

## NOTE

**Non-occurrence of free-living *Paramoeba invadens* in water and sediments of Halifax Harbour, Nova Scotia, Canada**

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**ABSTRACT:** Seawater and sediment samples from various sites in or near Halifax Harbour were cultured for *Paramoeba*, as were tissues of the natural sea urchin host *Strongylocentrotus droebachiensis* sampled from Halifax Harbour. An isolate of *Paramoeba* from a Halifax Harbour water sample was not virulent on injection into healthy sea urchins. However, some sea urchins in cages near the outfall pipe of Dalhousie University's seawater system were infected during the annual peak of the seawater temperature cycle. Results indicate that (1) there is no evidence of a free-living endemic population of *Paramoeba invadens* in or near Halifax Harbour, (2) *S. droebachiensis*, the natural host for *P. invadens*, do not harbour a reservoir population of this organism at temperatures sub-optimal for paramoebiasis, and (3) *Paramoeba* which are morphologically indistinct from laboratory stocks of *P. invadens* were recovered near the outfall pipe. These organisms may have been released via the outfall pipe into Halifax Harbour and subsequently lost virulence.

A pathogenic marine amoeba, *Paramoeba invadens* Jones, 1985, was found to be the causative agent of sea urchin *Strongylocentrotus droebachiensis* mass mortalities along the Atlantic coast of Nova Scotia from 1980 to 1983 (Miller & Colodey 1983, Scheibling & Stephenson 1984, Jones & Scheibling 1985, Jones et al. 1985). Most species of the genus *Paramoeba* are free-living and ubiquitous in the marine environment, and are commonly found in the surface microlayer, water column and bottom sediments in a range that extends from the North Atlantic to the Gulf of Mexico (Page 1970, 1973, 1976, 1983, Davis et al. 1978, Bovee & Sawyer 1979, Sawyer 1980). Only 2 species in this genus are known to be parasitic (Page 1983, Jones 1985). *Paramoeba perniciosus* appears to be an obligate parasite (Sprague et al. 1969, Page 1983) in the blue crab *Callinectes sapidus*. *P. invadens* is known only

from the tissues of diseased sea urchins and their surrounding seawater (Jones & Scheibling 1985), but it can be cultured on bacteria and is therefore thought to be a free-living opportunistic parasite (Jones 1985, Jones & Scheibling 1985, Jones et al. 1985, Jellett & Scheibling 1988b). The only known species of free-living *Paramoeba* morphologically similar to *P. invadens* is *P. aestuarina* (Page 1983). Recent observations by Rogerson (1988 and pers. comm.) may bring into question their position as different taxonomic species.

The origin (endemic or exotic) of the infective population of *Paramoeba invadens* involved in the sea urchin mass mortalities along the Nova Scotia coast has remained unresolved (Jones & Scheibling 1985, Scheibling 1988).

Though field and laboratory studies (Scheibling & Stephenson 1984, Jellett & Scheibling 1988a), a direct relationship was found between temperature and the sea urchin disease, and between temperature and growth rates of *Paramoeba invadens* in monoxenic culture. These studies showed that temperatures exceeding 10 to 12°C are necessary for elevated growth rates of *P. invadens* in vitro and for transmission and/or progression of sea urchin paramoebiasis in vivo. However, *P. invadens* can survive, but does not grow or cause disease in sea urchins, at low temperatures (2 to 5°C).

Paramoebiasis has not been seen in nature since 1983. Since that time, the disease has been maintained in vivo (in *Strongylocentrotus droebachiensis*) in a temperature-controlled flow-through seawater system (after Scheibling & Stephenson 1984). The untreated effluent from the aquaria containing diseased sea urchins drains into the outflow from Dalhousie University's Aquatron system which is pumped into the Northwest Arm of Halifax Harbour.

The purpose of this study was to determine (1) if *Paramoeba invadens* released from the Aquatron can

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survive and maintain virulence in Halifax Harbour, (2) if *P. invadens* can be recovered from sediment or seawater away from the discharge site, thereby indicating the free-living and possibly endemic nature of this marine amoeba, and (3) if *Paramoeba* exists as a low-grade (non-pathogenic) parasite in *Strongylocentrotus droebachiensis* at temperatures which are sub-optimal for disease occurrence.

