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Toxicity assessment of peptaibols and contaminated sediments on *Crassostrea gigas* embryos

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Abstract:

Peptaibols are known membrane-modifying peptides that were recently detected in marine sediments and mussels collected from a shellfish farming area (Fier d'Ars, Atlantic coast, France). In this investigation, embryotoxicity bioassays with oysters (*Crassostrea gigas*) were performed to assess acute toxicity of alamethicin and different groups of peptaibols produced by a *Trichoderma longibrachiatum* strain isolated from marine environment. *C. gigas* embryos appeared very sensitive to all the metabolites examined with higher toxic effects for long-sequence peptides (EC_{50} ranging from 10 to 64 nM). D-shaped larvae with mantle abnormality were particularly noticed when peptaibol concentrations increased. Disturbances of embryogenesis were also observed following exposure to organic and aqueous extract of sediments from Fier d'Ars (EC_{50} = 42.4 and 6.6 g L^{-1} dry weight, respectively). Although peptaibol concentrations measured in these sediments could explain only a part of the toxic effects observed, this study suggests that these mycotoxins can induce larval abnormalities in a population of exposed animals at environmentally realistic concentrations. Their detection in coastal areas devoted to bivalve culture should be taken into account.

Keywords: Marine fungi; Mycotoxins; Bivalve bioassay; Embryotoxicity; Risk assessment

Introduction

The peptaibols are small linear peptides produced by widely distributed filamentous fungi including terrestrial and marine species (Degenkolb et al., 2003). They constitute the main subgroup (approximately 86%) of the constantly growing family of peptaibiotics including more than 450 reported compounds (Whitmore and Wallace, 2004b). Most of the peptaibiotics (≈ 80%) have been isolated from fungal strains belonging to the genus *Trichoderma* or its teleomorph *Hypocrea*. They range between 500 and 2200 Da and show a high content of α aminoisobutyric acid which represents the main common characteristic of these diversified molecules (Chugh and Wallace, 2001). Peptaibols possess an acylated N-terminus and a C-terminal residue that consists of a free 2-amino alcohol (Degenkolb et al., 2003; Degenkolb et al., 2006).

Peptaibols and other peptaibiotics present unique physico-chemical and biological activities depending on particular structural properties. Their helical structures allow them to interact with natural and artificial bilayers to form pores or voltage-dependent ion channels increasing membrane permeability (Rebuffat et al., 1999; Peltola et al., 2004; Whitmore and Wallace, 2004a). Related to this particular ability, a variety of biological activities such as antibacterial, antifungal, and occasionally antiviral, insecticidal and antiparasitic activities have been described. Bioactivity of peptaibiotics includes also uncoupling of oxidative phosphorylation in mitochondria, stimulation of catecholamine secretion from adrenal cells, activation of membrane-bound enzymes such as adenylate and guanylate cyclases, cytotoxicity, neurotoxicity and neuroleptic effects (for reviews, see Szekeres et al., 2005 and Krause et al., 2006). The increasing exploitation of peptaibol-producing strains as biological

control agents against phytopathogens illustrates the great interest of these peptides (Degenkolb et al., 2006; Xiao-Yan et al., 2006).

In a marine area devoted to bivalve culture (Fier d'Ars, Atlantic coast, France), the presence of peptaibols and peptaibols-producing *Trichoderma* sp. strains has recently been detected in sediments which showed a high toxicity for mussel larvae (*Mytilus edulis*) in the absence of significant contaminations (metals, PCBs, HAPs, pesticides, antibiotics) or eutrophication (Amiard-Triquet et al., 2003; Poirier et al., 2007b). The ability of *Trichoderma* sp. to grow in hypersaline environment and to produce such metabolites has been already demonstrated in laboratory marine-like culture conditions (Landreau et al., 2002; Mohamed-Benkada et al., 2006). Identification of peptaibols in marine natural samples can be considered as a proof of the development of these saprophytic fungi in marine sediments. Presence of these peptides was also reported in marine bivalves (Poirier et al., 2007b) and they were shown to be accumulated by filter-feeder molluscs in experimental contamination inducing physiological stress (Sallenave-Namont et al., 1999). The existence of such compounds in shellfish farming areas could represent a risk for shellfish populations and poisoning risks through the consumption of contaminated shellfish.

The present study was designed to establish a causal relationship between peptaibol concentrations measured in sediments and potential toxic effects for bivalve organisms. Different bioassays using various marine organisms have been proposed to assess the toxicity of pollutants and the biological quality of waters and sediments in coastal areas. The embryotoxicity test with the oyster *Crassostrea gigas* is recognized as one of the most sensitive of all classically used (His et al., 1999). Eggs, embryos and larvae are less tolerant to toxic compounds than adults and therefore represent the critical life stages for toxicological

studies (Connor, 1972; Martin et al., 1981). Acute toxicity bioassays using embryo-larval development of *C. gigas* were performed on alamethicin and different groups of peptaibols produced by a strain of *Trichoderma longibrachiatum* isolated from marine environment. To know the potential effects caused by environmental levels, the embryotoxicity of purified extract of Fier d'Ars sediment and its elutriate was also studied.

