Received Date : 28-Jul-2015

Revised Date : 11-Oct-2015

Accepted Date : 21-Nov-2015

Article type : Original Article

Seasonality of QPX disease in the Raritan Bay (NY) wild hard clam (*Mercenaria mercenaria*) population

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Abstract

Quahog Parasite Unknown (QPX) is a potentially lethal pathogen of the hard clam *Mercenaria mercenaria* along the northeastern coast of the United States. In the Raritan Bay wild clam population, QPX prevalence and infection intensity at three sites were examined by both histology and quantitative PCR in 2006. At two of three sites, QPX infection showed a seasonal pattern, with prevalence and weighted prevalence increasing through the spring, peaking in the summer and declining in the fall, while at the other site, the highest QPX prevalence and infection intensity were observed in the spring although overall prevalence at this site was generally low. Our data suggested that temperature may be an important environmental factor regulating the seasonal pattern of QPX disease in wild clams but also demonstrated that seasonal patterns vary from site to site, possibly linked to the clam density or other environmental factors. Over-winter sampling and further investigations focusing on environmental factors, clam density and clam mortality as related to QPX infection are needed to better characterize and understand the seasonality of QPX disease.

Keywords: Quahog Parasite Unknown, bivalve, epizootic, infection, temperature, quantitative PCR, qPCR

Introduction

Quahog Parasite Unknown (QPX) is a potentially lethal pathogen of the hard clam *Mercenaria mercenaria*. Since the 1960s, QPX disease has been documented in aquaculture and wild clam populations in various locations of the American and Canadian Atlantic coasts (Ford *et al.*, 2002; MacCallum and McGladdery, 2000; Ragone Calvo *et al.*, 1998; Smolowitz *et al.*, 1998; Whyte *et al.*, 1994). In New York State, mortalities associated with QPX disease were first observed in the summer of 2002 in a wild clam population in Raritan Bay off the coast of Staten Island (Dove *et al.*, 2004), leading to the suspension of the Raritan Bay transplant fishery until 2005, when the fishery resumed on a limited basis.

Effective management of hard clam populations and fisheries is hindered by incomplete understanding of the factors regulating the occurrence and severity of QPX disease. For

example, there are conflicting reports about whether QPX disease prevalence exhibits a seasonal pattern. Ragone Calvo *et al*. (1998) reported higher disease prevalence during May in Virginia as compared to other sampling periods, and preliminary observations in New York showed prevalence generally peaking during summer, then declining until the following spring, suggesting a role of seasonal environmental factors in disease dynamics (Allam *et al*., 2005). In contrast, another study by Ragone Calvo *et al*. (2007) did not detect seasonal patterns of QPX disease in clams deployed in New Jersey and Virginia. Using a compilation of published and unpublished data, Lyons *et al*. (2007) were also unable to identify any seasonal pattern in QPX disease prevalence. Temperature, the most obvious potential driver of seasonal patterns, has recently been shown to strongly affect both QPX growth *in vitro* and disease development *in vivo*, although with a different thermal maximum for each (23 ^{o}C and 13 ^{o}C, respectively) (Perrigault *et al*. 2010; Dahl *et al*. 2011; Perrigault *et al*. 2011).

Most of the available QPX prevalence data is from histological examination of clam tissues. Histopathological surveillance of Raritan Bay clams following the 2002 mortality event revealed that QPX prevalence was generally below 10% (Allam and Pawagi, 2005). Unless large numbers of clams are examined, this low prevalence makes detection of seasonal (and other) patterns in QPX disease statistically challenging. Additionally, because QPX lesions often display a focal distribution in clam tissues, the histological technique appears to underestimate QPX prevalence, yielding a high number of false negative results if the infection site is missed by the small amount of tissue section examined (Liu *et al*., 2009). The sensitivity of a new quantitative PCR (qPCR) assay allows us to detect a relatively low QPX abundance in an aliquot of homogenized clam tissue and reveals that prevalence could be much greater than indicated by histological examination alone even when the same amount of tissue is examined by both techniques (Liu *et al*. 2009). In this study, both histological and qPCR techniques were used to determine QPX prevalence and infection intensity in the Raritan Bay clam population in an attempt to more precisely reveal the seasonal and spatial dynamics of QPX disease.

