Cytotoxicity of quahog parasite unknown (QPX) toward hard clam (*Mercenaria mercenaria*) haemocytes and interactions between different pathogen isolates and host strains

MICKAEL PERRIGAULT *and* BASSEM ALLAM*

School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook. NY 11794-5000, USA

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SUMMARY

The ability of pathogens to neutralize host defence mechanisms represents a fundamental requisite in the successful establishment of an infection. Host-pathogen interactions between quahog parasite unknown (QPX) and its hard clam host are poorly understood. Our prior *in vivo* investigations have shown that different QPX isolates display varying levels of pathogenicity toward clams. Similarly, field investigations and laboratory transmission studies revealed some variations in the susceptibility of different hard clam stocks to QPX infection. An *in vitro* approach was developed in this study to evaluate the toxicity of QPX cells and extracellular products toward haemocytes using a neutral red uptake assay. Results demonstrated that QPX produces virulence factors that are cytotoxic to *M. mercenaria* haemocytes. This cytotoxicity appears to be induced by clam factors, suggesting that it may play an important role in supporting QPX infection and proliferation within the host. Moreover, application of this technique to different QPX isolates and clam broodstocks indicates variations of QPX cytotoxicity in agreement with previous *in vivo* experiments, strengthening the existence of different QPX strains.

Key words: *Mercenaria mercenaria*, hard clam, haemocyte, QPX, thraustochytrid, neutral red uptake, extracellular products, virulence factors, cytotoxicity.

INTRODUCTION

Quahog parasite unknown (QPX) is a parasite member of the phylum Labyrinthulomycota (Maas *et al*. 1999; Ragan *et al*. 2000; Stokes *et al*. 2002). Despite the ubiquitous nature of this phylum in an aquatic environment, Labyrinthulomycota have been poorly studied (Raghukumar, 2002) and only few pathogens have been described in this group (Bower, 1987; Muehlstein *et al*. 1988). QPX has been associated with mass mortalities of cultured and wild *Mercenaria mercenaria* along the Northeastern coasts of the United States (Calvo *et al*. 1998; Smolowitz *et al*. 1998; Ford *et al*. 2002; Dove *et al*. 2004) and Maritime Canada (Whyte *et al*. 1994; Ragan *et al*. 2000).

Despite the economic importance of *M. mercenaria*, little is known about the interactions between hard clam and QPX at cellular and molecular levels, as most information has been gained from histological observations of infected clam tissues. These observations have clearly shown that QPX infections cause a significant inflammatory response in clams, sometimes leading to efficient neutralization of parasite cells and healing (Calvo *et al*. 1998; Smolowitz *et al*. 1998; personal observations). Histological observations also suggest that QPX displays virulence activities that facilitate the colonization of clam tissues. For instance, QPX produces mucus, forming lucent areas around parasite cells in clam tissues. It has been hypothesized that this mucus plays a role in virulence, acting as a physical barrier to both phagocytosis, as well as diffusion of clam defence molecules (Smolowitz *et al*. 1998; Anderson *et al*. 2003). Lucent areas around parasite cells could also result from tissue degradation by proteases produced and released by QPX (Anderson *et al*. 2006). As a matter of fact, it is not uncommon to identify necrotic haemocytes near active QPX foci (Allam, unpublished observations).

The balance between parasite virulence and host resistance factors is probably linked to the fact that QPX is present at extremely low prevalence in some geographical areas (below 0·1%), while it causes significant mortalities in other locations (Smolowitz *et al*. 1998; Allam, unpublished observations). This led Ford (2001) to designate QPX as an opportunistic pathogen that only infects clams under unfavourable conditions. Interestingly, host genetic makeup has been clearly shown to be associated with clam susceptibility, as both field (Ford *et al*. 2002; Calvo *et al*.

* Corresponding author: Tel: +1 631 632 8745. Fax: +1 631 632 3072. E-mail: Bassem.Allam@stonybrook.edu

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2007) and laboratory (Dahl *et al*. 2008) experiments have demonstrated higher susceptibility toward QPX of southern clam strains (originating from South Carolina and Florida) as compared to northern strains (Massachusetts, New York and New Jersey).

