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Environmental distribution and persistence of Quahog Parasite Unknown (QPX)

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ABSTRACT: Quahog Parasite Unknown (QPX) is the cause of mass mortality events of hard clams *Mercenaria mercenaria* from Virginia, USA, to New Brunswick, Canada. Aquaculture areas in Massachusetts, USA, have been particularly hard hit. The parasite has been shown to be a directly infective organism, but it is unclear whether it could exist or persist outside of its clam host. We used molecular methods to examine water, sediment, seaweeds, seagrass and various invertebrates for the presence of QPX. Sites in Virginia and Massachusetts were selected based upon the incidence of QPX-induced clam die-offs, and they were monitored seasonally. QPX was detectable in almost all of our different sample types from Massachusetts, indicating that the parasite was widely distributed in the environment. Significantly more samples from Massachusetts were positive than from Virginia, and there was a seasonal pattern to the types of samples positive from Massachusetts. The data suggest that, although it may be difficult to completely eradicate QPX from the environment, it may be possible to keep the incidence of disease under control through good plot husbandry and the removal of infected and dying clams.

KEY WORDS: Quahog Parasite Unknown · QPX · Environmental detection · Remediation

INTRODUCTION

The culture of marine shellfish makes up a large percentage of seafood production and is the fastest growing segment within the United States aquaculture industry. Bivalve mollusks, including clams, are the primary shellfish species produced and routinely compete with salmon as the highest valued marine product. In 2003, clam aquaculture production in the USA was estimated at approximately 36.7 million dollars (Olin 2006). In addition to the commercial importance clams have in the seafood market, they have also received significant attention as a species for stock enhancement. Clams are an essential component of the marine environment, and several restoration efforts designed to supplement wild populations are underway in the USA. However, just as is the case for several other important commercially grown species (e.g. oysters), disease has become a significant bottleneck for successful production and is potentially the largest threat to wild populations besides overfishing.

One of the deadliest diseases associated with clams is caused by an organism referred to as Quahog Parasite Unknown (QPX), a directly infective protistan parasite of the hard clam or northern quahog *Mercenaria mercenaria*. This labyrinthomorphid protist was first reported in the 1960s and, since its discovery, has caused significant economic damage to hard clam aquaculture from New Brunswick, Canada to Virginia, USA (Whyte et al. 1994, Ragone-Calvo et al. 1998, Smolowitz et al. 1998, Dove et al. 2004). QPX disease can result in devastating mortality events in clam populations (less than 10% survival), and death usually occurs when the clams are approaching the final

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stages of growth prior to market distribution (Smolowitz et al. 1998). QPX epizootics have decimated clam-growing areas, resulting in cessation of local aquaculture development in some regions. In other locales, following detection of QPX disease in selected clam beds, shellfish growers voluntarily destroyed potentially infected seed clams, worth hundreds of thousands of dollars in marketable clams, in an attempt to minimize the impact of the disease.

Recent studies of the parasite have reported on the development of molecular methods to detect its presence (Ragone Calvo et al. 2001, Stokes et al. 2002, Lyons et al. 2005a, 2006, Gast et al. 2006), the mechanisms of transmission (Smolowitz et al. 2001, Ford et al. 2002, Dahl & Allam 2007), the general lack of genetic variation between isolates (Qian et al. 2007) and the potential for macroalgae to support growth of the parasite (Lyons et al. 2005b, Bugge & Allam 2007). Currently, there are also studies in progress to elucidate aspects of the parasite physiology, pathogenicity and disease development in clams. The identification of the QPX parasite in environmental reservoirs may provide a better understanding of the associated disease and allow development of more appropriate clam aquaculture management techniques. There has been no reasonable way to identify the parasite in water or sediment samples, and therefore it was not known if QPX persisted in the environment outside of clams or whether it was also found in other organisms. Molecular detection tools are appropriate for searching for QPX in various environments and other species, since these tools are specific to all potential QPX life stages, regardless of host or reservoir species. We used an improved method for the molecular detection of the protist (Gast et al. 2006), along with *in situ* hybridization (Stokes et al. 2002), to investigate the distribution of the QPX organism in environmental samples from impacted regions in Massachusetts and Virginia.

MATERIALS AND METHODS

Environmental samples and plots. Samples of seawater, sediment, invertebrates, seagrass and algae were collected at sites near clam beds in both Massachusetts (MA) and Virginia (VA) during 2004 and 2005.

Virginia: During each year of the study, 4 sites were sampled along the Eastern Shore of Virginia. In 2004, the studied sites were Wachapreague and Smith Island, where QPX infections had previously been reported in local clam populations, and Cherrystone and Metompkin, sites that had no prior evidence of QPX infections. All sites were located on the Eastern Shore seaside, except for Cherrystone, which was located on Chesapeake Bay. The sites differed in their salinity regime, with Wachapreague and Smith Island being exposed to high salinity (>25 psu), and Cherrystone and Metompkin to moderate salinity (15 to 25 psu) conditions. In 2004, all 4 sites were sampled in the summer, and Wachapreague and Cherrystone were sampled again in the fall. In 2005, the Metompkin site was replaced by a clam bed known as Plantation Creek, located on the Chesapeake Bay side and with a higher salinity (20 to 25 psu). These sites were sampled once in the summer and once in the fall. Samples collected from each site during the first study year included clams, water samples, surface sediment, materials scraped from shell surfaces, gastropods, crustaceans, bryozoans, sponges and macrophytes (Table 1). Based on results obtained during the first year, environmental samples collected during the second year were limited to clams, algae, water and sediment samples, and materials scraped from surfaces.

