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In vitro interactions between several species of harmful algae and haemocytes of bivalve molluscs

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Abstract Harmful algal blooms (HABs) can have both lethal and sublethal impacts on shellfish. To understand the possible roles of haemocytes in bivalve immune responses to HABs and how the algae are affected by these cells (haemocytes), in vitro tests between cultured harmful algal species and haemocytes of the northern quahog (= hard clam) *Mercenaria mercenaria*, the soft-shell clam *Mya arenaria*, the eastern and Pacific oysters *Crassostrea virginica* and *Crassostrea gigas* and the Manila clam *Ruditapes philippinarum* were carried out. Within their respective ranges of distribution, these shellfish species can experience blooms of several HAB species, including *Prorocentrum minimum*, *Heterosigma akashiwo*, *Alexandrium fundyense*, *Alexandrium minutum* and *Karenia* spp.; thus, these algal species were chosen for testing. Possible differences in haemocyte variables attributable to harmful algae and also effects of haemolymph and haemocytes on the algae themselves were measured. Using microscopic and flow cytometric observations, changes were measured in haemocytes, including cell morphology, mortality, phagocytosis, adhesion and reactive oxygen species (ROS) production, as well as changes in the physiology and the characteristics of the algal cells, including mortality, size, internal complexity and chlorophyll fluorescence. These experiments suggest different effects of the several species of harmful algae upon bivalve haemocytes. Some harmful algae act as immunostimulants, whereas others are immunosuppressive. *P. minimum* appears to activate haemocytes, but the other harmful algal species tested seem to cause a suppression of immune functions, generally consisting of decreases in phagocytosis, production of ROS and cell

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adhesion and besides cause an increase in the percentage of dead haemocytes, which could be attributable to the action of chemical toxins. Microalgal cells exposed to shellfish haemolymph generally showed evidence of algal degradation, e.g. loss of chlorophyll fluorescence and modification of cell shape. Thus, in vitro tests allow a better understanding of the role of the haemocytes and the haemolymph in the defence mechanisms protecting molluscan shellfish from harmful algal cells and could also be further developed to estimate the effects of HABs on bivalve molluscs in vivo.

Keywords Bivalve · Haemocyte · Harmful algae · HAB · Immune response · Phagocytosis

Introduction

Internal defence in invertebrates, such as bivalve molluscs, is mainly based upon a non-adaptive, non-specific, innate immune system (Janeway 1994). More recently, Rowley and Powell (2007) demonstrated the possibility of a specific immune response of invertebrates, suggesting the potential existence of an acquired immune response. The innate immune system, however, remains the more recognized and understood among invertebrate groups although heterogeneous and complex (Loker et al. 2004). In molluscs, innate immune responses are mediated by haemocytes, the main defence cells, and humoural factors, such as antimicrobial peptides (Cheng 1996; Hine 1999; Bachère et al. 2004), lysozymes, lectins and the alternative complement pathway (Medzhitov and Janeway 2002). Indeed, as pathogens encounter the external protective barrier of the mollusc, the host recognizes their specific molecular pattern (pathogen-associated molecular patterns) by its pattern recognition proteins (Medzhitov and Janeway 2002) and initiates haemocyte-mediated responses such as phagocytosis and oxidative burst to accomplish complete elimination of invading pathogens.

Numerous publications have demonstrated the effects of pathogens, pesticides and other toxic pollutants on the immune system of bivalves (Chu and Lapeyre 1993a, b; Anderson et al. 1995; Lapeyre et al. 1995; Anderson 1996, 1999; Fisher et al. 1999, 2000; Oliver et al. 2001; Fournier et al. 2001; Sauvé et al. 2002; Hamoutene et al. 2004; Villalba et al. 2004; Gagnaire et al. 2006; Bado-Nilles et al. 2008; Morga et al. 2009; Hannam et al. 2010a, b), and among the environmental agents that may activate or modulate the immune system of bivalves are harmful or toxic microalgae (Hégaret and Wikfors 2005a, b; Hégaret et al. 2007a, b; da Silva et al. 2008; Galimany et al. 2008a, b; Haberkorn et al. 2010a). Harmful algal blooms occur routinely in locations where bivalve molluscs are present and can have profound effects, including mass mortalities, leading to both economic and ecological impacts (Shumway 1990; Burkholder 1998; Hoagland et al. 2002; Matsuyama and Shumway 2009). There are many different species of harmful algae which invoke various toxic or noxious mechanisms. These microalgae can be toxic to shellfish and also to human consumers of biotoxin-contaminated shellfish (reviewed in Shumway 1990; Landsberg 2002). As bivalve molluscs are filter feeders, these harmful algae can contact gill, digestive-epithelial and other tissues during ingestion, which allows for interaction with haemocytes found throughout the bivalve open circulatory system.

Harmful algal cells have been observed in tissues of bay scallops (Leibovitz et al. 1984; Wikfors and Smolowitz 1993). Therefore, it may be feasible for bivalves to identify algal cells as foreign invaders. The most likely effect of harmful algae upon the bivalve immune system is through release of biochemical toxins (i.e. saxitoxins, venerupin, gymnodimine, brevetoxins; reviewed by Shumway 1990; Landsberg 2002), haemolytic toxins (Jenkinson and Arzul 2000), other extracellular, organic compounds (Twiner et al. 2004, 2005; Gentien et al. 2007) or reactive oxygen species (Marshall et al. 2005a, b). Toxins or toxic compounds associated with HABs are species specific. Accordingly, assessing the direct interactions between haemocytes and individual species of harmful algae can provide insights into the responses of bivalves as they are exposed to harmful algal blooms.

Experiments reported here document in vitro interactions between haemocytes of (1) the northern quahog, or hard clam, *Mercenaria mercenaria*, the soft-shell clam *Mya arenaria* and the eastern oyster *Crassostrea virginica* interacting with three harmful algal species: *Alexandrium fundyense*, *Heterosigma akashiwo* and *Prorocentrum minimum*; (2) the Pacific oyster *Crassostrea gigas* and the harmful dinoflagellate *Alexandrium minutum* and (3) the Manila clam *Ruditapes philippinarum* and the harmful algae *Karenia selliformis* and *Karenia mikimotoi*. These species-specific

combinations were selected as they represent commercial shellfish species and dominant HAB species in the Northeastern United States and western France that can interact in the natural environment. These interactions were also selected based upon observations of feeding behaviour in various HAB–bivalve pairs that revealed differences (Hégaret et al. 2007b). An understanding of these in vitro interactions, i.e. in the absence of confounding physiological and environmental effects possible during in vivo exposures, can help to identify the specific roles of the haemocytes in defence mechanisms when living bivalves are exposed to HABs in the environment.

Materials and methods

Bivalve molluscs

Northern quahogs (hard clams, *M. mercenaria* Linnaeus—shell length (s.l.), 50–60 mm) and soft-shell clams (*M. arenaria* Linnaeus, s.l. 60–80 mm) were collected in Milford Harbor, CT, USA. Eastern oysters, *C. virginica* Gmelin (s.l. 50–60 mm), were received from Fisher's Island Oyster Farm, Fisher's Island, NY, USA. Bivalves were acclimated for at least 1 week and maintained in 18°C and 33 ppt with a continual flow of seawater in the Milford Laboratory prior to experiments. Manila clams, *R. philippinarum* Adams and Reeve (s.l. 35–45 cm), were collected the Morbihan Golfe in Brittany (NW France). Manila clams were acclimated for at least 1 week with a continual flow of seawater of 35 ppt at 16°C prior to experiments. Pacific oysters, *C. gigas* (Thunberg 1793; 60–70 mm), were collected from bay of Brest (Brittany, France) and acclimated for 1 week with continual flow of filtered (1 μm) sea water of 35 ppt at 16°C.

