The effects of feeding Karlodinium veneficum (PLY # 103; Gymnodinium veneficum Ballantine) to the blue mussel Mytilus edulis

Eva Galimany[1]

Allen R. Place[2]

Montserrat Ramón[3]

Maria Jutson[4]

Richard K. Pipe[4]

[1]IRTA-St. Carles de la Ràpita, Crta. Poble Nou s/n St. Carles de la Ràpita 43540, Spain

[2]University of Maryland Biotechnology Institute., Center of Marine Biotechnology, Baltimore, Maryland, United States of America

[3]IEO-Centre Oceanogràfic de Balears, Moll de Ponent s/n Palma de Mallorca 07015, Spain

[4]Marine Biological Association, Citadel Hill Plymouth PL1 2PB, United Kingdom

Corresponding author:

Eva Galimany

Centre d'Aqüicultura-IRTA

Crta. Poble Nou s/n

St. Carles de la Ràpita 43540

Spain

Ph: (0034) 977 745 427

Fax: (0034) 977 744 138

E-mail: eva.galimany@irta.es

Abstract

The effects of exposure to the type species for Karlodinium veneficum (PLY # 103) on immune function and histopathology in the blue mussel Mytilus edulis were investigated. Mussels from Whitsand Bay, Cornwall (UK) were exposed to K. veneficum (PLY # 103) for 3 and 6 days. Assays for immune function included total and differential cells counts, phagocytosis and release of extra cellular reactive oxygen species. Histology was carried out on digestive gland and mantle tissues. The toxin cell quota for K. veneficum (PLY #103) was measured by liquid chromatography-mass spectrometry detecting two separable toxins KvTx1 (11.6 ± 5.4 ng/ml) and KvTx2 (47.7 ± 4.2 ng/ml). There were significant effects of K. veneficum exposure with increasing phagocytosis and release of reactive oxygen species following 6 days exposure. There were no significant effects on total cell counts. However, differential cell counts did show significant effects after 3 days exposure to the toxic alga. All mussels produced faeces but not pseudofaeces indicating that algae were not rejected prior to ingestion. Digestive glands showed ingestion of the algae and hemocyte infiltration after 3 days of exposure, whereas mantle tissue did not show differences between treatments. As the effects of K. veneficum were not observed in the mantle tissue it can be hypothesized that the algal concentration was not high enough, or exposure long enough, to affect all the tissues. Despite being in culture for more than 50 years the original K. veneficum isolate obtained by Mary Parke still showed toxic effects on mussels.

Key words: blue mussel, harmful algal blooms, histopathology, immunology, Karlodinium veneficum.

1. Introduction

The dinoflagellate genus Gymnodinium forms a large group of unarmoured species which was recently sub-divided into four genera, Karenia (Hansen and Moestrup), Karlodinium (Larsen), Akashiwo (Hansen and Moestrup), with the description of Gymnodinium emended (Daugbjerg et al., 2000). Karlodinium and Karenia contain several species with the potential for forming large toxic blooms which have been responsible for killing fish and molluscs, resulting in huge economic losses (Anderson et al., 2000).

Gymnodinium veneficum was first isolated from a region near Plymouth Sound, UK in 1950, by Parke and subsequently described and named by Ballantine (1956). The strain, PLY # 103, was described to produce a powerful toxin which was lethal to nearly all marine organisms tested (Abbott and Ballantine, 1957, Place et al., 2005). The bivalve Lasaea rubra was shown not to filter a suspension of G. veneficum (Ballantine and Morton, 1956). A recent re-examination of the PLY # 103 strain, using light and electron microscopy and partial LSU rDNA, found it to be identical to K. micrum and thus necessitating a change of name for both G. veneficum and K. micrum to K. veneficum (Bergholtz et al., 2005). Laboratory experiments have shown this organism causes mortality in juvenile cod and reduced growth in mussels (Nielsen and Strømgren, 1991, Nielsen, 1993). Despite being in culture for more than 50 years PLY#103 was shown to still produce toxins which were similar to karlotoxins described for North American isolates of K. veneficum (Kempton et al., 2002, Place et al., 2005, Deeds et al., 2006).

It has been shown that filter feeding bivalves, including blue mussels Mytilus edulis, are affected when exposed to toxic microalgae presenting: growth rate reduction, production of mucus, lesions in different tissues, clearance-rate reduction, immune responses and high mortality rates (Shumway and Cucci, 1987, Nielsen and Strømgren,

1991, Smolowitz and Shumway, 1997, Keppler et al., 2005, Wikfors, 2005). The effects can be of great importance, not only for natural communities but also for aquaculture facilities (Shumway, 1990, Anderson et al., 2000).

The present study investigated whether exposure to K. veneficum (PLY #103) would show adverse effects, especially on immune function and histopathology in Mytilus edulis.