Massachusetts: We sampled 2 sites on Cape Cod monthly from April to November 2004, and then again in April and June 2005. It was not possible to access our sites during the winter months because of ice. Barnstable Harbor is an area of intensive quahog aquaculture that suffers annual outbreaks of QPX disease. Pleasant Bay is also an active aquaculture site, but there has not been a large-scale outbreak in several years. All sites had salinities >26 psu (usually between 28 and 30) and temperatures that ranged from below 0°C in January and February to 25°C (or higher) in July and August in Barnstable Harbor. Pleasant Bay temperatures were between 20 and 25°C during the summer of 2004, which was generally about 5°C less than Barnstable Harbor. At these sites, we placed 2 sets of 2 nets (1.25 × 1.85 m in size) to sample the flora and fauna that accumulate on aquaculture structures (nets) without having to sample from growers' plots. In each harbor, we identified a positive site as being in close proximity to plots of infected clams (or previously infected clams) and a negative site as being distant from any active or previous infections. Clams were not planted under the nets.

A third site on Cape Cod, Wellfleet Harbor, was sampled in the summers of 2005 and 2006 in response to their first reported QPX-associated die-off in late fall of 2004, which was followed by an extensive clean-up effort by many of the fishermen. Growers in Wellfleet Harbor removed all clams from plots showing signs of QPX infection and then left those plots empty for the following 2 yr. In the summer of 2005, samples of seawater, sediment, seaweeds, seagrasses and shells were collected from 2 of the sites where infections were detected, and from 1 site with no history of QPX infection. In the summer of 2006, only samples of seawater and sediment were collected from 6 sites — 2 were the same QPX-positive sites

Table 1. Types of environmental samples collected in Massachusetts and Virginia

Plant and algal samples

Marsh grass	Green algae	Brown algae	Red algae
Ruppia maritima	*Bryopsis plumose*	*Acrothrix gracilis*	*Agardhiella subulata*
Spartina sp.	*Bryposis hypnoides*	*Ectocarpus* sp.	*Callithamnion pseudobyssoides*
Zostera sp.	*Cladorphora* sp.	*Fucus vesiclosis*	*Ceramium* sp.
	Codium fragile spp.	*Laminaria saccharina*	*Chondira* sp.
	tomentosoides		*Centroceras clayulatum*
	Enteromorpha intestinalis		*Ceramium cimbricum*
	Ulva lactuca		*Dasya baillouviana*
			Gracilaria sp.
			Hypnea sp.
			Lomentaria baileyana
			Polysiphonia denudata
			Polysiphonia harveyi
			Polysiphonia sp.
			Spermothamnion repens

Invertebrate samples

Bivalves	Gastropods	Miscellaneous invertebrates	Ascidians (mixed)	Amphipods
Clinocardium ciliatum (cockle)	*Crepidula* sp. (slipper shell)	Annelids	*Botryllus* sp.	Unidentified species
Ensis directus (razor clam)	*Ilyanassa obsoleta* (mudsnail)	*Balanus* sp. (barnacles)	Unidentified species	
Mercenaria mercenaria	*Littorina littorea* (periwinkle)	*Cancer* sp. (crab)		
Mya arenaria (soft shell clam)	*Lunatia* sp. (moon snail)	*Eurypanopeus* sp. (mud crab)		
Mytilus edulis (blue mussel)	Whelk	Polychaetes		
		Pagurus sp. (hermit crab)		
		Paleomonetes sp. (glass shrimp)		

from 2005, 2 were additional sites where QPX was detected and remediated, and 2 sites were treated as negative controls, due to a lack of aquaculture activity. Salinity levels ranged from 20 psu at a negative site to 27 to 29 psu at the harbor sites.

Sample processing: All samples were collected in the field and placed in coolers for transportation back to the laboratory. For the MA sites, seawater and sediment samples were processed within 24 h of collection, while samples of shells, invertebrates and aquatic vegetation were either processed the same day or stored at 4°C and processed within 2 d. For the VA sites, samples were brought back to the laboratory, identified and placed at –20°C until DNA extraction. Seawater was collected in sterile, acid washed 1 l polycarbonate bottles. Following this, 1 l (MA) or 2 replicate samples of 100 ml (VA) were filtered onto 47 mm diameter, 0.8 µm (MA) or 1.2 µm (VA) pore size polycarbonate filters. Sediment samples were collected using 50 ml conical tubes as coring devices, and 0.25 to 0.5 g of the top 3 mm was subsampled. Seaweeds and seagrasses (macrophytes) were sampled by macerating or scraping the surface of a piece approximately 1 to 2 cm long with sterile razor blades. In MA, scraped material was recovered using sterile cotton swabs. The cotton portion of the swab was removed, placed in sterile 1.5 ml microcentrifuge tubes and frozen. Macerated algal material was collected using sterile spatulas, placed in sterile 1.5 ml microcentrifuge tubes and frozen. Surfaces of shells from live and dead bivalves and gastropods were swabbed with sterile cotton swabs, and the cotton portion removed and placed in sterile 1.5 ml microcentrifuge tubes and frozen. In VA, scraped material and surfaces of shells were recovered with sterile blades, and the collected material was placed in sterile 1.5 ml microcentrifuge tubes and frozen. Snails were removed from their shells, rinsed in sterile water, placed in microcentrifuge tubes and frozen. Bivalve shells were rinsed with water and ethanol and then shucked. Mantle or gut tissue (0.5 g) was removed using sterile razor blades, placed in microcentrifuge tubes and frozen. For hard clams, we collected mantle tissue near the incurrent siphon. For razor clams and mussels, we collected mantle and gut tissue. Clams collected in VA were examined by histology to determine QPX prevalence and average infection intensity. Infection intensity was categorized as described by

Ragone-Calvo et al. (1998), where infection intensity is based on the number of parasite cells per total tissue section area: rare (1 to 10 cells), light (11 to 100 cells), moderate (101 to 1000 cells) and heavy (>1000 cells).