Our prior investigations also showed that QPX virulence *in vivo* was specific to the parasite isolate (Dahl *et al*. 2008). In that study, 4 clam strains were experimentally challenged with 2 QPX isolates originating from New York (NY) and 1 parasite isolate from Massachusetts (MA). Results showed that the NY isolates of the parasite were more virulent than the MA isolate when injected into clams originating from Florida, New York and Massachusetts. In clams originating from Virginia, however, the MA isolate of QPX caused significantly higher disease prevalence and mortality. This was one of a few studies dealing with bivalve diseases that combined investigations on resistances of different host strains toward different parasite isolates. Bushek and Allen (1996*a*) previously reported variations of both virulence of *Perkinsus marinus* strains and resistance of geographically distinct populations of the host, *Crassostrea virginica*. In *M. mercenaria*, our findings raise questions about the biological bases of QPX virulence and clam resistance.

Because histological observations of infected clam tissues suggest a role for haemocytes during pathogenesis (see above), we initiated research investigating the interactions between haemocytes and QPX on cellular levels. In this study, we developed a technique, using the neutral red (NR) uptake assay, to evaluate the cytotoxicity of QPX cells and extracellular products (ECP) toward haemocytes. This assay is based on the lysosome's ability to retain weakly cationic supravital dye (NR). The dye readily diffuses through the plasma membrane and concentrates in the lysosomes of undamaged cells. Alterations of the cell surface or lysosomal membrane under the effect of cytotoxic compounds (including contaminants and pathogen-derived compounds) lead to gradual leakage of NR from cells to the surrounding medium (Borenfreund and Puerner, 1985*b*). This is the first study to report the cytotoxicity of QPX products to clam haemocytes. Additionally, we compared the cytotoxicity of ECP obtained from different QPX isolates against haemocytes collected from 2 clam strains, and discussed results in light of prior information obtained during *in vivo* pathogenicity studies.

MATERIALS AND METHODS

QPX cultures

Three QPX isolates were used in this study. Two isolates, NY0314220AC5 (NY1-QPX) and NY0313808BC7 (NY2-QPX) were established from 2 infected clams collected from 2 field sites in New York (Qian *et al*. 2007). QPX isolation and culturing were performed in Minimal Essential Medium Eagle (MEM, Sigma M06440) as described by Kleinschuster *et al*. (1998). The third isolate (MA-QPX), originally isolated from Massachusetts hard clams in 1997 (Kleinschuster *et al*. 1998), was purchased from ATCC (Number 50749) as a cryopreserved sample, thawed and maintained in culture the same way as the new isolates. QPX isolate cultures were propagated in MEM, or in clam tissue homogenates (1000 $\mu g.ml^{-1}$ protein of muscle homogenates) according to the method of Perrigault *et al*. (2009). Subculturing of each QPX isolate in both culture media was performed weekly for at least 6 months before use in the cytotoxicity experiments. Under these conditions, cultures in the exponential phase of growth are typically obtained after 1 week at 23 °C. QPX growth in cultures was monitored by measuring parasite biomass as described by Buggé and Allam (2005). Briefly, 100 μl of culture aliquots were plated in a black 96-well microplate and 50 μM fluorescein diacetate (FDA) were added before fluorescence was measured at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader).

Haemolymph sampling

Adult hard clams (45–55 mm in length) from New York (NY) and Massachusetts (MA) were obtained from commercial sources and transferred overnight to our laboratory in refrigerated containers. All clams belonged to the same year class (~2·5 to 3 years old) and were descendants from broodstocks cultured by these companies in each site for several generations. They were maintained in tanks equipped with a closed recirculating seawater system (20 °C, 28 ppt) and fed daily with live algae (DT Plankton, IL, USA). Clams were acclimated for 2–4 weeks before haemolymph collection. Haemolymph was withdrawn from the adductor muscle with a 1 ml plastic syringe equipped with a 25-gauge needle. According to each assay, haemolymph samples were used individually or pooled in a 15-ml tube held on ice. The number of haemocytes per ml of haemolymph was microscopically determined using a Neubauer chamber.