**Methods.** Bottom and surface seawater samples were collected on July 21, 1987 from the following locations in Halifax Harbour: at the outfall of the Dalhousie Aquatron seawater system in the Northwest Arm (Fig. 1, Site 6), 30 m north and south of the outfall, 60 m north and south of the outfall, and at Eastern Passage (Fig. 1, Site 11). In addition, between September 10 and 15, 1987, nearshore surface seawater samples were collected from a total of 14 sites in and around Halifax

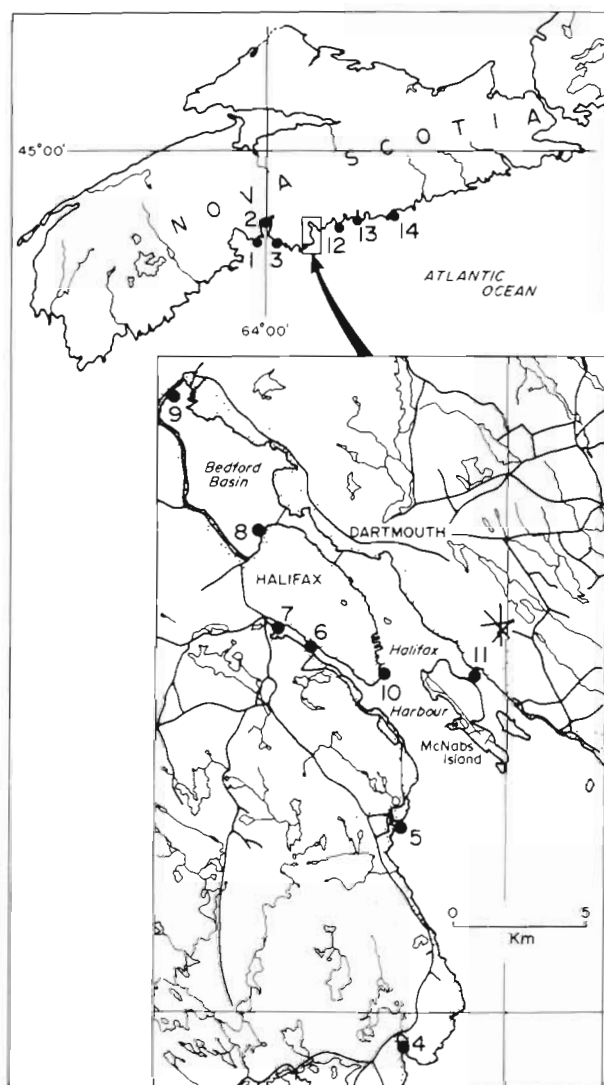


Fig. 1. Sampling locations (Sites 1 to 14) in (inset) and around Halifax Harbour. Aquatron outfall is Site 6

Harbour (Fig. 1). Surface water samples were collected by hand in clean polypropylene bottles at all sites. Bottom water samples (1 m off the bottom) were collected at 7.6 m at the outfall sites using a Kemmerer sampler fitted with a thermometer, and at 21 m at Eastern Passage using SCUBA. All seawater samples were gravity-filtered through 5  $\mu\text{m}$  membrane filters (Millipore Type SM) to the maximum volume filterable before clogging (660 to 2300 ml).

*Paramoeba* was cultured by placing the filters onto artificial seawater (ASW) agar (Jellett & Scheibling 1988b) in 100 mm disposable plastic petri dishes with ca 20 ml of ASW overlay (after Page 1983). Cultures were incubated at 15°C for 3 wk in complete darkness. The cultures were inspected using phase contrast microscopy (400  $\times$ ) for amoebae. Cultures containing amoebae conforming in size and morphology to *Paramoeba* (Page 1983) were examined at 1000  $\times$  with differential interference contrast optics to confirm the presence of one or more parasomes, the identifying feature of the genus *Paramoeba*. The estimated minimum number (EMN) of *Paramoeba* in the sample was determined by assuming the presence of at least one *Paramoeba* per original volume of filtered seawater in positive cultures, and in negative cultures as less than one *Paramoeba* per volume of filtered seawater. All filtered volumes were then equated to 1 l for comparison.

