*Journal of Fish Biology* (2008) **72,** 168–188
doi:10.1111/j.1095-8649.2007.01698.x, available online at http://www.blackwell-synergy.com

# Effects of the toxic dinoflagellate, *Alexandrium fundyense* on three species of larval fish: a food-chain approach

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*(Received 13 December 2006, Accepted 12 September 2007)*

Sublethal behavioural effects of exposure to paralytic shellfish toxins (PST; saxitoxin and its derivatives) from the toxic dinoflagellate *Alexandrium fundyense* were investigated in newly settled winter flounder *Pseudopleuronectes americanus*, larval sheepshead minnow *Cyprinodon variegatus* and larval mummichog *Fundulus heteroclitus* through an *A. fundyense*–copepod–fish food chain. Consumption of as few as six to 12 toxin-containing copepods was lethal to the fishes. After consuming fewer toxin-containing copepods, all three fish species exhibited sublethal effects from vector-mediated exposure. Prey-capture ability of mummichogs was reduced in larvae that had consumed toxic copepods, *Coullana canadensis*. After consuming toxic *C. canadensis* or mixed copepods, mummichog larvae had reduced swimming performance. Swimming activity was also significantly reduced in winter flounder after consuming toxic copepods, including time spent in motion and distance travelled. Prey capture and predator avoidance were reduced in first-feeding sheepshead minnow larvae that had consumed toxic dinoflagellate cells. Adverse effects on prey capture or predator avoidance may reduce larval survival and facilitate the transmission of PST through the food web. This study provides baseline information on sublethal effects of PST exposure on fishes using a novel food-chain approach with zooplankton as vectors.


Key words: *Alexandrium fundyense*; *Cyprinodon variegatus*; *Fundulus heteroclitus*; harmful algal bloom; *Pseudopleuronectes americanus*; saxitoxin.

## INTRODUCTION

The toxigenic dinoflagellate *Alexandrium fundyense* Balech is present along the north-eastern Atlantic coast. This dinoflagellate produces paralytic shellfish poisoning toxins (PST: saxitoxin and derivatives), and has been implicated in fish kills during bloom conditions (White, 1980; Mortensen, 1985; Montoya *et al.*, 1996; Cembella *et al.*, 2002). Blooms of *Alexandrium* spp. generally occur

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from May to October from the Gulf of St Lawrence in Canada to Massachusetts (Franks & Anderson, 1992) and coastal regions of the Gulf of Maine have experienced bloom events in most years since 1972 (Shumway *et al.*, 1988). The persistence and recurrence of *Alexandrium* spp. blooms may have damaging effects on aquatic communities at many different levels, most of which have not been rigorously investigated. The dinoflagellates are at the base of the aquatic food web and are directly consumed by a large number of phytoplanktivorous organisms. Effects of the toxic algae on marine organisms vary from no obvious impacts to death, and there is evidence of vertical transmission of PST through the food web (White, 1980, 1982; Shumway *et al.*, 1988; Geraci *et al.*, 1989; Shumway, 1990; Runge, 1992; Scarratt, 1992; Teegarden & Cembella, 1996; Turner & Tester, 1997; Ramsdell *et al.*, 2005). Many herbivorous zooplankton and filter-feeding organisms can accumulate large quantities of PST without suffering mortality, but may pass lethal toxins to higher-order predators such as fishes, birds, marine mammals and humans. Single blooms of *Alexandrium* spp. have been implicated in the death of millions of adult fishes (White, 1980, 1981*a*; Mortensen, 1985; Montoya *et al.*, 1997; Costas & Lopez-Rodas, 1998). Larval and embryonic stages of fishes are generally more sensitive than adults to environmental stressors. Lingering bloom conditions (up to several weeks), the diversity of toxin vectors and the persistence of PST in exposed organisms increase the likelihood of exposure of larval fishes residing in bays and estuaries to PST. Larval exposure to PST may occur directly through consumption of toxic *Alexandrium* spp. cells or indirectly through consumption of other exposed organisms that have accumulated the toxin, for example, copepods that can serve as vectors.