Setup of the neutral red uptake assay

The neutral red uptake assay was adapted from Borenfreund and Puerner (1985*a*). A neutral red working solution (250 $\mu g \cdot ml^{-1}$) was freshly prepared for each assay by diluting a stock solution of neutral red (Sigma N4638 at 20 $mg \cdot ml^{-1}$ in dimethyl sulfoxide) in filtered sterile seawater (FSSW). Haemolymph from NY hard clams was plated in flat bottom 96-well plates, in 4 replicates, at concentrations ranging from $3{\cdot}5 \times 10^3$ to 7×10^4 haemocytes

per well. Haemolymph samples were serially diluted with FSSW and control wells were made by substituting haemolymph with FSSW. Plates were incubated for 1 h at room temperature (RT) to allow haemocyte adhesion to the plates. The supernatant was carefully removed and cells were washed with 200 μl of FSSW. Plates were then incubated at RT with 100 μl of test media (FSSW for controls and setup experiments, QPX cells and ECP for cytotoxicity experiments, see below). After 1 h, liquids were carefully removed and haemocytes were washed with 200 μl of FSSW. Cells were then incubated 1 h at RT with 100 μl of neutral red work solution and washed twice with FSSW. Neutral red in viable cells was eluted into 100 μl of solvent consisting of glacial acetic acid:ethanol:water (1 : 50 : 49 by volume). Plates were sealed, agitated for 10 min and absorbance was recorded at 560 nm. Haemocyte viability and morphology were monitored under phase-contrast microscopy before and after cell staining with neutral red.

Effect of QPX cells and supernatants

Exponentially growing cultures of QPX (isolate NY0313808BC7) in MEM or tissue homogenates were diluted with their respective media at 1×10^5 cell·ml^{-1} and centrifuged at 1000 ***g*** for 20 min. Supernatants (henceforth called extracellular products or ECP) were collected and filtered through 0·22 μm syringe filters. Pellets made of QPX cells were washed, centrifuged at 1000 ***g*** for 10 min and resuspended in the appropriate culture medium (volume equal to initial culture volume at 1×10^5 cell·ml^{-1}). Haemolymph from 6 NY clams was pooled and plated at a final density of 5×10^4 haemocytes per well. After 1 h at RT, plasma was discarded and haemocyte preparations were challenged with 100 μl of QPX ECP or resuspended parasite cells. Both undiluted or diluted (1/5 and 1/10 dilution by volume in the appropriate medium) QPX cells or ECP were added to each well, resulting in 1 : 5 (undiluted), 1 : 25 or 1 : 50 QPX (cells or cell equivalent for ECP) : haemocyte ratios. Sterile MEM and tissue homogenate were added to control wells (empty wells and wells containing the haemocyte layer). All preparations were made in triplicate. Neutral red uptake assay was performed as described above and absorbance was recorded after 1 and 4 h of interaction between haemocytes and QPX cells or ECP. Data are presented as percentage of optical density of test media (QPX suspension or ECP) to control preparations made with culture media (MEM or tissue homogenates).

Comparison of QPX isolates and clam populations

Cytotoxicity of ECP from 3 QPX isolates (NY1-QPX, NY2-QPX and MA-QPX) on haemocytes collected from clams originating from NY and MA were investigated. Haemolymph from 5 clams from each population was withdrawn individually and diluted with FSSW at 5×10^5 haemocytes·ml^{-1}. Then 100 μl of haemolymph dilution from each clam was plated in 3 replicates and allowed to adhere to the plate for 1 h. QPX cultures (NY1-QPX, NY2-QPX and MA-QPX) grown in tissue homogenates were diluted with sterile media to obtain 1×10^5 cells·ml^{-1}, and suspensions were processed as previously to collect ECP. Tissue homogenates were chosen as culture media to produce ECP rather than MEM based on results from preliminary experiments (moderate and consistent cytotoxicity of QPX ECP, see Results section). Challenges were initiated by adding 100 μl of ECP to experimental wells, while control wells were made up with sterile culture medium. After 1 h of challenge, QPX ECP were removed and the NR uptake assay was performed as described above.