2. Materials and methods

Mussels, Mytilus edulis, (40-55 mm shell length) were collected at low tide from Whitsand Bay, southeast Cornwall, UK, an exposed open ocean site. They were immediately transported to the laboratory where epiphytes and other encrusting organisms were removed from the shells. A hundred mussels were placed in each of two experimental tanks, containing 8 l of filtered sea water at the same temperature as the sampling site, and left for 24 h to acclimate. The mussels were then exposed to either K. veneficum or Dunaliella primolecta (PLY # 81). Cell diameters averages were 11.4 ± 0.02 µm for PLY # 103 and 5.9 ± 0.05 µm for PLY # 81. Mussels were removed on day 0, before exposure to the algae, day 3 and day 6 of exposure, 30 each time, and used for hemocyte counts, immune assays and histology.

2.1. Algal cultures

The algal cultures (PLY#103 and PLY #81) used for the exposure experiments were maintained in Erd-Schreiber medium (Bruce et al., 1940) at 15°C. Both culture cell concentrations were measured with an improved Neubauer hemocytometer and adjusted to give a final concentration of 6.25×10^4 cell/l daily in each tank. Seawater in experimental tanks was changed daily and the tanks rinsed thoroughly to avoid build up of mussel biodeposits. The algae were added daily to each tank after cleaning. After

removing the mussels for sampling on day 3, the algal concentration was adjusted to the remaining number of bivalves so they were exposed to the same concentration throughout the experiment.

2.2 Toxin measurement

Karlotoxin (KvTx) concentrations were measured by liquid chromatography – mass spectrometry (LC-MS) (Bachvaroff et al., 2007) using the fact that karlotoxin binds to teflon (PTFE) filters quantitatively. Aliquots (2 and 10 ml) of the cultures were filtered on 13 mm PTFE syringe filters (Whatman, 0.2 mm pore size). The filtrate was discarded and the filters eluted with 1 ml HPLC grade methanol into glass test tubes containing 2 ml dH_2O. Toxin samples were injected onto a C8 (LiChrosphere 125 mm x 4 mm 5 mm bead size RP-8, Waters Corp.) column and subjected to a 1 ml/min 10% to 95% methanol : water gradient over 25 min using an HP/Agilent 1100 HPLC. Toxin peaks were detected at 225 nm or 235 nm as appropriate for KvTx1 or KvTx2 toxins, respectively. A portion of the mobile phase (1/3 to 1/6) was then passed to the electro-spray nozzle of the MS (Agilent G1956A SL or VL) for ionization. A 1% formic acid in methanol solution (0.1 ml/min) was added to provide appropriate pH conditions for positive mode ionization. Peaks previously determined to have hemolytic activity were quantified as a mass (pg) based on calibration curves determined with pure karlotoxin standards.

2.3 Hemocyte counts

Hemolymph samples were withdrawn from the posterior adductor muscle into an equal volume of Baker's formol calcium, containing 2% NaCl. Total cell counts were measured with a Neubauer hemocytometer. Differential cells counts were prepared using a Shandon cytocentrifuge with 200 µl of hemolymph . Cells were post fixed with methanol and stained using Wright's stain (Parry and Pipe, 2004). After air drying, the

samples were mounted with Canada balsam. Eosinophilic and basophilic cells were identified (Friebel and Renwrantz, 1996) and relative numbers were calculated by counting 200 blood cells per sample. The counts were carried out for 5 mussels from each treatment.

2.4 Immune assays

2.4.1 Detection of extracellular superoxide anion

Hemolymph from 5 mussels per treatment was extracted from the posterior adductor muscle into an equal volume of Tris-buffered saline (TBS), pH 7.6 containing 2% of sodium chloride. Six 100 µl aliquots of each hemolymph sample were pipetted into microplate wells. An equal volume of cytochrome-C solution (80 µM ferricytochrome-C in TBS containing 2% sodium chloride) was added to three wells. Cytochrome-C solution containing 300 units ml^{-1} of superoxide dismutase (SOD) was pipetted into the other three wells (100 µl). The cytochrome-C solutions (with and without SOD) were also aliquoted into wells without blood cells and, in addition, cells in only buffer were used as controls.

The optical density (OD) was read immediately and every 30 sec for 20 min using a 550 nm filter and a kinetics package. Results are expressed in optical density (OD) per milligram hemocyte protein which was analyzed using a bicinchoninic acid (BCA) protein assay (Pierce Chem. Co).

2.4.2 Phagocytosis

Hemolymph from 5 mussels per treatment was withdrawn from the posterior adductor muscle into an equal volume of TBS, pH 7.6 containing 2% sodium chloride. Aliquots of 50 µl of each hemolymph sample were pipetted into 4 replicate wells of one plate and 1 well of another for the protein assay. After 10 min, an equal volume of neutral red-stained zymosan suspension was added (Pipe et al., 1995a). Hemocytes fixed with

Baker's formol calcium containing 2% sodium chloride were aliquoted and zymosan added to be used as blanks. Zymosan in buffer only was used as negative control (same volumes as samples). After incubating the plates for 30 min at 15ºC, 100 µl of Baker's formol calcium was added to each well to stop the reaction. The plate was spun at 70 g for 5 min, the supernatant discarded and the cells re-suspended in 100 µl of TBS buffer. This washing procedure was repeated until there was no evidence of zymosan remaining in the wells of the negative controls (~6 washes). Just before the last spin, 50 µl of standard zymosan suspensions of known particle concentrations were pipetted into duplicate wells, using serial dilutions of the zymosan from a stock suspension, 5.0×10^6 particles per well and 50 µl TBS added to each well to provide a standard curve. Finally, the neutral red was solubilised by adding 100 µl of 1% acetic acid in 50% ethanol to each well and incubating for 30 min. After shaking the plate, the OD was read with a 550 nm filter and results expressed as particles of zymosan phagocytosed per milligram hemocyte protein. Hemocyte protein was analyzed using a BCA protein assay (Pierce Chem. Co).