Nucleic acid extraction and nested PCR amplification. Seawater, seaweeds, seagrasses, shell swabs and invertebrates from MA were processed for the extraction of nucleic acids using the hot detergent method (Gast et al. 2004). Nucleic acids were extracted from MA sediment samples using a UltraClean™ soil or PowerSoil™ DNA kit (Mo Bio Laboratories) within 12 h of the sediment collection and without freezing the sample. The QIAamp® stool kit (Qiagen) was used to extract nucleic acids from samples collected in VA.

A positive control for the QPX organism was generated from 1 ml of washed QPX culture (Anderson et al. 2003). The cells were pelleted at 14 000 rpm for 10 min, then nucleic acids were extracted following the hot detergent protocol for seawater samples (Gast et al. 2004). Extract (1 µl) was used in amplifications to establish the correct size for the cultured QPX PCR product on agarose gels and to generate a positive marker for the QPX organism on denaturing gradient gels.

A nested PCR amplification detection procedure, previously described by Gast et al. (2006), was used to analyze the DNA of collected samples. Briefly, 3 rounds of PCR amplification were done, with the final round generating a product for denaturing gradient gel electrophoresis (DGGE) analysis. Negative controls for contamination without added template DNA were run with every PCR experiment. The primers used range from selective for the thraustochytrid group (Mo et al. 2002) to specific for the QPX organism (Stokes et al. 2002). Products from the DGGE amplification were detected on 1% agarose gels stained with ethidium bromide prior to confirmation as QPX on denaturing gels.

Denaturing gradient gel electrophoresis. Products from 960GC/QPXR2 amplification were precipitated overnight at –20°C with 0.3 M sodium acetate and 0.6 volumes of isopropanol. Products were pelleted by microcentrifugation at maximum speed (usually 15 000 × *g*) for 10 min. The pellets were allowed to air dry before resuspension in 5 µl of sterile distilled water and 5 µl of DGGE loading dye (40% Ficoll 400, 10 mM Tris pH 7.8, 1 mM EDTA, 0.1% bromphenolblue). A volume of 3 to 5 µl was loaded per lane on the gel. DGGE gels were run on 8% acrylamide with a denaturing gradient of 45 to 75% (100% denaturing defined as 7 M urea and 40% formamide) at 60°C and 95 V overnight (16 h) using the CBS Scientific model DGGE-2000 gel apparatus. Bands were visualized by staining the gel in 1× TAE (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA, pH adjusted to 7.4 with acetic acid) with ethidium bromide for 10 min, followed by 20 min destaining in distilled water. Digital images were obtained using the ChemImager™ System (Alpha Innotech).

Sequence analysis. Bands were recovered from the DGGE gel by touching the band with a sterile, aerosol resistant pipette tip, and then pipetting up and down several times in 5 µl of sterile distilled water. Using 960fb (non-GC clamped primer) and QPXR2, 2 µl were reamplified. These products were precipitated with isopropanol as described above to remove excess primers, then resuspended in 10 µl of sterile Milli-Q water. We sequenced 5 µl of product directly using ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Mix and the 960fb or QPXR2 primers. Sequencing reactions were run on an ABI 377 (PE Applied Biosystems) and analyzed using Sequencher™ 4.2.2 (Gene Codes Corporation).

***In situ* hybridization.** Only samples that were determined to be positive for the presence of QPX via nested amplification and DGGE were examined by *in situ* hybridization (ISH). We chose to examine samples from MA from October, August and November 2004. Samples were either directly shared for both molecular analysis and ISH by dividing a collected specimen in half, or duplicates were collected near each other in the field. Tissues to be stained by ISH were fixed in 10% formalin in sea water (Howard et al. 2004). Two types of preparations were made for ISH staining. First, superficial scrapings of the macrophytes and snail shells or 1 ml of the fixed detritus were suspended in 1 ml of sterile seawater. This was centrifuged at 5000 × *g* for 2 min, then washed twice in sterile phosphate buffered saline (PBS), centrifuged, and washed again. The resulting plug of material was prepared for ISH as previously described (Lyons et al. 2005a). Material was smeared evenly on Superfrost®/ Plus microscope slides (Fisherbrand), air dried and then stained using the ISH staining method (Stokes et al. 2002). Alternately, fixed tissues from macrophytes and snails were decalcified using formic acid, and then, if needed, processed in paraffin, sectioned at 6 µm, deparaffinated (Howard et al. 2004) and stained using the ISH method. Resulting slides were examined and photographed using a Zeiss Axioskop2 with attached digital camera.

The ISH staining procedure used to stain QPX organisms was previously described by Stokes et al. (2002). Briefly, the method uses a cocktail of 2 digoxigenin-labeled DNA oligonucleotide probes (4 ng μl^{-1} each of QPX 641 and QPX1318) that target the small subunit ribosomal RNA of the QPX organism. For a negative control, hybridization buffer was substituted for the probe. Nitroblue tetrazolium and 5-bromo-

4-chloro-3-indoyl phosphate are used to develop color after probe incubation. Color development was stopped by rinsing in TE buffer. Counterstaining was not used because the samples (macrophytes, detritus, decalcified shells) could be seen well without it. Slides were coverslipped using an aqueous mounting medium and the edges of slips were sealed with nail polish.

