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Effect of environmental factors on survival and growth of quahog parasite unknown (QPX) *in vitro*

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ABSTRACT

Quahog parasite unknown (QPX) is a protistan microorganism associated with mass mortalities of hard clams (*Mercenaria mercenaria*) along the northeastern coasts of the United States and maritime Canada. Because several studies indicate modulatory effects of prevailing environmental parameters on disease outbreaks, this study tested the effect of major environmental parameters (temperature, salinity and oxygen concentration; individually or combined) on QPX survival in artificial seawater and parasite growth in culture media *in vitro*. Three QPX isolates from two different geographic locations were compared. Results indicated that *in vitro* growth of QPX was optimal in standard culture medium at 34 ppt between 20 °C and 23 °C. Additionally, significant differences in temperature optima were observed for geographically distinct QPX isolates ($p < 0.001$) confirming previous studies suggesting the existence of different QPX strains (or ecotypes). When tested in seawater, QPX exhibited opposite trends with higher survival at 15 °C and 15 ppt. Results also demonstrated limited survival and growth of QPX under anoxic conditions. Additionally, results showed that the parasite is able to survive extreme temperatures (−12 °C to 32 °C) suggesting that QPX could overcome short periods of extreme conditions in the field. These results contribute to a better understanding of interactions between QPX and its environment, but potential impacts of environmental conditions on QPX disease development need further work as it also involves clam response to these factors.

1. Introduction

Quahog parasite unknown (QPX) is a protistan pathogen of the hard clam *Mercenaria mercenaria*, infecting both cultured and wild populations. QPX disease has resulted in hard clam mortalities along the east coast of North America from Virginia to maritime Canada (Dove et al., 2004; Ford et al., 2002; Ragone Calvo et al., 1998; Smolowitz et al., 1998; Whyte et al., 1994). QPX is a member of the phylum Labyrinthulomycota within the thraustochytrid family (Ragan et al., 2000). Labyrinthulomycota are ubiquitous in aquatic environments and the ecology of several species belonging to this phylum has been investigated (Raghukumar, 2002). QPX ecology remains relatively understudied. The parasite was successfully detected in seawater and sediment (Gast et al., 2008; Liu et al., 2009) and previous studies also demonstrated its ability to grow on degraded macroalgae material *in vitro* (Buggé and Allam, 2007) but available information on the effects of environmental parameters on the parasite is limited (Brothers et al., 2000).

Environmental parameters strongly affect host–pathogen interactions by modulating host defenses and pathogen survival and virulence. Temperature and salinity have been shown to be key environmental factors controlling infection and progression of several pathogens of marine bivalves, including *Perkinsus marinus*, *Haplosporidium nelsoni*, and *Bonamia* sp. (Auzoux-Bordenave et al., 1995; Carnegie et al., 2008; Chu and Greene, 1989; Ford and Haskin, 1988). Indications that temperature and salinity affect QPX distribution and/or associated disease development were previously reported. For instance, higher QPX prevalence and associated clam mortalities occur during summer in Canada, Massachusetts and New York (Dove et al., 2004; MacCallum and McGladdery, 2000; Smolowitz et al., 1998). On the other hand, Ragone Calvo et al. (1998) found QPX disease to be absent from areas with moderate salinities (15–25 ppt) and surveys of hard clams in New York suggested that low dissolved oxygen could be associated with higher QPX prevalence (Allam, unpublished). Additionally, QPX was identified in different locations along the east coast of the United States but never south of Virginia despite the fact that *in vivo* and *in vitro* studies demonstrated that southern clam stocks were more susceptible to QPX infection than northern stocks (Dahl et al., 2008; Ford et al., 2002; Perrigault and Allam, 2009; Ragone Calvo et al., 2007).

Recent studies suggested the presence of different stains of QPX exhibiting variable virulence *in vivo* (Dahl et al., 2008) and *in vitro* (Perrigault and Allam, 2009). In the case of *P. marinus*, Bushek and Allen (1996) also suggested that isolates obtained from different

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locations represented different strains exhibiting various degrees of virulence and environmental tolerances.

This study was designed to investigate the effect of three major environmental factors (temperature, salinity, and oxygen concentration) on QPX survival and growth *in vitro*. The growth of QPX isolates from different geographical locations was compared under ranges of temperatures and salinities to identify specific adaptations of each QPX isolate. Additionally, some environmental parameters were associated to evaluate their combined effects on QPX survival and growth. Finally, the ability of QPX to survive under extreme temperature conditions that occasionally occur in the field was also investigated.