Materials and methods

Clam sampling and processing

Clams were collected from Raritan Bay sites RB1, RB2 and RB3 (Figure 1) on 5 sampling dates at 6-week intervals from April to September 2006 and at site RB4 in May and August in 2006, giving a total of 17 groups of clams (~30 clams per group). At each sampling site, clams were collected by a patent tong (total area 1 m^2 per grab). Clam density was estimated by dividing the total number of live and recently dead (hinged shells with clean inner surface) clams by the number of grabs. Mortality rates were estimated by dividing the number of recently dead clams by the total of all collected (live and recently dead) clams. From all clams collected at each site, 30 clams were haphazardly selected; multiple grabs were performed to collect 30 live clams when clam density was lower than 30 m^{-2}. Clams were immediately placed on ice, transported to the laboratory, stored at 4 ºC and processed within 6-48 hours. For each site, the physical-chemical characteristics of bottom seawater (temperature, salinity and dissolved oxygen concentration) were measured using a YSI instrument. Depth ranged from 7 to 8m for RB1, RB2 and RB3 and was 5m for RB4.

Shell size (length and width) and external shell characteristics (e.g. gaping, chips etc.) were noted for each animal. The clams were then shucked and examined for gross abnormalities in tissues such as nodules or swelling, which could be signs of QPX infection. Each clam was further dissected and diagnosed for QPX infection using both standard histological techniques and qPCR assay.

Histopathological analysis

For histopathology, a thin cross section (3-5 mm in thickness) of clam soft tissue, containing mantle, gills and visceral organs (e.g. digestive glands, stomach, gonad, heart and kidney), was taken. A transverse slice of tissue from the base of the siphon, where QPX infections have been suggested to be initiated (Smolowitz *et al*., 1998), and any visible nodule or swelling tissue was taken as well. The tissues were transferred to a histo-cassette, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 to 6 μm and mounted on slides. The histological slides were stained (Harris's hematoxylin for 2 min and Eosin Y for 1 min) and examined with a light microscope. The abundance and distribution of QPX was determined for each of the four tissue types within an individual section (siphon, mantle, gill and visceral mass). Signs of old lesions or "healing", as

indicated by the presence of lesions with dead and degrading parasite cells (Dahl and Allam, 2007), were also recorded when observed. The QPX infection intensity was scored based on the total number of QPX organisms in all tissue types per slide section, as described in Ragone Calvo *et al*. (1998), as rare (1-10), light (11-100), moderate (101-1000) or heavy (>1000) (no clam was categorized as heavily infected by histology in this study).

Quantification of QPX in clam samples by qPCR assay

For qPCR assay, the mantle and siphon tissues were targeted because they represent the main infection sites (Smolowitz *et al*., 1998). Mantle and siphon tissues remaining after histological sampling were drained on a clean paper towel, weighed and preserved in 100% ethanol at -80 °C. To recover DNA, ethanol-preserved clam tissues were washed twice using phosphate-buffered saline (PBS) and mechanically homogenized in 10 volumes of PBS (e.g., 1 g tissue in 10 ml PBS). A 1 ml aliquot of tissue homogenate from each clam, containing 100 mg clam tissue, was transferred to a 1.5 ml centrifuge tube for DNA extraction. DNA was extracted by following the protocol described in Liu *et al*. (2009) and diluted in 100 µl molecular grade water. One microliter DNA (representing 1 mg clam tissue) was used as template for duplicate qPCR reactions. For each sample, a positive QPX signal was determined by measuring at least 10 QPX internal transcribed spacer (ITS) copies per qPCR reaction and any PCR inhibition effect was corrected for each sample as described in Liu *et al*. (2009). The original abundance of QPX cells in each clam sample was then calculated by $(\#QPXinitial \times a \times b)/(c \times d \times e)$ as described in Liu *et al*. (2009). For Raritan Bay clams examined in this study, *#QPXinitial* is the corrected number of QPX ITS copies in 1 µl DNA template; *a* (dilution of template in qPCR assay) typically equals 1 or 10; *b* (total DNA elution volume) equals 150 or 300 µl; *c* (target gene copy number) equals 181 copy $cell^{-1}$; *d* (DNA extraction efficiency) is 16.31% (Liu *et al*., 2009); and *e* (wet weight of extracted tissue) equals 100 mg. The typical detection limit of the qPCR assay was calculated to be 0.5 cells mg^{-1} tissue based on all clam samples assayed.