Statistical analysis. Differences between the prevalence of positive results for each location, month and year of sample collection, and sample type were evaluated based on the counts for each category (positive and negative) with the nonparametric chi-square test of independence (χ^2). Yates continuity correction was applied for 2×2 contingency tables. Fisher's exact test was used when the sample size was small or when the minimum expected counts were <5. In all cases, the null hypothesis (H_0: no difference between observed frequency and expected frequency) assumed an even distribution of results among categories. Statistical tests were conducted using SPSS software.

RESULTS

Environmental samples collected

In our effort to document the presence of the QPX organism in the environment, seawater, and sediment, many different types of samples were collected for testing (Table 1). These environmental samples represented the organisms present at each site on the sampling date, and were not necessarily the same between sites or months. For example, Barnstable Harbor had large numbers of mudsnails, while Pleasant Bay did not. Macrophyte species (including seaweeds and seagrasses) collected also varied depending on the site and the season. In total, 779 samples were collected and analyzed from MA (403 at Barnstable Harbor, 196 at Pleasant Bay, and 180 at Wellfleet Harbor), and a total of 605 samples were collected and analyzed from the VA sites (182 at Wachapreague, 161 at Cherrystone, 146 at Smith Island, 61 at Plantation Creek, and 55 at Metompkin).

Sample analyses

Samples were counted as being positive for QPX if an amplification product of the correct size (approximately 250 bp) was observed on an agarose gel after the nested PCR amplification process. Confirmation that the PCR products were from the QPX organism was accomplished by running the products on DGGE gels, using an amplified PCR product from the QPX culture as a positive control (Fig. 1). All of the positive samples from MA had bands that matched the QPX control (Fig. 1A). Sometimes additional bands were present that yielded sequences very similar to the QPX target. Only 7 of the VA samples gave amplification products by nested PCR, several of which tended to show different bands than the QPX control on both agarose and DGGE gels.

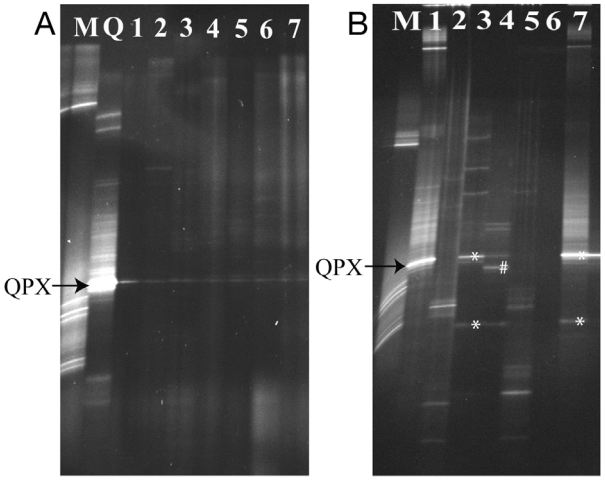

Fig. 1. Denaturing gradient gel results. M = gel markers, Q = QPX band from culture. (A) QPX positive environmental samples from April 2004 in Barnstable Harbor. Lane 1: shrimp swab; lane 2: *Mytilus edulis* shell swab; lane 3, lane 4: *M. edulis* guts (note: same animal sampled for lanes 2 , 3 and 4), lane 5: razor clam shell swab; lane 6: *Codium fragile* spp.; lane 7: *Mytilus* guts. (B) PCR positive environmental samples from Virginia. Lane 1: *C. fragile* spp. from Wachapreague July 2004; lane 2: *Agardhiella subulata* from Smith Island August 2004; lane 3: *Ceramium* sp. from Smith Island August 2004; lane 4, lane 5: *Ilyanassa obsoleta* shell swab from Smith Island August 2004; lane 6: sediment from Metompkin August 2004; lane 7: sediment from Smith Island August 2004. *: bands most similar to QPX; #: band with sequence most similar to uncultured thraustochytrid by BLAST analysis

Virginia

Table 2 shows the QPX prevalence and infection intensity in clams collected from the 5 VA sites. None of the environmental samples collected in 2005 or the fall of 2004 were positive for QPX, but 3 sites yielded putatively positive samples in the summer of 2004. The site of moderate QPX infection intensity in clams was Wachapreague, but only 1 environmental sample was positive (green alga, *Codium* sp. sample, Fig. 1B, lane 1). Smith Island had clams with light QPX infection intensity and yielded 5 potentially positive environmental samples in 2004. These included a sediment sample (Fig. 1B, lane 7), 2 gastropod *Ilyanassa obsoleta*