2.5 Histology

Digestive gland and mantle tissues were dissected from 10 individuals on day 0, before exposure to the algae, and on days 3 and 6 after the exposure and fixed in Baker's formol calcium for 48 h. The tissues were then rinsed in tap water and transferred to 70% alcohol. Samples were dehydrated and embedded in paraffin. After processing, sections were cut (5 µm thickness), stained using a hematoxylin-eosin staining procedure and examined under a light microscope. The thickness of the digestive gland tubular epithelium was measured for 5 mussels from each treatment. The measurements involved 4 readings from each of 10 tubules chosen for each individual using the Image-Pro Plus image analysis software package (Media Cybernetics, L. P.).

2.6 Statistical analysis

Results of hemocyte counts, immune assays and digestive gland tubule thickness were analyzed using Statistica 98 Edition (StatSoft Inc., 1998). Pair-wise comparisons between tanks at each experimental time were done using t-test considering that each mussel was a unit of replication. Results were significant with a probability (*P*) value of <0.05.

3. Results

3.1 Algal toxicity

Two major toxic peaks (KvTx1 and KvTx2) were obtained upon methanol gradient elution for PLY # 103. Both were hemolytic to rainbow trout erythrocytes and found in nearly equivalent cell quotas (0.93 pg/cell vs 1.25 pg/cell) (unpublished data). The UV spectra of the two toxins differed with KvTx1 having a peak at 225 nm while KvTx2 had a UV absorption maximum at 235 nm. LC/MS analysis of the two peaks found masses of 1208.8d and 1267.8d for KvTx1 vs 1242.7d and 1301.8d for KvTx2. The concentration of the two toxins measured for the K. veneficum culture (4.0 x 10^6 cell/l) was KvTX1 11.6 ± 5.4 ng/ml and KvTX2 47.7 ± 4.2 ng/ml. After dilution in the tank for feeding to the mussels, the concentrations were KvTX1 < 0.4 ± 0.0 ng/ml and KvTxX2 < 2.7 ± 0.0 ng/ml.

3.2 Hemocyte counts

There were no significant differences between treatments for the total blood cell counts ($P > 0.05$); however, there were significant differences for the differential blood cell percentages between treatments ($P < 0.05$) on day 3 but not on day 6 (Tab. 1). On day 0, prior to algal exposure, the ratio of eosinophilic to basophilic cells was 60:40. This ratio did not vary with time for the mussels exposed to the non-toxic alga. On day 3, the

mussels exposed to toxic algae did show significant differences with eosinophilic cells decreasing from 60% to 30% and basophilic cells increasing from 40% to 70%. These values changed again on day 6 of the experiment and both cell types reached a final concentration of 50% of the total hemolymph content.

3.3 Immune assays

The cytochrome-C reduction assay to detect extracellular superoxide anion showed significant differences between treatments ($P < 0.05$) at both times. Blood cells from mussels fed with the toxic algae showed an increase in the release of reactive oxygen species on both day 3 and day 6 in comparison with the mussels fed with the non-toxic alga (Fig. 1).

Phagocytosis of zymosan showed significant differences between treatments on day 6 ($P < 0.05$) but not on day 3 (Fig. 2). Hemocytes from mussels exposed to the toxic algae showed significantly more phagocystosis compared with both pre-exposed mussels and those exposed to the non-toxic algae.

3.4 Histology

The mantle tissues contained ripe gametes in all cases and no differences were detected between treatments, gender or time. Hemocyte infiltration and oocyte atresia were not observed in the mantle tissues of any of the samples examined. The digestive gland, however, did show histological differences with time and treatments. Hemocyte infiltration appeared on days 3 and 6 in mussels exposed to the K. veneficum whereas the mussels exposed to the non-toxic algae, remained the same throughout the experiment (Photo 1). The lumina of the digestive glands tubules were full in both treatments throughout the exposures showing that the algae were not rejected prior to ingestion. Intact K. veneficum cells were present in the digestive gland tubules on days 3 and 6 (Photo 2) but intact cells of D. primolecta were not apparent in the tubules.

Measurements of the digestive tubule thickness did not show differences with treatment at both times ($P < 0.05$) (Fig. 3).

Discussion

This study demonstrates that exposure to pre-bloom concentrations of PLY # 103, for up to six days, results in measurable effects on the blue mussel, Mytilus edulis. During bloom conditions of K. veneficum cell densities can reach 10 to 100 times those used in this experiment (Fensin, 2004, Goshorn et al., 2004).