Materials and methods

1. Bioassay procedure

The bioassay followed the method reported by His et al. (1997) and Quiniou et al. (2005). Mature adults of *C. gigas* were induced to spawn by thermal stimulation (alternating immersion in seawater of 15°C and 28°C for 30 min each time). Spawing males and females were individually isolated in beakers with 0.2 µm natural filtered seawater. The oocytes and sperm of different oysters were observed under an inverted microscope, and the best reproductive pair (regular oocytes and very mobile spermatozoa) was selected for the experiment. Oocytes and sperm solutions were respectively sieved trough a 100 µm and a 32 µm mesh to remove debris. The oocytes were fertilized using a few milliliters of the sperm-dense solution. Fifteen minutes after fertilization, the embryos were counted and placed in 30 mL transparent polypropylene vessels filled with the different media to be tested (1000 eggs; three replicates per treatment). The embryos were incubated at 24 °C for 22 h until D-larvae stages were obtained. Cupric sulphate ($CuSO_4$, 5 H_2O) was used as a reference toxicant and concentrations of 0 (controls), 20, 40, 60 and 80 µg L^{-1} were tested on *C. gigas* embryos.

After incubation, 0.5 mL of 8% buffered formalin was added to each vessel, and abnormalities were determined by direct observation of 100 individuals (chosen at random from the 1000 in each vessel). According to His et al. (1997), the categories of abnormal larvae included: segmented eggs, normal or malformed embryos that had not reached the D-larval stage; and D-larvae with shell abnormalities (convex hinge, indented shell margins, incomplete shell) or protruded mantle.

2. Peptaibol treatments

2.1. General

Embryotoxicity tests were conducted with different group of peptaibols, alamethicin and microheterogeneous mixtures of peptaibols isolated from a marine strain of *T. longibrachiatum*. In all cases, peptaibols were added to incubation media just before addition of the embryos (≈ 1 min). Peptaibol ethanolic solution (20 µL) was added in 30 mL filtered seawater. Concentrations of ethanolic solutions were prepared in order to test the final following concentrations: 0 (ethanol for control), 1, 10, 50, 100, and 500 µg L^{-1}. For each nominal concentration tested, 0.5 mL of incubation medium was sampled at the beginning (t = 0) and the end (t = 22h) of the experiment to determine peptaibol concentrations by high performance liquid chromatography (HPLC) / mass spectrometry (MS) according to the method described by Poirier et al. (2007a). Aqueous aliquot was washed with dichloromethane/methanol mixture with the following proportion: dichloromethane/methanol/aqueous aliquot 2:2:1 (v/v). The aqueous phase containing salts was washed two other times with dichloromethane. The totality of organic phases were then combined and evaporated to dryness before HPLC/MS analysis.

2.2. Alamethicin

Alamethicins are 20-residue peptaibols isolated from a strain of *Trichoderma viride* (Sigma Aldrich, Ref. A4665). The mixture used consisted of analogues F50/5, F50/6a, F50/7 and F50/8b (Kirschbaum et al., 2003; Poirier et al., 2007a).

2.3. Peptaibol isolated from a *T. longibrachiatum* marine-related strain

Fungal strain and cultures

The strain used for peptaibol production was a *Trichoderma longibrachiatum* Rifai isolated from mussels (*Mytilus edulis*) collected in a marine area devoted to bivalve culture from the estuary of the Loire river (Tharon, France – 47° 10’ N 2° 10’ W). The original isolate was deposited in the SMAB Marine Fungal collection of University of Nantes as MMS 151 reference. The identification of this strain was based on molecular methods and reported by Mohamed-Benkada et al. (2006). Fungal cultures were performed on Dextrose Casein Agar medium prepared with seawater (dextrose 40 g L^{-1} ; enzymatic digest of casein 10 g L^{-1} ; agar 15 g L^{-1}) in 20 cm Petri dishes (125 mL of medium/dish). Cultures were incubated for 10 days at 27°C.

Extraction and purification of peptaibols

Fungal biomass (mycelium and conidia) was scraped from the agar surface and steeped three times in dichloromethane/methanol mixture (1:2, 2:1 then 1:1, v/v) for 2 h at room temperature. The combined organic phases were filtered under vacuum (0.45 µm PTFE membrane filters, Sartorius, Göttingen, Germany), washed with water and evaporated to dryness. Chromatography on an open silica gel column (30 x 300 mm, 60 Å 35-75 µm, SDS, Peypin, France) was performed on the crude extract obtained with dichloromethane, acetone and methanol as eluants. Two groups of peptaibols (A and B) were eluted in the acetonic and

methanolic phases, respectively. A second chromatographic separation on a silica gel column (20 mm x 300 mm) was then performed on each group. Elution was realized with various dichloromethane/methanol mixtures (90:10, 88:12, 85:15, 80:20, v/v). As final step, a preparative HPLC-UV on a modular apparatus including a ConstaMetric III pump (LDC S.A., Paris, France), a SpectroMonitor® D (LDC) and a 4.6 x 250 mm Inertsil ODS-3 column (Interchim, Montluçon, France) was carried out only on group B fractions to achieve their purification. The mobile phase consisted of a methanol/H_20 (85:15, v/v) mixture delivered at a constant flow rate of 5 mL/min. Detection was performed at 230 nm.

