

Diatom cultures exhibit differential effects on larval metamorphosis in the marine gastropod *Ilyanassa obsoleta* (Say)

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Abstract:

Adult *Ilyanassa obsoleta* recruit from the plankton to surface sediments of intertidal mudflats along the U.S. East Coast as a result of metamorphic induction of planktonic larvae by environmental cues. A previous researcher discovered that seawater extracts of mud from juvenile habitats could induce metamorphosis and provided evidence that the inductive agent was of biological origin. Because juvenile *I. obsoleta* are herbivorous and grow when fed diets of benthic diatoms, we hypothesized that benthic diatoms or their associated microflora might be responsible for producing a metamorphic stimulus. We tested extracts of a culture of mixed diatoms and extracts of cultures of 6 diatom species isolated from mudflat sediments along the coast of North Carolina in which sexually mature and immature adult (juvenile) *I. obsoleta* commonly co-occur. We also tested extracts of one diatom culture isolated at the Friday Harbor Laboratories (Friday Harbor, WA). We found differential effects among the diatom species tested. One centric diatom, *Coscinodiscus* sp., induced metamorphosis at levels close to those triggered by a known neuroactive compound, serotonin. Several species of benthic diatoms displayed neutral effects, showing little or no inductive capability. In one experiment, an unidentified pennate diatom species appeared to inhibit spontaneous metamorphosis that occurred in some competent larvae. We hypothesize that *Coscinodiscus* sp. or its associated microflora, is part of a suite of benthic species that is either indicative of a favorable juvenile environment or is itself part of the post-metamorphic diet. Our results also indicate that larval *Ilyanassa* demonstrated positive, neutral, and perhaps even negative responses to potential metamorphic cues.

Keywords: Caenogastropod, *Coscinodiscus*, Mollusc, Prosobranch, Snail, Tychopelagic

Article:

1. Introduction

Metamorphically competent larvae of most marine invertebrates are generally small in size and thus, to a large extent, are affected by meso-scale features of their oceanic or estuarine environments (Jackson, 1986; Hadfield, 2000). While passive interactions with their environments can explain some of the observed depositional patterns seen in certain species, particularly in sessile organisms like barnacles (Wetthey, 1986), it is now widely accepted that many larvae can and do respond to the physical and biological characteristics of their habitats, selecting those environments that favor their long-term survival and post-metamorphic reproductive success (reviewed in Hadfield, 1998). For many species that require specific juvenile or post-metamorphic habitats, inductive cues often include odorants that largely arise from 1 of 3 major biological sources (Rodríguez et al., 1993): (1) conspecific juveniles or adults (Pechenik, 1980; McGee and Targett, 1989; Tamburri et al., 1992; Zimmer and Butman, 2000; Tamburri et al., 2008), (2) microbial biofilms (Wieczorek and Todd, 1998; Zhao and Qian, 2002; Lau et al., 2005), or (3) juvenile prey or food items (Hadfield and Scheuer, 1985; Morse, 1990, 1991; Boettcher and Targett, 1996; Ito and Kitamura, 1997; Bryan and Qian, 1998; Krug and Manzi, 1999; Roberts et al., 1999; Gallardo and Buen, 2003; Lam et al., 2005). Motile carnivorous and herbivorous molluscs, including adult *Ilyanassa obsoleta* (Say, 1822, synonymous with *Nassarius obsoletus*), are highly dependent on their olfactory abilities to find prey or food items (Carr, 1967a,b; Atema and Burd, 1975; Kohn,

1983; Dorsett, 1986). Newly metamorphosed juveniles of this species need a readily available food source, so we suggest the possibility that larval *I. obsoleta* might also use odorants arising from juvenile food items simultaneously to detect suitable metamorphic sites and trigger the metamorphic process.

Ilyanassa obsoleta, a caenogastropod, inhabits mudflats along much of the East Coast of North America (Scheltema, 1961; Gosner, 1971). This snail has been used for over 130 years as a model organism for the study of early developmental processes, mechanisms of molluscan metamorphosis and more recently, molecular genetics (Collier, 2002). Various aspects of its ecology and natural history, including its distribution, feeding preferences and olfactory capabilities have also received noteworthy attention (Collier, 2002). Adult *I. obsoleta* can graze selectively on unattached migratory diatom cells (Connor and Edgar, 1982; Connor et al., 1982) and historically, these snails have been considered to be herbivorous deposit feeders utilizing sediment microorganisms (Scheltema, 1964). Nonetheless, *I. obsoleta* is more correctly viewed as an obligate omnivore or facultative carnivore that requires a high protein diet for reproductive success (Scheltema, 1964; Curtis and Hurd, 1979, 1981; Connor and Edgar, 1982; Curtis, 1985; Hurd, 1985; Brenchley, 1987). Conversely, as juveniles, these marine snails are reported to be obligate herbivores (Brenchley, 1987; Lin and Leise, 1996) and anecdotally, have been raised for over 12 months on diatom films in laboratory aquaria (Scheltema, 1964).

Previously, Leise and coworkers discovered that endogenous production of the gaseous neurotransmitter, nitric oxide (NO), is required to maintain the larval state in *I. obsoleta* (Froggett and Leise, 1999; Thavaradhara and Leise, 2001). When larval metamorphosis is initiated by the reception of an appropriate pharmacological stimulus in the laboratory, the ensuing morphological and physiological transformations include a decrease in the expression of the gene coding for nitric oxide synthase, the enzyme that produces NO (Froggett and Leise, 1999; Leise et al., 2001, 2004; Gifondorwa and Leise, 2006; Hens et al., 2006). Leise and colleagues also confirmed Levantine and Bonar's (1986) discovery that the classical neurotransmitter serotonin (5-HT) can induce metamorphosis in competent larvae, and they provided pharmacological data supporting the idea that exogenously applied 5-HT mimics the action of serotonergic neurons in the larval nervous system (Couper and Leise, 1996; Leise et al., 2001, 2004). Our studies of the cellular and physiological mechanisms that bring about metamorphosis in this species continue, but to understand the regulation of this process from an environmentally relevant viewpoint, we need specific knowledge of how *I. obsoleta* reacts to a natural inducer. We can then investigate how natural induction of metamorphosis affects the opposing actions of NO and 5-HT. Furthermore, as *I. obsoleta* is an important and widespread agent of disturbance, impacting the structure and complexity of temperate western Atlantic mudflats (Connor et al., 1982; Hunt et al., 1987; Cranford, 1988; Kelaher et al., 2003), we need to understand more fully how metamorphic and juvenile requirements affect its widespread intertidal distribution.

