Marine Environmental Research 69 (2010) 337–344

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Combined effects of a parasite, QPX, and the harmful-alga, *Prorocentrum minimum* on northern quahogs, *Mercenaria mercenaria*

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ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form 18 December 2009
Accepted 22 December 2009

Keywords:
Bivalve mollusc
Shellfish
Dinoflagellate
Harmful-algal bloom
Hemocyte
Quahog
Mercenaria mercenaria
Parasite
Prorocentrum minimum
QPX

ABSTRACT

Northern quahogs, *Mercenaria mercenaria* (L.), frequently are infected with the parasite Quahog Parasite Unknown (QPX, Labyrintohomorpha, Thraustochytriales), which can cause morbidity and mortality of the quahogs. Possible interactions between this parasitic disease and exposure to the harmful dinoflagellate *Prorocentrum minimum* in *M. mercenaria* were studied experimentally. Quahogs from Massachusetts with variable intensity of QPX infection were exposed, under controlled laboratory conditions, to cultured *P. minimum* added to the natural plankton at a cell density equivalent to a natural bloom. After 5 days of exposure, individual clams were diagnosed histologically to assess prevalence and intensity of parasitic infection, as well as other pathological conditions. Further, cellular defense status of clams was evaluated by analyzing hemocyte parameters (morphological and functional) using flow-cytometry. Exposure of quahogs to *P. minimum* resulted in: a lower percentage of phagocytic hemocytes, higher production of reactive oxygen species (ROS), larger hemocyte size, more-numerous hemocytic aggregates, and increased numbers of hemocytes in gills accompanied by vacuolation and hyperplasia of the water-tubular epithelial cells of the gills. Quahogs had a low prevalence of QPX; by chance, the parasite was present only in quahogs exposed to *P. minimum*. Thus, the effect of QPX alone on the hemocyte parameters of quahogs could not be assessed in this experiment, but it was possible to assess different responses of infected *versus* non-infected quahogs to *P. minimum*. QPX-infected quahogs exposed to *P. minimum* had repressed percentage of phagocytic hemocytes, consistent with immuno-modulating effect of *P. minimum* upon several molluscan species, as well as smaller hemocytes and increased hemocyte infiltration throughout the soft tissues. This experiment demonstrates the importance of considering interactive effects of different factors on the immunology and histopathology of bivalve shellfish, and highlights the importance of considering the presence of parasites when bivalves are subjected to harmful-algal blooms.

1. Introduction

Northern quahogs, *Mercenaria mercenaria* (L.), from a portion of the east coast of North America (Virginia to Prince Edward Island) have demonstrated variable infection with the protozoan parasite, Quahog Parasite Unknown, QPX, Labyrintohomorpha, Thraustrochytriales (Smolowitz and Leavitt, 1997), in some locations resulting in variable and sometimes high mortalities (Smolowitz et al., 1998; Ford, 2001; Ford et al., 2002; Dahl et al., 2008). Pathogenesis of this disease begins with appearance of parasite cells in mantle and gill tissues, which induces hemocyte migration into the area of infection to isolate and destroy the QPX cells (Smolowitz et al., 1998). Progression of the disease may include large, focal lesions or multifocal, granulomatous, inflammatory responses induced by the parasites, which increase with the severity of infection (Smolowitz et al., 1998). Eventually, necrosis and bacterial/fungal decomposition of infected tissues occurs, implying that immune functions may become impaired by QPX infection. The prevalence of the parasite in quahogs correlates with the mortality rate of the animals, and also is related to the origin of the quahog broodstock, i.e., specific genotypes appear to have higher susceptibility to the parasite (Ragone Calvo et al., 2007).

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doi:10.1016/j.marenvres.2009.12.008

Studies of how QPX disease modifies the morphology and functions of hemocytes, circulating cells involved in defense, in quahogs have already been performed (Hégaret et al., 2008a; Perrigault and Allam, 2009). Another thraustochytrid protist, phylogenetically related to QPX but without the envelope of secreted mucoid material present around QPX, isolated from *M. mercenaria*, C9G, has been demonstrated to activate the hemocyte phagocytic response, but to have no effect on production of ROS in quahog hemocytes (Anderson et al., 2003a). This selective response suggests that hemocytes were able to kill the parasite, but without involving oxygen-dependant mechanisms (Anderson et al., 2003a). Other parasites also have demonstrated effects on bivalve hemocytes (Anderson et al., 1995; Allam et al., 2001; Cochennec-Laureau et al., 2003; Goedken et al., 2005a, b). Thus, the presence of a parasite can be expected to affect the immune status of a bivalve, modifying responses to other environmental changes such as harmful-algal blooms (HABs) (Hégaret et al., 2007a; da Silva et al., 2008). Harvell et al. (1999) listed HABs as one of the growing concerns that may enhance the impact of diseases and parasites on marine organisms and the food webs supporting them.