Table 2. QPX. Prevalence and infection intensity at Virginia sites

Year	Site	Sampling date	Salinity (psu)	No. of clams examined	QPX prevalence (%)	Average infection intensity
2004	Wachapreague	30 Jul	>30	25	4	Moderate
		13 Oct	33	30	3.3	Light
	Smith Island	16 Aug	25	29	3.4	Light
	Cherrystone	16 Aug	15	30	0	
		12 Oct	19	30	0	
	Metompkin	17 Aug	21	30	0	
2005	Wachapreague	21 Jul	31	30	3.3	Light
		20 Sept	31	30	3.3	Rare
	Smith Island	16 Aug	28	15	0	
		19 Sept	30	30	0	
	Cherrystone	20 Jul	16	30	0	
		20 Sep	24	30	0	
	Plantation Creek	16 Aug	20	30	0	
		19 Sept	25	30	0	

shell swab samples (Fig. 1B, lanes 4 & 5), and samples from the red algae *Agardhiella* sp. (Fig. 1B, lane 2) and *Ceramium* sp. (Fig. 1B, lane 3). Only the sediment sample (lane 7), 1 of the gastropod samples (lane 4), and the *Ceramium* sp. sample (lane 3) had a band at the QPX position. Finally, Metompkin, a site where no QPX infections were detected in clams, yielded an amplification result from a sediment sample. However, the band was larger than expected for QPX on agarose gels, and there was no band visible by DGGE (Fig. 1B, lane 6). We saw a similar result in the MA environmental samples, and when this band was sequenced, it represented a very different organism (Gast et al. 2006). Attempts to recover and sequence this particular band from the VA sample were unsuccessful. Other bands have been recovered from the VA samples on the DGGE gel (marked on gel image), including the one marked as QPX in Fig. 1B. All of the other bands were similar to either QPX or other thraustochytrid sequences from the database. Plantation Creek yielded no QPX-infected clams, and none of the environmental samples collected there were positive using the nested-PCR protocol. Despite the overall low number of VA samples positive for QPX, they represent the same sample types that regularly gave positive QPX results during MA sampling. A summary of the VA results based upon sample type is given in Table 3. Overall, the percentage of positive results differed for samples collected in VA (0.66%) as compared to MA (38.4%), and this difference was significant ($\chi^2 = 268.0$, df = 1, n = 1386, $p < 0.001$]. The very low number of positive results from VA samples (4 positive out of 605 tested) makes a valid statistical evaluation of this subset of data difficult. Consequently, only the results from MA samples were evaluated further.

Massachusetts

In contrast to the results from VA, QPX was detected at all of the MA sites (Table 3, Fig. 2) at all sampling times (Fig. 3). Barnstable Harbor and Pleasant Bay were selected as sample sites because of their infection intensity levels in clams (generally high and generally low, respectively). Although these levels were not directly determined throughout the present study, we made these distinctions based upon the history of reported QPX-related clam mortalities, general observations (clams and shells at the sediment surface) at the sites each time we sampled, and the initial testing of clams at the start of the study (from Barnstable Harbor only). Using histology, 44% of buried clams and 100% of clams on top of the sediment collected from a nearby plot in Barnstable Harbor were found to be infected with QPX in the spring of 2004 (R. Smolowitz unpubl. data). Barnstable Harbor had significant QPX-related clam die-offs in the spring and fall of 2004 as well as the spring of 2005. Pleasant Bay did not suffer a significant QPX outbreak during our sampling period, although a low level of QPX infection was potentially present, and in previous years clams in the adjacent culture site were severely affected by the disease. When comparing the percentage of total samples positive for QPX between the 2 sites, Barnstable Harbor tended to be higher than Pleasant Bay at most of the sampling points with significant peaks in June of both years (Fig. 2A; Fisher's exact test, n = 599, $p < 0.05$). There were no significant differences in the percentage of positive samples between the event positive and negative sites within each bay ($\chi^2 = 6.2$, df = 3, n = 599, p = 0.104) (Fig. 2B).

Table 3. QPX-prevalence in Massachusetts and Virginia sample types (na = not assessed)

Sample type		No. of samples tested		No. of samples positive for QPX		% of samples positive for QPX	
		MA	VA	MA	VA	MA	VA
Seawater		55	30	30	–	54.5	–
Sediment		70	32	32	1	45.7	3.1
Macrophytes		178	245	77	3	43.3	1.2
All seagrasses		30	11	21	–	70.0	–
All seaweeds		127	234	47	3	37.0	1.3
	Green algae	63	78	29	1	46.0	1.3
	Brown algae	21	14	8	–	38.1	–
	Red algae	43	142	10	1	23.3	0.7
	Decaying macrophytes	21	na	9	na	42.9	na
	Fresh macrophytes	157	na	68	na	43.3	na
Invertebrates		297	298	87	1	29.3	0.34
All bivalves		71	30	25	–	35.2	–
All gastropods		187	131	50	1	26.7	0.76
All other invertebrates		39	137	12	–	30.8	–
	Amphipods	7	na	–	na	0	na
	Ascidians	11	8	1	–	9.1	–
	Crab, shrimp, barnacles	21	129	11	–	52.4	–
All shell samples		107	115	44	1	41.1	0.87
	Bivalve shells	54	16	21	–	38.9	–
	Gastropod shells	53	46	23	1	43.4	2.1
	Other shells	na	53	na	–	na	–
	Bivalve shells not rinsed	49	na	21	na	42.9	na
	Bivalve shells rinsed	5	na	–	na	0	na
All gut samples		151	99	31	–	20.5	–
	Guts from bivalves	17	14	4	–	23.5	–
	Guts from gastropods	134	85	27	–	20.1	–