Using a micromanipulator, a single *Paramoeba* was isolated from a culture from outfall bottom water and grown on 1.2% ASW agar preinoculated with *Pseudomonas nautica* as a food source (after Jellett & Scheibling 1988b). Infection experiments with this environmental isolate of *Paramoeba* (from the outfall) were performed by the method of Jones & Scheibling (1985). Individuals in 2 groups of 5 sea urchins *Strongylocentrotus droebachiensis* were each injected with 1.0 ml of antibiotic-treated *Paramoeba* from culture containing 1240 amoebae  $\text{ml}^{-1}$ . Antibiotics (100 IU  $\text{ml}^{-1}$  penicillin and 100  $\mu\text{g ml}^{-1}$  streptomycin, Gibco) suppress the bacteria which serve as the amoebic food source. As controls, individuals in 2 groups of 5 sea urchins were each injected with 1.0 ml of antibiotic-treated *P. nautica* and individuals in another 2 groups of 5 were each injected only with 1.0 ml of antibiotic solution. Temperature-controlled (16°C) seawater flowed over each group of injected sea urchins and into a separate aquarium containing 6 healthy *S. droebachiensis*. Injected sea urchins and those in the aquaria (waterborne exposure) were monitored daily for signs of paramoebiasis, particularly loss of tube foot attachment (Jones & Scheibling 1985). After 30 d all sea urchins were dissected, and a segment of each radial nerve and 1.0 ml of coelomic fluid were removed and cultured on 0.6% ASW agar for *Paramoeba* after the method of

Jellett & Scheibling (1988b). After 3 wk of incubation at 15°C, the radial nerve and coelomic fluid samples were examined for amoebae. Radial nerve tissues from diseased sea urchins (maintained at 16°C in a flow-through seawater system after Scheibling & Stephenson 1984), and sediment samples (600 ml gravity-filtered) from aquaria containing diseased sea urchins, were cultured for *Paramoeba* as described above.

A preliminary experiment was performed to determine if pathogenic *Paramoeba invadens* were being released into Halifax Harbour via the Aquatron outfall. Between September 1 and 24, 1987, during the peak (9.9 to 16.5°C) of the annual temperature cycle, vexar cages (1 m diameter, 40 cm height, 16 mm mesh aperture), each containing 50 healthy *Strongylocentrotus droebachiensis* (collected from Bedford Basin) and kelp as food, were placed on the bottom at the outfall, and at Sites 1 and 2 km south of the outfall. Sea urchins were monitored for signs of paramoebiasis by lifting the cages at weekly intervals and examining the urchins for loss of tube foot attachment or other abnormalities. Sea urchins showing any pathological signs were removed and dissected. Segments of each radial nerve and 1.0 ml samples of coelomic fluid were cultured on 0.6% ASW agar for *Paramoeba*. After 22 d in the cages, all sea urchins were removed and radial nerve and coelomic fluid samples were cultured for *Paramoeba* as described above. Culture dishes were incubated for 3 wk at 15°C and then examined for amoebae.

The environmental isolate of *Paramoeba* and stock-cultured *Paramoeba invadens* were further examined and photographed for comparison after staining with DAPI (Rogerson 1988) using a Wild Leitz Fluorescence microscope equipped with an A2 filter system.

One hundred apparently healthy sea urchins *Strongylocentrotus droebachiensis* were collected on January 21, 1988 from Bedford Basin by divers using SCUBA. Seawater temperature at the time of collection was ca 2°C. The sea urchins were maintained in ambient, flow-through seawater. They were dissected within 7 d and a segment of each radial nerve and 1.0 ml of coelomic fluid were cultured for *Paramoeba*. After incubation at 15°C for 3 wk, cultures were examined for amoebae. Any positive cultures were further examined at 1000 × with differential interference contrast optics.

**Results and Discussion.** Diseased sea urchins have been maintained continuously in the Dalhousie seawater system (Aquatron) since October 1983 (Scheibling & Stephenson 1984, Jones & Scheibling 1985, Jellett & Scheibling 1988b, Jellett et al. 1988a, b, this study). *Paramoeba invadens* is known to be transmitted in seawater (Scheibling & Stephenson 1984, Jones & Scheibling 1985) and in this study, *Paramoeba* was isolated from sediment collected from an aquarium

containing infected sea urchins. Water and sediment from aquaria containing diseased sea urchins drains into the Aquatron outfall pipe.

The effluent from the Aquatron discharges into a blind inlet, the Northwest Arm of Halifax Harbour, which is 4.75 km long, receives no freshwater input and has a tidal flux of 1 m. The outfall (Fig. 1, Site 6) is a point-source discharge situated ca 19.0 m offshore, 0.5 m above the bottom at a water depth of ca 10 m. The volume of effluent discharged from the system depends on current usage and is a mixture of both freshwater and saltwater.