Mass spectrometry analysis

The purified fractions of both groups were analyzed on a modular HPLC system consisting of a Spectraphysics Spectra System P2000 pump, an AS 100XR autosampler (Thermo Separation Products, San Jose, CA, USA) equipped with a Kromasil C-18 5-µm reverse-phase 2.0 × 250 mm column (Interchim) heated to 40°C and coupled to a Finnigan Matt LCQ ion-trap mass spectrometer with an electrospray interface (Thermo Separation Products). The mobile phase consisted of a methanol/H_20 (85:15, v/v) mixture delivered at a constant flow rate of 0.2 mL/min. Sample injection volume was 5 µL. All mass analyses were performed in the positive mode. The mass spectrometer parameters were previously adjusted to ensure optimal detection of peptaibols (Poirier et al., 2007a). The spray voltage was set to 4.50 kV, the capillary temperature to 266°C and the capillary voltage to 42 V. The nitrogen flow rates were respectively 89 and 37 (arbitrary units) for sheath and auxiliary gas. Parameters of ion optic transmission were adjusted to 55 V for Tube Lens Offset, -3.50 V for Multipole 1 Offset, -6 V for Multipole 2 Offset and 400 V for Multipole RF Amplifier (peak to peak). MS^n analyses were carried out with a collision energy of 32% and an isolation width of 1 u.

All spectra acquisitions and reworks were done using LCQ Xcalibur 1.3 software (Thermo Separation Products). The purest fractions were chosen for bioassays.

3. Sediment treatments

Sediments from Fier d'Ars (Ré Island – 46° 13' N 1° 29' W) were collected in March 2006. All samples were transported from the site to the laboratory in isothermic containers and frozen at -20°C until bioassays. The bioassay was conducted with organic extract of sediment and elutriate.

A purified organic extract was prepared according to the method reported in Poirier et al. (2007a). Briefly, the steps were as follows: extraction of 10 g of sediment wet weight (ww) with 3 × 25 mL of acetone / acetic acid 1% (v/v); sonication of the sediment-solvent mixture for 15 min; centrifugation at 700 g for 5 min; filtration; evaporation to dryness; purification of crude extracts by vacuum liquid chromatography on a diol-silica gel column (Supelco, Bellefonte, PA, USA); elution performed with successive dichloromethane/ethanol mixtures (100:0, 98:2, 90:10 and 50:50, v/v). The concentrations of peptaibols were determined in each fraction by HPLC/MS analysis and previously reported by Poirier et al. (2007a). Fractions at 10 and 50% of ethanol, eluting peptaibols, were combined, evaporated and frozen at -20°C prior to the bioassay.

As reported for peptaibol treatments (see section 2.1. of Materials and methods), this organic extract was taken up by ethanol and added to incubation media just before the embryos by addition of ethanolic solution (20 µL) in vessels filled with 30 mL filtered seawater. Concentrations of ethanolic solutions were prepared in order to test the final

following concentrations equivalent to 0 (ethanol for control), 0.4, 2, and 4 g L^{-1} of sediment dry weight (dw).

Elutriate was prepared using a modified Melzian method (1990) according to Geffard et al. (2004). Frozen sediments were shaken mechanically at 500 rpm in glass bottles with filtered seawater at a ratio of 1:4 (sediment/water, v/v) for 8 h and allowed to decant for a further 8 h period before removal of the supernatant. For toxicity test, an aliquot was diluted with filtered seawater to the concentrations equivalent to 0 (control), 0.15, 1, 7, 14, 71 and 143 g L^{-1} of sediment dw.

4. Toxicological data analysis

Mean percentage of abnormalities and standard deviation were calculated for each treatments and corrected for effects in control tests by applying Abbot's formula (Emmens, 1948). If abnormalities in the controls (seawater and ethanol) were 20% or more, the test was judged invalid and repeated.

Bartlett's test was used to examine homogeneity of variance. If the data failed this test, arcsin$\sqrt{p}$ transformations were applied to achieve homogeneity. The null hypothesis (peptaibol and sediment treatments had no effects on embryogenesis) was tested by comparison of percentages of abnormal larvae at each concentration to the controls using a one-level ANOVA. Whenever the null hypothesis was rejected ($\alpha = 0.05$), non-linear regressions were performed in order to assess $EC_{50} \pm SD$ by using Macro REGTOX program (version 7.0.5), according to Vindimian et al. (1983).

Mann Whitney U-tests were performed to examine differences between mean percentage of abnormalities in seawater and ethanol controls, and between mean coefficients

of variation (CV) calculated for the peptaibol and copper treatments. Covariance analysis was used to test the difference between the two regression lines established between the two sedimentary treatments.

Results

1. Validity of bioassays

In the experiments carried out, the seawater controls showed 84 ± 4% of normally developed embryos of *C. gigas*. No significant difference (Mann-Whitney U-test, $P>0.05$) was found in development to D-larvae in the ethanol controls (82 ± 0.2%).