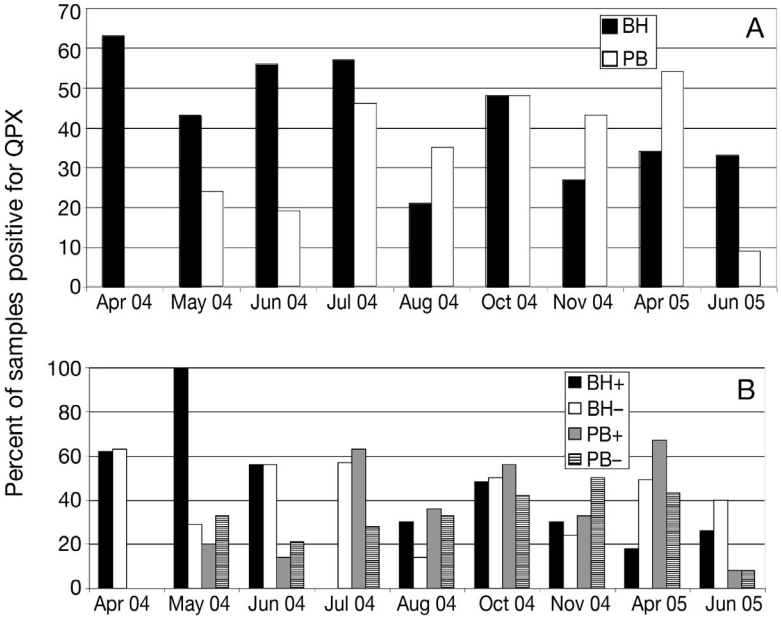

Fig. 2. Percentage of QPX-positive samples at Massachusetts sites Barnstable Harbor (BH) and Pleasant Bay (PB) throughout the study period. (A) Percent samples positive at each aquaculture area. No samples were collected from PB in April 2004. (B) Percent samples positive within each aquaculture site at designated positive (BH+ and PB+) and negative (BH– and PB–) net sites. No samples were collected at BH+ site in July 2004

Of the 599 samples tested from Barnstable Harbor and Pleasant Bay, 230 were positive for QPX, yielding an overall expected percentage of positive results of 38.4% (solid vertical lines, Figs. 3 & 4). Overall, there was no difference in the percent of positive results between Barnstable Harbor and Pleasant Bay ($\chi^2 = 1.93$, df = 1, n = 599, p = 0.15). When results were evaluated by month of collection (Fig. 3) there was a significantly higher than expected number of positive results for April, July and October 2004 ($\chi^2 = 32.14$, df = 8, n = 599, p < 0.001). When results were evaluated by sample type, there were significant differences in the percent of positive results in different seasons. Season was pooled as spring (April 2004, 2005 and May 2004), summer (June 2004, 2005, July and August 2004) and fall (September, October, and November 2004). There was a significantly higher than expected number of positive results for macrophyte and seawater samples collected in the spring ($\chi^2 = 13.8$, df = 3, n = 599, p =

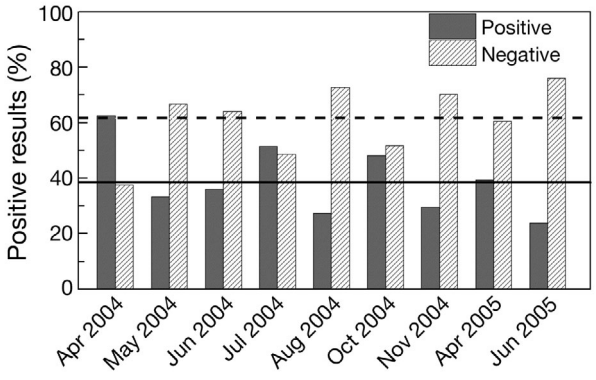

Fig. 3. Massachusetts samples. Observed percentage of positive QPX results per month (cases; dark bars) fluctuated throughout the year with higher than expected percentages (38.4%, solid horizontal line) in April, July and October of 2004. Samples collected in 5 out of 9 mo had higher than expected percentages (61.6%; dashed horizontal line) of QPX-negative results (controls; hatched bars), with the largest difference recorded in June 2005

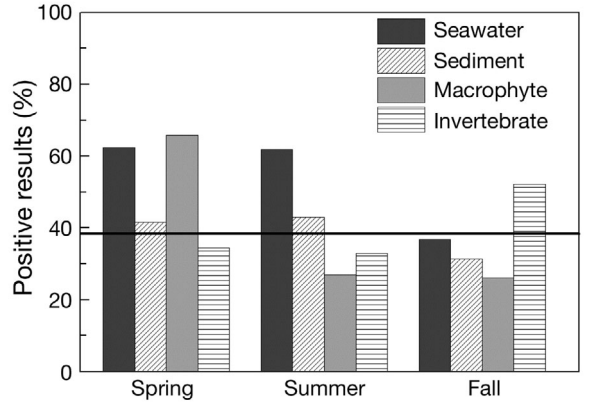

Fig. 4. Massachusetts samples. Observed percentage of positive results varied seasonally for each sample type (bars). Season was defined as spring (April 2004, 2005 and May 2004), summer (June 2004, 2005, July and August 2004) and fall (September, October, and November of 2004). There were higher than expected (38.4%, solid horizontal line) positive results for: seawater, sediment, and macrophyte samples in spring; seawater and sediment in summer; and only invertebrate samples in fall

0.003), for seawater and sediment samples collected in the summer (χ^2 = 10.2, df = 3, n = 599, p = 0.017), and for invertebrate samples collected in the fall (χ^2 = 9.1, df = 3, n = 599, p = 0.028).

The distribution of QPX in the marine environment of MA was very broad. Almost every sample type yielded a positive result at some point in our sampling regime (Table 3). The only exceptions were the shells rinsed with fresh water prior to swabbing and amphipods. The sample type showing the highest total percentage of positive results for QPX presence was seawater, followed by sediment, macrophytes and invertebrates, respectively. Within the environmental sample types (macrophytes and invertebrates), relatively high percentages of positive results were detected for samples of seagrass, green algae and shell swabs. Seaweeds were also analyzed by division (greens, browns, reds), because it was difficult to consistently collect the same genera from each site. The results indicated that red algae had the lowest percentage of positive results for QPX, while green algae had the highest. There was no difference in the presence of QPX for decomposing versus fresh macrophyte samples, but the sample size was relatively low for decaying macrophytes (21 out of 178 samples).