Harmful-algal blooms can have diverse, deleterious effects on bivalve species (reviewed in Shumway (1990) and Landsberg (2002)), resulting in morbidity to mortality. Several studies have highlighted effects of harmful-algal species upon bivalve hemocytes (Hégaret and Wikfors, 2005a, b; Hégaret et al., 2007a, b; da Silva et al., 2008; Ford et al., 2008; Galimany et al., 2008a, b). Specifically, the dinoflagellate *Prorocentrum minimum* has been shown to cause morphological and functional changes in hemocytes of several bivalve species (Hégaret and Wikfors, 2005a, b; Galimany et al., 2008a; Hégaret et al., 2008b, 2009). This dinoflagellate is present throughout the world (Heil et al., 2005) and has been reported to affect filtration, growth, survival, or organ and tissue development of northern quahogs, bay scallops and juvenile eastern oysters (Leibovitz et al., 1984; Shumway et al., 1985; Luckenbach et al., 1993; Wikfors and Smolowitz, 1993, 1995, reviewed in Wikfors (2005)). Blooms of *P. minimum* have been recorded on the East Coast of the United States (Freudenthal and Jijina, 1985), indicating that this phytoplankter is sympatric with QPX-infected quahogs. Possible combined effects of *P. minimum* and QPX on quahogs, however, have never been assessed.

Objectives of the present study were to determine (1) if *in vivo* exposure of northern quahogs, *M. mercenaria*, to *P. minimum* would impart immunological or pathological changes, (2) whether or not the presence of the parasite QPX could affect any responses observed following a harmful-algal exposure, and thus assessing if there could be any combined effect of these both stresses.

2. Materials and methods

2.1. Experimental clams

Northern quahogs, *M. mercenaria* (45–55 mm shell length), were collected on August 10th 2006 from a low-intertidal sand flat near Scudder's Lane in Barnstable, MA, a location where the prevalence of QPX varies from 30% to 70% in 2 year-old quahogs (Smolowitz, unpubl. obs.). Quahogs were acclimated in flow-through seawater tanks for one week before the experiment in unfiltered seawater containing natural plankton assemblages pumped from Vineyard Sound, just off shore of Woods Hole, MA. The natural plankton assemblage was examined micro scopically to ensure the absence of any natural, harmful-algal bloom. The dinoflagellate *P. minimum* is not known to occur at bloom levels in this area. All the water coming out of the flow through system was collected, treated with sodium hypochlorite and discarded into the fresh water sewer system to prevent the potential spreading of the disease and the harmful-alga.

2.2. Algal cultures

The *P. minimum* (Pavillard) Schiller strain JA-98-01 (isolated from the Choptank River, Chesapeake Bay, Maryland, USA), was obtained from the Milford Microalgal Culture Collection. As inconsistent responses of bivalves to *P. minimum* have been observed in nature and in experiments (Wikfors, 2005), the strain JA-98-01 was chosen for its toxicity to juvenile bay scallops, *Argopecten irradians*, used as a bioassay to test algal toxicity; mortality of juvenile bay scallops occurs after 24 h exposure to stationary-phase cells, but log-phase cells are less toxic (Hégaret and Wikfors, 2005a; Galimany et al., 2008a). Cultures of *P. minimum* were grown in EDL7 medium, a modified version of the enriched-seawater E-medium (Ukeles, 1973) that contains L-1 trace metals, double the EDTA of the standard E formulation, KNO_3 rather than $NaNO_3$, and soil extract. The microalga was cultured in 20 L glass carboy assemblies using aseptic technique (Ukeles, 1973). Cultures were maintained at 20 °C with 24 h light, and harvested semi-continuously to maintain consistency in culture quality over the course of the study. Cells were harvested in stationary phase, usually approaching a concentration of $1–5 \times 10^5$ cells mL^{-1}. Algal cell densities were determined by hemocytometer counts under a light microscope.

2.3. Experimental design

Sixty quahogs were distributed randomly into twelve 1 L basins, i.e. five clams per basin. Six replicates of two different treatments were done in this experiment:

(1) Clams fed only the natural plankton, a community of 2–5 μm cyanobacteria and non-motile eukaryotic cells at approximately 10^4 cells ml^{-1}.
(2) Clams fed *P. minimum* at 2×10^4 cells ml^{-1}, added to the natural plankton.

Each replicate group of clams was fed continuously 5 mL min^{-1} for 5 days using a self-contained, integrated apparatus for exposing aquatic organisms to different water sources (Smith et al., 2006). Briefly, this integrated apparatus contains 12 flow meters, feeding twelve 1 L basins, which are themselves contained in a much larger 80 L basin. The overflow of this basin is collected by one-single drain to be treated before disposal. The twelve 1 L basins connected to individual flow meters can each receive a different algal mix. In this experiment, the two algal mixes were fed to the clams using gravity; the flow was controlled using 12 individual float-ball flow meters (Cole-Parmer). The algal suspension was provided to each basin, six receiving the natural plankton, and the other six the natural plankton to which *P. minimum* had been added. As the algal mixes were continuously added to the basins, they overflowed into the large basin and treated with Chlorox. Previous experiments showed that effects of *P. minimum* on eastern oysters hemocytes could be observed after 5 days of exposure (Hégaret and Wikfors, 2005a). Thus, after 5 days of exposure, the clams were removed from the apparatus; hemocytes were analyzed using flow-cytometric methods, and presence of Quahog Parasite Unknown (QPX) and other pathological conditions were assessed by histology.

2.4. Analysis of hemocyte parameters

Hemolymph was withdrawn with a needle and 1 mL syringe from the adductor muscle of each quahog, filtered through 75 μm mesh, and stored temporarily before use in an Eppendorf