Algal cultures

The algal species to which quahogs, soft-shell clams and eastern oysters were exposed were obtained from the NOAA, Milford Laboratory (CT, USA) collection: *A. fundyense* Balech (strain BF2, isolated from the Gulf of Maine, USA), *P. minimum* (Pavillard) Schiller (strain JA-98-01, isolated from the Choptank River, MD, USA) and *H. akashiwo* (Hada) Hada ex Sournia (strain OL, isolated from NJ, USA). The RHODO strain of *Rhodomonas* sp. was used as a non-toxic, control alga. Cultures of *A. fundyense* were grown in F/2-enriched (Guillard and Ryther 1962; Guillard 1975) Milford seawater; *H. akashiwo* and *Rhodomonas* sp. were cultured in E-medium (Ukeles 1973) and *P. minimum* was grown in EDL7 medium, a modified version of the enriched seawater E-medium that contains L-1 trace metals, double the EDTA of the standard E formulation, KNO_3 rather than $NaNO_3$ and soil extract. The algae were maintained at 20°C on a 12:12-h light/dark cycle and used in log-phase.

The dinoflagellates *Karenia* (= *Gymnodinium*) *mikimotoi* (Miyake and Kominami ex Oda) Hansen et Moestrup (Stock GM95TIN, isolated in 1995 at Tinduff, Rade de Brest, France) and *K. selliformis* (= *Gymnodinium maguelonnense*, Strain GM94GAB, isolated from Gulf of Gabès, Tunisia) were obtained from the Dyneco Department of IFREMER (Brest, France) and grown in the IUEM laboratory in sterile, 6-L carboys. Medium used for these cultures was F/2-enriched, seawater from the Argenton hatchery, filtered to 1 μm and autoclaved. Cultures of *Karenia* spp. were maintained at 18–20°C on a 12:12-h light/dark cycle and used in log-phase.

Cultures of *A. minutum* Halim (strain AM89BM, isolated in 1989 in the bay of Morlaix, Brittany, France) and *Heterocapsa triquetra* (Ehrenberg) Stein (strain HT99PZ, isolated in 1999 in the bay of Morlaix, Brittany, France) were grown in 1-l batch culture using autoclaved seawater filtered to 1 μm and supplemented with L1 medium (Guillard and Hargraves 1993) and maintained at 16±1°C, with a dark/light cycle of 12:12 h. Cells of *A. minutum* and *H. triquetra* were harvested in exponential growth phase after 12 days of culture.

Algal cell densities were determined by haemocytometer counts under light microscope. For all experiments, algal densities in exposure tubes were adjusted to a concentration corresponding to a cell density ten times higher than a natural bloom to simulate the concentration of cells that occurs during filtration: 10^4 cells ml^{-1} for *A. fundyense* (Shumway et al. 1988; Townsend et al. 2005), 10^5 cells ml^{-1} for *H. akashiwo* (Rensel and Whyte 2004), 10^5 cells ml^{-1} for *P. minimum* (Hégaret and Wikfors 2005a), 5.10^4 cells ml^{-1} for *A. minutum* (REPHY; Haberkorn 2009) and 10^5 cells ml^{-1} for *Rhodomonas* sp. and 5.10^4 cells ml^{-1} for *H. triquetra* as controls. Concentrations of algae were adjusted by diluting the cells in their *spent* medium (filtered at 0.2 μm). Filtrate was obtained by centrifuging (200×*g*,

5 min, 16°C) the culture before filtering supernatant using syringe filters (0.2 μm diameter). In the case of *K. mikimotoi* and *K. selliformis*, the concentration of algal cells was 4×10^3 cells ml^{-1} for the first experiment and 7×10^3 cells ml^{-1} for the second experiment; these densities could not be higher as they corresponded to the highest cell counts achieved in the cultures of *K. selliformis*. For the first experiment, *K. mikimotoi* was diluted 2.2 times in F/2 medium, to reach the same concentration as *K. selliformis* for the haemocyte exposures.

For analyses involving the effect of the media on the haemocytes, the algal samples were filtered (0.2 μm filter), and the *spent* media (culture media after filtration/elimination of the algal cells) were collected for analyses. Algal sizes ranged from approximately 20–25 μm diameter for *A. fundyense*, *A. minutum*, *H. triquetra* and *P. minimum* to 15 μm for *H. akashiwo* and *Rhodomonas* sp., which also corresponds to the size of the haemocytes. The harmful algal species used in this study all have demonstrated toxicity to finfish or shellfish (Shumway and Cucci 1987; Erard-Le-Denn et al. 1990; Luckenbach et al. 1993; Wikfors and Smolowitz 1993, 1995; Arzul et al. 1995; Lush et al. 1996; Jenkinson and Arzul 2000; Guillou et al. 2002; Bricelj et al. 2005).

Experimental design

Bivalve haemocytes from five or six individual animals were exposed to the different species of harmful algae (Table 1) for 1, 2, 3 or 4 h (n=5–6). Results of the first set of experiments indicated that the effects of the harmful algal cells on haemocytes and vice versa occurred very rapidly, often before 4 h of incubation (data not shown), but that the results had reached a stable end point by 4 h. Thus, results after 4 h of interaction are presented here. Control analyses were also carried out on haemocytes in 0.2-μm filtered seawater (FSW) only.

As the different algal species were cultured in different seawater media, possible effects of the media on the haemocytes were also tested to ascertain whether the observed effect was attributable to the algae itself or the media in which it was grown. To accomplish this, haemocytes were exposed to each culture medium at a quantity equivalent to microalgal culture exposures.

Flow cytometric analyses

Haemocyte analyses

Haemolymph was withdrawn from the adductor muscle of bivalves using a 5- or 1-ml syringe, then screened through 80-μm mesh and stored temporarily in microcentrifuge tubes on ice before use. Haemocyte analyses were conducted on haemolymph collected from individual bivalves.

Haematoimmunological parameters measured were haemocyte characterization, in terms of size–FSC detector and internal complexity–SSC detector

Table 1 Harmful algae–bivalve haemocyte interactions tested

	Mercenaria mercenaria	*Mya arenaria*	*Crassostrea virginica*	*Crassostrea gigas*	*Ruditapes philippinarum*
Alexandrium fundyense	Culture, media	Culture	Culture		
Alexandrium minutum				Culture, media	
Heterocapsa triquetra				Culture, media	
Heterosigma akashiwo	Culture, media	Culture	Culture		
Prorocentrum minimum	Culture, media	Culture	Culture		
Rhodomonas	Culture	Culture	Culture		
Karenia selliformis					Culture, media
Karenia mikimotoi					Culture

Empty cells are the interactions that were not assessed

Culture the whole culture was tested, *media* the spent medium was tested

according to Hégaret et al. (2003a), as well as some of their immune functions:

(a) Haemocyte mortality, as percentage of dead haemocytes, using propidium iodide (Sigma, final concentration 20 μg/ml) according to Hégaret et al. (2003b)
(b) Phagocytosis of fluorescent microbeads (Fluoresbrite YG Microspheres, 2.00 μm, Polysciences) by haemocytes, as percentage of highly phagocytic (>2 beads) haemocytes according to Hégaret et al. (2003b)
(c) Haemocyte production of reactive oxygen species (ROS) with potential to kill non-self, engulfed particles was assessed using 2′,7′-dichlorofluorescein diacetate (Sigma) described in Buggé et al. (2007)
(d) Adhesion of the haemocytes was measured by assessing the proportion of haemocytes that detach from the surface of experimental chambers after incubation with potential toxins as previously described for clams by Choquet et al. (2003). The assay was conducted in 24-well plates

FACScalibur or FACScan (BD Biosciences, San Jose, CA, USA) flow cytometer was used for all haemocyte analyses.