Statistical analysis

Data were analysed using SigmaStat (Ver3.11. Systat Software, Inc., San Jose, CA) statistical software. Effects of the time-of-incubation and the dilution factor of QPX cells and ECP on NR uptake by haemocytes were analysed by a two-way ANOVA. A two-way ANOVA was also used to compare differences of cytotoxicity among QPX isolates on haemocytes withdrawn from MA and NY clams. ANOVA treatments that generated probability values below 0·05 were systematically followed by a Holm-Sidak post-hoc test comparing different data points. Differences between data points were considered statistically significant at $P < 0{\cdot}05$.

RESULTS

Setup of the neutral red uptake assay

Under the tested experimental conditions, the assay indicated a linear relationship between neutral red uptake and the number of haemocytes in the range of $3{\cdot}5 \times 10^3$ to 7×10^4 haemocytes per well (Fig. 1, $R^2 = 0{\cdot}99$). Higher densities of haemocytes enhanced cell aggregations and haemocyte concentrations were no longer proportional to the absorbance (data not shown).

Cytotoxicity of QPX cells and ECP produced in tissue homogenates

Addition of QPX cells or ECP from parasite cultures caused an increase in cell debris and decrease in the uptake of neutral red in the exposed haemocytes (Fig. 2). Challenged haemocyte layers displayed discoloured cytoplasm, sometimes lacking intracellular complexity when compared to control

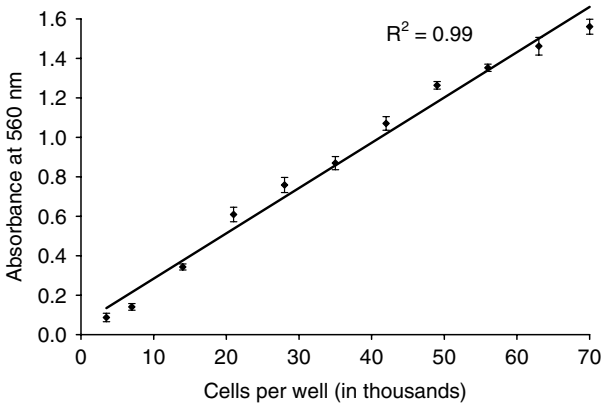

Fig. 1. Neutral red uptake (absorbance at 560 nm) as a function of haemocyte counts in experimental wells. Linear relationship ($R^2 = 0{\cdot}99$) was measured for haemocyte concentrations ranging from $3{\cdot}5 \times 10^3$ to 7×10^4 cells per well. Mean ± S.E.M. (4 replicates/data point).

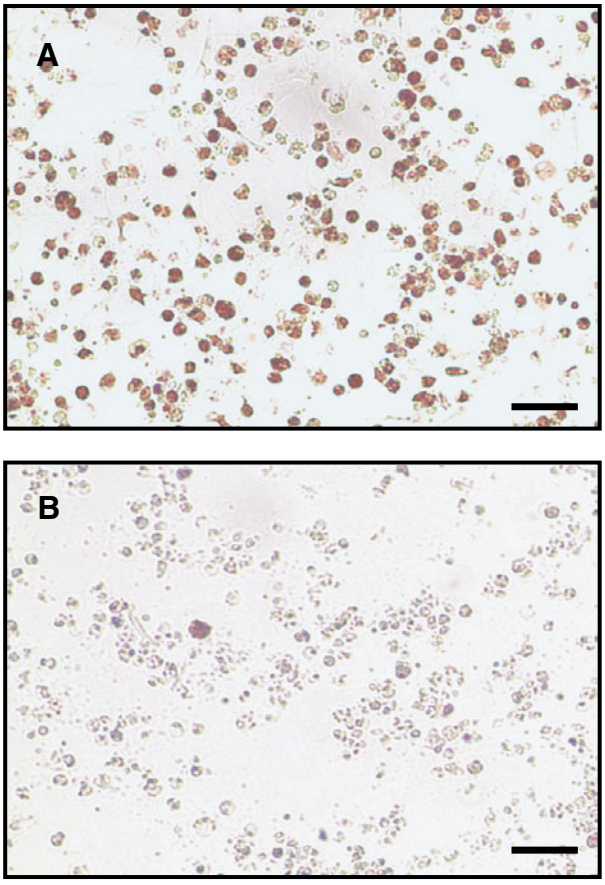

Fig. 2. Neutral red uptake by haemocytes exposed for 4 h to (A) sterile culture media or to (B) supernatants of an exponentially growing QPX culture in tissue homogenates (isolate NY0313808BC7). Scale bar = 50 μm.