2. Materials and methods

2.1. QPX cultures

Two New York isolates of QPX, NY0313808BC7 (NY1-QPX) and NY0314220AC6 (NY2-QPX), were obtained from nodules of infected hard clams collected in Raritan Bay, NY in October 2003 (Qian et al., 2007). Massachusetts QPX isolate (MA-QPX) was obtained from the American Type Culture Collection (ATCC 50749). Isolation and subculture were performed in 25-cm^2 culture flasks at 23 °C using Minimal Essential Medium Eagle (MEM, Sigma M06440) adjusted to 34 ppt according to the methods described by Kleinschuster et al. (1998). In all experiments, survival and growth of QPX *in vitro* were measured using a fluorometric technique based on quantification of QPX biovolume. This technique uses the dye fluorescein di-acetate (FDA) to measure esterase activity of live cells as previously described by Buggé and Allam (2005) and Perrigault et al. (2009).

2.2. Effect of temperature on the growth of different QPX isolates

QPX isolates (NY1-QPX, NY2-QPX and MA-QPX) were subcultured in MEM (34 ppt) at 23 °C for 7 days to reach exponential growth phase. Aliquots (100 µl) of each culture were transferred in quadruplicate to 24-well plates containing 2.5 ml of MEM culture medium and incubated at 3, 8, 17, 20, 23, 29, and 32 °C. QPX biovolume was measured by the FDA technique at t_0 and days 1, 3, 7, and 14. Briefly, 100 µl of each culture was transferred into a black 96-well plate (in duplicate) and 12 µM of FDA was added to each well. Plates were incubated in the dark at room temperature (RT) for 30 min and fluorescence was measured at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader). Data are presented as percentage of the maximal fluorescence measured for each isolate.

2.3. Effect of salinity on the growth of different QPX isolates

A similar assay was designed to measure QPX growth under different salinity conditions *in vitro*. Modified culture medium at 19 ppt (MEM-19 ppt) was prepared by removing NaCl from the standard preparation and the different treatments were made by supplementing MEM-19 ppt with specific amount of NaCl to reach 22, 25, 28, 31, 34, and 37 ppt. Aliquots (100 µl) of exponential culture of each QPX isolate were transferred in triplicate into the wells of a 24-well plate and supplemented with 2.5 ml of MEM adjusted to the specific salinities. Plates were incubated at 23 °C and QPX biovolume was measured at t_0 and days 3, 7, and 14 using the FDA technique as described above. Data are presented as percentage of the maximal fluorescence measured for each isolate.

2.4. Combined effects of temperature and salinity on QPX

Combined effects of temperature and salinity on *in vitro* QPX growth in MEM were investigated in black 96-well plates according to Perrigault et al. (2009). Briefly, an exponential culture of NY1-QPX was harvested by centrifugation (15 min at 600*g*) and QPX cells were resuspended in filtered artificial seawater (FASW) (Perrigault et al., 2009). Five hundred QPX cells suspended in FASW were transferred into the wells of a black 96-well plate containing MEM adjusted to 15, 25, and 35 ppt (final salinities). Replicate plates were incubated at 15 °C or 23 °C. Each condition (temperature/salinity combination) was performed in triplicate. QPX biovolume was measured at t_0 and days 2 and 4 by the FDA technique. Data are presented as relative fluorescence unit (RFU) after removing background fluorescence generated by QPX-free MEM.

A second assay was performed to investigate the combined effects of temperature and salinity on *in vitro* QPX survival in artificial seawater without MEM supplement. As previously, an exponential culture of NY1-QPX was harvested and QPX cells were resuspended in FASW. One hundred microliter of QPX suspension (1×10^5 QPX cells) were transferred into 24-well plates containing 1.9 ml of FASW adjusted to 15, 25, and 35 ppt (final salinities). Plates were incubated at 15 °C and 23 °C. Each condition (temperature/salinity combination) was performed in duplicate. QPX biovolume was measured at t_0 and days 4, 7, and 10 by the FDA technique. Data are presented as relative fluorescence unit (RFU) after removing background fluorescence generated by QPX-free FASW.

2.5. Effect of anoxia on QPX

To evaluate the effect of anoxia on *in vitro* QPX growth, NY1-QPX was subcultured in quadruplicate in 2.5 ml MEM (34 ppt) in 24-well plate and incubated for 25 days in an anoxic glove-box incubator with a 5% CO_2, 10% H_2, and 85% N_2 gas mix at room temperature (22 ± 1 °C). Controls (NY1-QPX in MEM at 34 ppt) were incubated in 24-well plate under ambient air conditions at room temperature. The FDA technique was used to measure QPX biovolume at t_0, day 7 and day 25.