QPX prevalence and weighted prevalence

QPX prevalence in each group of clams was calculated as the percentage of QPX-positive clams in all sampled animals. QPX prevalence determined by the histological method is based on the individuals containing QPX cells in the tissue section examined by microscopy. QPX prevalence determined by qPCR assay is the proportion of clams producing a positive QPX signal in duplicate qPCR reactions.

To describe the average QPX infection intensity determined by qPCR in each group of clams, QPX infection intensity in each individual clam was rated based on the estimated number of QPX cells in each milligram mantle tissue, from 0 (below detection limit of qPCR assay), 1 (rare infection, detection limit to 10), 2 (light infection, 11-100), 3 (moderate infection, 101-1000), to 4 (heavy infection, >1001). QPX weighted prevalence was then calculated by dividing the sum of individual QPX infection intensity by the total number of clams examined.

Statistical analysis

Prevalence and mortality values were arcsine transformed. Paired t-tests were performed to determine if there was a significant difference between prevalence determined by histological and qPCR methods for each site. A Spearman Rank correlation test was performed to examine the correlation between the prevalence data generated by these two diagnosis methods. Water temperature measured by a nearby USGS weather station (USGS 01407081 Raritan Bay at Keansburg, New Jersey, Figure 1) up to 150 days before the sampling date was tested for time-lagged correlation with weighted prevalence. Dissolved oxygen and mortality were not tested for time-offset correlations due to insufficient data. All differences were considered statistically significant at $p < 0.05$.

Results

Environmental conditions

In 2006, bottom seawater temperature at Raritan Bay sites RB1, RB2 and RB3 varied seasonally from 9.3 °C to 22.7 °C during the study period (Table 1). At site RB4, which is located inside Great Kills Harbor (Figure 1), the temperature was up to 4 °C higher than the other 3 sites. Salinity ranged from 22.8 to 25.7, and the salinity of site RB4 was not significantly different from the other sites (data not shown). The dissolved oxygen concentrations of bottom water at sites RB1, RB2 and RB3 ranged from 12.1 mg L^{-1} in

April to 4.7 mg L^{-1} in September (Table 1). At site RB4, dissolved oxygen concentrations in June and August were up to 4.4 mg L^{-1} less than the average dissolved oxygen concentration at the other three sites (minimum of 2.1 in August), but were similar to the other sites in April, May and September.

QPX prevalence

Fifteen groups of clams (~30 clams per group) collected on 5 sampling dates in 2006 from three Raritan Bay sites (RB1, RB2 and RB3) were analyzed by both histological and qPCR methods (Figure 2). At sites RB1 and RB3, no QPX was detected by histology in clams sampled in April (Figure 2A and 2B) and the highest histological prevalence for both sites was observed in August (6.6% at site RB1 and 13.3% at site RB3), then decreased again in September. At both sites, QPX prevalence determined by qPCR assay was significantly greater than prevalence determined by histology (Student's t-test, $p<0.01$), but the two were also significantly correlated (Spearman Rank correlation test, $p<0.05$), revealing a similar temporal pattern. QPX infection was detected by qPCR in April (Figure 2D and 2E) with a relatively low prevalence (3.3% at site RB1 and 10% at site RB3), and prevalence reached a peak in August (20% at site RB1 and 40% at site RB3), then declined in September. Although these two sites generally showed a similar temporal pattern, prevalence dropped to 0 by histology (Figure 2A) and 3.4% by qPCR (Figure 2D) at site RB1 from May to June, while QPX prevalence by histology was unchanged and by qPCR almost tripled at site RB3 (Figure 2E) during the same time.

QPX prevalence determined by histology showed a different temporal pattern at site RB2 (Figure 2C), with the highest prevalence (13.2%) in April and the lowest in August. As at sites RB1 and RB3, qPCR assay detected a significantly higher prevalence (Student's test, $p=0.012$) than histology at site RB2. Although the correlation between qPCR prevalence determined by histology and qPCR was not statistically significant, the seasonal pattern was similar, with the highest (20.7%-23.3%) prevalence detected by qPCR in April and June, followed by 13.3%-13.7% in May and August, and 10% in September (Figure 2F). The QPX prevalence determined by qPCR at site RB2 showed less variation over time than sites RB1 and RB3.

Clams collected from site RB4 in May and August (30 clams per group) were also examined by both histological and qPCR assays. No QPX infection was detected in these clams by either assay.