Differential blood cells counts showed differences with treatments and time, with circulating eosinophilic cells decreasing in the K. veneficum exposed mussels. Eosinophilic hemocytes include only granular cells whereas the basophilic hemocytes include both agranular and granular cells with small granules (Pipe et al., 1997). The basophilic blood cells are less phagocytic than the eosinophilic cells (Carballal et al., 1997, Pipe et al., 1997, Hine, 1999). It appears that the eosinophilic cells were stimulated by the presence of the toxic algae resulting in a rapid decline from circulation, possibly due to movement into the tissues to phagocytose damaged cells. In support of this hypothesis, larger eosinophilic cells were observed on days 3 and 6 in the mussels exposed to the toxic algae, the cells appeared to be granular hemocytes containing phagocytosed material. By day 6 of the study the baseline levels of eosinophilic and basophilic hemocytes had almost recovered. This result is similar to the findings of Hégaret and Wikfors (2005a) who exposed Crassostrea virginica and Argopecten irradians to the toxic alga Prorocentrum minimum. They observed that the percentage of granulocytes decreased at the very beginning of their exposure but increased after a few days.

The results from the phagocytosis assays support the differential blood cells counts, as there was a significant increase in phagocytosis on day 6 in mussels exposed to the toxic algae compared with pre-exposure and controls. On day 3 of the study there was also an increase in phagocytosis in mussels exposed to the toxic algae, despite the reduced numbers of eosinophilic hemocytes; however, it should be noted that there was also an increase in phagocytosis in the controls which may reflect a tank effect. Hégaret and Wikfors (2005b) also found an increase in phagocytosis when exposing Crassostrea virginica to toxic Prorocentrum minimum.

The release of reactive oxygen species by blood cells from mussels fed with the toxic algae was higher on both days 3 and 6 compared with mussels fed on the non-toxic algae. In general, exposure to low concentrations of chemical contaminants has resulted in an enhanced response for release of reactive oxygen species in bivalve hemocytes (Pipe and Coles, 1995b, Dyrynda et al., 1998, 2000). With increasing levels of pollutant exposure there has tended to be an inhibition of reactive oxygen release (Pipe and Coles, 1995b, Pipe et al., 1999). The decrease in values for release of reactive oxygen species for both toxic and non-toxic treatments on day 3 compared with pre-exposure levels could again reflect a tank effect, although it should be noted that the toxin exposed mussels did show a significant increase compared with the controls.

Wootton et al. (2003) recently compared immune function in M. edulis with two other bivalve molluscs (the edible cockle, Cerastoderma edule, and the razor-shell, Ensis siliqua). M. edulis hemocytes were much more active in phagocytosis and superoxide generation than hemocytes from the other two species. Although the authors were not exposing the bivalves to toxic algae, it can be suggested that the enhanced levels of phagocytosis and release of reactive oxygen species in the present study indicate a stimulation of immune response in the mussels following exposure to the toxic algae.

Digestive gland tissues showed hemocyte infiltration following toxic algal exposure in mussels sampled on both days 3 and 6. Similar results were found when exposing bivalves to toxic Prorocentrum minimum (Wikfors and Smolowitz, 1995). The karlotoxins produced by K. veneficum act by depolarization of cell membranes and are able to lyse rainbow trout erythrocytes and cause extensive gill damage in exposed fish (Abbot and Ballantine, 1957, Deeds et al., 2006). Hemocyte infiltration was generally associated with the connective tissues surrounding the tubules and could be related to phagocytosis of tissues damaged by the toxin. Interestingly, despite the cytotoxic effects of K. veneficum, histology revealed that the digestive gland tubules were full throughout the exposure, indicating that feeding was not inhibited by the toxic algae. Feeding on toxic algae by other bivalves resulted in a decrease of digestive gland wall thickness, dilation of the digestive gland tubules and no evidence of algae within the tubule lumen (Bricelj et al., 2004, Pearce et al., 2005). In the present study, there were no differences observed in the digestive tubules between treatments or over time, indicating that all mussels were able to continue feeding at this algal density with no apparent direct effect on the digestive cells. In addition, faeces were found each day throughout the experiment without any evidence of pseudofaeces, suggesting that the mussels were able to filter and digest both the toxic and non-toxic algae. Further experiments at higher algal densities will be necessary to determine whether similar findings are found under bloom conditions.

The mantle tissue showed ripe gametes in all cases and no differences were detected between treatments or time. The fact that the reproductive tissue did not appear to be affected by the K. veneficum may be due the relatively short exposure time together with the low concentration of the toxic algae ingested. Karlotoxins exhibit very steep dose response curves to hemolysis or fish death (Deeds et al., 2006). Notwithstanding,

the results are in accord with those found by Franchini et al. (2003) who localized yessotoxins in hemocytes and digestive glands of M. galloprovincialis but did not detect toxin in the gonads.