To study the effect of anoxia on *in vitro* QPX survival, 100 µl of QPX cultures exposed to anoxic conditions for 7 and 21 days as well as controls (ambient conditions for 21 days) were transferred to new culture flasks containing 4 ml MEM (34 ppt) and submitted to standard culture condition (ambient air, 23 °C, three replicates). QPX biovolume was assessed after 7 days by the FDA technique.

2.6. Tolerance of QPX to extreme temperatures

QPX cultures were also exposed to extreme high and low temperatures that may occasionally occur in the natural environment. Exponential cultures of NY1-QPX were transferred to fresh MEM (34 ppt) and exposed to extreme temperatures for increasing periods of time. Cultures were incubated at −12 °C (inter-tidal freezing conditions), 3 °C (sub-tidal winter), or 32 °C (inter-tidal summer). After exposure to temperatures for the allotted time period (6 h, 24 h, 48 h, 72 h, 1 week, and 3 weeks), 100 µl culture aliquots were transferred to new culture flasks containing 4 ml MEM (34 ppt) and incubated at 23 °C (in triplicate). These transferred cultures were monitored under microscope on a daily basis for 4 weeks to determine whether QPX cells could survive the tested temperatures and propagate when returned to standard conditions.

2.7. Statistical analysis

Data were analyzed using SigmaStat (Ver. 3.1, Systat Software, Inc., San Jose, California, USA) statistical software. Analysis of variance (1- or 2-way ANOVA according to each data set) was used to compare the effects of environmental parameters (temperature, salinity, and oxygen concentration) individually or combined on QPX growth and survival within each sampling time. Effect of incu-

bation time within each treatment on QPX growth and survival was analyzed by repeated ANOVA. ANOVA treatments that generated probability values below 0.05 were followed by a Holm-Sidak post hoc test comparing different conditions. All differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effect of temperature on the growth of different QPX isolates

Results demonstrated a clear effect of the temperature on the *in vitro* growth of QPX with an optimal proliferation between 20 and 23 °C (Fig. 1). MA-QPX isolate reached maximal biovolume (as measured by fluorescence) at 20 °C, whereas NY isolates had optimal biovolume at 23 °C (Fig. 1). Statistical analysis showed that both NY-QPX isolates were significantly different from MA-QPX at 23 °C ($p < 0.001$, Holm-Sidak post hoc test). Incubation of all QPX isolates at temperatures below or above these optima reduced the proliferation of QPX to about 6% and 9% (of maximal biovolume) at 3 °C and 32 °C, respectively.

3.2. Effect of salinity on the growth of different QPX isolates

The growth of QPX at different salinities demonstrated the importance of this parameter on parasite proliferation *in vitro* over the 14 days of culture (Fig. 2). However, the range of tested salinities caused limited effect on QPX biovolume compared to temperature effects reported above, with a minimal proliferation of MA-QPX at 22 ppt. Overall, no significant differences were observed between NY-QPX and MA-QPX (2-way ANOVA) and all tested QPX isolates exhibited maximal biovolume at 34 ppt. However, differences of trends were observed among isolates: MA-QPX showed higher susceptibility to low salinity (22–28 ppt) with biovolumes ranging from 44% to 54% of the maximal biovolume measured at 34 ppt as compared to NY-QPX isolates (55–68%). Interestingly, trends were inverted at higher salinity (37 ppt) with higher biovolume for MA-QPX compared to NY-QPX isolates (Fig. 2).

3.3. Combined effects of temperature and salinity on QPX

The third experiment investigating the combined effects of temperature and salinity on *in vitro* QPX growth confirmed previous assays. For instance, higher QPX biovolumes were observed at 35 ppt and 23 °C and decreasing the salinity to 25 ppt and 15 ppt or the reduction of temperature to 15 °C resulted in a significant

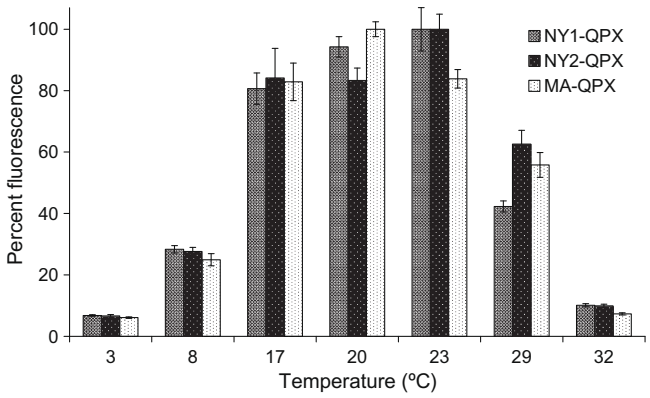

Fig. 1. Biovolume of QPX isolates cultured in MEM (34 ppt) and incubated at a range of temperatures from 3 °C to 32 °C. Data are presented as percentage of the maximal fluorescence measured for each isolate at Day 14 (Mean ± SEM, $n = 4$ replicates).