QPX infection intensity

Like prevalence, the average QPX infection intensity determined by qPCR, evaluated by weighted prevalence, showed a similar temporal pattern at sites RB1 and RB3 (Figure 2D and 2E). At both sites, weighted prevalence increased from April through August reflecting changes in both prevalence and infection intensity (except an interruption by the decline of QPX prevalence in June at site RB1). Weighted prevalence was lowest in April when only rare QPX infection intensities (0.5-10 cells mg^{-1} tissue) were detected in QPX-positive clams. Weighted prevalence increased in May, due to the appearance of more clams with greater infection intensities (10-100 and/or 101-1000 cells mg^{-1} tissue), and reached its peak in August when more QPX-positive clams were detected, including some with heavy infection intensities (>1001 cells mg^{-1} tissue). From August to September, weighted prevalence declined as no heavily infected individuals were detected and rare to moderate infections were also detected in fewer clams. It should be noted that weighted prevalence dropped to the same value in September at both sites, though clams at site RB1 exhibited a lower prevalence, but with heavier infections than clams at site RB3.

The pattern of QPX infection intensity in clams at site RB2 was different from site RB1 and RB3 (Figure 2F). Weighted prevalence was highest in April through June. From June to August, in contrast to the increasing weighted prevalence observed at sites RB1 and RB3, clams at site RB2 exhibited a decrease in QPX infection intensity, due to the absence of detection of moderately and heavily infected clams.

Relationship of QPX disease to environmental conditions

Correlation analysis showed different lag times between QPX weighted prevalence determined by qPCR and water temperature at each site (Figure 3). The best fit (maximum R^2) at site RB3 (R^2=0.961) had no time lag, and the best fit (R^2~0.6) at site RB1 had a 0 to 30 day lag, while QPX weighted prevalence at site RB2 was best correlated with the temperature 120 days before the clam sampling date (R^2=0.986).

Dissolved oxygen concentration and mortality recorded on the sampling date were not significantly correlated with weighted QPX prevalence at any site (data not shown).

Discussion

Hard clams at Raritan Bay sites RB1 and RB3, except for the June sample at RB1, showed an increase in both prevalence and intensity of QPX infection from spring into summer and then a decline into the fall (Figure 2). This pattern was consistent with the seasonal pattern in QPX prevalence determined by histological analysis at the same sites from 2002 to 2005 (Allam and Pawagi 2005). Increases in infection intensity presumably reflect progression of infections from lighter to heavier, while increases in prevalence could reflect either the acquisition of new infections or the progression of previously undetectable infections. Decreases in prevalence and infection intensity could reflect the death of heavily infected clams, the partial or complete healing of infected clams, or a combination of both. The direct relationship between weighted prevalence and current or recent (up to 30 days prior) temperature for sites RB1 and RB3 (Figure 3) suggests that temperature may be an important environmental factor regulating seasonal progression of QPX disease. This is not surprising since temperature is well known to modulate host-parasite interactions in several bivalve species such as the oyster *Crassostrea virginica* (Audemard *et al*., 2006; Burreson and Ragone Calvo, 1996; Oliver *et al*., 1998; Ragone Calvo *et al*., 2003), the clam *Ruditapes philippinarum* (Paillard *et al*., 2004) and the cockle *Cerastoderma edule* (Desclaux *et al*., 2004). In the specific case of QPX, Perrigault *et al*. (2010) found that *in vitro* cultured parasite cells grew best in the range between 17 and 23 °C. In contrast, laboratory experiments have shown higher QPX disease prevalence and intensity in clams kept at 13 °C for 4 months as compared to those held at 21 °C or 27 °C, as well as a reduction of QPX prevalence in clams transferred from 13 °C to 21 °C (Dahl *et al*. 2011; Perrigault *et al*. 2011), indicating a major effect of temperature on clam immunity and resistance to QPX disease. Importantly, in laboratory investigations, several months were needed for QPX to establish histologically detectable infections and progress to mortality (Dahl *et al*., 2008, Dahl *et al*., 2011). Consistent with these laboratory results, at sites RB1 and RB3 QPX infections became more numerous and intense through late spring and early summer, when bottom water temperature was below 21 °C (Table 1), while QPX infections

