

Invertebrate Resting Eggs as Secondary Aquatic Invasion Vectors

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This project was completed in CY2007

Overview

The Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990, as amended (16 U.S.C.4701 et seq.) requires the Secretary of Commerce to conduct a ballast treatment technology demonstration program to develop information and approaches that will help prevent aquatic nonindigenous species from being introduced via ballast water. NOAA has been mandated by Congress via appropriations to carry out this responsibility. Section 4722(c) of the Act also requires members of the ANS Task Force, of which NOAA is co-chair, to identify aquatic invasion pathways, assess the risk associated with those pathways, and evaluate measures to prevent introductions via those pathways.

At present, ballast treatment approaches being tested by various groups in the United States and elsewhere fall into categories of physical removal (filtration; hydrocyclone or ultracentrifuge) and/or some form of biocide exposure (UV radiation, heat, ozonation, deoxygenation, and various chemical biocides). However, most tests of various treatment technologies and approaches have not looked at resting stages (see below), which may be resuspended and entrained with local sediments during ballast water intake, or produced by organisms that came in with the ballast water. Based on the results from the Great Lakes NOBOB Program (below), resting eggs pose a potential invasion threat to both marine coastal areas and the Great Lakes.

2005 Plans

- Publish results of SeaKleen evaluation.
- Conduct bioassays assessing the sensitivity of resting eggs to Heat, UV, Gluteraldehyde and Hypochlorite.
- Compile a resting egg catalogue that includes size, appearance, and unique morphological characteristics of resting eggs by species and taxa, to the extent that such information is available in the literature.

2004 Progress

The progress made on this project on FY04 includes the initial start-up phase of laboratory work. Although funding was previously obtained and general methodology described in the proposal, specific procedures were not in place. This reflects the novelty of the investigation, namely to test dose-response of invertebrate resting stages to biocides. In order to get the project off the ground, I had to overcome two major methodological hurdles: 1) obtaining

suitable test subjects in viable resting eggs, and 2) manipulating resting eggs in the laboratory. Both of these hurdles required exploratory creation and testing of new methods.

Obtaining Resting Eggs

To be useful in the laboratory, resting eggs had to be available in large quantities, hatch quickly and at a good hatching rate, and be free of extraneous material. Three potential sources existed: collecting eggs from sediments, culturing eggs in the laboratory, and buying eggs from suppliers. As might be expected, only some of these sources were useful.

Collection of lake sediment was limited by existing cruises and opportunistic sampling by colleagues at other institutions. Opportunities for collection of ballast tank sediment was similarly limited. Moreover, lake sediments had to be collected as early in the season as possible, in order to avoid collecting material after eggs hatched naturally. Collection could not begin before May. I collected sediment from Lake Michigan, Muskegon Lake, and one ballast tank. I obtained Lake Michigan sediment from Tom Nalepa, and Lake Erie sediment from Don Schlosser of USGS, and Tom Bridgeman of The University of Toledo. I evaluated sediment using a sugar flotation and centrifugation procedure. This separated inorganic sediments from organic matter including resting eggs and allowed opportunistic testing of organisms depending on what taxa happened to be present. For example, I was able to test copepod resting eggs because copepod *nauplii* emerged from one sediment sample. Testing of various sediment samples successfully identified sediment with a high density of resting eggs. This sediment is now being used for bioassays.

Laboratory culturing required trying to raise large population sizes and then inducing the production of resting stages. I considered this a risky endeavor which could fail outright should no eggs be produced. I reared cultures of *Daphnia pulicaria*, *Daphnia mendotae*, *Ceriodaphnia* sp., and *Bosmina longirostri*. I successfully induced resting egg production in one species, *Daphnia pulicaria*. I then focused on this species, increased culture population sizes, and am now inducing resting egg production monthly. The eggs are currently being stored in the cold room to simulate a refractory period prior to their use in bioassays.

Several invertebrate species were available for purchase in the resting life stage, including the cladocerans *Daphnia magna* and *Daphnia pulex*, the rotifers *Brachionus calyciflorus* and *Brachionus plicatilis*, and the brine shrimp *Artemia franciscana*. Both *Daphnia* species were not suitable for use in the laboratory, due to high cost and low hatching rate. I have successfully run bioassays with *Brachionus calyciflorus* and *Artemia franciscana*.