haemocytes exposed to sterile culture media (Fig. 2). The cytotoxicity of QPX cells and ECP from parasite cultures grown in clam tissue homogenates was dose dependent, with NR uptake higher at higher

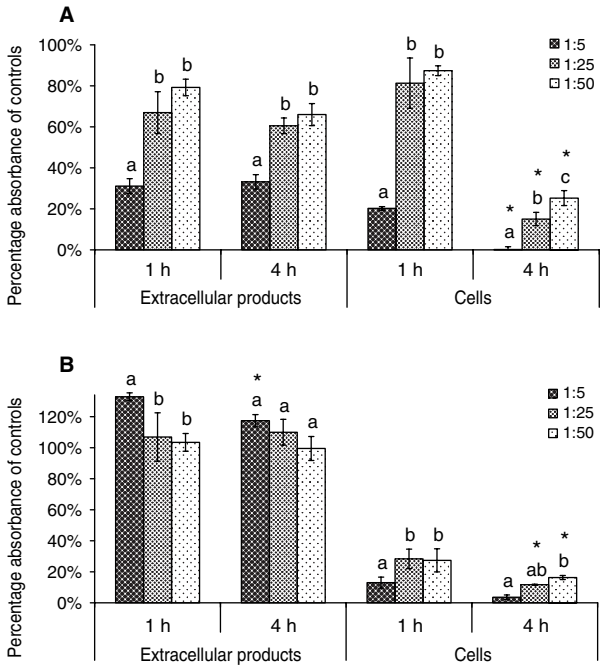

Fig. 3. Neutral red uptake by haemocytes exposed to serial dilutions of QPX cells and ECP from isolate NY0313808BC7 grown in tissue homogenate (A) and in MEM (B). Ratios correspond to 1 : 5, 1 : 25 and 1 : 50 QPX (cells or cell equivalent for ECP) : haemocyte ratios. Results are expressed as percentage of absorbance of unchallenged controls at 560 nm. Lower NR uptake/absorbance indicates higher QPX cytotoxicity. Letters (a, b and c) represent differences among different dilutions for each time and symbols (*) indicate significant differences between incubation times for each dilution (two-way ANOVA, $P < 0{\cdot}05$). Mean ± S.E.M. (3 replicates/data point).

dilutions of QPX cells or ECP (Fig. 3A, Table 1). When haemocytes were incubated with undiluted (1 : 5 ratio, see Materials and Methods section) QPX ECP (Fig. 3A), the decrease in NR uptake was maximal ($31{\cdot}12 \pm 3{\cdot}57$% of the controls) after 1-h exposure to ECP; further incubation (4 h) induced a barely significant decrease of NR uptake by haemocytes (Table 1, $P = 0{\cdot}047$).

Interestingly, a different trend was observed in haemocytes exposed to QPX cells: NR uptake was significantly lower at longer incubation periods (4 h), as compared to those exposed for 1 h ($P < 0{\cdot}001$). This was the case for haemocytes challenged with both undiluted and diluted parasite cells (Fig. 3A, Table 1).