became less numerous and intense at sites RB1 and RB3 from August to September (Fig. 2), when bottom water temperature was above 21 °C (Table 1). At the same time that development of QPX disease is slower and clams with lighter infections can heal at warmer temperatures (Dahl *et al*., 2011; Perrigault *et al*., 2011), clams with heavier infections may die because of the combined effect of QPX infection and natural stressors such as increased metabolic demands at higher temperature. Increasing metabolic demands during summer have been recognized as an aggravating factor for infectious diseases in several marine mollusks such as abalone (Travers *et al*., 2008) and oysters (Li *et al*., 2009; Samain *et al*., 2007; Sauvage *et al*., 2009). Mortality was higher at both RB1 and RB3 in August than in September (Table 1). Thus, the absence of clams with the heaviest infections (by qPCR) in the September sample could be related to the death of the most severely infected clams present in August. The concurrent decrease in clams with all intensity levels of infection could also reflect healing of lighter infections.

The general pattern of QPX prevalence and weighted prevalence was different at site RB2, where the highest QPX prevalence and infection intensity were observed in April to June (Figure 2). The relationship between weighted prevalence and temperature was also different at site RB2, with the best correlation found with a 120 day lag (Figure 3). It is unclear what might cause two different seasonal patterns in three sampling sites which experienced similar environmental conditions, but it appears that some other factor(s) were more important than recent temperature at RB2. RB2 had greater clam density, estimated as 90 clams m^{-2} versus 30 clams m^{-2} or less at the RB1 and RB3 (Table 1). Positive correlations between clam density and QPX prevalence have been reported in wild (Allam and Pawagi, 2004) and cultured (Ford *et al*., 2002) clam populations. Lyons *et al*. (2007) also suggested that clam density could be important in the transmission of QPX, and Dahl and Allam (2015) showed a reduction in QPX disease prevalence in a field study after clam density was experimentally reduced. At site RB2, infection acquisition and progression may start earlier because the higher clam density may increase the chances of QPX spreading from one clam to another. Physiological stress associated with high clam density may also result in less healing and more rapid disease progression, which may cause QPX-infected over-winter survivors to more quickly develop the heavier infections found in April samples. Mortality at RB2 in April was the

highest recorded (Table 1), with over 20% of the recovered shells from recently dead clams, consistent with greater stress and disease at this site. Future studies that include sampling during the late winter/early spring months would be required to examine these possible differences between sites. Overall, our findings may indicate that site RB2 serves as a reservoir for QPX, although this scenario remains highly speculative until a higher resolution picture of disease pattern between fall and the next spring is established.

Interestingly, QPX disease has not been detected in this study or in previous surveys (over 800 clams processed by histopathology; Allam, unpublished data) at site RB4, which is located in a relatively shallow embayment (Great Kills Harbor in Staten Island, NY) and harbors a very high clam density (~370 clams m-2) (Figure 1, Table 1). During our sampling in 2006, water temperature at site RB4 was always higher, and dissolved oxygen concentration was lower in summer, compared to other sampling sites in Raritan Bay. High temperature at this site may be related to the lack of disease since previous experimental data showed a reduction in disease prevalence and intensity when clams are exposed to chronic or acute pulses of increased temperature (Dahl *et al*. 2011; Dahl *et al*., 2015; Wang *et al*., 2014). Nevertheless, the factors that impede transmission of QPX or enhance the resistance of clams at this site are worth further study.

Significant mortalities from epizootics of QPX disease have been observed in hard clam aquaculture plantings in parts of Atlantic Canada, Massachusetts, New Jersey and Virginia (Lyons *et al.*, 2007; Ragone Calvo *et al.*, 1998; Ragone Calvo *et al.*, 2007; Smolowitz *et al.*, 1998). Previous field observations in the Raritan Bay area reported high clam mortality associated with severe QPX infection during summer (Dove *et al*., 2004). Although there were some intriguing patterns in the mortality data (see above), overall, clam mortality estimated during sample collection was not significantly correlated with QPX prevalence or weighted prevalence at any site (Spearman Rank correlation test). Moreover, there was no significant correlation between mortality and any environmental parameter, including temperature (current or previous) and dissolved oxygen (data not shown). The differences in estimated clam mortality between RB1 and RB3 in May and June, and low estimated mortality in September at all sites (Table 1), suggest that other factors affect either mortality itself or our ability to estimate mortality. It is difficult to estimate hard clam mortality in the field: moribund hard clams tend to rise to the

sediment surface, but the time required for this process is unknown and may vary seasonally; additionally, the fragile shells of some small dead clams may disintegrate quickly and be lost from mortality counts by the time of sampling. Even in previous studies (Ragone Calvo *et al*., 2007; Kraeuter *et al*., 2012) using aquacultured clams where mortalities are relatively easier to approximate, there was still no definitive relationship between QPX infection and mortality: high mortality was often found associated with low QPX prevalence and intensity, suggesting that clam mortality could be the result of complex interactions of QPX infection with stressful environmental conditions and/or other unidentified factors.