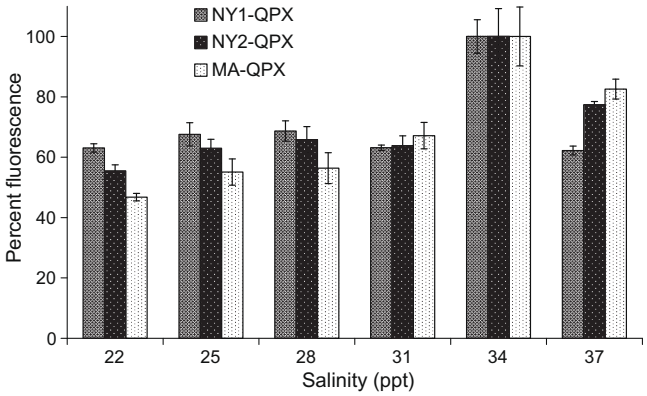

Fig. 2. Biovolume of QPX isolates cultured at 23 °C in MEM adjusted to a range of salinities from 22 ppt to 37 ppt. Data are presented as percentage of the maximal fluorescence measured for each isolate at Day 14 (Mean ± SEM, $n = 3$ replicates).

decrease of QPX biovolume at Day 4 ($p < 0.02$, 2-way ANOVA and Holm-Sidak post hoc test, Fig. 3, Table 1). Additionally, a large reduction in QPX growth was observed at 15 ppt for both tested temperatures with a significant decrease of QPX biovolume between t_0 and Day 4 at 23 °C ($p = 0.007$, repeated ANOVA). Interestingly, incubation of QPX cultures at 25 ppt and 35 ppt under sub-optimal temperature (15 °C) yielded similar growth levels in contrast to similar cultures submitted to 23 °C (Fig. 3).

Investigations of *in vitro* QPX survival in artificial seawater under combined environmental conditions showed different trends (Fig. 4). Statistical analysis by 2-way ANOVA at each sampling time demonstrated a significant effect of the temperature ($p < 0.03$, Table 2) on QPX survival as well as significant differences among different salinity treatments (15 ppt *versus* 25 ppt and 35 ppt), particularly driven by cultures maintained at 23 °C ($p < 0.01$). QPX biovolume was roughly constant over time at 25 ppt and 35 ppt under 15 °C, and slightly increased at 15 ppt. At 23 °C, parasite biovolume was unchanged over time at 15 ppt whereas a marked decrease of QPX biomass was noted as soon as 4 days at 25 ppt and 35 ppt ($p < 0.008$, repeated ANOVA and Holm-Sidak post hoc test, Fig. 4). Highest QPX biovolumes were observed at low salinity and temperature (Fig. 4). Microscopic observation of 35 ppt cultures maintained at 23 °C showed significant cell mortal-

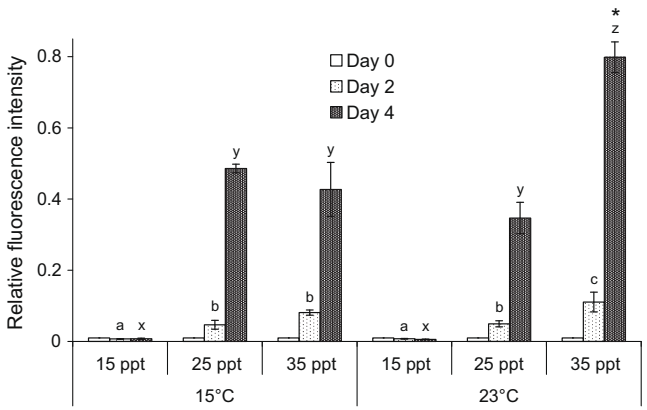

Fig. 3. Biovolume (relative fluorescence units, Mean ± SEM, $n = 3$ replicates) of NY1-QPX (8BC7) in MEM adjusted to different salinities and incubated at 15 °C or 23 °C. Fluorescence was measured at t_0 and after 2 and 4 days of incubation. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters; Holm-Sidak post hoc test, $p < 0.05$) QPX biovolume means among cultures at different salinities within each temperature condition at Day 2 (a–c), or Day 4 (x–z). *Indicates significantly higher biovolume in this treatment at Day 4 compared to its counterpart incubated at 15 °C.