Experimentations performed with copper as reference toxicant confirmed the good repeatability of each assay but highlighted differences of embryo sensitivity. For the assay realized to estimate sediment toxicity, a mean EC_{50} ± SD of 40 ± 0.4 µg of $CuSO_4$ L^{-1} was obtained (CV = 1%, n = 3), whereas a mean of 22 ± 1 µg of $CuSO_4$ L^{-1} (CV = 5%, n = 3) was calculated for the assay carried out to determine peptaibol toxicity. These values were both within the EC_{50} acceptability range (21-45 µg L^{-1}) (Quiniou et al., 2005).

2. Peptaibol treatments

2.1. Identification of peptaibols isolated from the *T. longibrachiatum* strain

Two main peptaibol fractions were obtained from the culture of *T. longibrachiatum* and purified by extraction of fungal biomass, silica gel chromatography and reverse phase HPLC.

When analyzed by HPLC/MS, the two-peptide groups appeared as complex mixtures of peptaibol analogues. MS/MS analysis allows identification of the main peptaibols by sequence comparison with literature data.

The first group (Fig. 1A) had $[M+Na]^+$ ions at m/z 1169.9, 1183.9, 1197.9 and 1211.9, suggesting a mixture of 11-residue peptides similar to trichobrachins A, trichorovins TV, trichorozins, hypomurocins A or harzianins HB and HK (Iida et al., 1995; Wada et al., 1995; Rebuffat et al., 1996; Augeven-Bour et al., 1997; Becker et al., 1997; Mohamed-Benkada et al., 2006).

In the second group (Fig. 1B-D), doubly charged $[M+2Na]^{2+}$ ions at m/z 991.2, 991.7, 998.3 and 1002.6 were observed, indicating a mixture of long-sequence peptides with 20 residues, close to longibrachins and trichokonins (Huang et al., 1995; Huang et al., 1996; Leclerc et al., 1998; Leclerc et al., 2001; Landreau et al., 2002).

2.2. Observation of abnormalities

Among the different abnormalities observed, abnormal D-shaped larvae presenting a protruding mantle associated to an irregular shell were frequently recorded with peptaibol treatments (Fig. 2). Compared to the controls for which mantle abnormalities represent 28 ± 4% of observed deformities, the percentage of larvae developing this category of abnormality significantly increased according to a linear relation with the percentage net response obtained following exposures to the different groups of peptaibols and consequently with the peptaibol concentrations in incubation media ($r = 0.74$, $n = 57$). It reached 66 ± 13% when the percentage of abnormalities was over 90%.

279 **2.3. Embryotoxicity of peptaibols**

280 The results of peptaibols quantification in tested media showed an important and variable
281 decrease of solubilized compounds during the experiment. Expressed as a percentage of loss
282 after 22h (y), this decrease was significantly correlated to the initial peptaibols concentration
283 (x) by the following non linear regression ($r = 0.85$, $n = 10$):

284 $$y = -14.67 \, Ln(x) + 108.79 \quad (1)$$

285 Thus, the concentrations of peptaibols in incubation media were expressed in effective
286 concentrations measured at the beginning of the experiment.

287

288 The effects of each group of peptaibols were significant on *C. gigas* embryogenesis
289 ($P<0.0001$). In general, an important variability between responses of replicates was
290 observed. The mean coefficient of variation (35 ± 19%) was significantly superior to the mean
291 CV obtained for copper test (6 ± 7%) (Mann-Whitney U-test, $P<0.01$) indicating that this lack
292 of repeatability was certainly related to the variation of compound solubility in incubation
293 media.

294 Alamethicin showed 22 ± 4% of abnormalities at the lowest concentration tested (0.5 nM;
295 Fig. 3). Its toxicity increased strongly to gain 64 ± 38% at 23 nM. No significant increase of
296 abnormalities was observed at higher concentrations. The calculated EC_{50} was 31 ± 3 nM.

297 Toxic effects of the long-chain peptaibols isolated from *T. longibrachiatum* were not
298 significantly different from the controls at low concentrations (up to 1.2 nM) (Fig. 3).
299 Percentage of abnormalities increased significantly to reach a maximum of 95 ± 5% at 61 nM.
300 The EC_{50} was estimated to be 10 ± 3 nM.

301 Short-sequence peptaibols isolated from this strain had also significant effects on oyster
302 larval development (Fig. 3). Toxic effects (24 ± 12%) were noticed at lowest concentration

(0.4 nM). Above 4 nM, toxicity reaches a maximum and seems to be independent of the peptide concentration. An EC_{50} of 64 ± 8 nM was extrapolated.

3. Embryotoxicity induced by sediment extracts

Purified extract of sediments from Fier d'Ars had significant effects on *C. gigas* embryogenesis ($P<0.002$). No differences were observed between the controls and the lowest concentration tested (0.4 g L^{-1} dw). Abnormalities increased slowly at the higher concentrations and reached 20.5 ± 5.5% at the highest level tested (4.2 g L^{-1} dw). An EC_{50} of 42.4 ± 2.7 g L^{-1} dw could be extrapolated.