Our data showed seasonal patterns of QPX disease in wild clams but also demonstrated that the seasonal pattern may vary from site to site, possibly linked to the clam density and mortality in the field. It is impossible to compare our results to previous studies because no prior seasonal surveys of QPX infections in wild clams exist. Previous studies in aquacultured clams did not show a clear seasonal pattern in disease development in different clam strains planted in experimental plots in Massachusetts, New Jersey and Virginia (Ragone Calvo *et al*., 2007; Kraeuter *et al*., 2012), although differential susceptibility of various clam strains toward the infection may confound disease development patterns as suggested by the findings of Dahl *et al*. (2010). Another factor that may lead to different disease patterns in the aquacultured stocks monitored by Ragone Calvo *et al*. (2007) is clam density, which was more than 500 clams/m^2, 5-20 times higher than the natural clam densities in our QPX-positive Raritan Bay sites. Further experiments focusing on environmental factors, clam density and clam mortality as related to disease prevalence and intensity will be important to differentiate the factors responsible for differences in transmission and development of QPX disease in clams and to better characterize the seasonality of QPX disease.

Acknowledgements

This research is a resulting product from projects R/XG-15, R/FBM-33 and RX/G-19 funded under awards NA16RG1645 and NA07OAR4170010 from the National Sea Grant College Program of the U.S. Department of Commerce's National Oceanic and Atmospheric Administration, to the Research Foundation of the State University of New

York on behalf of New York Sea Grant. The statements, findings, conclusions, views and recommendations are those of the authors and do not necessarily reflect the views of any of those organizations. The qPCR assays were performed in the Molecular Evolution of Adaptation and Diversity (MEAD) lab, founded at Stony Brook University with funding from the National Science Foundation (DBI-0400829) to JLC and John True. The authors thank the New York State Department of Environmental Conservation for field support and staff of the Marine Animal Disease Laboratory for help with sample processing.

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Figure Legend

Figure 1. Locations of the Raritan Bay (RB) sampling sites south of Staten Island (NY). The star indicates the location of the USGS weather station at Keansburg, New Jersey.

Figure 2. QPX prevalence and infection intensity determined by histopathology (A, B, C) and qPCR assay (D, E, F) at site RB1, RB3 and RB2. Numbers in stacked columns represent the percentage of clams with different infection intensities determined by each method. The total prevalence determined by each method is the sum of all numbers in the column. The number on the top of each qPCR column indicates the weighted prevalence.

Figure 3. R^2 values for correlation between average daily water temperature and QPX weighted prevalence determined by qPCR assay at three sampling locations in Raritan Bay. Temperature used is daily mean value for each day for the previous 6 years (calculated from 10/01/2000 to 09/30/2006), measured at USGS 01407081 Raritan Bay weather station at Keansburg NJ (Figure 1)

Table 1. Characteristics of sampling sites in Raritan Bay (RB), New York, in 2006