Cytotoxicity of QPX cells and ECP produced in MEM

The trends of NR uptake were noticeably different in haemocytes exposed to QPX cells and ECP produced in MEM (Fig. 3B). Significant differences between the tested ECP dilutions were detected by ANOVA analysis (Table 1). However, these

Table 1. Summary of multifactor ANOVA testing the effects of QPX cells and ECP (isolate NY0313808BC7) grown in tissue homogenates and MEM on neutral red uptake by haemocytes from NY clams

Culture medium	Source of Variation	Degrees of freedom	Sum of squares	Mean squares	*F*	*P*
Clam extracts	QPX cells					
	Time	1	1·102	1·102	361·915	<0·001
	Dilution	2	0·727	0·363	119·386	<0·001
	Time × Dilution	2	0·197	0·098	32·306	<0·001
	QPX ECPs					
	Time	1	0·016	0·016	4·907	0·047
	Dilution	2	0·542	0·271	85·922	<0·001
	Time × Dilution	2	0·018	0·009	2·807	0·1
MEM	QPX cells					
	Time	1	0·069	0·069	37·012	<0·001
	Dilution	2	0·065	0·032	17·400	<0·001
	Time × Dilution	2	0·004	0·002	1·177	0·341
	QPX ECPs					
	Time	1	0·013	0·013	1·866	0·197
	Dilution	2	0·177	0·089	12·438	0·001
	Time × Dilution	2	0·026	0·013	1·829	0·203

differences were mostly attributed to effects of undiluted ECP. NR uptake in haemocytes exposed to diluted ECP was not statistically different from control haemocytes exposed to sterile MEM. Surprisingly, haemocytes exposed to undiluted ECP displayed higher NR uptake than haemocytes exposed to diluted ECP samples (1 h) or control haemocytes (1 h and 4 h).

On the other hand, QPX cells cultured in MEM exhibited a strong cytotoxicity toward haemocytes after 1 h of challenge and appeared more toxic to haemocytes than parasites grown in tissue homogenates, particularly when diluted parasite cells were used (Fig. 3). Both dilution- and time-dependent effects were significant (Table 1).

Comparison of QPX isolates and clam populations

ECP produced by different QPX isolates grown in tissue homogenate displayed different levels of cytotoxicity toward haemocytes (Fig. 4). Multifactor ANOVA analysis indicated variations of cytotoxicity between tested QPX isolates ($P<0{\cdot}001$) and differences in haemocyte resistance between MA and NY clams ($P=0{\cdot}008$). No specific interactions between specific QPX isolates and clam strains were detected ($P=0{\cdot}57$, Table 2).

Among all clams, NR uptake by haemocytes was systematically lower when NY isolates of the parasite (particularly isolate NY1-QPX) were used as compared to the MA isolate (MA-QPX). Absorbance readings obtained with ECP from the NY isolates ranged from $39{\cdot}02\pm5{\cdot}6$ to $68{\cdot}49\pm9{\cdot}6$% of control values while ECP from the MA isolate resulted in a smaller reduction of NR uptake (ranging from $76{\cdot}09\pm4{\cdot}1$ to $92{\cdot}15\pm5{\cdot}76$% of control absorbance).

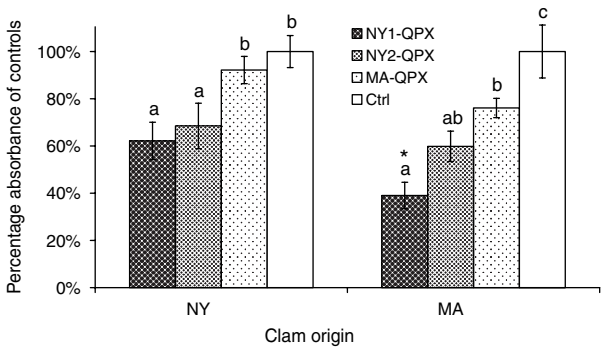

Fig. 4. Neutral red uptake by haemocytes from 2 clam stocks exposed to ECP from 3 different QPX isolates. Results are expressed as percentage absorbance of corresponding controls at 560 nm. Lower NR uptake/absorbance indicates higher QPX cytotoxicity. The letters a, b and c represent differences among treatments for the same clam strain while symbol (*) represents difference among clam strains exposed to the same QPX isolate (two-way ANOVA, $P<0{\cdot}05$). Mean ± S.E.M. (5 replicates).

Significant differences of ECP cytotoxicity were observed between NY1-QPX and MA-QPX for both MA and NY clams ($P<0{\cdot}005$), whereas the 2 NY isolates of the parasite exhibited no significant difference when compared to each other.