Algal analyses

Characterization of microalgal cells (size–FSC detector, complexity–SSC detector and chlorophyll fluorescence–FL3 detector) was assessed by flow cytometry for each exposure. The percentage of dead algal cells was also assessed by flow cytometry using Sytox Green nucleic acid stain (Molecular Probes, S7020; Veldhuis et al. 1997), which selectively stains dead algal cells, with fluorescence detected by the cytometer FL1 detector.

To analyse interactions between haemocytes and harmful algae, microscopic observations, as well as flow cytometric analyses, were conducted for five to six replicates. The density of algae compare to haemocytes was from one to ten haemocytes per algal cell, according to the combination of species tested. The volume ratio was 1:3 (haemolymph/algal culture).

Statistical analyses

Results were analysed statistically with *t* tests, contrasting FSW controls with each treatment after 4 h of incubation, using Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA).

Results

Preliminary experiments confirmed that no algal species was phagocytic, engulfing neither beads nor haemocytes—activities that could have confounded interpretation of cytograms.

Effect of the media on haemocytes of the several bivalve species

The haemocyte variables of quahogs, soft-shell clams and eastern oysters were assessed with haemocytes incubated with FSW (control), F/2-enriched Milford seawater (*A. fundyense* medium) and E-medium (*Rhodomonas* sp., *H. akashiwo* and *P. minimum* media). The haemocyte parameters of Manila clams also were assessed with haemocytes incubated with FSW (control) and F/2 medium (*Karenia* spp. medium). These analyses confirmed that any effects of algal culture were attributable to the algae themselves and not to the media in which the cells were grown. Only one haemocyte variable was affected by the E-medium: The percentage of phagocytic haemocytes decreased when quahog haemocytes were incubated in E-medium. None of the other haemocyte parameters tested was affected by the presence of the media compared to haemocytes incubated in FSW (*t* test, $P>0.05$). Primary, flow cytometric values for parameters measured when haemocytes were exposed to FSW are presented in Table 2.

Quahog (= hard clam) haemocytes—whole cultures and cell-free (= *spent*) media—of *A. fundyense*, *H. akashiwo*, *P. minimum* and *Rhodomonas* sp. as control

Haemocytes exposed to FSW or to *Rhodomonas* sp. did not show any measurable differences after 4 h of incubation (Fig. 1); thus, effects of *spent* media from these algae were not assessed. Only the whole culture of the raphidophyte *H. akashiwo* caused mortality of haemocytes after 4 h (Fig. 1). Haemocyte morphology was affected only by *P. minimum*, which induced a slight decrease in size and complexity (Fig. 1) of the

Table 2 Control values for the several haemocyte parameters of the five bivalve species tested, after 4 h of incubation with FSW, mean and SE, $n=5$

	Percentage of dead haemocytes		Percentage of phagocytosis		Percentage of adhered cells	
	Mean	SE	Mean	SE	Mean	SE
Crassostrea gigas	5.48	2.86	8.19	1.07	35.80	12.47
Crassostrea virginica	1.63	0.38	19.56	3.10		
Mercenaria mercenaria	8.02	2.16	35.36	1.21	94.93	3.14
Mya arenaria	7.12	1.60	10.71	1.43	87.87	2.63
Ruditapes philippinarum	14.73	2.51	24.19	2.73	81.10	6.04

haemocytes. Adhesion of haemocytes was significantly inhibited by cultures of *A. fundyense* (Fig. 1). Similarly, haemocytes incubated in *H. akashiwo spent* medium were less able to adhere, whereas the *spent* medium of *P. minimum* enhanced the adhesion of haemocytes (Table 3). The whole culture and *spent* media of the dinoflagellates *A. fundyense* and *P. minimum* caused significant decreases in percentages

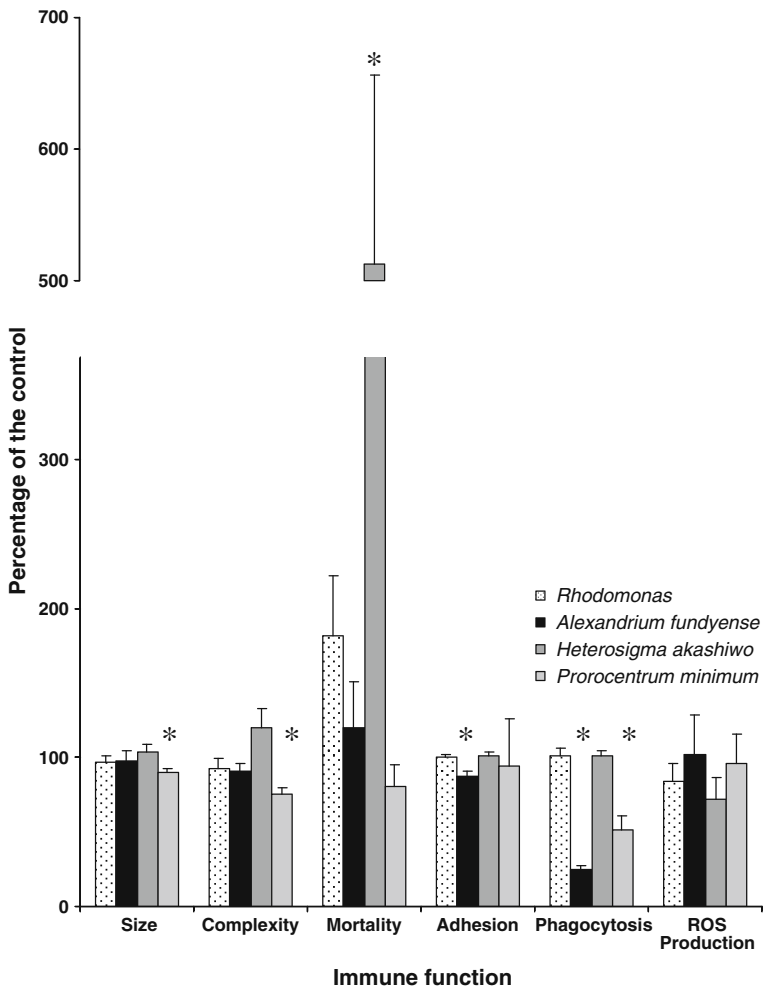

Fig. 1 Effects of in vitro exposure of whole culture of *Rhodomonas* sp., *A. fundyense*, *H. akashiwo* and *P. minimum* upon haemocyte parameters of Northern quahogs *M. mercenaria* (results are presented as a percentage of the FSW control (mean, ±SE), cf. Table 2 for control values; *asterisk* indicates a significant effect, *t* test, $P<0.05$)

Table 3 Effects of the cell-free (=*spent*) media of algal species on haemocyte variables of quahogs *M. mercenaria*

	Haemocyte parameters of *Mercenaria mercenaria* tested											
	Size		Complexity		Mortality		Phagocytosis		Production ROS		Adhesion	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
A. fundyense medium	91.7	10.7	97.0	7.8	81.3	42.3	80.8[a]	10.7	119.6	27.6	86.3	3.9
H. akashiwo medium	96.7	7.8	91.6	12.4	40.7	15.2	63.9[a]	14.0	97.3	13.2	31.6[a]	19.4
P. minimum medium	99.3	2.2	90.3	5.3	77.0	14.8	60.0[a]	3.8	139.0	31.4	114.1[a]	12.6

Results are presented as a percentage of the FSW control

[a] Indicates a significant effect, *t* test, $P<0.05$, $n=5$

of phagocytic haemocytes (Fig. 1; Table 3); however, the effect observed with *P. minimum* may be attributable to the E-medium and not to the algae themselves, as E-medium alone also inhibited phagocytosis. No significant effect of any harmful algal species tested on a loss of haemocyte counts within the tube during the time of exposure or on production of ROS (Table 3; Fig. 1) was found.