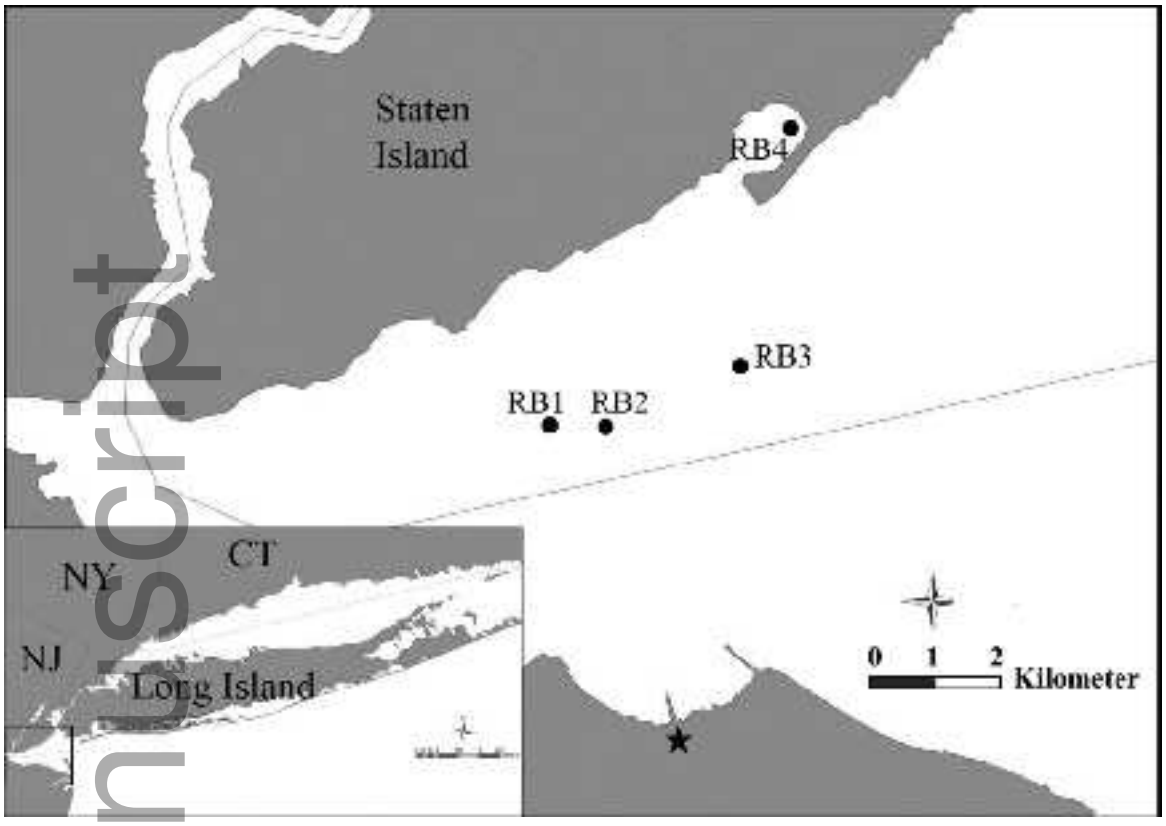
Sampling site	Bottom water temperature (ºC)					Dissolved oxygen concentration (mg L^{-1})					Estimated clam mortality (%)					Clam density (clams/m^2) [b]	Historical QPX prevalence (%)[c]
	Apr[a]	May	Jun	Aug	Sep	Apr	May	Jun	Aug	Sep	Apr	May	June	Aug	Sep		
RB1	9.6	15.5	20.7	22.6	21.3	12.1	5.7	5.2	6.2	4.7	6	11.3	10.3	9.8	1.5	30 ± 14	9.2 ± 6.6
RB2	9.5	15	20.4	22.7	22	12.0	6.1	4.9	5.8	7.5	23.3	13.2	10.2	9.2	1.1	93 ± 21	6.7 ± 7.1
RB3	9.3	15.5	20	22.3	22.4	11.3	7.3	5.0	7.6	8.8	4.9	2.4	0	10	0	14 ± 4	4.2 ± 4.4
RB4	10.3	16.5	24	24.2	22.6	10.1	6.0	2.3	2.1	8.9	n/a[d]	5.7	2.3	n/a	n/a	369 ± 152	0

[a] The actual sampling dates were April 11, May 17, June 9, August 9 and September 19 in 2006.

[b] Average estimated density (Mean ± standard deviation) over all sampling dates in 2006. Clam density was estimated as (# clams $_{\text{live}}$ + #clam $_{\text{newly dead}}$)/# grab.

[c] Average histological QPX prevalence (Mean ± STD) over all samples from 2002 to 2006; data from Allam and Pawagi (2006).