The corresponding elutriate also had significant adverse effects on embryogenesis ($P<0.0001$). Its toxicity was not significant at concentrations ≤ 1.4 g L^{-1} dw but increased at higher values to reach 100% of abnormalities at 14.3 g L^{-1} dw. The EC_{50} was 6.6 ± 0.2 g L^{-1} dw.

Fig. 4 shows the linear relationships established between the concentrations of sediment extract or elutriate and the percentage of abnormalities. Covariance analysis revealed no significant difference between the two regression lines, indicating that toxicity of the sedimentary extract and elutriate was similar ($P>0.05$).

Discussion

1. Embryotoxicity of peptaibols

Long and short-sequence peptaibols were shown to disturb embryogenesis of *C. gigas* in the nanomolar concentration range. Toxicity of the fungal peptides was 1.3 - 8 times higher than the toxicity induced by copper for which an EC_{50} of 86 ± 4 nM was calculated. Although no significant differences between the different groups of peptaibols can be highlighted because of the important variations between replicates, toxic effects based on the estimation of EC_{50} appeared appreciably different. Indeed, compared to short-sequence peptaibol mixture showing an EC_{50} of 64 ± 8 nM, long-sequence peptaibols were associated with a higher toxicity. EC_{50} of 10 ± 3 nM and of 31 ± 3 nM were, respectively, estimated for peptaibols isolated from the *T. longibrachiatum* strain and for alamethicin mixture. Peptaibols are known to have membrane-modifying properties which are considered as the basis of their broad spectrum of bioactivity. They interact with biological membranes, modify their permeability and form voltage-dependent transmembrane ion-channels (Boheim et al., 1978; Sansom, 1993; Cafiso, 1994; Lucaciu et al., 1997; Duclohier, 2004). Leakage of cytoplasmic material can occur through such channels, leading to cell death (Chugh and Wallace, 2001). With regard to the peptides studied in our work, membrane-modifying properties were demonstrated for the long-sequence peptaibols, longibrachins, trichokonins, alamethicins, like for the short-sequence ones, trichorovins, trichorozins, harzianins HB and HK (Cafiso, 1994; Huang et al., 1994; Iida et al., 1995; Wada et al., 1995; Cosette et al., 1999; Rebuffat et al., 1999; Leclerc et al., 2001). The permeabilization process was shown to increase with the helix length and global hydrophobicity of peptaibols (Lucaciu et al., 1997; Rebuffat et al., 2000; Kropracheva and Raap, 2002; Berg et al., 2003; Grigoriev et al., 2003). Consequently,

the lower embryotoxicity of short-sequence peptaibol mixture observed in our experiments could be explained by the shorter peptidic chain length.

The augmentation of peptaibol concentration in incubation media lead to a significant increase of mantle abnormalities. The different categories of abnormal larvae were defined by His et al. (1997) on the basis of a study of *C. gigas* larvae isolated from natural environment. In more than 30 years of observations, all of these larvae were classified as perfectly normal straight-hinge D-larvae (His et al., 1999). D-shaped larvae with convex hinges have been described by Nice et al. (2000) as deformity related to exposure to 4-nonylphenol, an endocrine disrupter perturbing calcium metabolism. Therefore, in this investigation, protruding mantle abnormality often associated to irregular shell could be reasonably assumed to be the result of particular chemical interference of peptaibols with embryolarval development in relation to their membrane interaction properties.

Among all their activities against different target organisms, some effects of peptaibols have been reported on animal larval models. Neurotoxicity of trichokonins was reported on blowfly larvae in millimolar concentration range (Landreau et al., 2002). The acute toxicity of fungal biocontrol agent metabolites was recently evinced using invertebrate bioassays (*Artemia salina* and *Daphnia magna*) (Favilla et al., 2006). Alamethicin was confirmed as the most toxic among the peptaibol toxins tested (alamethicin, paracelsin and antiamoebin) with LC_{50} in the low micromolar range. Matha et al. (1992) reported the mosquitocidal activity of different representatives of the peptaibol group on *Culex pipiens* larvae in the nM-µM range. These authors demonstrated that the mode of action of peptaibols was mediated through the damage to mitochondria, as a consequence of the total lysis of the internal mitochondrial contents induced by inhibition of phosphoryl transfer and uncoupling of respiration. In bivalve embryo-larval development bioassay, we could supposed that toxic effects of

peptaibols on *C. gigas* larvae were more acute during the first phase of the development of bivalve corresponding to the embryonal stages from the fertilized egg to the ciliated blastula (His et al., 1999).

Micellization studies have demonstrated that alamethicin has a limited solubility in aqueous media and aggregates above concentrations of 10-20 µM (Archer et al., 1991). A decrease of solubilized compounds in incubation media was observed during our experiment for each group of peptaibols tested and for concentrations inferior to µM. This phenomenon was shown to be strongly dependent of the initial concentration. The percentage of compounds solubilized in the water column at the end of the experiment was respectively of 20 ± 30% for the lowest concentrations tested (<50 nM) and 70 ± 10% for the highest ones (>200 nM). An adsorption of the amphipathic peptides on the vessels could be encountered on a limited number of fixation sites. In these conditions and since embryotoxic effects were significantly observed from the lowest concentrations ranging from 0.4 to 6 nM, the hypothesis of an early action of peptaibols during the first embryonal stages can be supported.