Total haemolymph from quahogs *M. mercenaria* had significant effects on the harmful algal species (Table 4). Quahog haemolymph affected the morphology of some algal cells species, e.g. the complexity of *A. fundyense* increased. Microscopic observations showed the presence of numerous temporary cysts of *A. fundyense* when the algal cells were incubated with quahog haemolymph (Fig. 2); the algal cells were losing cell wall plates and transforming into temporary cysts. The size of *H. akashiwo* increased, whereas its chlorophyll fluorescence and complexity decreased in the presence of quahog haemolymph, and *H. akashiwo* cells appeared to lose shape and chlorophyll fluorescence and to degrade very quickly. Chlorophyll fluorescence of the three algal species tested (*H. akashiwo*, *P. minimum* and *A. fundyense*) also decreased significantly in the presence of quahog haemolymph. Quahog haemo-

Table 4 Effect of the haemocytes of several Molluscan bivalve species on harmful algal species

Harmful algal species	Mollusc bivalve species	Algal parameters tested							
		Size		Complexity		Fluorescence		Mortality	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Alexandrium fundyense	*Crassostrea virginica*	108.5	3.3	115.1[a]	3.9	97.9[a]	0.7	335.2[a]	73.6
	Mercenaria mercenaria	106.9	6.4	115.1[a]	2.6	100.6	1.4	54.0	15.1
	Mya arenaria	111.4[a]	2.6	106.8[a]	1.8	99.7	1.9	260.7[a]	58.4
Heterosigma akashiwo	*Crassostrea virginica*	100.3	1.3	100.7	2.8	93.9[a]	2.1	309.8	146.0
	Mercenaria mercenaria	121.9[a]	6.6	81.8	11.1	88.2[a]	2.3	424.1[a]	76.1
	Mya arenaria	114.3[a]	4.4	86.8[a]	1.8	83.9[a]	3.9	5,799.8[a]	2,344.3
Prorocentrum minimum	*Crassostrea virginica*	102.8[a]	1.2	96.5	1.4	98.4[a]	0.7	64.4[a]	10.9
	Mercenaria mercenaria	109.7	6.7	116.5	6.9	89.9[a]	1.6	198.2[a]	26.5
	Mya arenaria	96.6	10.4	105.2	3.7	96.5	2.8	491.6[a]	104.4
Karenia mikimotoi	*Ruditapes philippinarum*	156.8[a]	4.1	174.4[a]	4.1	89.6[a]	2.0		
Karenia selliformis	*Ruditapes philippinarum*	171.5[a]	10.0	274.5[a]	30.3	97.5	8.9		

Results are presented as a percentage of the FSW control

[a] Indicates a significant effect, *t* test, $P<0.05$, $n=5–6$

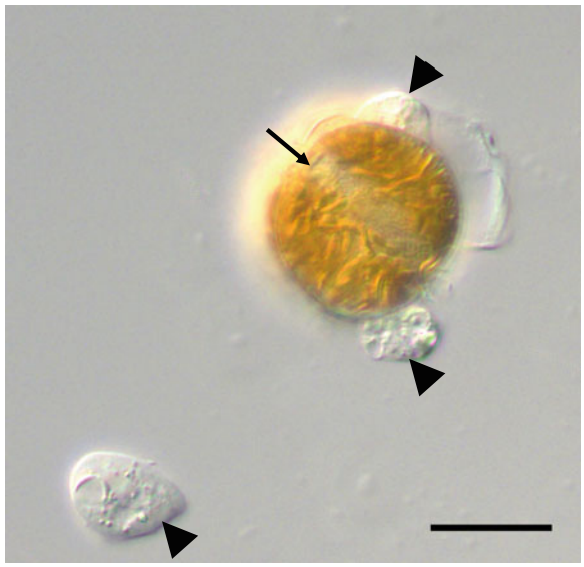

Fig. 2 Temporary cyst of *A. fundyense* (*arrow*) exposed to quahog haemocytes (*arrowhead*; *scale bar*=20 μm)

lymph also increased the percentage of dead algal cells in *P. minimum* and *H. akashiwo*; conversely, the percentage of dead *A. fundyense* cells decreased. Microscopic observations also indicated the presence of red (= chlorophyll) fluorescence in individual haemocytes incubated with *H. akashiwo* (Fig. 3) and the presence of aggregates of haemocytes surrounding *P. minimum* cells (Fig. 4).

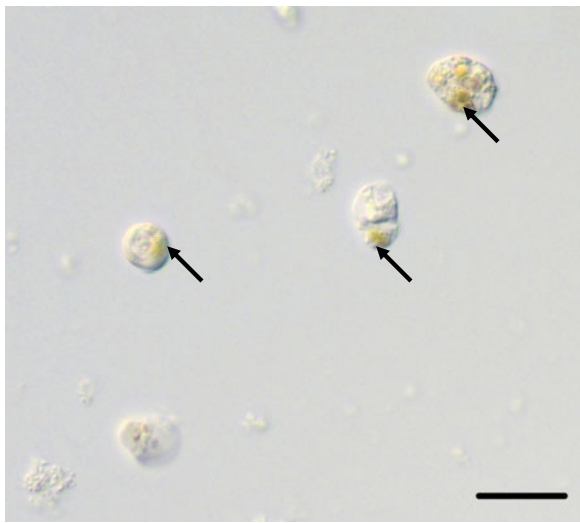

Fig. 3 Cells of *H. akashiwo* engulfed by haemocytes of quahogs *M. mercenaria*. *Arrow* indicates chlorophyll fluorescence inside haemocytes (*scale bar*=20 μm)

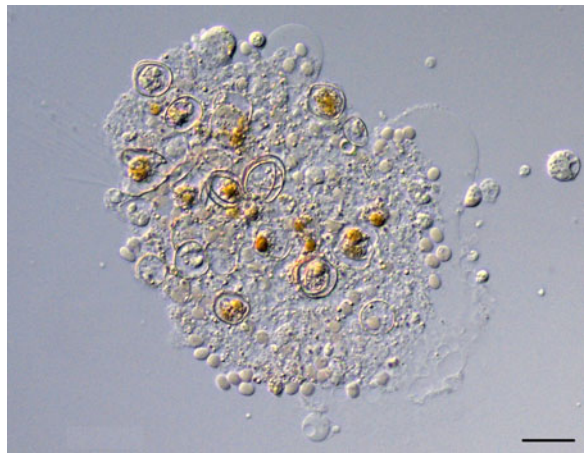

Fig. 4 Aggregates of quahog haemocytes surrounding *P. minimum* cells (*scale bar*=20 μm)

Soft-shell clam haemocytes—whole cultures of *A. fundyense*, *H. akashiwo* and *P. minimum* and *Rhodomonas* sp. as control

Haemocytes from soft-shell clams *M. arenaria* exposed to FSW or to *Rhodomonas* sp. did not show any measurable differences after 4 h of incubation, except for a significant increase in adhesion of haemocytes (Fig. 5). Haemocytes from soft-shell clam exposed to three different species of harmful algae showed some significant effects as well. The production of ROS by soft-shell clam haemocytes (Fig. 5) significantly increased in the presence of *H. akashiwo*. Conversely, *A. fundyense* and *P. minimum*, which did not affect the production of ROS by haemocytes, significantly decreased the percentage of phagocytic haemocytes (Fig. 5). The haemocyte adhesion (Fig. 5) was not affected by any of the harmful algae tested. The percentage of dead haemocytes after incubation with *A. fundyense* and *P. minimum* did not change either (Fig. 5). Flow cytometric studies did not allow assessment of the effect of the *H. akashiwo* upon haemocyte viability; algae incubated with the haemocytes lost chlorophyll fluorescence and shape; therefore, it was not possible to distinguish between the red fluorescence of chlorophyll and the red fluorescence of dead haemocytes (stained with propidium iodide). The observations of the cytograms plotting haemocytes and *H. akashiwo* indicated that the algal cells either lost complexity or fluorescence or that the haemocytes engulfed algal cells, thereby acquiring chlorophyll fluorescence.