Conclusion

Mussels ingested and digested the toxic algae as the digestive glands tubules were full throughout the experiment, faeces were found daily and the toxin levels in the experimental tank decreased with addition of the mussels. These results indicate that M. edulis did not reject or avoid the toxic algae by closing down and stopping filtration. The karlotoxins seemed to affect the mussel tissues and cells that were in first contact with the algae including the hemocytes and digestive gland cells. Observations on gill tissues are highly recommended for further studies. There was no observed effect on the mantle tissues, possibly due to the low algal concentration and the short exposure time. The differential blood cells counts varied with time due to stimulation of the immune system by the toxins and movement of the granular eosinophilic cells from circulation into the tissues to phagocytose damaged cells. This result is supported by the increased capacity of the hemocytes to phagocytose zymosan in the toxin exposed mussels. The blood cells from mussels exposed to the toxic algae also showed an increase in the release of reactive oxygen species. A possible tank effect was observed on day 3, suggesting that the acclimation period should be increased for future experiments.

The possibility cannot be dismissed that a difference in the two tanks used, not related to the experimental K. veneficum exposure, could be confounding results attributed to Karlodinium. Nevertheless, immunological measurements and histological data are consistent with the sequence of hemocyte proliferation and then infiltration into tissues suggesting that the observed effects did result from exposure to the toxic algae.

Although faeces where not examined for the presence of intact K. veneficum cells, it is clear that M. edulis were affected by the toxic algae due to differnent responses obtained between treatments. Longer exposure experiments would be of interest to see whether the effects increase with time or whether the mussels may be able to adapt over a longer time period. Similarly, exposure to bloom densities of K. veneficum may have more immediate and pervasive affects.

Acknowledgements

This study has been supported by a grant from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) to the first author and partially financed by the RTA04-023 INIA research project. This work was funded in part by grants from National Oceanic and Atmospheric Administration Coastal Ocean Program under ECOHAB award #NA04NOS4780276 to University of Maryland Biotechnology Institute, and Grant # U50/CCU 323376, Centers for Disease Control and Prevention and the Maryland Department of Health and Mental Hygiene. This is contribution # 07-170 from the UMBI Center of Marine Biotechnology, and # 219 from the Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) program.

References

Abbott, B.C., Ballantine, D., 1957. The toxin of *Gymnodinium veneficum* Ballantine. J. Mar. Biol. Ass. U. K. 36, 169-189.

Anderson, D.M., Kaoru, Y., White, A.W., 2000. Estimated annual economic impacts from harmful algal blooms (HABs) in the United States. Woods Hole Oceanographic Institution Technical Report, WHOI-2000-11, pp. 97.

Bachvaroff, T. R., Adolf, J. E., Squier, A., Harvey, H. R., Place, A. R., 2007. Liquid chromatographic-mass spectroscopic characterization, detection and quantification of karlotoxins. Harmful Algae (In Review).

Ballantine, D., 1956. Two new marine species of *Gymnodinium* isolated from the Plymouth area. J. Mar. Biol. Ass. U. K. 35, 467-474.

Ballantine, D., Morton, J. E. 1956. Filtering, feeding, and digestion in the labellibranch Lasaea rubra. J. Mar. Biol. Ass. U. K. 35, 241-274.

Bergholtz, T., Daugbjerg, N., Moestrup, O., Fernández-Tejedor, M., 2005. On the identity of *Karlodinium venefi*cum and description of *Karlodinium armiger* sp. nov. (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition. J. Phycol. 42, 170-193.

Bricelj, V.M., MacQuarrie, S.P., Smolowitz, R., 2004. Concentration-dependent effects of toxic and non-toxic isolates of the brown tide alga *Aureococcus anophagefferens* on growth of juvenile bivalves. Mar. Ecol. Prog. Ser. 282, 101-114.

Bruce, J.R., Knight, M., Parke, M.W., 1940. The rearing of oyster larvae on an algal diet. J. Mar. Biol. Ass. U. K. 24, 337-374.

Carballal, M.J., López, C., Azevedo, C., Villalba, A., 1997. In vitro study of a phagocytic ability of *Mytilus galloprovincialis* Lmk. haemocytes. Fish Shellfish Immunol. 7, 403-416.

Daugbjerg, N., Hansen, G., Larsen, J., Moestrup, O., 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39, 302-317.

Deeds, J. R., Reimschussel R., and Place, A. R., 2006. Histopathological effects in fish exposed to the toxins from *Karlodinium micrum* (Dinophyceae). J. Aquat. Anim. Health 18; 136-148.

Dyrynda, E.A., Pipe, R.K., Burt, G.R., Ratcliffe, N.A., 1998. Modulations in the immune defences of mussels (*Mytilius edulis*) from contaminated sites in the UK. Aquat. Toxicol. 42, 169-185.

Dyrynda, E.A., Law, R.J., Dyrynda, P.E.J., Kelly, C.A., Pipe, R.K., Ratcliffe, N.A., 2000. Changes in immune parameters of natural *Mytilus edulis* populations following a major oil spill (Sea Empress, Wales, UK). Mar. Ecol. Prog. Ser. 206, 155-170.

Fensin, E. E., 2004. Occurrence and ecology of the dinoflagellate *Karlodinium micrum* in estuaries of North Carloina, USA. In: Steidinger, K. A., et al. (Ed.), Harmful Algae 2002 Proceedings. St. Petersberg, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, pp 62-64.