2. Embryotoxicity of sediments from Ars

A high toxicity increasing with the concentration was observed following the exposures of *C. gigas* embryos to the two extracts of sediments from Fier d'Ars (organic extract and elutriate).

The organic extract corresponds to an acetonic/acetic acid sedimentary extract purified on diol phase gel. Peptaibols related to longibrachins and trichokonins were detected in this extract with a concentration of 7.1 ng g^{-1} dw (Poirier et al., 2007a). Consequently, an EC_{50} of

401 0.16 ± 0.01 nM of long-sequence peptaibols could be extrapolated to *C. gigas* embryogenesis.
402 Sediment elutriate corresponds to an aqueous extract of sediments containing suspended
403 inorganic and organic particles as well as any contaminant associated with the particles or the
404 dissolved fraction (Melzian, 1990). As is likely for many organic compounds, peptaibols may
405 adsorb on particulate organic matter resuspended during elutriation and thus become available
406 for *C. gigas* embryos. The pattern of toxicity appeared to be similar between the two
407 treatments, in spite of an estimated EC_{50} higher for the elutriate than that extrapolated for the
408 organic extract. However, according to the EC_{50} determined for long-sequence peptaibols (10
409 ± 3 nM) and the environmental peptaibol concentrations, the toxic effects observed following
410 exposure of embryos to both sedimentary treatments can not be exclusively induced by the
411 fungal peptides.
412
413 In a previous study (Amiard-Triquet et al., 2003), the sediments from Fier d'Ars were
414 considered as "clean" sediments since the total concentrations of metals and organic
415 contaminants are below the first level recommended by the French regulations about sediment
416 quality controls. We also reported a surprising high toxicity of these sediments for
417 embryogenesis in mussel *Mytilus edulis* larvae, in comparable levels to those observed in the
418 present work. Others sources of disturbance as ammonia concentrations, eutrophication or
419 antibacterial drugs used in fish farming were examined but none can be responsible for the
420 observed toxicity.
421 Although none of the biotic or abiotic stressors studied are in sufficient concentrations
422 to explain the high toxicity of these sediments on bivalve embryogenesis, interaction
423 involving chemical stress and other biotic factors such as peptaibols can occur in the marine
424 environment and could be considered to explain this apparent discrepancy. Furthermore, the
425 toxicological data obtained with pure compounds, slightly water soluble, may not represent

426 the real extent of peptaibol embryotoxicity. The bioavailability of these molecules could be
427 augmented when they are co-solubilized with suspended particulate organic matter.

Conclusion

The study presented herein provides the first toxicological data on peptaibol embryotoxicity on marine bivalve development. *C. gigas* embryos appeared to be very sensitive to fungal peptides compared to other animal larval models. It suggests that these molecules at environmentally realistic concentrations can disturb *C. gigas* embryogenesis and can cause an increase in larval deformities in a population of exposed animals. Further studies are necessary to evaluate whether the abnormality found in the larvae of *C. gigas* is disabling, or indeed lethal, and whether this deformity has arisen as a result of membrane permeability perturbations. Concentrations measured in sediments from Fier d'Ars did not fully explain the embryotoxicity observed for these sediments. Additive or synergistic toxic effects with others stressors such as phycotoxins, bacterial toxins or other chemicals usually analysed should be examined.

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Figure Captions

Figure 1 : MS spectra of the two main peptaibol fractions isolated from *T. longibrachiatum* marine strain. A : $[M+Na]^+$ ions of short-sequence peptaibol mixture (respectively: t_R = 8.2, 9.5, 11.2 and 12 min); B-D: doubly charged $[M+2Na]^{2+}$ ions of long-sequence peptaibol mixture observed at respectively t_R of 5.1, 8.8 and 9.8 min.

Figure 2 : Normally developed D-shaped larva (A) and D-shaped larva with a protruding mantle associated to an irregular shell margin (B). Scale bars: 10 µm.

Figure 3 : Peptaibol concentration effects on percentages of abnormal *Crassostrea gigas* development (± SD). Alamethicin (—●—), long-sequence peptaibol mixture (---■---), short-sequence peptaibol mixture (····▲····).

Figure 4 : Effect of the sediment treatments on percentages of abnormal *Crassostrea gigas* development (± SD): organic extract (--○--) (r = 0.97, n = 4), elutriate (—●—) (r = 0.98, n = 5).