Table 1
Summary of 2-way ANOVA testing the effect of temperature and salinity on QPX growth in MEM.

Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*	*P*
Day 2					
Temperature	1	0.019	0.019	0.782	0.394
Salinity	2	3.865	1.933	80.101	<0.001
Temperature × salinity	2	0.004	0.002	0.077	0.926
Day 4					
Temperature	1	0.001	0.001	0.066	0.801
Salinity	2	14.047	7.024	649.398	<0.001
Temperature × salinity	2	0.168	0.084	7.777	0.007

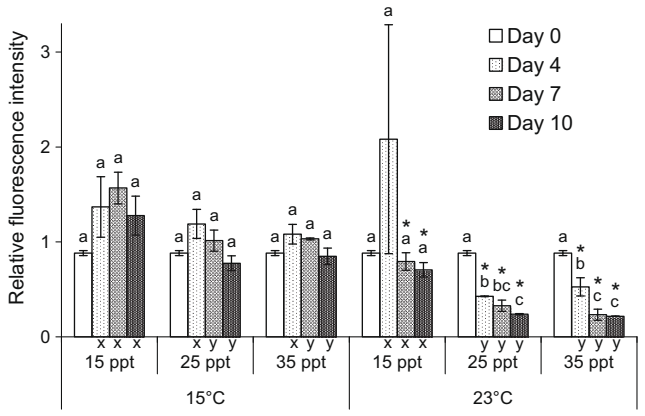

Fig. 4. Survival of NY1-QPX (8BC7) in artificial seawater adjusted to different salinities and incubated at 15 °C or 23 °C. Biovolume (relative fluorescence units, Mean ± SEM, $n = 2$ replicates) was measured for 10 days. Letters (a–c) indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters; Holm-Sidak post hoc test, $p < 0.05$) QPX biovolume means over time within each culture. Similarly, within each temperature, x and y indicate statistical differences in QPX biovolume means among cultures made at different salinities at the same time interval. *Indicates significantly lower biovolume in this treatment compared to its counterpart incubated at 15 °C.

ity characterized by the presence of cell debris as opposed to those held at 15 °C (Fig. 5), confirming that lower FDA uptake resulted from a reduction of QPX survival.

3.4. Effect of anoxia on QPX

Anoxic conditions significantly reduced *in vitro* QPX growth ($p < 0.001$, 2-way ANOVA, Fig. 6a) without altering the pH of the culture medium as evaluated by change in culture color. Incubation of QPX cultures under anoxic conditions for 7 days slightly increased parasite biovolume compared to t_0 ($p < 0.03$, repeated ANOVA) but QPX growth was significantly reduced compared to controls ($p < 0.001$, 1-way ANOVA). At day 25, QPX biomass under anoxic conditions was no longer different from t_0. Additionally, QPX cultures exposed to anoxic conditions for 7 days displayed limited ability to resume proliferation after transfer to fresh medium under standard conditions (ambient air) compared to normoxic control cultures (Fig. 6b, $p < 0.001$, 1-way ANOVA). Longer exposure of QPX to anoxic conditions (21 days) further reduced parasite's survival and subsequent proliferation under standard conditions (Fig. 6b).

3.5. Tolerances of QPX to extreme temperatures

Results from experiments exposing QPX cultures to extreme temperatures for varying periods of time showed that parasite cells can survive incubation at −12 °C for up to 24 h (Table 3). Cells from cultures incubated at −12 °C for longer periods did not propagate when transferred to fresh MEM (34 ppt) and incubated at the standard temperature of 23 °C. QPX was able to tolerate incubation at 3 °C for up to 1 week without any observed change in the microscopic appearance of cultures. After 3 weeks of incubation at 3 °C, QPX cells displayed morphological alterations characterized by a loss of membrane integrity and subcellular structures. These cells were unable to resume proliferation after transfer to standard culture conditions. Similarly, tolerance of QPX to 32 °C was limited to 1 week and no parasite proliferation was noted after subsequent transfer of cultures to standard conditions.