Franchini, A., Milandri, A., Poletti, R., Ottaviani, E., 2003. Immunolocalization of yessotoxins in the mussel *Mytilus galloprovincialis*. Toxicon 41, 967-970.

Friebel, B., Renwrantz, L., 1996. Application of density gradient centrifugation for separation of eosinophilic and basophilic haemocytes from *Mytilus edulis* and characterization of both cell groups. Comp. Biochem. Physiol. A 112, 81-90.

Goshorn, D., Deeds, J., Tango, P., Poukish, C., Place, A.R., McGinty, M., Butler, W., Luckett, C., and Magnien, R., 2004. Occurrence of *Karlodinium micrum* and its association with fish kills in Maryland estuaries. In: Steidinger, K. A., et al. (Ed.), Harmful Algae 2002 Proceedings. St. Petersberg, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, pp. 361-363.

Hégaret, H., Wikfors, G.H., 2005a. Time-dependent changes in haemocytes of eastern oysters, *Crassostrea virginica*, and northern bay scallops, *Argopecten irradians*

irradians, exposed to a cultured strain of *Prorocentrum minimum*. Harmful Algae 4, 187-199.

Hégaret, H., Wikfors, G.H., 2005b. Effects of natural and field-simulated blooms of the dinoflagellate *Prorocentrum minimum* upon hemocytes of eastern oysters, *Crassostrea virginica*, from two different populations. Harmful Algae 4, 201-209.

Hine, P.M., 1999. The inter-relationship of bivalve haemocytes. Fish Shellfish Immunol. 9, 367-385.

Kempton, J.W., Lewitus, A.J., Deeds, J.R., McHugh Law, J., Place, A.R., 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. Harmful Algae 1, 233-241.

Keppler, C.J., Hoguet, J., Smith, K., Ringwood, A.H., Lewitus, A.J., 2005. Sublethal effects of the toxic alga *Heterosigma akashiwo* on the southeastern oyster (*Crassostrea virginica*). Harmful Algae 4, 275-285.

Nielsen, M.V., Strømgren, T., 1991. Shell growth response of mussels (*Mytilus edulis*) exposed to toxic microalgae. Mar. Biol. 108, 263-267.

Nielsen, M.V., 1993. Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. Mar. Ecol. Prog. Ser. 95, 273-277.

Parry, H.E., Pipe, R.K., 2004. Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). Aquat. Toxicol. 69, 311-325.

Pearce, I., Handlinger, J.H., Hallegraeff, G.M., 2005. Histopathology in Pacific oyster (*Crassostrea gigas*) spat caused by the dinoflagellate *Prorocentrum rhathymum*. Harmful Algae 4, 61-74.

Pipe, R.K., Coles, J.A., Farley, S.R., 1995a. Assays for measuring immune response in the mussel *Mytilus edulis*. In: Stolen, J.S., et al. (Ed.), Techniques in Fish Immunology.

Immunology and Pathology of Aquatic Invertebrates. SOS publications, Fair Haven, New Jersey, pp. 93-100.

Pipe, R.K., Coles, J.A., 1995b. Environmental contaminants influencing immune function in marine bivalve molluscs. Fish Shellfish Immunol. 5, 581-595.

Pipe, R.K., Farley, S.R., Coles, J.A., 1997. The separation and characterisation of haemocytes from the mussel *Mytilus edulis*. Cell & Tiss. Res. 289, 537-545

Pipe, R.K., Coles, J.A., Carissan, F.M.M., and Ramanathan, K., 1999. Copper induced immunomodulation in the marine mussel, *Mytilus edulis*. Aquatic Toxicol., 46, 43-54.

Place, A.R., Bachvaroff, T.R., Adolf, J.E., 2005. The toxin from *Gymnodinium veneficum* Ballantine-rediscovered: it's a karlotoxin. 3rd Symposium on Harmful Algae in the U. S., California.

Shumway, S.E., Cucci, T.L., 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. Aquat. Toxicol. 10, 9-27.

Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aquaculture. J. World Aquac. Soc. 21, 65-104.

Smolowitz, R., Shumway, S.E., 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve molluscs. Aquac. Int. 5, 291-300.

Wikfors, G.H., Smolowitz, R., 1995. Experimental and histological studies of four life-history stages of the eastern oyster, *Crassostrea virginica*, exposed to a cultured strain of the dinoflagellate *Prorocentrum minimum*. Biological Bulletin 188, 313-328.

Wikfors, G.H., 2005. A review and new analysis of trophic interactions between *Prorocentrum minimum* and clams, scallops, and oysters. Harmful Algae 4, 585-592.

Wootton, E.C., Dyrynda, E.A., Ratcliffe, N.A., 2003. Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*). Fish Shellfish Immunol. 15, 195-210.

Figure legends

Table 1: Percentage of eosinophilic (eos) and basophilic (bas) cells throughout the experiment for both toxic (T) and non-toxic (NT) treatments. Numbers are expressed in mean ± SE.