Haemocytes also presented different levels of resistance to ECP according to the clam stock from which they were withdrawn (Fig. 4, Table 2). For each specific QPX isolate, haemocytes from NY clams exhibited higher resistance to ECP than haemocytes from MA clams. However, pairwise comparisons demonstrated statistically significant variations of resistance only during challenge with

Table 2. Two-way ANOVA results testing the effects of ECP from three QPX isolates on clam haemocytes from two geographical locations, and interactions between variables

Source of variation	Degrees of freedom	Sum of squares	Mean squares	*F*	*P*
Clam	1	0·191	0·191	8·244	0·008
QPX	2	0·569	0·285	12·305	<0·001
Clam × QPX	2	0·0262	0·0131	0·566	0·575

ECP from NY1-QPX (Holm-Sidak post-hoc test, $P=0{\cdot}02$, Fig. 4).

DISCUSSION

Neutral red (NR) uptake assay was initially developed by Borenfreund and Puerner (1985*a*) to test cytotoxic effects of chemical agents on cells in a monolayer culture. This method was successfully used in bivalves to investigate cytotoxic effects of metals (cadmium, copper, nickel) or chemical compounds (polycyclic aromatic hydrocarbons, tributyltin) (Cajaraville *et al*. 1996; Grundy *et al*. 1996; Pipe *et al*. 1999; Chu *et al*. 2002; Matozzo *et al*. 2002). Alteration of NR uptake usually reflects damage to the membrane or, alternatively, changes in the volume of the lysosomal compartment. These alterations are therefore characterized by either a decrease or an increase in NR uptake by haemocytes of exposed bivalves. For instance, an increased NR uptake was reported in haemocytes of the mussel *Mytilus edulis* exposed to cadmium (Coles *et al*. 1995), whereas the same heavy metal caused a decrease in NR uptake in *M. mercenaria* (Zaroogian *et al*. 1992). Neutral red assay uptake, however, has been rarely used to investigate cytotoxic effects of parasites or parasite products on cells (Le Sueur *et al*. 2005; Pichardo *et al*. 2006). The increase of NR uptake in haemocytes challenged by ECP from QPX grown in MEM might be related to the effect of by-products resulting from MEM degradation as supported by our ongoing studies (data not shown). The observed reduction in NR uptake following exposure to QPX products in this study is, however, a clear indication of the parasite's cytotoxic effects on clam haemocytes. However, the NR uptake technique does not allow for identification of cytotoxicity mechanisms, since reduction in the uptake may result from modification of lysosomal compartment volume, loss of haemocyte adherence, or increased fragility of membrane integrity. Microscopic observations supported alterations of cell membranes and cell death in haemocyte monolayers exposed to QPX ECP. Pathogens are known to produce and sometimes release cytotoxic substances that interfere with normal activities of bivalve haemocytes. For instance, the exposure of haemocytes from the clam *Ruditapes philippinarum* to ECP produced by its pathogen *Vibrio tapetis* caused significant reduction in haemocyte viability and phagocytic activity (Allam *et al*. 2002). *Vibrio* spp. cells can also induce morphological alterations of bivalve haemocytes, leading to cell rounding and loss of adherence (Lane and Birkbeck, 2000; Choquet *et al*. 2003). Similarly, ECP of *P. marinus* altered the motility of *C. virginica* haemocytes (Garreis *et al*. 1996).

Interestingly, variations of cytotoxicity induced by QPX cells and ECP were observed according to culture conditions. ECP from parasite cultures grown in tissue homogenates exhibited significantly higher cytotoxicity compared to ECP from cultures made in MEM. These findings suggest that the presence of clam factors in the culture medium enhanced the production/release of cytotoxic compounds into the surrounding environment. Thraustochytrids are well known to produce and release a large variety of extracellular compounds (Jain *et al*. 2005) and Anderson *et al*. (2006) demonstrated the presence of serine protease in QPX ECP. Similar mechanisms have already been described in the oyster parasite *P. marinus*, in which the supplementation of parasite cultures with oyster tissue extracts or plasma significantly induced the secretion of low-molecular weight serine proteases (MacIntyre *et al*. 2003) and enhanced infectivity of the parasite (Earnhart *et al*. 2004).