Samples of seawater, sediment, seaweeds, seagrass and shells collected in Wellfleet Harbor during the summer of 2005 indicated that the pathogen was present at the 3 locations sampled (27% Indian Neck, 38% Harbor Dock, 80% Little Island). Neither of the 2 original sites (Indian Neck and Little Island) was positive for the presence of QPX in the summer of 2006. From the 4 new sites, 2 yielded samples positive for QPX (Site 1: 1 out of 3 water samples; Site 2: 1 out of 3 sediment samples and 2 out of 3 water samples).

In situ hybridization staining

QPX cells were detected at very low numbers directly in or on environmental samples (Fig. 5). In both paraffin-embedded samples of macrophyte tissues and scrapings of detritus, fewer than 5 cells per slide were usually detected. Qualitatively, this indicates that, although QPX was widespread in the environment, it was likely present at relatively low numbers in the evaluated samples and tended to be patchy in its distribution.

DISCUSSION

Distribution and persistence of the pathogen

The present study demomstrates that the QPX organism is present in the marine environment outside of its clam host throughout the year. QPX was previously detected associated with marine aggregates (Lyons et al. 2005a), but this current study has vastly expanded the environmental reservoirs to include seawater, sediments, seaweed, seagrass, shell surfaces and invertebrate gut contents. These results, coupled with QPX growth experiments (Lyons et al. 2005b, Bugge & Allam 2007), provide strong evidence for the facultative nature of QPX. Facultative parasites are difficult to eradicate because they are not dependent on a

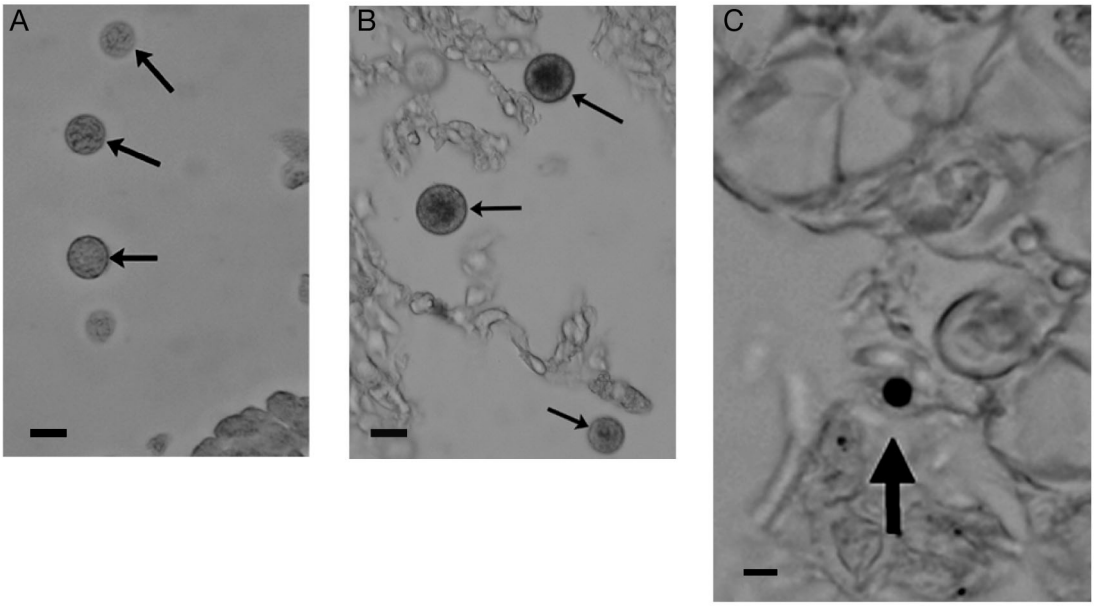

Fig. 5. *In situ* staining. Arrows point to QPX cells approximately 10 µm in diameter. (A) Negative control, no probe added, no staining of QPX organisms. (B) Positive control, probe added, staining of QPX organisms. (C) Staining of QPX on sectioned sample of *Ilyanassa obsoleta* shell surface. Scale bars ≈ 10 µm (at 400×)

parasitic way of life and can reproduce independently of their hosts. Rinsing samples, such as shell surfaces, prior to sampling or analysis tended to reduce the recovery of QPX DNA (Gast et al. 2006), suggesting the potential importance of washing rakes, gear, boots and boats to minimize transfer of QPX between beds within a harbor and between harbors.

Hard clam aquaculture in both MA and VA has been impacted by QPX-associated die-offs of clams (Ragone-Calvo et al. 1998, Smolowitz et al. 1998). Our survey of environmental reservoirs included samples from both locations in an effort to determine whether similar organisms or sample types harbored the QPX parasite. Our DGGE and preliminary sequence results suggest that QPX strains may be slightly different between MA and VA sites, which agrees with some histopathological observations that the disease symptoms are also slightly different between infected clams from MA compared to VA (Ragone-Calvo et al. 1998, Smolowitz et al. 1998). In addition, although fewer samples were positive from the VA sites, they were of the same sample types that were often positive for the presence of QPX in MA (e.g. sediment, seaweeds, shell swabs). This supports the speculation that the QPX organism would be able to grow or at least persist in either location and that it facultatively causes infections in clams, rather than being an obligate parasite of clams. Laboratory experiments (M. M. Lyons pers. obs.) demonstrated the ability of QPX to grow on seaweeds, most notably common representatives from green and brown algal divisions. A study by Bugge & Allam (2007) also reported the ability of the parasite to grow on brown and red macroalgal homogenates, but the one green alga (*Ulva lactuca*) that they tested did not support growth. This is in contrast to our environmental samples; approximately half of our *Ulva* samples were positive for the presence of QPX, and green algae showed the highest positive results for QPX compared to brown or red algae. The difference may lie in the method of homogenate preparation used by the Bugge study, where the release of large amounts of macroalgal compounds by massive cell lysis could inhibit QPX growth. In the environment, QPX may not be exposed to high levels of these compounds. What all of these studies do support is the potential for macroalgae to serve as a resource for QPX persistence outside of the clam. They may not grow rapidly or to high numbers, but they can grow.