[d] mortality data not available



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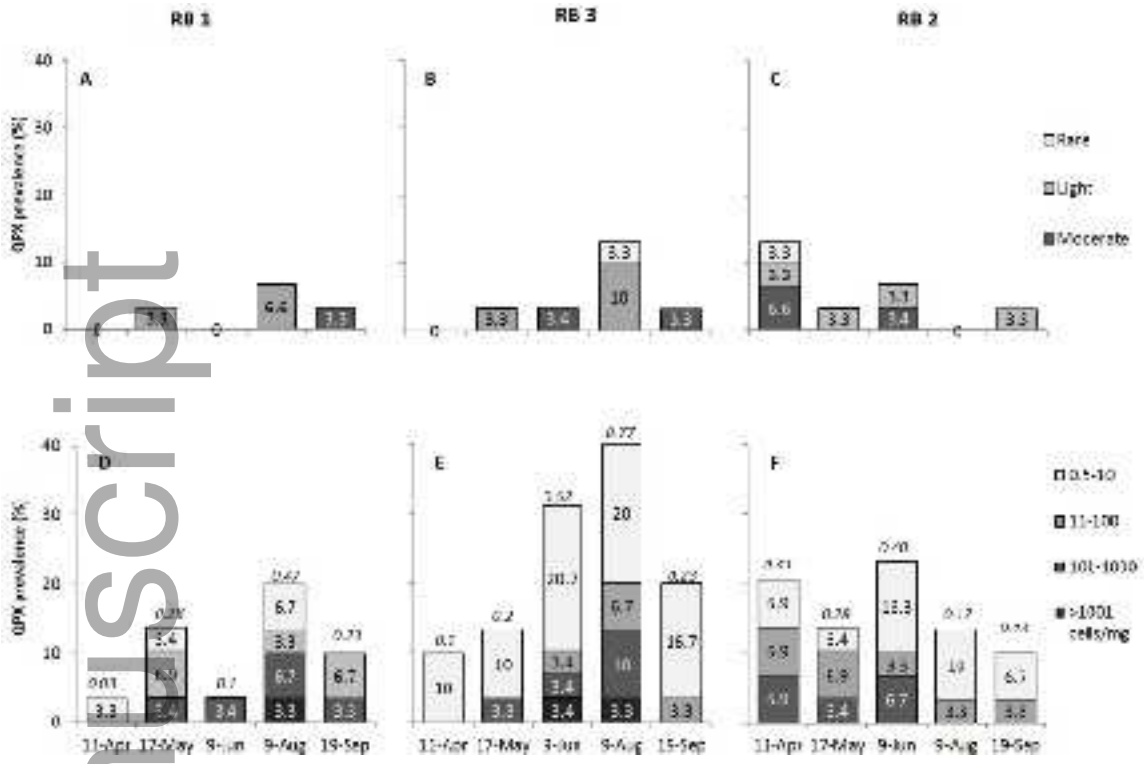

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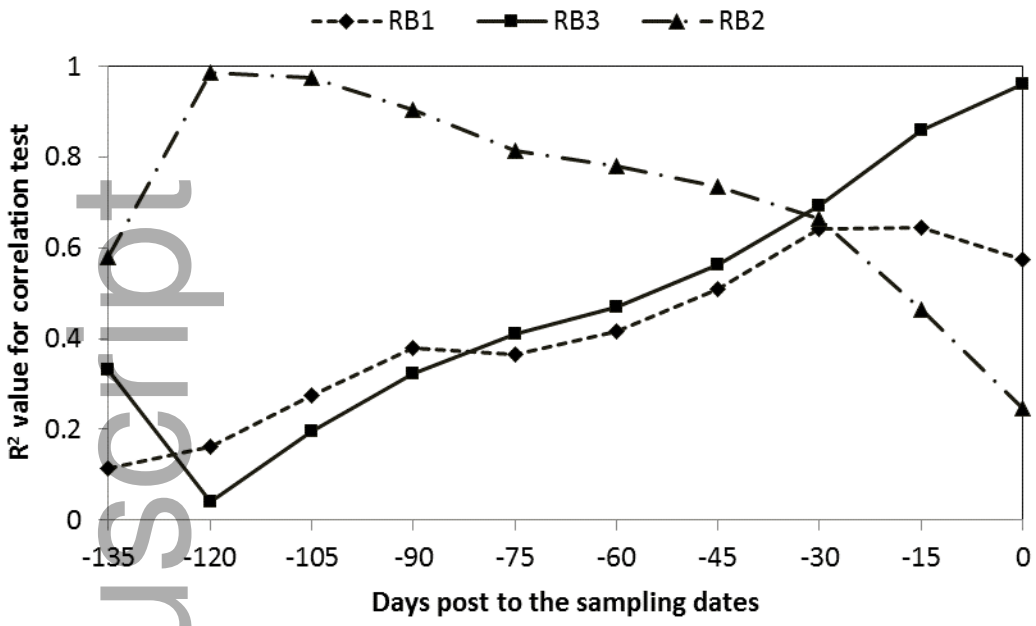

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