4. Discussion

Our experiments testing the effects of temperature, salinity, and oxygen concentration demonstrated that all tested parameters influence the *in vitro* growth of QPX. All QPX isolates were able to grow under the tested range of salinities (22–37 ppt at 23 °C) with an optimal growth at 34 ppt (Fig. 2). Salinities below and above this optimum decreased QPX biovolume up to about 40% of controls and only salinity as low as 15 ppt appeared to inhibit

Table 2
Summary of 2-way ANOVA testing the effect of temperature and salinity on QPX survival in seawater.

Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*	*P*
Day 4					
Temperature	1	0.124	0.124	8.019	0.03
Salinity	2	0.311	0.155	10.036	0.012
Temperature × salinity	2	0.194	0.097	6.258	0.034
Day 7					
Temperature	1	0.693	0.693	165.455	<0.001
Salinity	2	0.289	0.144	34.475	<0.001
Temperature × salinity	2	0.064	0.032	7.626	0.023
Day 10					
Temperature	1	0.615	0.615	330.77	<0.001
Salinity	2	0.314	0.157	84.452	<0.001
Temperature × salinity	2	0.0612	0.031	16.463	0.004

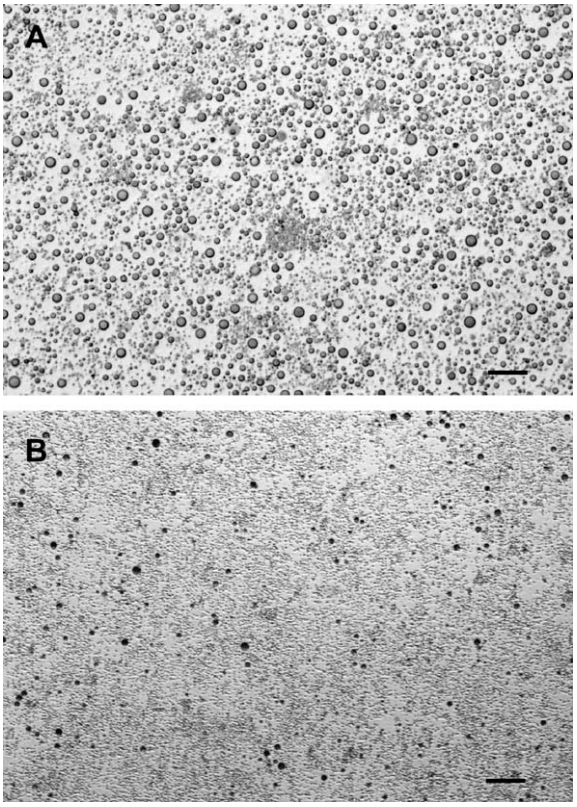

Fig. 5. NY1-QPX cells observed after 7 days in seawater at 35 ppt and incubated at 15 °C (A) or 23 °C (B). Scale bar = 50 μm.

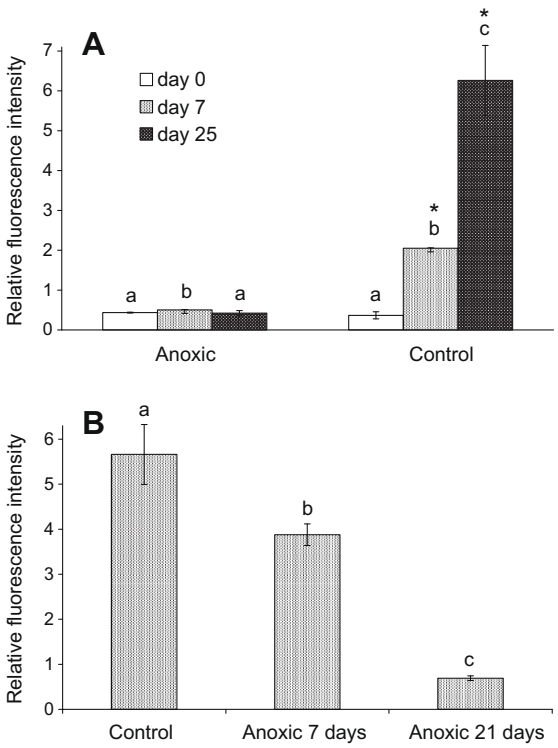

Fig. 6. Effects of anoxic conditions on QPX survival and growth. (A) Biovolume (relative fluorescence units, Mean ± SEM, $n = 3$ replicates) of NY1-QPX (8BC7) in MEM (34 ppt) incubated under anoxic or standard conditions for 25 days at room temperature. Letters indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters, Holm-Sidak post hoc test, $p < 0.05$) QPX biovolume means within each culture over time. *Indicates significant differences between both treatments for the same time interval. (B) QPX abilities to grow under standard conditions after incubation in anoxic conditions for 7 and 21 days. Biovolume (relative fluorescence units, Mean ± SEM, $n = 3$ replicates) was measured after 7 days of incubation in normoxic conditions. Letters (a–c) indicate that all treatments are significantly different from each other (Holm-Sidak post hoc test, $p < 0.05$).

parasite growth in culture medium (Fig. 3). Temperature optima were also relatively narrow (20–23 °C) and variation of temperature below 15 °C and above 29 °C significantly reduced QPX growth to less than 50% of the optimal growth measured for all tested isolates (Figs. 1).