The results from the nested PCR of VA samples were also qualitatively different from those recovered from MA. There were an increased number of bands on both agarose and DGGE gels, but when bands were recovered and sequenced, it was found that they were usually products of DNA from either QPX-like or thraustochytrid-like organisms. The thraustochytrid primers of Mo et al. (2002) are not specific for that group, but selective. Using the protocols presented here, however, these primers enhanced the detection level 10-fold. The QPX primers of Stokes et al. (2002) may also not be as specific for the QPX organism as

originally thought, although they do primarily amplify DNA sequences from organisms that are closely related to the parasite. We have not observed the same level of amplification diversity in the MA samples as was found in VA, which may suggest that populations of related thraustochytrids in VA are more diverse, or simply different, from those in MA.

The DGGE analysis has been a very useful tool in determining whether the additional products amplified by the nested PCR were from DNA of organisms related to the parasite, or whether they represented very different organisms (Gast et al. 2006). We were able to rapidly determine whether the QPX parasite was present in a sample, and how many other related organisms might also be present (Fig. 1). The primers used for our analysis target a variable region of the small subunit ribosomal gene, but the sequence was conserved among the QPX sequences available in the database. A recent study examining the genetic variation in multiple isolates of QPX indicated that the small subunit ribosomal genes were essentially identical in all of the isolates (Qian et al. 2007). The only sequences that showed substantial variability in that study were ITS 1 and ITS 2, but the variability observed within the same isolate was almost as great as that observed between different isolates. This may ultimately limit the ability to distinguish between different strains of QPX that might be present in an environmental sample. We used a method that allows the general, but very sensitive, detection of QPX DNA in the environment.

As the detection method used for this study was not quantitative, it was not possible to determine and compare the concentration of pathogens in the different samples. Nonetheless, the overall data suggests a seasonal trend in the prevalence of QPX present in the environment, with sample types showing different patterns. In the spring, there were more positive results from seawater, seagrasses and seaweeds than from sediment and invertebrate samples. In the summer, there were relatively more positive results from seawater and sediment samples, while in the fall there were relatively more positive results from the invertebrate samples. Besides being found in a wide range of environmental reservoirs, our results also demonstrated that QPX is present in the marine environment throughout the year and in areas where large scale clam die-offs are not actively occurring, indicating that complete eradication of this parasite from an area, once it is established, may be very difficult, if not impossible. We detected the QPX organism in samples collected from an area where no hard clam aquaculture was occurring (Little Island, MA), although it was near a site that had a history of clam transplantation and wild clam sets (Quissett Harbor, MA; data not shown). Overall, these observations emphasize that the QPX organism can be a natural component of coastal marine environments, as are many other species of labyrinthomorphids (Raghukumar 2002).

Results of the *in situ* staining of environmental samples indicated that QPX does not normally exist at high numbers in the marine environment. This suggests that a relatively large number of clam hosts may be needed for efficient spread of the parasite and development of disease outbreaks. The extremely high densities of clams that are planted in aquaculture efforts may create conditions favorable to the growth and release of large numbers of the parasite, especially since this is considered a directly infective disease (Smolowitz et al. 2001). It is well known that a high density of susceptible organisms will favor the transmission and increased occurrence of a disease. When a diseased clam dies of QPX infection, it may release thousands of the pathogens into the water column. A high density of clams either within or between plots would increase the potential for another clam to encounter high densities of the pathogens before they were dispersed or diluted by currents. Crowding of a population also causes stress, which can increase susceptibility to disease by suppressing function of the host's immune system.

Despite the potential difficulty associated with eradicating QPX from the environment if it can grow and persist without infecting clams, results of this study suggest management measures that might be implemented to help control the disease. Good plot husbandry may provide a mechanism to keep the pathogen at levels below those necessary to cause infection. When shellfish growers in Wellfleet experienced a large die-off of clams in the fall of 2004, they went through extensive efforts to remove the dead and dying clams from plots. They also left these plots fallow for 2 yr. Our survey of environmental samples from the area 7 to 8 mo later showed the presence of QPX at a frequency similar to that seen for our other Massachusetts sites. Seawater and sediment from those and additional sites in Wellfleet about 19 mo after the initial die-off indicated that QPX was below our detection levels (50 cells l^{-1} in seawater, 10 cells g^{-1} in sediment; Gast et al. 2006) at almost all of the sites. Removing the clams is only a first step towards effective control of the infection, and leaving plots fallow for 1 to 2 yr may be necessary to ensure success. There have been no QPX infections or die-offs since the remediation effort in Wellfleet, even in plots adjacent to those that were infected. Planting of other plots has continued as usual, and densities of soon-to-be market-sized clams were extremely high in the summer of 2006.

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