Manipulation of Resting Eggs

With test subjects in hand, the next obstacle was manipulation of eggs in a quantitative manner in order to run replicated bioassay experiments. Counting resting eggs requires microscopy. Some cladoceran resting eggs can be manipulated with forceps, but others require scooping them up with a bit of mesh affixed to the end of a probe. Other eggs are too small even for that, and require manipulation with a pipette. But the primary difficulty was controlling the duration of exposure to the toxicant. Eggs had to be counted, distributed to test chambers, exposed to

water containing the toxicant for 24 hours, then transferred to water without toxicant. In order to avoid over-handling of eggs and risking damage or loss during this procedure, I devised a novel method. I created test chambers consisting of a plastic beaker modified by having a section of the bottom removed and replaced with mesh. Eggs were placed into this modified beaker. This beaker could then be placed inside an unmodified beaker containing the toxicant. After the specified exposure time, the inner beaker with eggs is then lifted out of the outer beaker, allowing the toxicant to drain through the mesh. The modified beaker with eggs is then placed in another beaker with clean medium. Bioassays with rotifers required a different set up. I cut a notch out of the side of a 35mm petri dish, and replaced it with mesh. Water with toxicant can then be wicked away from the petri dish, and eggs therein.

Results

To date I have run 18 bioassays using the potential biocide SeaKleen, a commercial product specifically designed to be used in ballast tanks, on a variety of organisms. After an initial range-finding experiment, bioassays were run between 2 and 10 $\mu\text{g/L}$. Data patterns have fit the logistic curve very well, allowing analysis with LOGIT in SYSTAT. SeaKleen is toxic to resting eggs, but at concentrations higher than that for adult pelagic life stages ($\sim 2 \mu\text{g/L}$). Larger eggs are more resistant, as are eggs with greater physical protection such as ephippia. Mortality was assessed by non-hatching and hatched but immobile nauplii. The latter class also included malformed neonates. Immobility peaked at $\sim 5 \mu\text{g/L}$, then fell as overall mortality increased with eggs not hatching at all. Teratogenic effects were visible at low rates, but increased with increasing concentration.

SeaKleen degrades quickly in sunlight, as indicated by analysis of light absorbance between 200 and 300 nm. Peaks at 245nm and 265 nm begin to drop after 24 hrs in sunlight, and by 219 hrs are gone. Simultaneously, a degradation product appears at 210 nm. Interestingly, the concentration of SeaKleen appears to rise after 24 hrs. in the dark. This may be due to reactions that produce products that absorb at the same wavelengths. After 219 hrs in the dark, SeaKleen begins to decay, with a degradation product appearing at 210 nm.

Patterns of light absorbance reflect the toxicity of SeaKleen under these conditions. After 24 hrs in sunlight, toxicity is reduced, and by 72 hrs it is essentially non-toxic. Interestingly the toxicity of SeaKleen appears to increase over the short term in darkness.

Resting Egg Studies

Life cycles of many marine and freshwater invertebrate and phytoplankton species include the production of resting stages (variously called cysts, ephippia, resting eggs, diapause eggs, or spores, according to taxon) as a strategy to assure long-term survival of the species. Resting eggs are fertilized embryos enclosed in one or more layers of protective casing, making them extremely resistant to various adverse conditions. They are known to survive burial in sediments for decades to centuries (Hairston et al, 1995; Hairston 1996), desiccation (Arnott & Yan, 2002), and passage through digestive tracts of fish (Jarnagin et al., 2000) and waterfowl. Some resting stages are buoyant and will remain in the water column, while many are dense enough to sink and accumulate as "seed banks" in sediment (Hairston et al., 1995). Resting stages may remain

viable in sediments in a virtual suspended metabolic state for decades or even centuries (Hairston et al., 1995), but can still germinate under favorable environmental conditions.