Scheltema (1961) identified mud from the juvenile habitat of *I. obsoleta* as supplying a potent, water-soluble metamorphic inducer of biological origin, and this was later confirmed by Levantine and Bonar (1986). While these researchers did not identify a specific origin for this inductive compound, Scheltema (1956, 1961, 1962b) hypothesized that organic material from the benthos, including adsorbed compounds, microflora, or microfauna, might be sources for a metamorphic trigger.

Juvenile *I. obsoleta* feed on benthic diatoms (Brenchley, 1987) and preliminary experiments in our laboratory indicated a lack of response by recently metamorphosed juveniles to fish carrion, which induces vigorous feeding by adults (Lin and Leise, 1996). Studies of soft sediment intertidal habitats report very high abundances of benthic diatoms (McIntyre et al., 1996; Cahoon et al., 1999), a food source for adult *I. obsoleta* and other benthic herbivores and omnivores (Haines and Montague, 1979; Hughes and Sherr, 1983). Moreover, benthic microflora are relatively protein-rich, with C:N ratios of approximately 7:1 (Cammen et al., 1982) (Cahoon, unpublished data). The distribution of benthic diatoms and other microalgae is very patchy in time and space, responding to both abiotic and biotic processes (Cahoon et al., 1999). The species composition of benthic microalgae also varies considerably with local depositional and erosional properties, sediment composition, and other factors (Amspoker and McIntire, 1978; Whiting and McIntire, 1985; McIntire and Amspoker, 1986). Owing to the importance and variable nature of the benthic food resource, and the importance to

metamorphosing *I. obsoleta* of selecting beneficial micro-habitats, we thought it likely that Scheltema's (1956, 1961, 1962a) benthic inducer hypothesis would be correct and that some species of benthic diatoms might produce inductive substances. Thus, we tested benthic microalgae, including a number of individual diatom species that occur commonly in coastal mudflats, where *I. obsoleta* are abundant, for their inductive capabilities.

We began by testing the effects of a seawater extract of raw sediment from mudflats inhabited by adult and juvenile *I. obsoleta*. Positive results from this experiment and from the exposure of competent larvae to an extract of a culture of mixed diatoms influenced us to examine the effectiveness of extracts of diatom cultures derived from single-celled isolates in an effort to define a potential source for a natural metamorphic trigger. In this paper, we report our experimental results, which indicate that larval *I. obsoleta* can display positive, neutral and perhaps negative responses to water-soluble extracts of cultured diatoms species isolated from assemblages that occur in coastal mudflats.

2. Materials and methods

Adult snails, sediment and most diatoms that were subsequently isolated and cultured were collected in 1996 from intertidal mudflats along the north side of the dock at UNC Wilmington's Center for Marine Science (CMS; 34°08'25.39N, 77°51'46.96W), near the Masonboro Sound IntraCoastal Waterway Marker 137, in the Myrtle Grove area in southeastern North Carolina, at low tide. Adult and juvenile *I. obsoleta* co-occur in these same mudflats. Sediment and diatoms were harvested in midwinter so that cultures could be raised in time to be tested on competent larvae during the spring and summer of 1996.

Microalgae living in the top 1–2 cm of sediment were cultured in the laboratory using 14:10 h light:dark fluorescent illumination in a constant temperature incubator (Fisher Scientific) at 20 °C in 0.45 μ m filtered seawater (~30 ppt salinity) that had been autoclaved and amended with an enhanced 'f/2' medium (doubled silicate and vitamins; R.R.L. Guillard, pers. comm.) (Stein, 1973). Three types of extracts were prepared and tested on metamorphically competent larvae of *I. obsoleta*: extracts of a mixed diatom culture, single-species isolates, and of the f/2 culture medium (a control). A mixed diatom culture was prepared by micropipeting a few diatom cells from small aliquots of surface sediments into filtered seawater and culturing them together as described above. Individual diatom isolates were prepared by micropipeting single cells from the original surface sediment samples through several washes in filtered seawater, then into culture tubes and cultured as above. One culture of benthic pennate diatoms that had been isolated at the University of Washington's Friday Harbor Laboratories (Friday Harbor, WA) in September 1995 was also grown as described above and assayed for its inductive abilities. No attempt was made to grow axenic cultures. Most diatoms in cultures were identified to genus and, when possible, to species, using standard reference keys (Hustedt, 1955; Smith, 1977). Unfortunately, these diatom cultures were ultimately lost because of the damage incurred at the UNCW facilities during Hurricane Fran (Sept. 4–8, 1996), precluding further experimentation and final identification of several cultures made from individual cell isolates.

Extracts of sediment and diatom cultures were filtered through glass fiber filters (nominally 1.0 μ m pore size) to remove the diatom cells, lyophilized at UNCW, shipped on dry ice to UNCG, and stored continuously at —60°C to —80°C until needed. Only the mixed diatom culture (DIAMIX, Fig. 1) was additionally filtered to 0.45 μ m before lyophilization. Lyophilized samples were reconstituted to their original salinity with distilled water that had been subjected to ultrafiltration through a Nanopure Ultrapure Water System, Model D4751 (Barnstead/Thermolyne, Inc.), just before experimentation. Reconstituted extracts were then filtered to 0.2 μ m to produce cell-free extracts just before use unless otherwise indicated. With the exception of extracts used in Fig. 5, where larvae were not immediately available, extracts were used within 3 weeks of being received at UNCG.

Procedures for the maintenance of breeding populations of adult *I. obsoleta* and the culture of larvae have been described previously in detail (Couper and Leise, 1996; Froggett and Leise, 1999). Briefly, egg capsules laid in laboratory aquaria by adult snails were collected weekly and newly hatched larvae were cultured at densities of about 1: 1.5 ml of larval seawater (1: 1 mixture of natural seawater and artificial seawater, filtered to 0.2 μ m).