Figure 1: Detection of extra cellular superoxide anion in mussel hemocytes sampled on days 0, 3 and 6 of the experiment (T: toxic, NT: non-toxic). Error bars are 1 SEM. Significant differences were found between time ($P < 0,05$) and treatment ($P < 0,05$).

Figure 2: Phagocytosis of zymosan by mussel blood cells sampled on days 0, 3 and 6 of the experiment (T: toxic, NT: non-toxic). Error bars are 1 SEM. Significant differences between treatments ($P < 0,05$) were found on day 6.

Figure 3: Digestive gland tubule thickness (mm). Error bars are 1 SEM. No significant differences were found (T: toxic, NT: non-toxic).

Photo 1: Optical micrographs of digestive gland tissues sampled on days 3 (A) and 6 (B), from mussels exposed to the toxic algae. Arrows show hemocyte infiltration. DG: digestive gland.

Photo 2: Optical micrograph of a digestive gland containing a cell of Karlodinium veneficum shown by an arrow. DG: digestive gland

Table 1

	D0	D3	D6
Eos T		29.85 ± 6.69	51.57 ± 2.41
Bas T		70.15 ± 6.69	48.43 ± 2.41
Eos NT	60.13 ± 2.54	58.09 ± 3.22	61.73 ± 5.50
Bas NT	39.87 ± 2.54	41.91 ± 3.22	38.27 ± 5.50