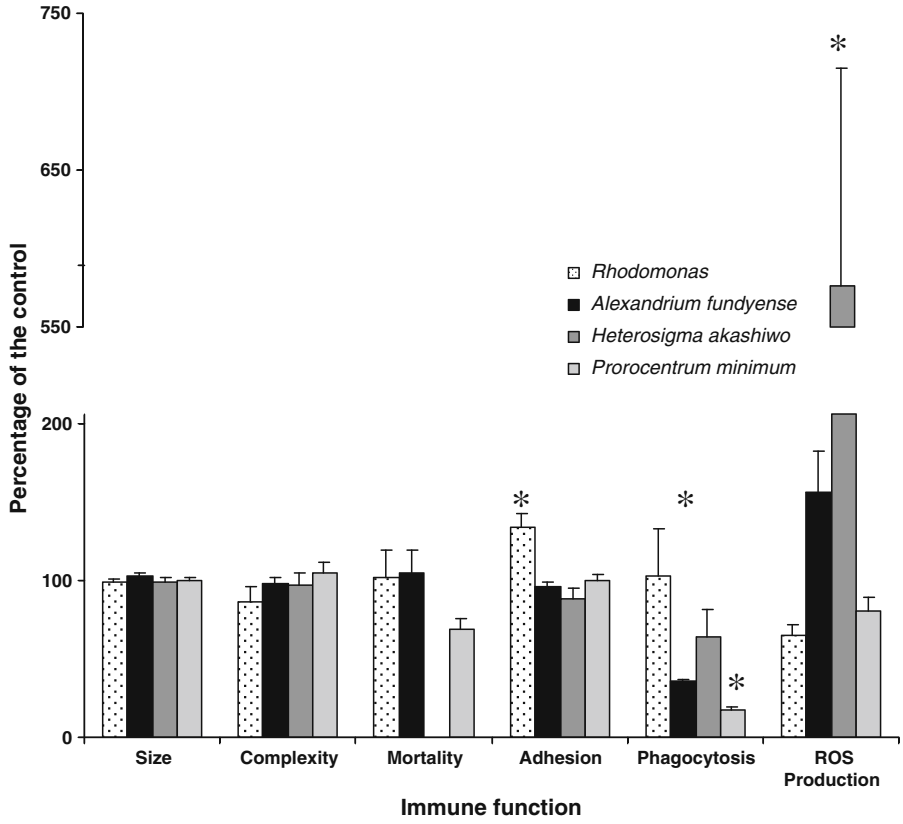

Fig. 5 Effects of in vitro exposure of whole culture of *Rhodomonas* sp., *A. fundyense*, *H. akashiwo* and *P. minimum* upon haemocyte parameters of soft-shell clams *M. arenaria* (results are presented as a percentage of the FSW control (mean, ±SE), cf. Table 2 for control values; *asterisk* indicates a significant effect, *t* test, $P<0.05$)

During this study, the effects of soft-shell clam haemolymph on algal cells were also assessed (Table 4). For the three algal species tested, the percentage of dead algal cells increased significantly in the presence of soft-shell clam haemolymph. Morphology and chlorophyll content in the dinoflagellate *P. minimum* were not affected by the presence of soft-shell clam haemolymph, but *A. fundyense* cells had significantly higher mean complexity and size in the presence of clam haemolymph. Finally, *H. akashiwo* was most affected by soft-shell clam haemolymph; counts of *H. akashiwo* cells in the tubes incubated with haemocytes strongly decreased whereas mortality increased, simultaneously, the size of the algal cells increased and the chlorophyll content and the complexity decreased significantly.

Oyster haemocytes—whole cultures of *A. fundyense*, *H. akashiwo* and *P. minimum* and *Rhodomonas* sp. as control

Results of experimental co-incubations of Eastern oyster *C. virginica* haemocytes and three microalgal species are presented Fig. 6. Two or three populations of haemocytes were detected with the flow cytometer: granulocytes and large and small hyalinocytes, which were not always distinguishable. Haemocytes exposed to *Rhodomonas* sp. did not show any significant changes from the FSW control. Results showed that *A. fundyense* and *H. akashiwo* caused an increase in the percentage of dead haemocytes (Fig. 6). Moreover, *A. fundyense* inhibited phagocytosis by oyster haemocytes (but not significantly; Fig. 6). *P. minimum* had no significant effects on oyster haemocytes.

Cells of *A. fundyense* in contact with oyster haemolymph tended to lose chlorophyll fluorescence, but percentages of dead algal cells and internal complexity both increased (Table 4). These observations are consistent with microscopic observations wherein we observed that *A. fundyense*, in the presence of haemolymph, was often in the form of temporary cysts and not vegetative cells (Fig. 2). In the presence of oyster haemolymph, the cells of *H. akashiwo* showed loss of chlorophyll fluorescence, but microscopic observations indicated that the algal cells were still motile. The dinoflagellate *P. minimum*

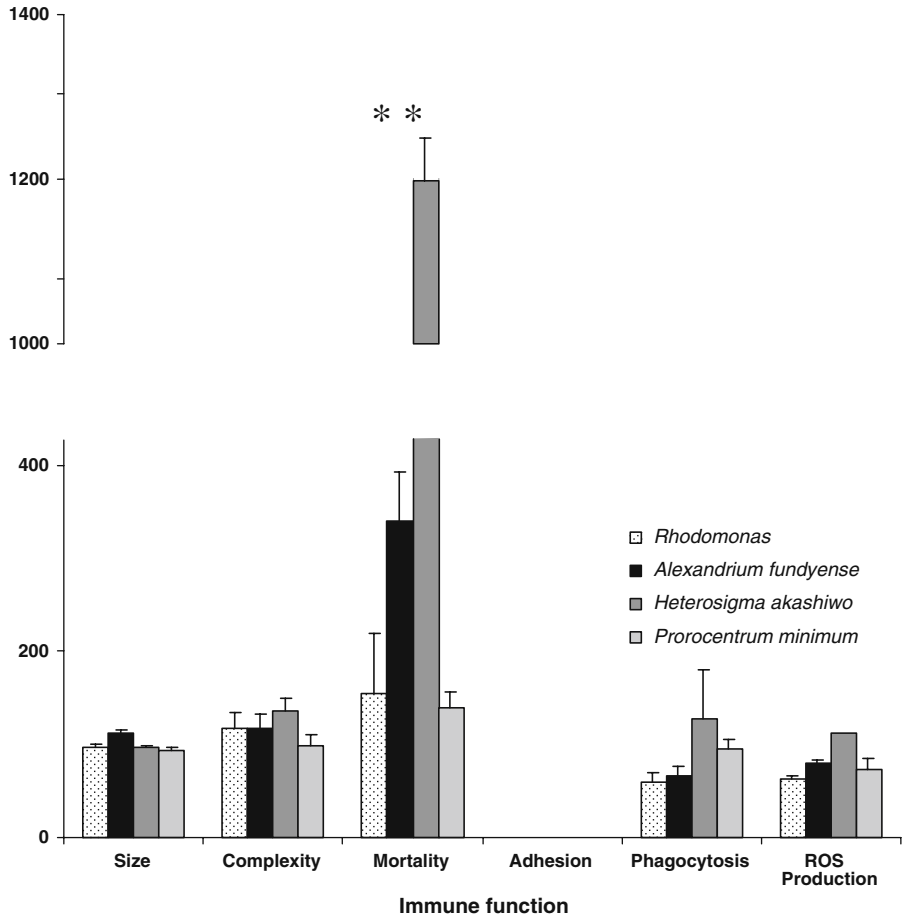

Fig. 6 Effects of in vitro exposure of whole culture of *Rhodomonas* sp., *A. fundyense*, *H. akashiwo* and *P. minimum* upon haemocyte parameters of Eastern oysters *C. virginica* (results are presented as a percentage of the FSW control (mean, ±SE), cf. Table 2 for control values; *asterisk* indicates a significant effect, *t* test, $P<0.05$)

lost chlorophyll fluorescence and increased in size when incubated in oyster haemolymph. The presence of haemolymph also caused a decrease in the number of *P. minimum* cells, as well as a decrease in the percentage of dead algal cells. Microscopic observations indicated that oyster haemocytes and *P. minimum* cells did not seem to have any major cell-to-cell interactions; the *P. minimum* cells remained motile.