Cultures typically include a mixture of larvae derived from multiple (5-15) adult females. Larvae are cultured in an airlift system, with antibiotics, at slightly elevated room temperature (25- 26°C) as previously described and fed daily with a mixture of two single-celled algae, *Dunaliella tertiolecta* and *Isochrysis galbana*. Experiments were conducted with metamorphically competent larvae cultured for 18-30 days after hatching. Just before use, the longest shell lengths were measured on 12-15 larvae per culture. Averages ranged from 540 to 657 μm . Bath experiments have also been described previously (Couper and Leise, 1996; Froggett and Leise, 1999; Gifondorwa and Leise, 2006). Briefly, competent larvae were tested in untreated 24-well Falcon® tissue culture plates at 10 larvae in 2 ml of experimental solution per well, at ambient light and temperature (~22°C). Results of unpublished experiments indicate identical results and no intra-larval effects for 1, 5 or 10 larvae per experimental well. Wells were assayed at 24 and 48 h for numbers of larvae and juveniles. As metamorphosis is an irreversible process in this species (Scheltema, 1961) and because newly competent larvae generally do not metamorphose spontaneously under our experimental conditions, only larvae that had lost their velar lobes were scored as metamorphosed. Loss of the velar lobes is the most obvious and dramatic of the external changes that occur at metamorphosis and is easily observed with a dissecting microscope (Scheltema, 1962a). In all experiments, artificial seawater made from Instant Ocean® or Reef Crystals® filtered to 0.2 μm (FIO) was used as a negative control and 100 μM serotonin (5-HT) in FIO, which induces metamorphosis (Levantine and Bonar, 1986; Couper and Leise, 1996), was the positive control. Experiments were conducted with larvae from 1-3 cultures and where multiple cultures were utilized, larvae from all cultures were exposed to each experimental treatment simultaneously. As indicated in the figures, with only 2 exceptions, control and experimental solutions were tested on 9 replicates of 10 larvae per well (i.e., $n = 9$) for a total of 90 animals per treatment.

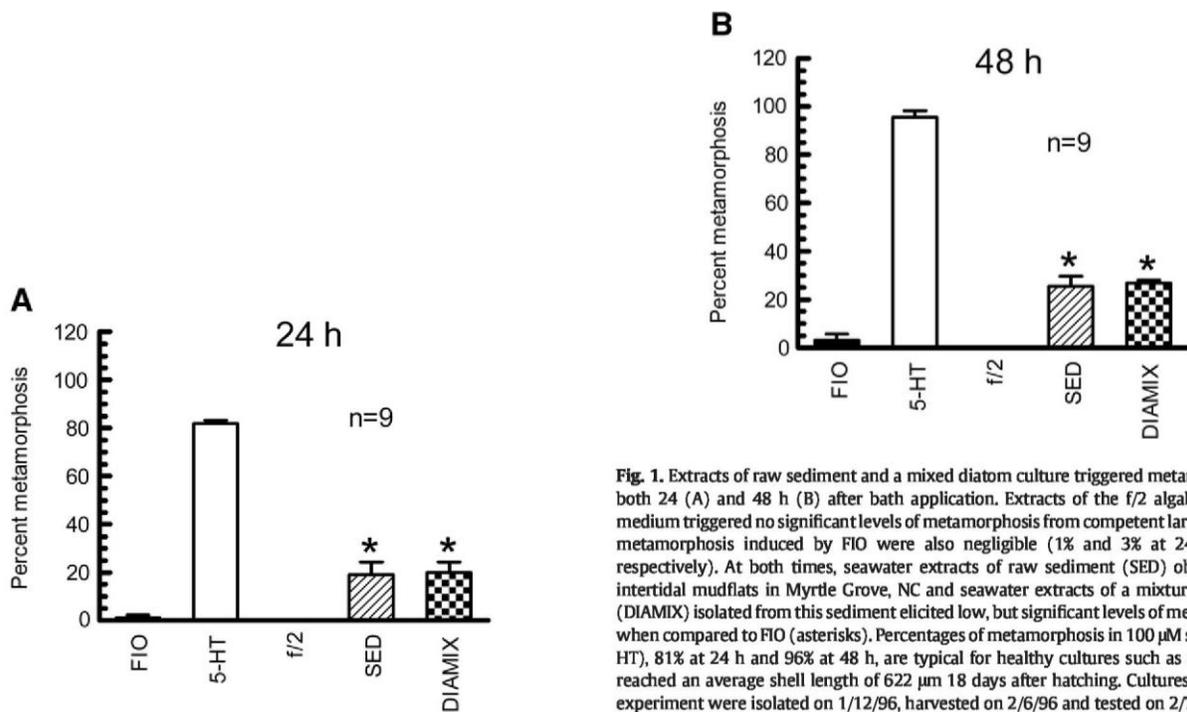


Fig. 1. Extracts of raw sediment and a mixed diatom culture triggered metamorphosis at both 24 (A) and 48 h (B) after bath application. Extracts of the f/2 algal enrichment medium triggered no significant levels of metamorphosis from competent larvae. Levels of metamorphosis induced by FIO were also negligible (1% and 3% at 24 and 48 h, respectively). At both times, seawater extracts of raw sediment (SED) obtained from intertidal mudflats in Myrtle Grove, NC and seawater extracts of a mixture of diatoms (DIAMIX) isolated from this sediment elicited low, but significant levels of metamorphosis when compared to FIO (asterisks). Percentages of metamorphosis in 100 μM serotonin (5-HT), 81% at 24 h and 96% at 48 h, are typical for healthy cultures such as these, which reached an average shell length of 622 μm 18 days after hatching. Cultures used in this experiment were isolated on 1/12/96, harvested on 2/6/96 and tested on 2/7/96.