MA-QPX isolate had optimal growth at a lower temperature (20 °C) than NY-QPX isolates (23 °C) (Fig. 1, $p < 0.001$). Similarly, differences between the NY and MA isolates were noted in their performance at salinities below and above 34 ppt (Fig. 2). These differences may be related to genetic or adaptive differences among the isolates to their environment. The existence of different QPX strains is also suggested by our previous studies that identified differences in *in vivo* (Dahl et al., 2008) and *in vitro* (Perrigault and Allam, 2009) virulence of the same isolates. The existence of different strains has been proposed for other parasites of marine bivalves. For instance, Bushek and Allen (1996) and Reece et al. (2001) demonstrated the existence of different *P. marinus* strains based on virulence and genetic variability they observed among different isolates. Attempts to discriminate *P. marinus* isolates based on their temperature optima were not conclusive, but important regional components to the proliferation rates of the pathogen were noted (Ford and Chintala, 2006).

Results from our *in vitro* investigations of temperature effects on QPX growth matched well with the timing of major QPX epizootic events in the field. MacCallum and McGladdery (2000) observed highest QPX prevalence during summer time with temperatures at about 20–25 °C which cover the range of optimal QPX growth *in vitro*. Similarly, major previous QPX epizootics also occurred during summer in Massachusetts (Smolowitz et al., 1998) and New York (Dove et al., 2004). On the other hand, the absence of QPX disease south of Virginia despite intensive clam aquaculture could be explained by higher temperatures in these locations limiting the presence of QPX. As a matter of fact, Gast et al. (2008) reported that QPX was more abundant in the environment in Massachusetts compared to more southern locations (Virginia). Similarly, QPX was not detected in moderate salinity waters (15–25 ppt) of the Chesapeake Bay while it was present in the more saline ocean-side bays of Virginia (Ragone Calvo et al., 1998). The distribution of other bivalve parasites has been also reported to be dictated by salinity. For example, *in vitro* studies by Chu and Greene (1989) clearly demonstrated the impact of salinity on the survival and sporulation of *P. marinus*. However, the extrapolation of *in vitro* studies to the distribution of QPX disease in the field (MacCallum and McGladdery, 2000; Ragone Calvo et al., 1998; Smolowitz et al., 1998) is not simple since QPX disease investigations do not take into account the impact of environmental factors on host fitness and immune performance.

Table 3
Effect of extreme temperatures on QPX survival. NY1-QPX (8BC7) cultures were submitted to −12 °C, 3 °C and 32 °C for different time intervals before transfer to standard culture conditions (MEM, 34 ppt, 23 °C) and monitoring of QPX proliferation over 4 weeks.

Treatment, °C	Duration					
	6 h	24 h	48 h	72 h	1 week	3 weeks
−12	+	+	−	−	−	−
3	+	+	+	+	+	−
32	+	+	+	+	+	−

(+) and (−) indicate presence and absence of QPX proliferation, respectively.

Our study showed about 20% growth reduction for MA-QPX at 23 °C compared to 20 °C while previous work by Brothers et al. (2000) concluded that cultured QPX originating from Massachusetts clams had optimal growth at 24 °C. Discrepancy between that study and our results could be related to the microscopic approach used by these authors to assess QPX growth which is problematic because of the production of viscous mucus by the parasite. Additionally, differences in temperature optima for MA-QPX may not be as contradictory as they appear, since Brothers et al. (2000) did not investigate the specific range of temperature used in our study. It is highly possible that they also would have found optimal growth at 20 °C if that temperature (±1 °C) was among their tested range of temperature. Similarly, these authors observed a regular increase of Massachusetts QPX growth between 20 ppt and 40 ppt and reported a maximal mucus production by the parasite at 34 ppt. It is unclear from our study whether the optimal proliferation of all isolates at 34 ppt truly reflects QPX requirements in the field or is more related to the *in vitro* maintenance of these cultures. For instance, all QPX isolates were routinely subcultured in MEM at 34 ppt for several months before their use in our study. Observed optima could be the result of QPX adaptation to the salinity of the standard culture medium. Long term subcultures at different salinities before testing might help determine if the optimal growth observed in our study at 34 ppt was related to intrinsic properties of QPX isolates or to specific adaptation to its culture environment.

