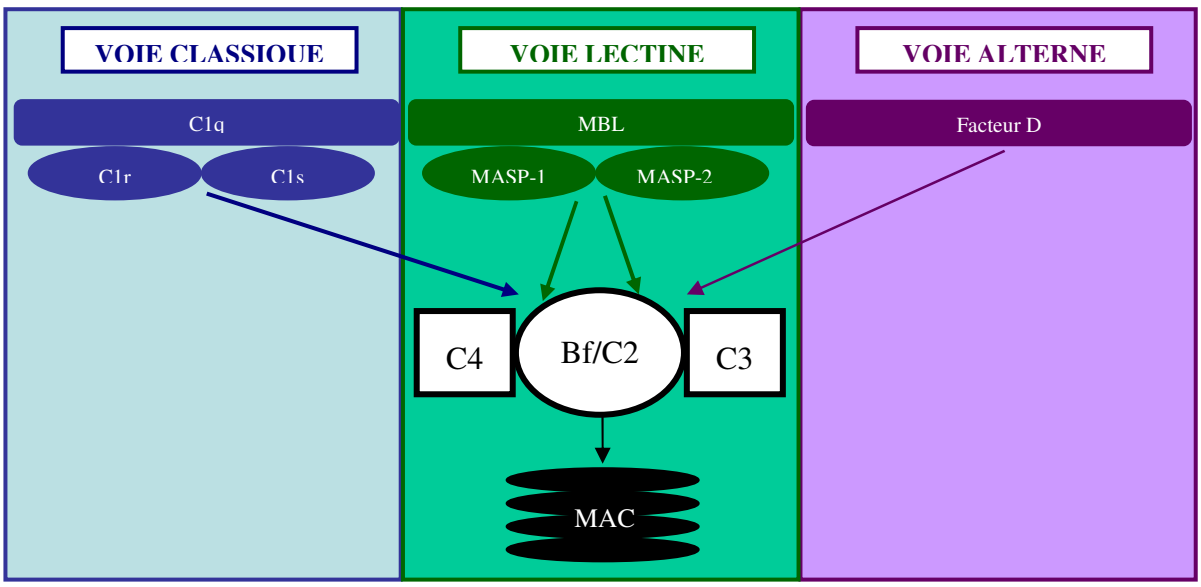


ANNEXE 4 : L'activité du complement

Représentation schématique simplifiée des trois voies d'activation du système du complément chez les Mammifères, modifié d'après Boshra *et al.* (2006) :



Représentation schématique simplifiée des trois voies d'activation du système du complément chez les poissons Téléostéens, modifié d'après Nonaka & Smith (2000) :



RESUME

Les effets des HAPs, parmi les plus toxiques de la liste de l'Agence de Protection Environnementale Américaine, sont testés *in vitro* et *in vivo* sur deux espèces commerciales des Pertuis-Charentais (Charente-Maritime, France) : le bar commun, *Dicentrarchus labrax*, et l'huître creuse, *Crassostrea gigas*. Cette étude, réalisée dans le cadre du projet européen EROCIPS, recherche de nouveaux descripteurs immunologiques d'une pollution occasionnelle par hydrocarbures. Lors d'expérimentations *in vitro*, le choix de polluants de type hydrocarbure et de descripteurs d'intérêt de l'immunité non spécifique chez les deux espèces étudiées est réalisé. Puis, des expositions *in vivo* à la fraction soluble du fioul lourd issus de l'*Erika* et de son fluxant, le light cycle oil, sont entreprises. Elles ont permis la validation de l'outil expérimental avec notamment la mesure des HAPs bioaccumulés et métabolisés et la détermination d'outils de diagnostic de type immunologique pertinents : l'activité phénoloxydase chez les Mollusques et l'activité hémolytique du complément voie alterne chez les poissons. Ces deux cascades enzymatiques sont proposées pour la première fois dans le cadre d'une évaluation d'une pollution occasionnelle par hydrocarbures pour des conditions réelles de terrain.

ABSTRACT

The effects of PAHs, considered among the most toxic by the United States Environmental Protection Agency, were tested *in vitro* and *in vivo* on two commercial species of the Pertuis-Charentais (Charente-Maritime, France): sea bass, *Dicentrarchus labrax*, and the Pacific oyster, *Crassostrea gigas*. This study was carried out as part of the European project EROCIPS with the aim of finding new immunological biomarkers caused by occasional pollution by hydrocarbons. During *in vitro* experimentation, pollutants and immunological biomarkers were choosen. Thereafter, the *in vitro* exposures to the soluble fraction of *Erika's* heavy fuel oil and its fluxant, light cycle oil, began. These exposures enable the validation of the experimental system used, with, in particular, the measurement of bioaccumulated PAHs and metabolites and of choice of the immune biomarkers. The phenoloxidase activity of molluscs and the haemolytic activity of the alternative complement pathway of fish were proposed, for the first time, as suitable biomarkers for the evaluation of pollutant risks in field conditions.