Manila clam haemocytes—whole culture of *K. mikimotoi* and whole culture and cell-free (= *spent*) medium of *K. selliformis*

As *K. selliformis* had a much stronger effect on clam haemocytes than *K. mikimotoi*, the experiment only assessed the effect of *K. selliformis spent* medium on Manila clams *R. philippinarum* haemocytes. Results of experimental co-incubations of Manila clam haemocytes and the two *Karenia* species show a significant effect of *K. selliformis* on haemocyte morphology, with a decrease in complexity (Fig. 7). Although haemocyte size was not affected, haemocyte functions of Manila clams exposed to *K. selliformis* were affected; percentage of dead haemocytes increased significantly (Fig. 7) while the percentage of phagocytic haemocytes and production of ROS and the adhesion decreased (Fig. 7). The alga *K. mikimotoi* had a much smaller impact on clam haemocytes but still inhibited adhesion (Fig. 7) and suppressed production of ROS (Fig. 7). The effect of the *spent* medium of *K. selliformis* on clam haemocytes was not as pronounced as for the whole culture but still caused a significant inhibition of the production of ROS and increased the mortality of haemocytes (Fig. 7).

Cells of *Karenia* spp. incubated with haemolymph of Manila clams for 4 h reacted with a highly significant increase in size and complexity (Table 4). The chlorophyll fluorescence also tended to decrease in both *Karenia* species, but the trend was only significant for *K. mikimotoi* (Table 4).

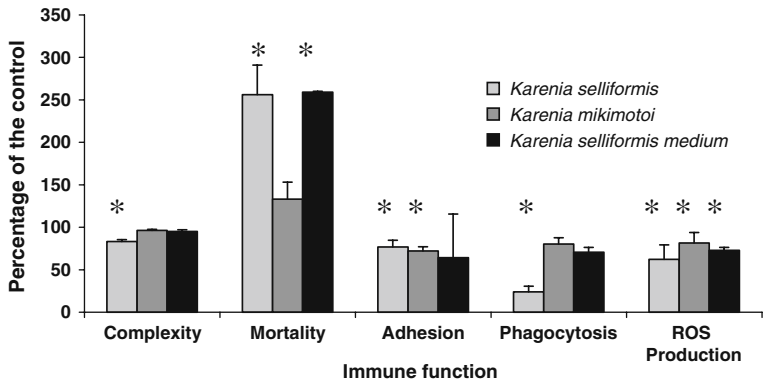

Fig. 7 Effects of in vitro exposure of whole culture of *K. selliformis* and *K. mikimotoi* and of the spent medium of *K. selliformis* upon haemocyte parameters of Manila clams *R. philippinarum* (results are presented as a percentage of the FSW control (mean, ±SE), cf. Table 2 for control values; *asterisk* indicates a significant effect, *t* test, $P<0.05$)

Pacific oyster haemocytes—whole culture and cell-free (= *spent*) medium of *A. minutum* and *H. triquetra*

Three populations of haemocytes were detected using flow cytometry: granulocytes, hyalinocytes and agranulocytes, but results presented here only show effects on the whole haemocyte population. Exposure for 4 h to *H. triquetra* or its *spent* medium did not significantly affect haemocyte parameters tested in comparison to the control (Fig. 8). Results show that *A. minutum* and its *spent* medium, however, caused an increase in the percentage of dead haemocytes and a decrease in their internal complexity (Fig. 8). Moreover, the whole culture of *A. minutum* significantly inhibited production of ROS by oyster haemocytes, whereas inhibition of the production of ROS by *A. minutum spent* medium was not significant (Fig. 8).

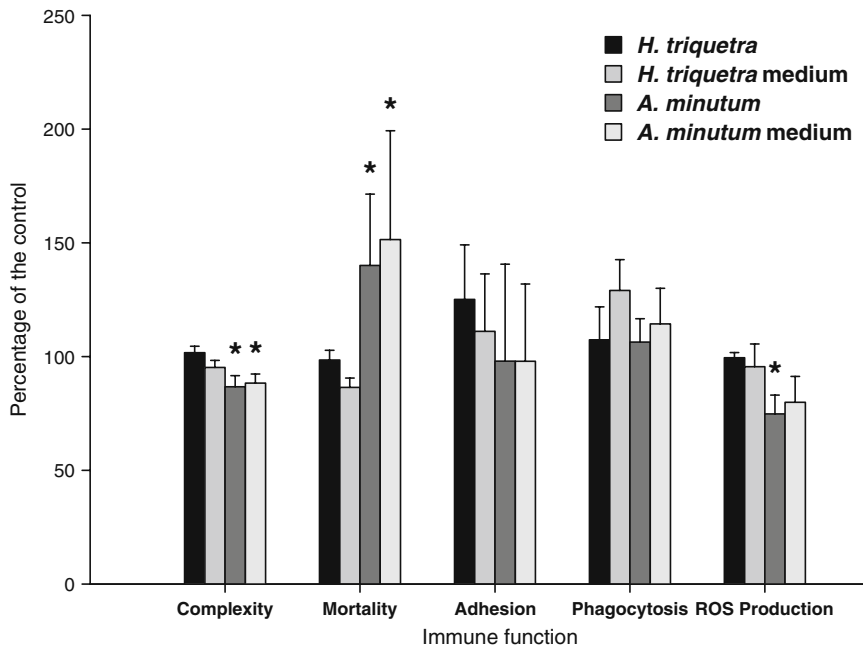

Fig. 8 Effects of in vitro exposure of whole cultures and *spent* media of *H. triquetra* and *A. minutum* upon haemocyte parameters of Pacific clams *C. gigas* (results are presented as a percentage of the FSW control (mean, ±SE), cf. Table 2 for control values; *asterisk* indicates a significant effect, *t* test, $P<0.05$)

Discussion

Results of the present study demonstrated that a non-harmful, microalgal species does not modulate the immune response of bivalve molluscs. In contrast, the harmful algal species investigated did cause changes in haemocytes, both morphological and functional, that can be expected to alter the physiological status and immunological responses of the shellfish. Our results also indicate that different harmful algal species may be affected differently when exposed to shellfish haemolymph.