Our results also demonstrated high cytotoxicity of QPX cells toward clam haemocytes for all tested conditions, with parasite cells grown in MEM tending to be more cytotoxic than QPX cells grown in tissue homogenates. The disparity between toxicity profiles of ECP and parasite cells in different culture media is intriguing and cannot be interpreted here without speculations, particularly since the factors responsible for the cytotoxic effects remain unidentified. Factors involved in QPX cell toxicity might be different from those mediating ECP toxicity (cell-cell interactions *versus* ECP proteases, for example). Alternatively, factors mediating ECP toxicity might be produced and stored in parasite cells and only released to the extracellular environment in the presence of host tissue extracts or haemocytes. This hypothesis could explain the strong cytotoxicity of ECP obtained in tissue homogenates and the rapid (within 1 h) and robust cytotoxicity

of QPX cells cultured in MEM. The delay of cytotoxicity observed between 1 h and 4 h with diluted QPX cells cultured in tissue homogenates may be attributed to the time required to *de novo* produce and release ECP by QPX. This hypothesis is supported by findings of Anderson *et al*. (2003), who reported that washed QPX cells were able to produce copious amounts of mucus (and probably virulence factors coupled within).

Our results revealed various levels of cytotoxicity among the 3 tested QPX isolates. New York isolates were significantly more cytotoxic to haemocytes (both clam strains combined) than the Massachusetts isolate ($P<0{\cdot}001$), but no significant difference was detected amongst both NY strains. These *in vitro* results closely matched *in vivo* observations that we previously reported, where experimental challenges produced higher mortality of naïve MA clams after exposure to QPX isolate NY0314220AC5 (here called NY1-QPX), followed by isolate NY0313808BC7 (NY2-QPX) and finally MA-QPX (Dahl *et al*. 2008). Obviously, it is difficult to make a link between *in vitro* cytotoxicity and *in vivo* pathogenicity, and potential relationships require further investigations. However, such variations among isolates suggest the existence of different QPX strains between Massachusetts and New York. Qian *et al*. (2007) observed substantial sequence variability in the rRNA operon intergenic spacers of different QPX isolates without being able to identify specific differences between isolates. The possible absence of sexual life-stages (zoospore stages) in QPX could enhance the speciation of the parasite. Investigations of virulence and environmental tolerance among *P. marinus* isolated from different geographical locations also demonstrated the existence of parasite races (Bushek and Allen 1996*b*; Reece *et al*. 2001).

Previous *in vivo* investigations demonstrated that clam resistance toward QPX disease is mediated genetically (Ford *et al*. 2002; Calvo *et al*. 2007; Dahl *et al*. 2008). Our *in vitro* results indicated higher susceptibility toward QPX ECP of haemocytes from MA clams as compared to NY clams. However, the significance of this difference was mostly supported by the effects of ECP from isolate NY1-QPX. Implying that *in vitro* resistance of haemocytes to QPX ECP is linked to higher *in situ* survival of clams exposed to QPX, prior and likely long term exposure of NY clams to NY1-QPX could explain higher resistance of haemocytes from these animals compared to haemocytes from MA clams, which had not been previously exposed to this strain. Additional investigations using a larger number of QPX isolates and clam populations from different geographical locations (particularly from areas where QPX disease is absent) are necessary to discriminate specific interactions between QPX isolates and haemocytes from different clam broodstocks.

This study demonstrated the cytotoxicity of QPX toward clam haemocytes and established the inducible nature of cytotoxic factors. The NR uptake assay allowed the comparison of QPX cytotoxicity among different parasite isolates. Results of these *in vitro* investigations corresponded well with results of prior *in vivo* studies comparing pathogenicity of different QPX isolates. The NR uptake technique described here appears to represent an efficient and sensitive tool for the investigation of QPX virulence factors and alternatively, for evaluation of haemocyte resistance to the parasite.

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