Interestingly, our *in vitro* experiments demonstrated modulatory effects of combined environmental parameters. For instance, salinity displayed major effect on QPX growth at 23 °C but parasite biovolume was similar at 25 ppt and 35 ppt when cultures were incubated at 15 °C (Fig. 3). As a matter of fact, QPX growth at 25 ppt was similar at 15 °C and 23 °C whereas parasite growth at 35 ppt increased only when cultures were maintained at 23 °C. This suggests that some environmental parameters could limit the impact of sub-optimal conditions and facilitate QPX presence in its environment.

Our results on the combined effects of salinity and temperature on QPX survival in seawater exhibited opposite trends compared to assays performed in standard culture media. Reduced survival of QPX in seawater (25–35 ppt) at 23 °C described here (Fig. 4) is in agreement with a prior report (Buggé and Allam, 2007). Interestingly, our results suggest that QPX survives well in seawater at low temperature, and even better when both salinity and temperature are low. Cold temperatures could decrease the metabolic rates of QPX and therefore delay mortality of the parasite in nutrient-poor seawater whereas in nutrient-rich standard culture medium, higher temperature (23 °C) might stimulate parasite's metabolism and growth rates. Similar contradictions were observed between the *in vivo* and the *in vitro* performances of the oyster pathogen *Bonamia ostreae* (Arzul et al., 2009). In that study, the survival of the parasite was higher at low temperature whereas *in vivo* studies highlighted the importance of warm temperature on seasonal parasite cycling (Carnegie et al., 2008). Alternatively, our fluorescein di-acetate-based biotest is based on the activity of esterase in QPX cells which is directly linked to parasite's biovolume (Buggé and Allam, 2005). Despite the fact that the technique was validated in several studies and that microscopic observations clearly supported differences observed here (Fig. 5), the impact of QPX starvation on FDA uptake has never been investigated. Similar remarks about limitations of metabolic assays to assess cell proliferation (variation in cell size and metabolic activity) were made by Ford and Chintala (2006) on another metabolic biotest developed by Dungan and Hamilton (1995) for *P. marinus*.

Our results showed that QPX is sensitive to anoxia and a prolonged anoxic episode significantly altered survival of the parasite *in vitro*. These results might not be surprising as anoxia induces severe alterations in many organisms. Various degrees of hypoxic conditions could provide additional information on QPX sensitivity to oxygen concentration in the environment. It is possible that very low levels of dissolved oxygen could limit the distribution of QPX in the marine environment. However, low levels of dissolved oxygen are known to depress immune systems of marine mollusks (Chen et al., 2007; Matozzo et al., 2005). Therefore, hypoxic conditions in the field could affect both QPX and hard clams and resulting disease development might be difficult to forecast from laboratory experiments alone.

Our results also showed that QPX is able to survive extreme warm and cold temperatures. QPX cells tolerated 32 °C and 3 °C for up to 7 days, and −12 °C for up to 24 h. The ability of QPX cells to survive 3 and 32 °C for up to 1 week suggests that QPX can tolerate very warm summer and cold winter temperatures that occur in the marine environment within the natural range of the parasite. These *in vitro* results could explain the persistence of QPX in enzootic areas (Gast et al., 2008).

In conclusion, this study provides new information about the effect of environmental parameters on QPX survival and growth *in vitro*. Most available information on QPX distribution in the field is based on disease prevalence in clams and may provide a partial picture of the real distribution of QPX itself in the environment. Despite this limitation, conditions for optimal growth of the parasite *in vitro* observed in this study matched well with temperature and salinity ranges observed during highest QPX prevalence *in situ*. Additionally, tested QPX isolates reacted differently to tested temperatures suggesting specific adaptation to their geographical location and confirming previous work proposing the existence of different QPX strains. Our results also demonstrated that the effects of temperature and salinity as well as other parameters (environmental or nutritional) on QPX were different whether these factors are applied individually or combined with each other. Differences in QPX performance in assays using standard culture media and seawater highlight the need for extreme care in the extrapolation to the field of *in vitro* studies made under "standard" conditions for marine pathogens available in culture. Overall, further knowledge on QPX ecology as well as on the effects of environmental factors on its host, *M. mercenaria*, are needed to better determine QPX distribution in marine environments and factors controlling QPX disease development in hard clams.

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