Sampling locations were selected near the outfall and extending through Halifax Harbour, and for some distance north and south of the Harbour mouth (Fig. 1). The seawater from the sampling sites varied in the amount of particulate matter, which determined the volume of water able to be gravity-filtered through a 5 µm filter.

*Paramoeba* was recovered from some morbid sea urchins which were removed from cages located on the sea bottom at the outfall and at 1 and 2 km south of the outfall. However, infection was not consistent at any one site nor was it restricted to the site closest to the outfall. This suggests that virulent *Paramoeba invadens* is released from the Aquatron outfall and can cause disease in the caged sea urchins. In previous laboratory studies (Scheibling & Stephenson 1984, Jellett & Scheibling 1988b), waterborne transmission of *P. invadens* at the seawater temperatures experienced at the caging sites (9.9 to 16.5°C) resulted in 100% mortality in *Strongylocentrotus droebachiensis* exposed to diseased conspecifics. However, incidence of infection by waterborne exposure or injection is known to be dosage dependent (Scheibling & Stephenson 1984, Jellett unpubl.). Two possibilities exist which could explain the sporadic infection of caged sea urchins in this study: (1) virulent amoebae are not released in sufficient quantity (through dilution in the outfall pipe and on subsequent release), or (2) some of those that are released have lost virulence (possibly through exposure to conditions in the outfall pipe).

Free-living *Paramoeba* were isolated from several of the water samples collected near the outfall site (Table 1). Specifically, *Paramoeba* was isolated from surface water samples collected up to 30 m, and from bottom water samples collected up to 60 m, from the point of discharge. On one occasion, *Paramoeba* also was isolated from surface and bottom water samples from another site within Halifax Harbour on July 21, 1987 (Table 1; Fig. 1, Site 11). However, on subsequent resampling between September 10 and 15, 1987, no *Paramoeba* was isolated from nearshore seawater surface samples from this site nor from any of the other of the 13 nearshore sites except for that nearest the out-

Table 1. *Paramoeba* recovered from Halifax Harbour seawater samples

Surface or bottom	Site	Temperature (°C)	<i>Paramoeba</i> recovered <sup>a</sup>
Surface	Outfall	15.2	–
	Outfall + 30 m S	15.2	+
	Outfall + 30 m N	15.2	+
	Outfall + 60 m S	15.2	–
	Outfall + 60 m N	15.2	–
	Eastern Passage	15.0	+
Bottom <sup>b</sup>	Outfall	11.0	+
	Outfall + 30 m S	11.0	–
	Outfall + 30 m N	11.0	+
	Outfall + 60 m S	11.0	+
	Outfall + 60 m N	11.0	+
	Eastern Passage <sup>a</sup>	2.0	+

<sup>a</sup> Estimated minimum number (EMN) of *Paramoeba* was <math>1\text{ l}^{-1}</math> when no amoebae were observed and >math>0.8\text{ l}^{-1}</math> when amoebae were isolated

<sup>b</sup> Bottom water samples were taken ca 1 m off the bottom at a water depth of 7.6 m (outfall) or 21 m (Eastern Passage)

fall. Since *Paramoeba* has been released via the Aquatron outfall since 1983, our results indicate that this organism has not established a free-living population in the environment in and around Halifax Harbour. However, it is possible that the amoeba might be present in hosts or habitats that we did not study.

The DAPI-stained environmental isolate of *Paramoeba* was morphologically similar to the stock-cultured *Paramoeba invadens* in both size and shape (Fig. 2) and consistent with the species description for *P. invadens* (Jones 1985). The parasome-nucleus complex is clearly displayed by both amoebae, with the diameters of both the nucleus and the parasome being 3 to 4  $\mu\text{m}$  in each organism. Like stock-cultured *P. invadens*, the organisms of the environmental isolate had only one nucleus and one parasome, unlike *P. pemaquidensis* which has supernumerary parasomes (Page 1983). Amoebae from both sources were pleomorphic, ranging from a small spherical floating form (Fig. 2a, b) to an extended locomotive form with dactylopodia or finely conical pseudopodia (Fig. 2c, d). The only known species of free-living *Paramoeba* morphologically similar to *P. invadens* is *P. aestuarina* (Page 1983). Recent observations by Rogerson (1988 and pers. comm.) may bring into question their position as different taxonomic species.