636 Figure 1

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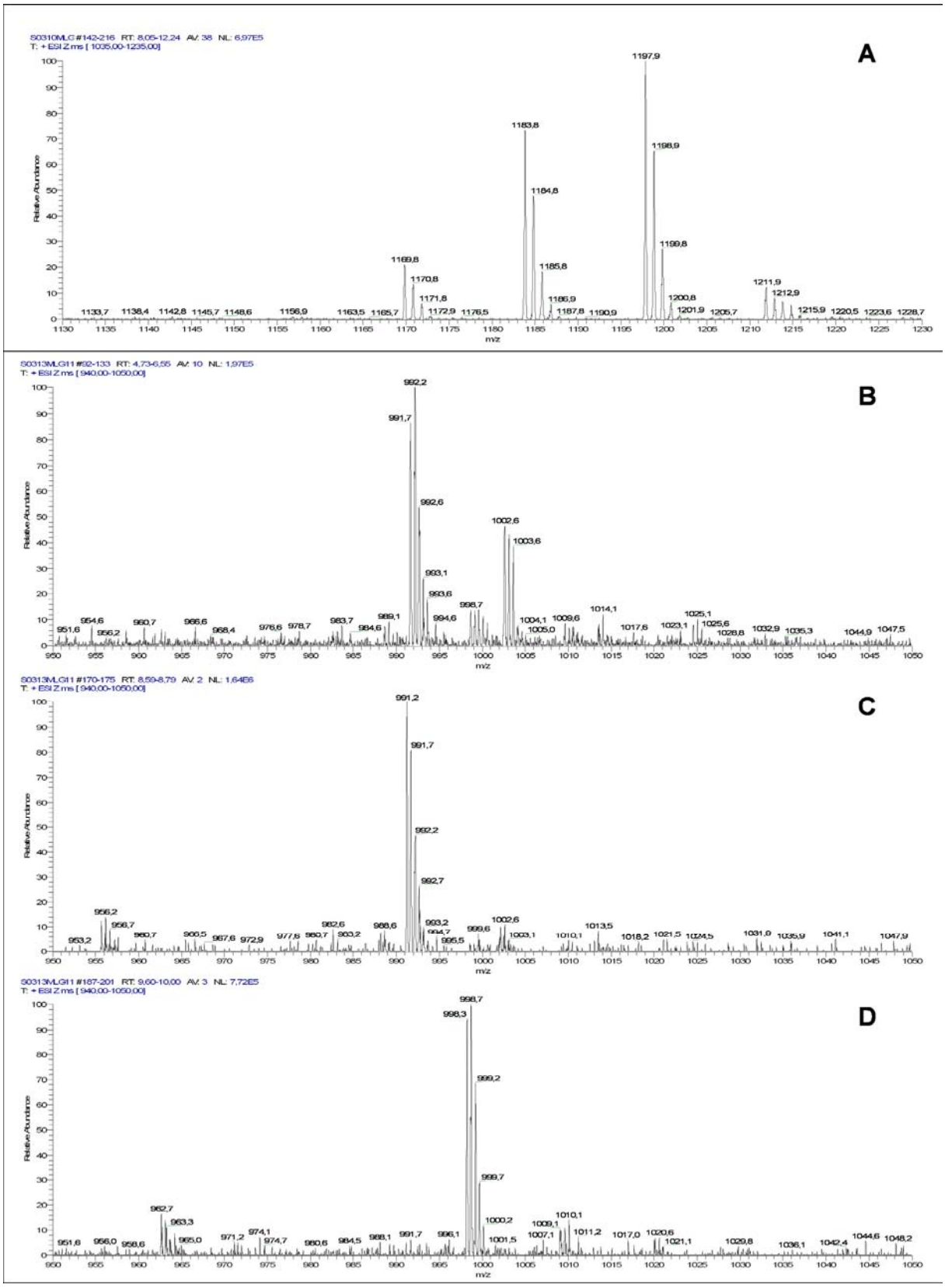

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641 Figure 2

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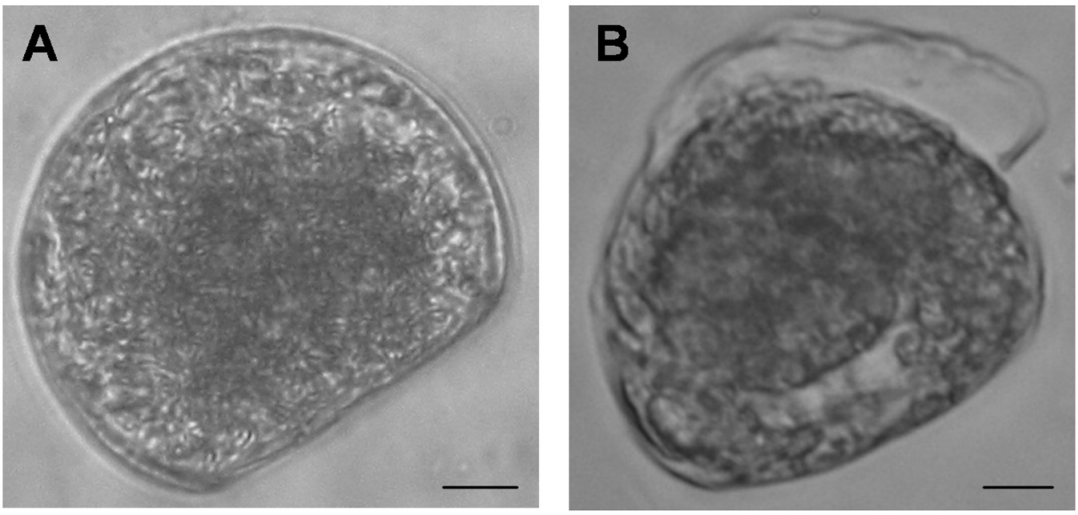