Resting eggs of invertebrates (zooplankton and benthos) are found in ballast tanks, and, in particular, in residual ballast found in the unpumpable water and sediment remaining after ballast tanks have been pumped out (Hallegraff & Bolch, 1991; Kelly, 1993; Hamer et al. 2000; Bailey et al, 2003). The Great Lakes NOBOB Program has found zooplankton egg densities ranging from 100-10,000 eggs/m² (~6000-600,000 eggs per metric ton of residual sediment). Egg viability ranged from 0-92%, averaging ~36%, based on hatching experiments. The primary species found were rotifers, cladocerans, and copepods. At least 30 freshwater species have been identified from among these groups in Great Lakes ballast tank residuals, of which 6 are not presently reported in the Great Lakes. The total number of ships tested represents only 5-6% of the total entering the Great Lakes from outside the system, and less than 0.1% of all ships entering U.S. ports. Extrapolating to the thousands of vessels that enter U.S. ports every year would suggest that resting stages might be a significant secondary vector by which some invasions may occur. For example, it may not be coincidence that the majority of recent zooplankton invaders found in the Great Lakes since the implementation of ballast water exchange are species that produce resting eggs.

Resting eggs in residual sediment and water in ballast tanks must either be physically removed or killed to eliminate them as an invasion threat. However, physical removal may be problematic, at least for some species. A limited search of the published literature shows that they can range in size from less than 50 microns for small zooplankton such as rotifers (Lewis, 1979) to several hundred microns for some cladocerans, such as *Daphnia* (Lewis, 1979; Jarnagin et al., 2000). Chen and Marcus (1997) reported resting egg sizes in the range of 75-150 microns for a selection of copepods. Treatment systems have yet to be developed and perfected that can remove particles smaller than several hundred microns and that are also practical for use aboard large commercial vessels. Thus, it is likely that the use of biocides of some form may be required to supplement physical removal if resting eggs are to be eliminated as potential invasion source. However, there have been few experiments to determine the efficacy of various potential biocides (such as hypochlorite, gluteraldehyde, SeaKleen, UV, heat) on resting eggs of various invertebrates. Landrum, for example, conducted a series of bioassays using gluteraldehyde, hypochlorite, and SeaKleen on live adult benthic organisms, but only on the resting eggs of one marine organism - *Artemia*.

Objectives

- Test the effect of various biocide treatments on the viability of a variety of zooplankton resting eggs obtained from natural populations, laboratory cultures, and ships of opportunity.
- Develop a resting stage key (appearance and size) for various invertebrate taxa, from literature searches and direct observations.

Approach and Methods

Research will be conducted under the general supervision of Drs. Reid and Landrum. Dr. Vanderploeg will provide guidance and expertise for sources and identification of resting eggs, lab techniques for hatching and viability experiments, and general ecology. Dr. Chip Blatchley (Purdue University) has graciously agreed to assist with design and performance of UV exposure experiments and also to make his lab available for conducting UV exposures.

Sources of Resting Eggs

Invertebrate resting eggs will be obtained from three possible sources:

1. From natural sediments in the Great Lakes - these are widely known to contain resting eggs for a variety of zooplankton (Yurista, 1997; Kerfoot, pers. comm.; Vanderploeg, pers. comm.)
2. GLERL maintains cultures of the Great Lakes caldoceran invaders *Bythotrephes* and *Cercopagis*, for another project and will provide resting eggs farmed from these cultures, as well as from natural pelagic populations collected from the Great Lakes water column at various times during the field season.
3. From ships of opportunity in connection with both the Great Lakes and Chesapeake Bay NOBOB Programs. The Great Lakes NOBOB program, co-managed by GLERL, will be entering several vessels during the 2003 shipping season in connection with in situ tank hatching experiments. Residual sediment will be collected from these vessels as opportunity permits. The Chesapeake Bay NOBOB Program is just starting its first field season and will be sampling ballast tank residuals from numerous vessels during 2003. Additional residual mud from those vessels will be provided for this project.

Egg Viability and Exposure

Eggs will be sorted into appropriate groupings for replicate control and exposure test sets. Control sets will be exposed to optimum hatching conditions as determined by a combination of procedures outlined by Bailey et al (2003), Landrum (pers. comm.), and other appropriate literature sources. The hatching success of replicate egg sets under optimized laboratory conditions will be compared against hatching success under similar optimum conditions but including exposure to different biocides (chlorination, SeaKleen, gluteraldehyde, heat, and UV) at various concentrations or doses. UV exposures will be conducted in the laboratory of Dr. Chip Blatchley (Purdue University) using his equipment. Two series of tests will be conducted for chemical exposures: one using eggs separated from sediment and placed in aqueous culture media, and one in which the eggs are gently mixed into a known sediment matrix which is then covered with aqueous culture media, the latter to explore the biocide effectiveness in the presence of sediments, as found in the bottom of many ballast tanks. In addition to recording the size and appearance of resting eggs obtained from various sources for this project, an extensive literature search will be conducted to compile a key that includes size, appearance, and unique morphological characteristics of resting eggs by species and taxa, to the extent that such information is available in the literature.