Figure 1

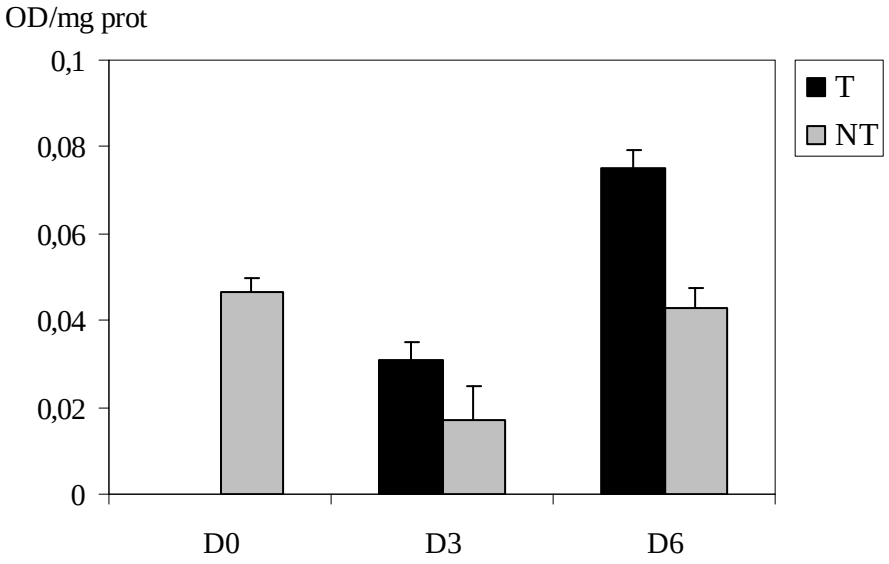

Figure 2

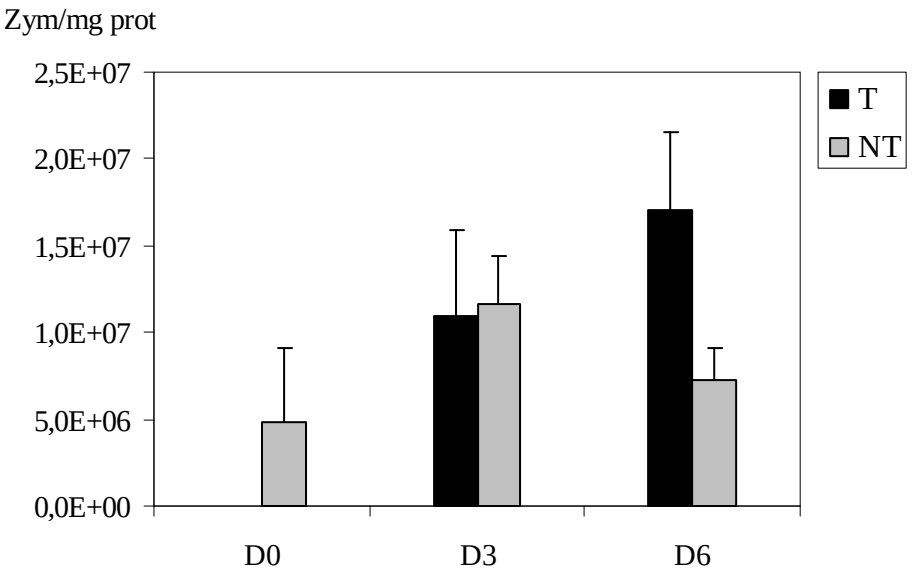

Figure 3

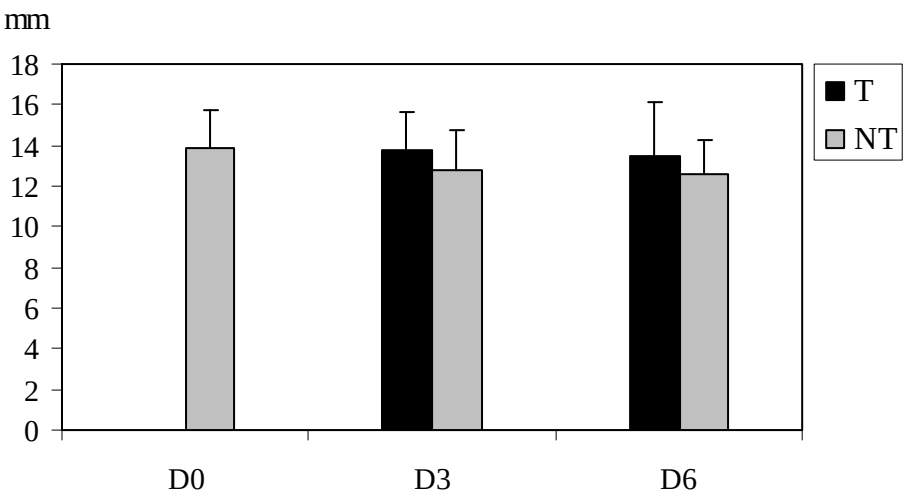

Photo 1

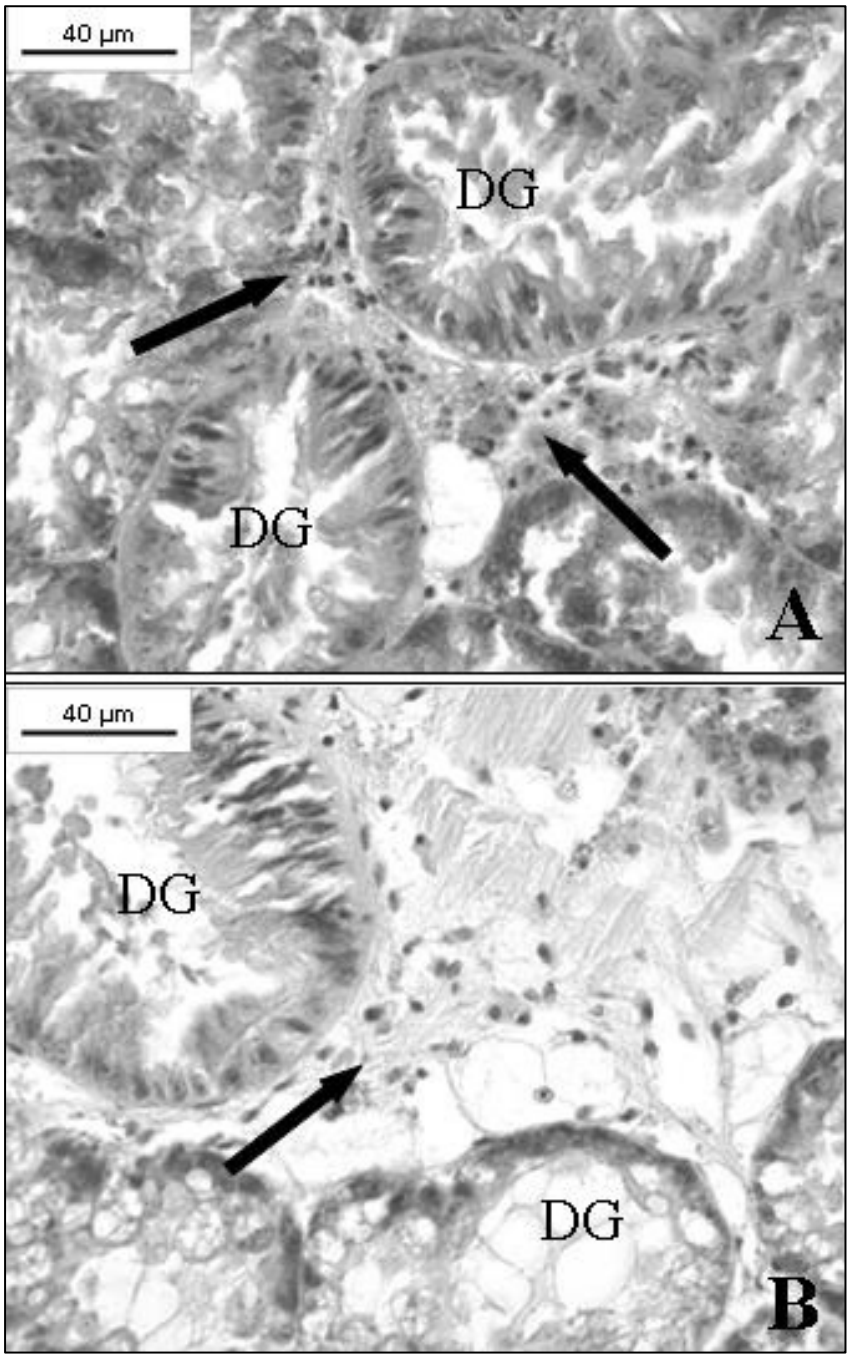

Photo 2