Results of each experiment were tested for statistically significant differences among treatments ($P = 0.05$) in 2×2 contingency tables (Sokal and Rohlf, 1995; Zar, 2010) using Fisher's exact test in GraphPad Prism Version 4.0 software (GraphPad Software, Inc.). Bonferroni's correction was used for multiple comparisons (Bland, 1995). Where Fisher's test was used, the corrected P value is presented. To test for effects of multiple levels of filtration, we also analyzed 4×2 and 3×2 contingency tables, as appropriate, with the Chi-square test. In these instances, we present the Chi-square statistic, also corrected by Bonferroni's method. We used contingency table analysis because it allowed us to test for statistical significance on raw, untransformed data (Sokal and Rohlf, 1995). These analyses require more robust data to detect statistically significant differences than do tests using analysis of variance. Graphs of untransformed means \pm the standard error of the mean (calculated on arcsine

transformed data, Sokal and Rohlf, 1995) were produced with GraphPad Prism Version 4.0 software (GraphPad Software, Inc.).

3. Results

We initially exposed competent larvae to seawater extracts of raw sediment and a mixture of diatoms cultured from these raw sediment samples to determine if the benthic diatom community might be a source of a metamorphic inducer for larval *I. obsoleta* (Table 1). These extracts induced a significant number of competent larvae to metamorphose (Fig. 1), although in both instances and at both time points, their inductive capability was significantly less than that of 5-HT. For both sediment (SED) and mixed diatom culture (DIAMIX) extracts, levels of metamorphosis increased from 24 to 48 h (e.g., 19% metamorphosis in SED at 24 h, $P < 0.0004$, rising to 26% at 48 h, $P < 0.0004$, compared to FIO). The DIAMIX extract induced slightly higher levels of metamorphosis at both times than did the SED extract, 20% metamorphosis at 24 h, rising to 27% at 48 h, but the differences between extracts were insignificant (Fig. 1). Extracts of the f/2 medium used to culture diatoms (Stein, 1973) induced metamorphosis in a maximum of 5.6% of competent larvae at 48 h (Fig. 2), also an insignificant effect ($P = 0.238$).

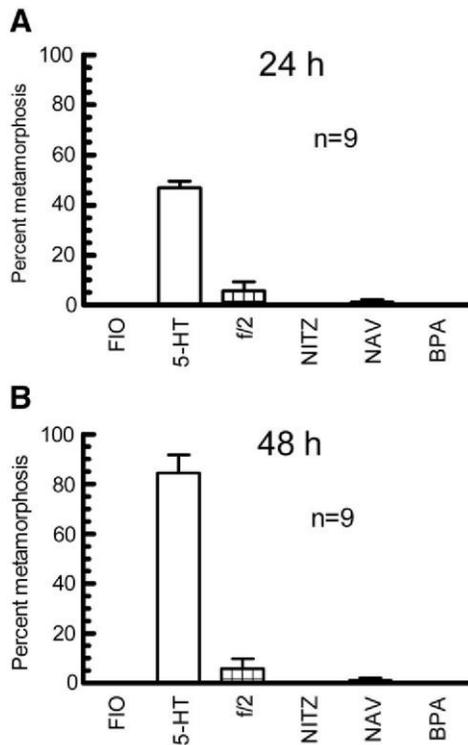


Fig. 2. Extracts of cultures of 3 diatoms, *Nitzschia closterium* (NITZ), *Navicula* sp. (NAV), and one unidentified species of attached benthic pennate (BPA) induced insignificant numbers of competent larvae to metamorphose. The extract of the f/2 medium and FIO also triggered negligible levels of metamorphosis at both 24 and 48 h. Cultures used in this experiment were isolated on 2/16/96, harvested on 3/13/96 and tested on 3/19/96.

Culture extracts of 3 diatoms common to the NC mudflats, *Nitzschia closterium*, *Navicula* sp., and an attached benthic pennate species (BPA), were not inductive (Fig. 2). To begin to determine if our typical filtration regimes might be decreasing the effectiveness of our culture extracts, two of the common species, *N. closterium* and *Navicula* sp., were tested at 3 levels of filtration, 5.0 pm, 1.2 pm and 0.2 pm (NITZ 5.0, NITZ 1.2, NITZ 0.2, NAV 5.0, NAV 1.2, and NAV 0.2, in Fig. 3, respectively), as was a third species, *Coscinodiscus* sp. (COS 5.0, COS 1.2, and COS 0.2, Fig. 3). Neither *N. closterium* nor *Navicula* sp. showed any inductive ability, at any level of filtration (Fig. 3). Conversely, at 24 h, *Coscinodiscus* sp. was as effective an inducer as 5-HT ($X^2_{0.05, 3} =$

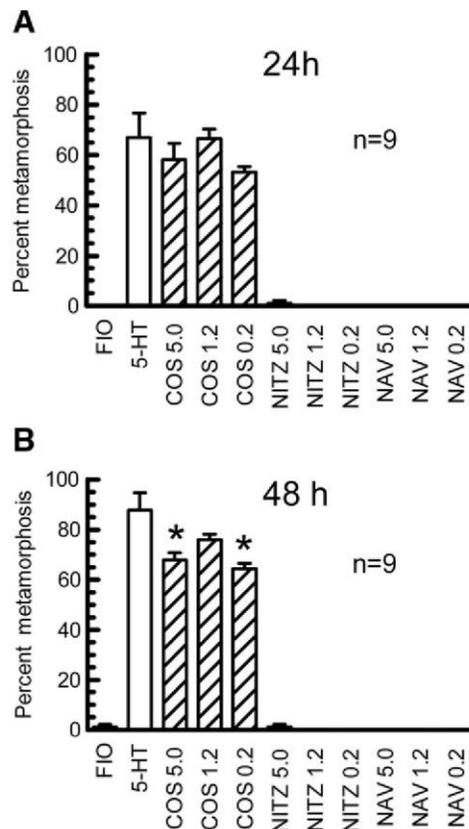


Fig. 3. At all levels of filtration (5.0 μ m, 1.2 μ m, and 0.2 μ m), extracts of *Coscinodiscus* sp. elicited metamorphosis from significant numbers of competent larvae. (A) Levels of metamorphosis with these extracts (COS 5.0, COS 1.2, COS 0.2) were similar to that elicited by 5-HT at 24 h. At 48 h (B), filtration through 5.0 and 0.2 μ m filters significantly reduced the amount of larval metamorphosis when compared to 5-HT (asterisks). Inductive capabilities of *Nitzschia closterium* and *Navicula* sp. remained negligible at all levels of filtration. (filtration to 5.0 μ m = NITZ5.0 or NAV 5.0, to 1.2 μ m = NITZ 1.2 or NAV 1.2, and to 0.2 μ m = NITZ 0.2 or NAV 0.2). Cultures used in this experiment were isolated on 1/12/96, harvested on 4/1/96 and tested on 4/11/96.