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664 Figure 3

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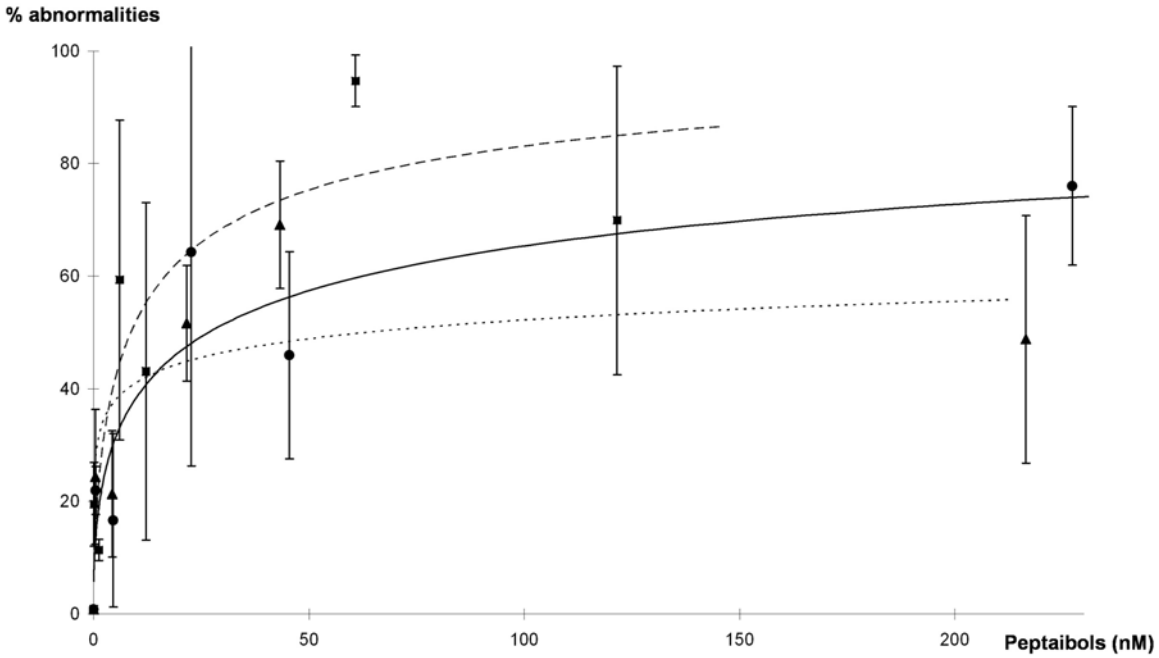

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682 Figure 4

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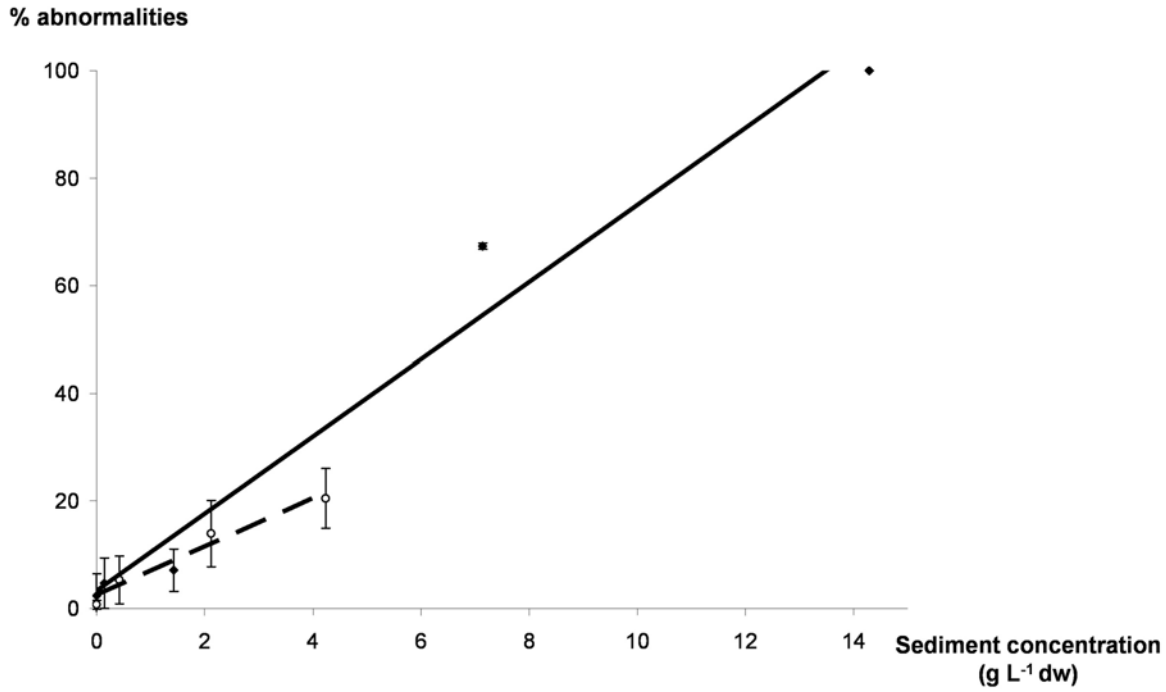

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