Effect of harmful algal cells on bivalve haemocytes and their functions

Taken together, our results suggest two main response patterns when haemocytes are incubated with harmful algae cells. Some harmful algae can act as immunostimulants (e.g. *P. minimum*) whereas others can cause a suppression of immune functions. Haemocytes of soft-shell clams and quahogs suffered a decrease in percentage of highly phagocytic haemocytes in the presence of *P. minimum*, though this may be due to the culture medium itself in the case of quahogs. Quahog haemocyte morphology changed during incubation with *P. minimum*. Such modification might be related to algal cells engulfment, as suggested by the acquisition of red fluorescence. Quahog haemocytes exposed in vitro to *P. minimum* form aggregates surrounding algal cells, confirming previous observations of Hégaret et al. (2008a), probably to isolate *P. minimum* cells from the host tissues. Similarly, in vivo exposures of quahogs, Manila clams, mussels and bay scallops exposed to *P. minimum* showed depression of phagocytosis, induced aggregation of haemocytes surrounding the harmful algae and a large inflammatory response characterised by a massive infiltration of haemocytes into the intestine (Wikfors and Smolowitz 1993; Hégaret and Wikfors 2005a; Galimany et al. 2008a; Hégaret et al. 2009, 2010). Present in vitro results support the hypothesis that this massive migration of haemocytes through the intestinal epithelium occurred to surround and isolate (via encapsulation) the cells of *P. minimum*. The active migration and activation of haemocytes might be induced and regulated by soluble molecules, released either by algal cells or by intestinal affected cells. Grzebyk et al. (1997) reported the presence of a water-soluble toxin in *P. minimum* cultures, and Wikfors (2005) suggested the existence of some still uncharacterized toxins. Such molecules might then act as activating chemical agents.

Contrastingly with clams, *P. minimum* cells did not induce any immunological effect over oyster haemocytes. Indeed, neither inhibition of phagocytosis nor formation of aggregates could be detected, as previously reported in vivo (Hégaret and Wikfors 2005a, b). Conversely, Wikfors and Smolowitz (1995) suggested that *P. minimum* cells interfere with cellular digestive processes. Indeed, eastern oysters, *C. virginica*, showed accumulation bodies within absorptive cells of the digestive tubules, indicating that *P. minimum* cells interfere with cellular digestive processes. Similarly, Pacific oysters, *C. gigas*, exposed in vivo to *Prorocentrum rhathymum* also had a reduction of the gut tubule epithelium, and some affected oysters displayed thinned digestive tubules containing sloughed cells (Pearce et al. 2005).

The in vitro exposure of Manila clam haemocytes to *K. selliformis* caused a decrease in haemocyte internal complexity. This loss of internal complexity could indicate a possible degranulation of haemocytes, releasing immunoactive enzymes into the haemolymph (Cheng 1996), or be a consequence of algal cytotoxicity. Indeed, adhesion, phagocytosis and production of ROS of Manila clam haemocytes were depressed, while the percentage of dead cells increased. Similarly, production of ROS and adhesion were altered in Manila clams exposed to both *Karenia* species. The *spent* medium of *K. selliformis* itself induced immune suppression, although in a lesser extent than algal cells. This indicates that the effect of *K. selliformis* is mainly attributable to the cells themselves, either through direct, physical contact with the cells (Uchida et al. 1999) or more likely from a fast-degrading toxin released by the cells (Gentien et al. 2007). *Karenia* sp. cells are known to produce allelopathic and ichtyotoxic compounds (Gentien and Arzul 1990) but also haemolysins, which in vitro induce red blood cells lysis (Arzul et al. 1995; Fossat et al. 1999; Sola et al. 1999; Jenkinson and Arzul 2000) and fast-degrading toxins responsible for autotoxicity in *K. mikimotoi* cultures (Gentien et al. 2007). Cells of *K. selliformis* also produce a well-characterised toxin referred to as gymnodimine (Seki et al. 1995, 1996; Mackenzie et al. 1996), which

caused death of oyster larvae exposed to whole cultures, culture filtrates or sonicated cell extracts of *Karenia* sp. after 7 to 24 h of exposure (Mackenzie et al. 1996). Gymnodimine, however, showed no haemolytic activity or cytotoxicity to mouse blood cells (Seki et al. 1996), and its mechanism of toxicity remains unclear. It is then unclear whether the observed effects on haemocytes could be attributable to gymnodimine, *Karenia* sp. cells or both. These in vitro exposures also revealed an intermediate reaction of haemocytes to *K. mikimotoi*, compared to *K. selliformis*, which was previously observed in vivo (Hégaret et al. 2007a). Jenkinson and Arzul (2000) also showed intermediate haemolytic properties in *K. mikimotoi*, compared to *K. selliformis*, in exposures of horse red blood cells.

The whole culture of the raphidophyte *H. akashiwo* caused a very large increase in the percentage of dead haemocytes from both quahogs and oysters, which was not significant with the *spent* medium indicating that the presence of the algal cells appeared necessary for cytotoxicity. Wang et al. (2006) showed an increase in mortality of scallop larvae exposed to *H. akashiwo* and suggested that its glycocalyx structures could be responsible for its toxicity. These glycocalyx structures were also reported to strongly inhibit swimming activity of brine shrimps, *Artemia salina* (Yan et al. 2004). Both entire culture and *spent* medium of *H. akashiwo*, however, triggered a decrease of phagocytosis from quahogs, which could be explained by the release of some chemical compounds by *H. akashiwo*, which may gradually accumulate and affect the ability of quahog haemocytes to adhere and phagocyte. Another hypothesis for reduced phagocytosis of fluorescent microbeads may be that haemocytes have been occupied engulfing harmful algal cells, as suggested by the chlorophyll fluorescence observed inside haemocytes. Moreover, *H. akashiwo* cells produce ROS (Marshall et al. 2005a) and secrete organic compounds (Twiner et al. 2004, 2005) that affect the metabolic activity of mammalian cells within a few hours and might then similarly affect bivalve haemocytes.

In vitro incubation of the dinoflagellates *A. fundyense* and *A. minutum* with bivalve haemocytes tends to induce immunosuppression. Phagocytosis of quahogs and soft-shell clams, as well as adhesion of quahog haemocytes, were depressed. Haemocytes of the Pacific oyster displayed a decreased internal complexity and an inhibited production of ROS. Finally, both *Alexandrium* species increased haemocyte mortality in both oyster species, *C. virginica* and *C. gigas*. *Alexandrium* spp. are known to produce paralytic shellfish toxins (PST), responsible for potentially lethal toxicity in mammals. Ford et al. (2008), however, reported a decrease of adhesion and phagocytosis of Manila clam and soft-shell clam haemocytes exposed in vitro to a non-PST producing *Alexandrium tamarense* strain, whereas another PST producing strain did not have any significant effect on these immune functions. Pacific oysters, *C. gigas*, exposed in vivo to *Alexandrium catenella* had an increase in percentage of dead haemocytes, which was not the case neither for *C. virginica* nor for *C. gigas*, respectively, exposed in vivo to *A. fundyense* and *A. minutum* (Hégaret et al. 2007b; Haberkorn et al. 2010b). Arzul et al. (1999) showed haemolytic, allelopathic and toxic activities of *Alexandrium* sp. over other algae and suggested the presence of PST-independent, chemical substances responsible for these effects. Moreover, a recent study reported that *Alexandrium leei* can secrete soluble polar ichthyotoxin(s), independent from PST which can cause lesions in and death of fishes (Tang et al. 2007). The production and release of chemical substances might then be responsible for the in vitro effects of *Alexandrium* sp. on bivalve haemocytes. Contrastingly, in vivo exposure of *C. virginica* and *C. gigas* to *A. fundyense* and *A. minutum*, respectively, did not induce immunosuppression nor toxicity (Hégaret et al. 2007b; Haberkorn et al. 2010b). Temporary cysts of *A. fundyense* and *A. minutum* have been observed in the stomach, digestive gland and biodeposits of bivalves (Shumway et al. 2006; Galimany et al. 2008b; Hégaret et al. 2008b; Haberkorn et al. 2010b), indicating that the algal cells may transform into cysts as they pass through the digestive system. The release of toxic substances might then be reduced, causing less effect on bivalve tissues and haemocytes during in vivo exposures compared with in vitro experiments.