3.928, critical value = 7.815). By 48 h, extracts of cultures of this tychopelagic, centric diatom had triggered a maximum of 76% metamorphosis in competent larvae, but filtration to 5.0 and 0.2 μm significantly reduced the effectiveness of this inducer when compared to 5-HT (e.g., $P = 0.0112$ for COS 5.0, Fisher's exact test with Bonferroni's correction, Fig. 3). We conducted a second experiment (Fig. 4) to confirm these results with COS and BPA (cf. Fig. 2) and to expand the search for other inductive diatoms.

As *I. obsoleta* has succeeded in colonizing West Coast mudflats (Kozloff, 1983) it seemed reasonable to test cultures of diatoms isolated from West Coast habitats. Thus, we tested an extract of a culture of a benthic pennate species (FHL) that had been isolated at the Friday Harbor Laboratories (Friday Harbor, WA). At 24 h, inductive capabilities for *Coscinodiscus* sp. and the Friday Harbor pennate species (FHL) were similar to those of 5-HT ($X^2_{0.05,2} = 5.989$, critical value= 5.991). By 48 h, FHL showed no inductive difference from 5-HT ($P = 0.516$, critical value= 5.991) while COS induced significantly fewer larvae to metamorphose than 5-HT ($P = 0.006$, Fisher's exact test with Bonferroni's correction).

In one experiment, we detected significant levels of spontaneous metamorphosis, 11% and 26% respectively, after 24 and 48 h in FIO, our negative control, in which we typically see under 5% metamorphosis. These larvae also displayed a strong response to 5-HT, which triggered 85% of larvae to metamorphose by 24 h and 96% by 48 h (cf. Figs. 2 and 5). Of the diatom extracts tested, 2 pennate species from Myrtle Grove (MGP I and MGP II) and the diatom culture isolated at Friday Harbor (FHL), at 24 h, only FHL significantly increased percentages of metamorphic induction above that in FIO ($P=0.0141$, star, Fig. 5A). However, by 48 h, levels of metamorphosis in response to MGP II and FHL were indistinguishable from that of FIO ($P=1.11$ and $P=1.85$, respectively). Competent larvae showed no response to MGPII at either time point, but after 48 h in the presence of another pennate diatom, MGP I, larvae displayed significantly reduced levels of metamorphosis when compared to those in FIO ($P = 0.0003$).

4. Discussion

Previous researchers found that sediments inhabited by adult and juvenile *I. obsoleta* contain seawater-soluble substances of biological origin that can induce metamorphosis in competent conspecific larvae (Scheltema, 1956,1961; Levantine and Bonar, 1986). Our results confirm and extend these findings; a seawater extract of raw sediment (SED) and an extract from a culture of mixed diatoms derived from this sediment (DIAMIX) elicited metamorphosis from significant proportions of larval *I. obsoleta* (Fig. 1). As stated, our sediment and diatom samples also originated from mudflats inhabited by adult and juvenile conspecifics. Percentages of metamorphosis in response to these extracts were lower than those induced by 5-HT or by extracts of a culture of *Coscinodiscus* sp. (cf. Figs. 1, 3 and 4). However, the levels of metamorphosis we observed were equivalent to those reported by Scheltema for larvae induced by seawater that was exposed to mud then filtered to 0.8 μm , if one subtracts his numbers for spontaneous metamorphosis (12.3%, Table VIII in Scheltema, 1961).

Table 1
Species list and relative abundance of diatoms (few cells, * to many cells, *****) in mixed diatom culture derived from raw sediment.

Species	Suborder	Abundance
Order Centrales		
<i>Biddulphia</i> sp.	Biddulphiineae	*
<i>Chaetoceros</i> sp.	Biddulphiineae	**
<i>Coscinodiscus</i> sp.	Coscinodiscineae	*
<i>Melosira</i> sp.	Coscinodiscineae	**
Order Pennales		
<i>Bacillaria</i> sp.	Biraphidineae	*
<i>Cocconeis</i> sp.	Monoraphidineae	*
<i>Licmophora</i> sp.	Araphidineae	*
<i>Navicula</i> sp.	Biraphidineae	*****
<i>Nitzschia closterium</i>	Biraphidineae	*****
<i>Nitzschia paradoxa</i>	Biraphidineae	**
<i>Nitzschia seriata</i>	Biraphidineae	***
<i>Pleurosigma</i> sp.	Biraphidineae	*****

This culture grew for several months in the laboratory before experimentation, so relative abundances of diatom species do not necessarily mimic those in the natural intertidal environment.

Our diatom mixture contained 12 species (Table 1), which is only a small subsample of the species occurring on these mudflats. Hustedt (1955) reported several hundred species of diatoms from two small samples from a coastal North Carolina habitat. Moreover, many diatoms found in the sediments also occur in the plankton, including *N. closterium* and some species of *Coscinodiscus*. Mud samples were obtained during the winter so that dense cultures, approximating surface algal mats that larvae might normally encounter, could be grown in time for testing on available larvae. Unlike phytoplankton populations, which display marked seasonality, benthic diatom populations maintain high biomass throughout the year (Nearhoof, 1994). The DIAMIX culture grew for about a month before being used, so the relative diatom abundances in it may not necessarily duplicate normal field occurrences of diatom species, cell sizes, or ages. Furthermore, the relatively low percentages of metamorphosis observed in responses to both SED and DIAMIX (Fig. 1) may have resulted from a potentially unnatural mixture of diatom cells or a preponderance of non-inductive or inhibitory species.

We tested the inductive actions of extracts of several diatom cultures grown from individual isolated cells to determine if diatoms and their associated bacteria might be a source for a water-soluble natural inducer. Of these, one unidentified species of *Coscinodiscus* triggered metamorphosis in a repeatable fashion, while another (FHL, Figs. 4 and 5) was less reliable.