Expected Outcomes

Scientific information about the efficacy of various biocide treatments (chlorination, gluteraldehyde, SeaKleen, heat, up exposure) to kill invertebrate resting eggs will be produced, including aqueous exposure in the absence of sediments and exposure in the presence of natural sediments of known composition. A key to resting egg size and morphology will help determine if physical removal treatments are likely to be effective, depending on the egg taxa.

Benefits

The goal of ballast water treatment is to have no live or viable biological material discharged from ballast tanks into the environment. This goal will be unattainable until the question of resting eggs is addressed and suitable treatment approaches are identified. Part I of this project will move towards that solution.

Chlorinated Ballast Tank Studies

During the second year of the Great Lakes NOBOB Program biological characterization, we discovered that several ships entering during 2002 had been required to chlorinate their ballast tanks before they were allowed to enter the port states of Brazil and Argentina. The NOBOB team was not prepared, at the time, for safe entry into recently chlorinated ballast tanks and therefore didn't attempt to sample them. Such ships provide a potentially outstanding opportunity to assess the effectiveness of operational chlorination on ballast tank biota, including resting stages.

Objectives

Sample residual ballast from up to five ships of opportunity that enter the Great Lakes (or possibly Chesapeake Bay) after having chlorinated one or more ballast tanks, and analyze for the presence of live organisms.

Experimental Approach

During 2003-2004 we intend to look for ships entering the Great Lakes with previously chlorinated ballast tanks and sample the residual material in those previously chlorinated tanks. This would provide a direct assessment of how effective the operational use of chlorination was. The shipping industry has indicated cooperation in identifying such ships (Fednav, Ltd., St. Lawrence Seaway authorities). Sampling in connection with the Chesapeake Bay NOBOB program is an alternate possible source.

Personnel specially trained in safe entry of ballast tanks will collect samples of residual water and sediment, as available, in previously chlorinated ballast tanks. Special safety and sampling protocols for chlorinated ballast tanks will be developed before first entry. Information about the procedure used to chlorinate the tanks will be obtained from the appropriate ship's officer, including history of the tanks, source of the water in the tanks at time of chlorination, amount and form of chlorine added, exposure time, and any testing that was performed.

Residual Water and Sediment Testing

Microbial Analyses

Samples will be collected under sterile conditions and sent to Dr. Fred Dobbs at Old Dominion University for analyses. Dr. Dobbs has indicated an interest and willingness to analyze a limited number of such samples at no cost to this project.

Phytoplankton and Invertebrates

Multiple treatments will be used for phytoplankton and invertebrate assessment. A-1) Replicate samples of residual water and (separately) samples of residual sediments will be placed directly in a sterile but nutritional culture media immediately upon collection and incubated at 20oC. A-2) Same as A-1, except the replicate samples will be gently mixed with the culture media and the culture media will be decanted and the procedure repeated - this to remove or reduce the concentration of any residual chloride. A final volume of culture media will be added and the samples incubated at 20oC. A-3) Same as (A-1), but, after the rinsing volumes, the samples of residual water and (separately) sediment will be placed in a final nutritional culture media to which live algal cells have been added to provide a food source for any viable zooplankton. All samples will be observed for evidence of live organism growth or activity for at least two weeks, and live organisms will be collected for identification; B) Samples of residual water and sediment will be placed in containers and processed for resting egg viability immediately upon return to the lab. Eggs will be separated, sorted, and placed in hatching experiments following existing protocols (see below).

Vertebrates

During tank sample collection, we will attempt to enumerate and collect any obvious vertebrates, alive or dead, for identification. Any vertebrates found in sediment or water samples will be separated and identified.

Expected Outcomes

This part of the project will provide data on the effectiveness of operational chlorination of ballast tanks.

Benefits

Results will supplement controlled lab-based exposure experiments and will provide an interesting opportunity to compare results of controlled experiments and operational chlorination not performed under well-controlled conditions.

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Products

Presentations

Acute toxicity of SeaKleen (Menadione) to Invertebrate Resting Eggs. 13th International Conference on Aquatic Invasive Species.