Effects of bivalve haemolymph on algae

The cellular morphology (size and complexity) of each algal species measured with flow cytometry changed when incubated with bivalve haemolymph. The most noticeable morphological modification

occurred with haemolymph of quahogs and oysters incubated with *A. fundyense*. Indeed, microscopic observations revealed the presence of temporary cysts which were not observed during incubation of algal cells with FSW. Temporary cysts are non-motile cells, surrounded by a pellicle and are produced by vegetative cells shedding their theca (ecdysis) in response to short-term or sudden adverse conditions. Thus, the transformation of *A. fundyense* cells into temporary cysts can be explained by a protective response to haemocytes. Similar in vitro observations of temporary cyst formation were made previously by Hégaret et al. (2008a), and temporary cysts of *A. fundyense* have been observed in vivo in the stomachs and biodeposits of bivalve molluscs (Persson et al. 2006; Hégaret et al. 2007c, 2008b; Galimany et al. 2008b).

In the majority of the HAB–bivalve pairs investigated in the present study, harmful algal cells lost chlorophyll fluorescence over time as they were incubated with haemolymph. The trend is particularly extreme with *H. akashiwo*, for which cells incubated with haemolymph of soft-shell clams and, to a certain extent, quahogs were degraded very quickly. Disappearance of degraded cells may be attributable to phagocytosis by the haemocytes, which would explain the decrease in concentration of *H. akashiwo* cells when incubated with haemolymph. These results would also confirm the observations of Hégaret et al. (2008a), who observed haemocytes with red fluorescence, probably of chlorophyll from previously engulfed *H. akashiwo* cells. Conversely, when exposed to oyster haemolymph, the degradation of the *H. akashiwo* cells did not seem as active. This is consistent with the partial shell closure and reduced filtration of oysters exposed to *H. akashiwo* (Hégaret et al. 2007c), which would limit contact with haemocytes.

Can an in vitro experiment be a good proxy for in vivo exposures?

Very few publications presenting results from in vivo exposure of bivalve molluscs to harmful algae can be found in the literature, which limits the possible comparisons between the same bivalve–HAB pairs in vitro and in vivo. It is, therefore, not always easy to assess whether or not in vitro experiments can be good proxies for in vivo exposures.

In the present study, an incubation time of 4 h was sufficient to detect the responses of haemocytes and harmful algal cells to co-incubation, confirming previous observations of in vitro interactions between bivalve haemocytes with harmful algal cells (Hégaret et al. 2008a) and chemical pollutants (Anderson et al. 1997; Gomez-Mendikute et al. 2002, Sauvé et al. 2002; Gagnaire et al. 2003, 2004, 2006).

The effects of *P. minimum* were previously tested in various in vivo bivalve–HAB interactions with eastern oysters *C. virginica* (Hégaret and Wikfors 2005a, b), bay scallops *Argopecten irradians* (Hégaret and Wikfors 2005a), blue mussels *Mytilus edulis* (Galimany et al. 2008a), quahogs *M. mercenaria* (Hégaret et al. 2010) and Manila clams *R. philippinarum* (Hégaret et al. 2009). Our results indicated that in vitro interactions between haemocytes and *P. minimum* are good *proxies* for in vivo experiments and show consistently different haemocyte responses, according to the bivalve tested, showing no measurable effects on fundamental haemocyte functions in oysters, a decrease in phagocytosis associated to the formation of large haemocyte aggregates surrounding the algal cells in quahogs, Manila clams and blue mussels (Galimany et al. 2008a; Hégaret et al. 2009, 2010). In addition, previous studies (Hégaret and Wikfors 2005a, b) also demonstrated that laboratory, in vivo experiments exposing bivalves to cultures of *P. minimum* were good *proxies* for field HAB exposures. Thus, an in vitro exposure between *P. minimum* and haemocytes of a bivalve might be used as a *proxy* for in vivo, natural and artificial exposures.

Exposure of Manila clam haemocytes in vitro to *K. mikimotoi* and *K. selliformis* affected haemocyte functions, reducing adhesion, phagocytosis and production of ROS, while increasing the percentage of dead haemocytes. Additionally, exposure to *K. selliformis* in vitro caused a decrease in complexity of Manila clam haemocytes, which confirms the in vivo exposure to both *Karenia* species (Hégaret et al. 2007b; da Silva et al. 2008). Flattened haemocytes were also observed in *A. irradians* exposed *in vivo* to *K. mikimotoi*, which tends to confirm present results (Smolowitz and Shumway 1997). Thus, in vitro exposure to *Karenia* spp. could also be used as a *proxy* for in vivo exposures. However, in vivo experimental exposures of clams to *Karenia* spp. resulted in fewer dead haemocytes (Hégaret et al. 2007b). Harmful algal cell densities used in vivo

were lower than for in vitro exposures, by a factor of 4 to 8, which may explain the difference in responses between in vitro and in vivo exposures. The toxins produced by *Karenia* spp. might also desintegrate before reaching the haemocytes in vivo, consistent with the hypothesis of a fast-degrading toxin (Gentien et al. 2007).

The percentage of dead haemocytes in *C. gigas* exposed in vitro to *A. minutum* increased whereas the percentage of dead haemocytes during in vivo exposure did not (Haberkorn et al. 2010a, b). Moreover, in vivo exposure of *C. gigas* to *A. minutum* resulted in immunostimulation, characterised by inflammatory response (Haberkorn et al. 2010a), whereas in vitro exposure did not show significant effects of *A. minutum*, besides a higher mortality and a decrease of the production of ROS. Such an inhibition of the production of ROS was similarly measured in *C. gigas* exposed in vivo to *A. minutum* in May, but the contrary was observed a month before on the same oyster population (Haberkorn et al. 2010b). These contradictory results underline the difficulties to extrapolate in vitro effects to in vivo exposure of *Alexandrium* sp. on oysters.

The dinoflagellates *A. fundyense* (this study; Hégaret et al. 2008a) and *A. tamarense* (Ford et al. 2008) in vitro also caused depressed phagocytosis and adhesion in clams (quahogs and soft-shell clams). Unfortunately, no in vivo exposures have been conducted with any of the clam species tested in vitro. Similarly, haemocytes of blue mussels exposed in vivo to *A. fundyense* were morphologically different, with lower size and complexity than the control, but no major changes of the haemocyte functions could be observed (Galimany et al. 2008b), but no data of in vitro exposure of haemocytes of mussels to *Alexandrium* sp. are available. Thus, further experiments are needed to ascertain whether or not in vitro experiments involving *Alexandrium* sp. can be good *proxies* for in vivo responses.

Conclusion

This study analysed the effects in vitro of harmful algal cells on haemocytes of several bivalve molluscs. Despite differences between the several harmful algae tested, results show that haemocytes, the immune defence cells in bivalves, respond to HABs in a somewhat species-specific manner, but HAB species often cause a consistent profile of immunomodulation in most bivalve species. HAB exposure generally is associated with increases in the percentages of dead haemocytes and decreases in haemocyte phagocytosis, production of ROS and adhesion (immunosuppression). In most cases, the production and secretion of chemical substances by algal cells is probably responsible for such effects. Contrastingly, some harmful algal species (e.g. *P. minimum*) can act as immunostimulants, activating a protective cellular immune response in bivalves.

This study also indicates that haemolymph and haemocytes can have measurable effects on algal cells, including changes in shape, chlorophyll fluorescence and mortality. Yet, further investigations will be needed to fully understand the cellular and molecular mechanisms of toxicity of harmful algae to bivalves and their effects on haemocyte responses.

Finally, our results compared to data from the literature suggested that in vitro analyses "have the potential to serve" as *proxies* for in vivo analyses, although further studies should be developed to carefully assess the correspondence between in vitro and in vivo interactions for some bivalve/algae pairs.

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