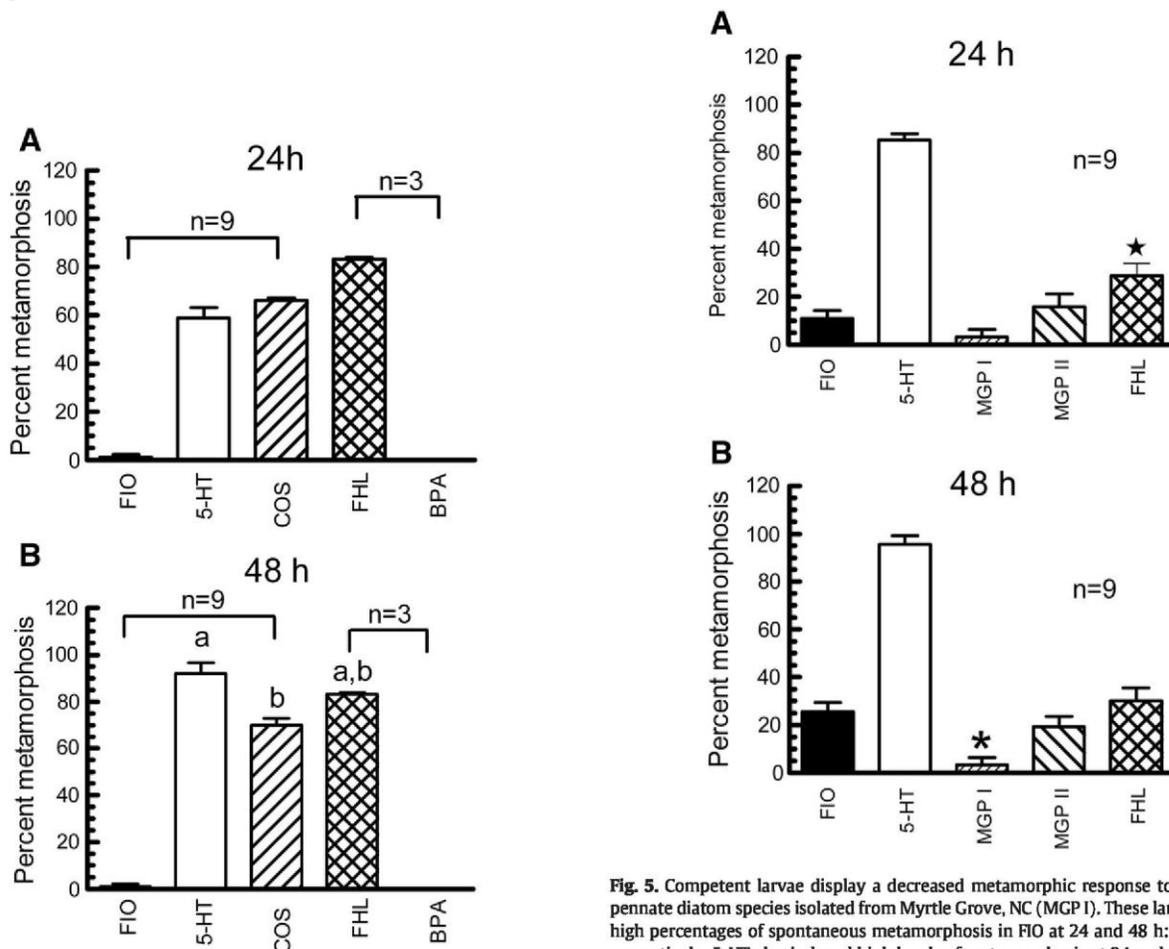


Fig. 4. Confirmation of inductive abilities of *Coscinodiscus* sp. (COS) and demonstration of induction by a pennate species (FHL) isolated at the Friday Harbor Laboratories. (A) At 24 h, COS and FHL both elicit metamorphosis at levels similar to those of 5-HT. (B) At 48 h, COS induced significantly fewer larvae to metamorphose than 5-HT. Letters above bars indicate statistically similar results ($P > 0.05$). Cultures used in this experiment were isolated on 1/12/96, harvested on 4/1/96 and tested on 4/16/96.

Fig. 5. Competent larvae display a decreased metamorphic response to extracts of a pennate diatom species isolated from Myrtle Grove, NC (MGP I). These larvae displayed high percentages of spontaneous metamorphosis in FIO at 24 and 48 h: 11% and 26%, respectively. 5-HT also induced high levels of metamorphosis at 24 and 48 h: 85% and 96%, respectively. (A) At 24 h, of the diatoms tested, only FHL induced metamorphosis in significantly greater numbers of larvae than the spontaneous metamorphosis seen in FIO (star). By 48 h, a pennate species isolated from mudflats in Myrtle Grove, NC (MGP II) and FHL showed no difference in inductive capabilities when compared to FIO. Another Myrtle Grove pennate (MGP I) induced significantly fewer larvae to metamorphose than did FIO at both 24 and 48 h (asterisk). Cultures used in this experiment were isolated on 1/12/96, harvested on 9/23/96 and tested on 12/4/96.

4. 1. *Coscinodiscus* sp. elicits metamorphosis

Extracts of cultures of a very large centric diatom, *Coscinodiscus* sp. (Bold and Wynne, 1985), which was isolated from intertidal sediments inhabited by juvenile and adult *I. obsoleta*, elicited relatively high levels of metamorphosis from newly competent larvae (Figs. 3 and 4). We think that this diatom species may play a

major role in producing a metamorphic cue in the natural environment. This tychopelagic diatom can be suspended in the plankton by vigorous turbulence, but settles to the benthos to become part of patchy mats under the relatively calm conditions that exist on mudflats behind the barrier islands along the coast. We hypothesize that competent larval *I. obsoleta*, interacting with substances diffusing from such patchy intertidal mats, will be induced to settle and metamorphose.