No signs of morbidity were observed within 30 d in any of the sea urchins injected with the environmental isolate or exposed by the waterborne route to the injected individuals. There also was no morbidity or mortality in any individual in either of the control groups. *Paramoeba* could not be cultured from radial

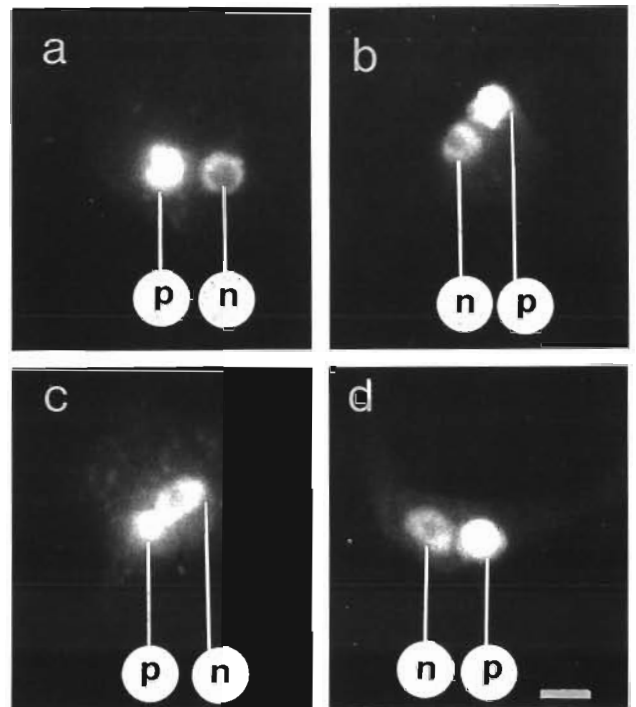


Fig. 2. *Paramoeba invadens*. Photomicrograph of amoeba stained with DAPI fluorescent dye. (a, c). Environmental isolate from an Aquatron outfall water sample. (b, d). Stock-cultured *P. invadens*. n: nucleus; p: parasome; bar = 3.0  $\mu\text{m}$

nerve or coelomic fluid samples from either injected or waterborne-exposed individuals. The injected dosage of amoebae used in this study was ca 5 times that used to consistently induce disease within 10 to 12 d post-injection (Jones & Scheibling 1985, Jellett & Scheibling 1988b). These results indicate that loss of virulence may occur in *Paramoeba* released via the Aquatron outfall. Factors leading to loss of virulence are unknown but may involve freshwater mixing or other conditions within the outfall pipe, or some combination of environmental factors in the Northwest Arm of Halifax Harbour after release. Loss of virulence of *P. invadens* is known to result after long-term (> 58 wk) culturing (Jellett & Scheibling 1988b).

*Paramoeba* was not isolated from radial nerve or coelomic fluid samples from any of 100 healthy sea urchins which were collected from Bedford Basin. Other amoebae (without parasomes) were isolated from one radial nerve sample from each of 7 individuals but not from any of the coelomic fluid samples. These results indicate that *Strongylocentrotus droebachiensis* do not harbour a reservoir population of *P. invadens* at times of the year when the temperature is below the threshold for paramoebiasis.

We have found no evidence to support or even indicate that the source of *Paramoeba invadens* involved in sea urchin mass mortalities between 1980 and 1983

along the Atlantic coast of Nova Scotia was an endemic population of this organism as previously suggested (Jones & Scheibling 1985, Scheibling 1988). Our data lend support to the alternate hypothesis (Jones & Scheibling 1985, Scheibling 1988) that the source of *P. invadens* is more likely outside of our immediate geographic area and that the organism was introduced at the time of the epizootics with warm water currents or via other oceanographic phenomena.

**Acknowledgements.** This research was supported by grants to J.A.N. and R.E.S. from the Natural Sciences and Engineering Research Council of Canada. J. F. J. was supported by predoctoral fellowships from the Natural Sciences and Engineering Research Council of Canada and the Killam Trust. The authors are grateful to Roy Hirtle for assistance with micromanipulation, and to Jay Bugden and Bruce Raymond for excellent field assistance.

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This article was presented by Dr M. R. Lewis, Washington, D.C., USA

Manuscript first received: June 17, 1988

Revised version accepted: May 10, 1989