Coscinodiscus spp. have been reported to be members of the benthic microflora in a variety of shallow marine habitats (Hustedt, 1955; Cahoon and Laws, 1993; Lucas et al., 2001; Ingole et al., 2006). Much of the organic material available to deposit feeders in such depositional environments might be detrital material of low quality. However, tychopelagic diatoms, such as *Coscinodiscus* sp., in such a well-illuminated, nutrient-rich environment, are likely to be physiologically vigorous and therefore of high nutritional quality (Sakshaug and Holm-Hansen, 1977), which, coupled with the relatively large size of *Coscinodiscus* sp. (>100 μm diameter), should make this an attractive food source for post-larval gastropods and lamellibranches (Yang, 1998). Such diatoms have been reported as frequent dietary components for other molluscan herbivores (Pastore et al., 1976; Nasr, 1984; Osorio et al., 1987; Kasigwa and Mahika, 1991; Cai et al., 1992). Thus, we presume that the presence of *Coscinodiscus* sp. indicates a favorable environment for juvenile snails. Although laboratory experiments can rarely duplicate the complexity of natural habitats, the strong responses we obtained from our larvae suggest that further experiments with this diatom genus are warranted. Data concerning the ingestion and digestibility of this diatom by young, post-metamorphic *I. obsoleta* would be most instructive.

Extracts of cultured *Coscinodiscus* sp. elicited lower percentages of metamorphosis than serotonin (Figs. 3 and 4) and several factors could account for this differential activity. First, *Coscinodiscus* sp. may produce only part of a natural metamorphic cue, with other microflora or microfauna adding necessary odorant components. Secondly, we may have lost inductive components of the extracts during the filtration necessary for our laboratory experiments. Our results show some support for this idea (Fig. 3), but as yet we have no answer for why filtration to 1.2 μm resulted in more metamorphic induction than filtration to 5.0 μm . Thirdly, in our laboratory experiments, larvae encounter dissolved substances in the water column, but never interact with diatom frustules as they would in the field. Finally, pharmacological reagents such as 5-HT, in conjunction with the laboratory setting, may induce an unusually high percentage of larval metamorphosis that does not mimic a natural inductive process.

Diatoms typically grow with specific associated bacteria (Grossart et al., 2005) and as we made no attempt to remove bacteria from our diatom cultures, our extracts may contain a mixture of bacterial and diatom exudates. Thus, we cannot identify the exact source of the metamorphic cue: the diatoms, their associated microflora, or both. Unlike other studies that used diatom cultures or biofilms with viable cells (Kawamura, 1996; Bryan and Qian, 1998; Sawatpeera et al., 2004; Chiu et al., 2007; Roberts et al., 2007), in our experiments, as mentioned, larval *I. obsoleta* were never directly exposed to living diatom cells. Bacteria can exert major influences on the growth of associated algal cells (Grossart and Simon, 2007), but an understanding of how such bacterially induced changes might affect the growth characteristics of diatoms in cultures, and thus, their inductive abilities, is beyond the scope of our experiments.

How a diatom inducer interacts with the known metamorphic pathway has not yet been studied, but we can erect reasonable hypotheses. Because exogenous 5-HT can elicit metamorphosis, presumably by mimicking the action of serotonergic neurons (Couper and Leise, 1996; Leise et al., 2001), we anticipate that 5-HT will potentiate larval responses to natural inducers. Conversely, because nitric oxide inhibits metamorphosis in competent larvae (Froggett and Leise, 1999; Leise et al., 2004; Hens et al., 2006), we expect this diatom inducer to antagonize this activity.

4.2. Larval *Ilyanassa* display no response to some diatoms

We tested extracts of cultures of 5 other diatom species, 2 of which, *N. closterium* and *Navicula* sp., are in commonly occurring genera. Both elicited no response from competent larval *I. obsoleta* (Figs. 2 and 3). *Nitzschia closterium* was investigated as a possible food source for juveniles of the South African abalone,

Haliotis midae, but these juveniles do not ingest it (Matthews and Cook, 1995). Other species in the above two diatom genera elicit metamorphosis from larval abalone, but not all species in these genera are inductive (Najmudeen and Victor, 2004). None of the other three diatom species, all pennate diatoms, that we investigated, could trigger larval metamorphosis (Figs. 4 and 5). As mentioned above, 2 of the most abundant species in the DIAMIX culture (Table 1) are non-inductive and may have been partly responsible for the limited metamorphic responses elicited by both SED and DIAMIX.

In larval abalone, metamorphosis on various species of diatoms often, but not always, reflects the nutritional value of the diatom as a juvenile food source (Kawamura, 1996). Thus, we hypothesize that neither *N. closterium*, nor our *Navicula* sp., will be part of the post-metamorphic diet of *I. obsoleta*, but that *Coscinodiscus* sp. will be. Like larvae of the Japanese abalone, *Haliotis discus hannai* (Kawamura et al., 1995; Kawamura, 1996), larval *I. obsoleta* can respond distinctively to different diatom genera and perhaps even to different congeneric species, displaying positive and neutral responses to a variety of local diatoms. We anticipate that tests of other local diatoms for their inductive effects will also reveal a range of metamorphic responses.

4.3. Is the natural cue a multi-component mixture?

Given the complexity and patchiness of mudflat environments (Qian et al., 2003), as well as the slightly reduced larval response to *Coscinodiscus* sp. in comparison to pharmacological induction, we expect a natural metamorphic cue to arise from multiple sources and to be multi-dimensional, with major and minor constituents. Such mixtures often elicit stronger responses than individual odorant molecules (Gentilcore and Derby, 1998), but we can only speculate that larval responses to odorants in the water column are distinct from their behaviors in the sediment, where they interact with small clusters of diatom cells in the selection of a final metamorphic site. The benefits of multi-component cues have been investigated previously. For example, films of the diatom *Navicula* sp. enriched with abalone mucus are better metamorphic inducers than the diatom films alone (Gallardo and Buen, 2003). Furthermore, if *I. obsoleta* has, as we suspect, evolved the ability to respond to odorants arising from several diatom species, this would help to explain its recruitment to novel intertidal environments.

Ilyanassa obsoleta has successfully colonized U.S. West Coast mudflats and oyster beds (Kozloff, 1983) and we have demonstrated that a benthic pennate species isolated at the Friday Harbor Laboratories can induce metamorphosis in this snail (Fig. 4), supporting our hypothesis that multiple diatom species may be capable of producing an inductive cue. However, the response to the West Coast diatom extract was variable (cf. Figs. 4 and 5). Our protocols suggest no obvious explanation for this lack of consistency, but we note that results displayed in Figs. 4 and 5 were obtained 8 months apart. Prolonged storage of this particular culture extract may have reduced its activity.

Our final reason for suggesting that multiple diatom species may be responsible for producing inductive cues in larvae is our knowledge of the importance of olfactory stimuli to adult *I. obsoleta*. Numerous laboratory and field investigations have demonstrated this snail's reliance on olfaction to find sources of protein and mates (Carr, 1967a, b; Atema and Burd, 1975; Curtis and Hurd, 1979). Whether or not competent larvae possess a functional osphradium is unclear, but they do possess a siphon and a sizeable osphradial ganglion, which is identifiable in larvae within 6 days of hatching, well before larvae become metamorphically competent (Lin and Leise, 1996). The cerebral ganglia, at the bases of the cephalic tentacles, as well as the apical ganglion, which has been implicated in the reception of metamorphic cues (reviewed in Leise et al., 2004), are likewise visible early in larval development and grow throughout the planktonic period. Thus, we suggest that competent veligers have the neural equipment necessary for processing olfactory signals that would reveal the relative merits of potential metamorphic, and thus juvenile, habitats.

4.4. Juveniles require digestible diatoms

Kawamura and colleagues (Kawamura et al., 1995, 1998; Kawamura, 1996; Roberts, 2001) have demonstrated that post-metamorphic *H. discus hannai* can ingest diatoms, but must be able to crack diatom frustules and extract cellular contents for digestion to occur. An inability to open some diatoms precludes long-term post-

metamorphic growth and survival. Experiments with *H. discus hannai* and *Haliotis iris* have also indicated that several diatom species can trigger metamorphosis (Kawamura, 1996; Roberts, 2001; Roberts et al., 2007), but prostrate, benthic diatoms are more likely to be used as substrates for larval metamorphosis than species that form 3-dimensional or filamentous mats. Of the 2 species that can trigger metamorphosis in competent larval *Ilyanassa*, *Coscinodiscus* sp. forms relatively flat mats in calm mudflat environments, but again, we do not know if this diatom is ingested by young juveniles. The other inductive species (FHL, Fig. 4) is an attached pennate with a somewhat upright growth pattern. Similarly, the juvenile digestibility of this species remains to be explored.

4.5. Potential inhibition of spontaneous metamorphosis

As larval *I. obsoleta* age in culture, some will spontaneously metamorphose and as the competence period progresses, the number of spontaneously metamorphosing individuals will increase (Scheltema, 1961). The aging of larvae in cultures, and their temporal progression through the competence period, depends upon a number of factors, including temperature, food availability, larval crowding, and age, so we included negative (FIO) and positive (5-HT) controls in each experiment to ensure that larvae from all cultures used in an experiment behaved in relatively comparable ways. Larval response to the negative and positive controls (0–5% in FIO, 75–100% in 5-HT) confirm that these experiments were conducted with larvae towards the beginning of their competence phase (Figs. 1–4). However, because culture conditions can vary, it is possible to use larvae that display significant levels of spontaneous metamorphosis, which may have allowed us to observe a negative response to diatoms that would have otherwise gone undetected (Fig. 5). The most parsimonious, but certainly not the only explanation for the low response to one pennate species, MGPI, after 48 h of exposure, is that extracts from this culture inhibited metamorphosis. However, we note that this experiment was not replicated, nor do we know if extracts of this culture would inhibit pharmacological induction of metamorphosis with 5-HT. How a larva would respond to a mixture of this culture and an inductive one like *Coscinodiscus* sp. remains to be determined.

Responses to naturally occurring environmental odorants by larvae and young juveniles generally include positive, neutral and negative responses, the latter leading to rejection of local environments as unacceptable or even to death (Woodin, 1991; Walters et al., 1996; Woodin et al., 1998). Inhibitory effects of diatoms on larval settlement and metamorphosis are not so well known as their positive effects, but several larval types display such negative responses (Dobretsov et al., 2006). Negative responses to particular diatoms could have decreased the effectiveness of the DIAMIX extract below that of the best inducer present within the mixture (c.f. Figs. 1 and 3). The significance of a negative response to a particular diatom culture is unclear—is this merely a poor juvenile food source or is it evidence of a dangerous or potentially toxic environment? This distinction requires further experimentation. However, we now tentatively add *Ilyanassa* to the growing list of marine invertebrate larvae capable of showing these 3 categories of behavioral responses to potential settlement habitats.

In Section 4.3, we discussed a likely reason for the low response to FHL displayed in Fig. 5, namely, a decrease in extract activity from prolonged storage. We present an alternative, that larval responses to natural inducers may decline during the end of the competence phase, as the end of larval life is reached. Doyle (1975), working with larvae of the polychaete *Spirorbis borealis*, predicted that larvae should display less substrate selectivity with age. For larval *Ilyanassa*, decreased selectivity is detectable as spontaneous metamorphosis that occurs without any inducer present. How larvae respond to *Coscinodiscus* sp. towards the end of the competence period is unknown, but there may be a loss of responsiveness as there is for FHL. And, although the response to 5-HT is high, it may be artificially elevated by the high levels of spontaneous metamorphosis observed in FIO. We speculate that late in the competence period, larvae may lose responsiveness because of degeneration of sensory neurons (Gifondorwa and Leise, 2006) or the lack of particular functional receptor molecules, but that they retain an ability to undergo a complete metamorphosis.

5. Conclusions

Water-soluble extracts of cultures of the centric, tychopeagic diatom *Coscinodiscus* sp. elicit metamorphosis from newly competent larvae of *I. obsoleta*. This diatom has been identified as a major component of mudflat

intertidal communities, but we can only speculate that it plays a role in the juvenile diet of this marine snail. A benthic pennate diatom isolated from the West Coast also induces metamorphosis from newly competent larvae, but is, to date, an unreliable inducer. In addition to these positive responses, several diatom species repeatedly elicited no response from these larval snails. Thus, like other larvae, *Ilyanassa* shows positive, neutral, and perhaps even negative reactions to environmental compounds that are indicative of potential juvenile environments, but the exact sources of such compounds, whether from the diatoms or their associated microbial species, remain to be determined.

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