The purpose of the present investigation was to determine sublethal effects of exposure to *A. fundyense* on newly settled juvenile-stage winter flounder *Pseudopleuronectes americanus* (Walbaum), larval sheepshead minnow *Cyprinodon variegatus* Lacépède and larval mummichog *Fundulus heteroclitus* (L.). All three of these fish species, as well as potential copepod vectors, occur in areas that experience regular and frequent blooms (Shumway *et al.*, 1988; Franks & Anderson, 1992; Robineau *et al.*, 1993; Anderson, 1997). To better understand the implications of exposure faced by early-stage fishes, different exposure situations were created using commonly occurring estuarine copepods as PST vectors, including *Coullana canadensis* (Willey) and a field-collected copepod assemblage (Fig. 1).

Winter flounder occur from Chesapeake Bay north to the Gulf of St Lawrence. They are one of the most abundant fish species throughout their range and support an important commercial and recreational fishery (Klein-MacPhee, 1978; Able *et al.*, 1989). Spawning occurs in estuaries, beginning around November in southern waters and proceeds progressively later in their more northern habitats (Kennedy & Steele, 1971). Adults return annually to their natal spawning ground (Klein-MacPhee, 1978), resulting in distinct, localized populations (Phelan, 1992; R. Schuck & S. Saila, unpubl. data). Winter flounder coexist temporally and spatially with toxic *Alexandrium* spp. during a vulnerable period in their life history. Settled juveniles begin to appear in estuaries and shallow embayments as early as April (in southern waters), where they remain for up to 2 years (Saucerman & Deegan, 1991). Phytoplanktivorous larvae risk exposure

 

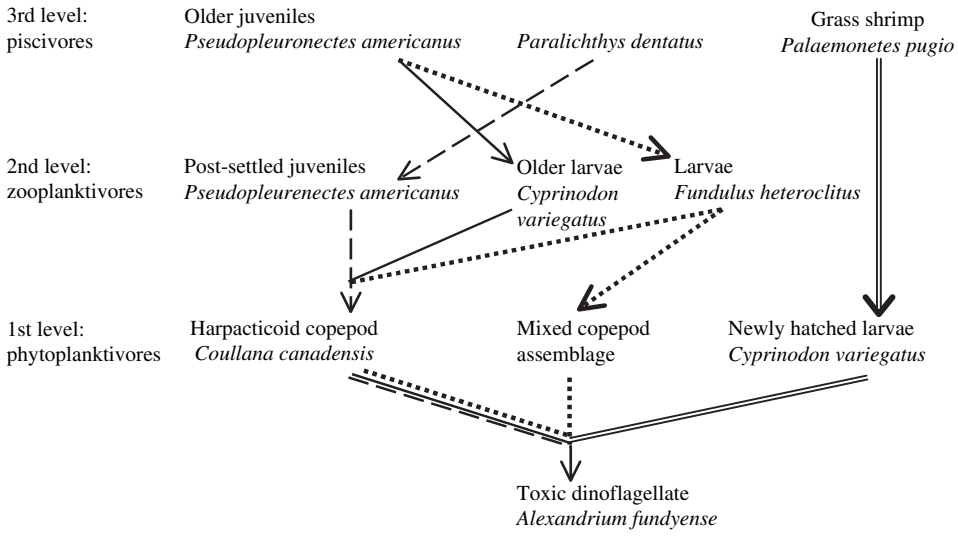


FIG. 1. The food webs used to investigate the lethal and sublethal effects of paralytic shellfish toxins to three species of fish. Individual food webs used for each fish species are designated by a specific line-style.

to PST *via* direct consumption of *Alexandrium* spp. cells, while juveniles risk indirect exposure by consuming organisms such as copepods that have consumed the toxic dinoflagellates. In previous laboratory studies, first-feeding larvae exposed to the toxic dinoflagellate *Alexandrium tamarense* (Lebour) Balech exhibited significant mortality (Mills & Klein-MacPhee, 1979; Robineau *et al.*, 1991*a*, *b*); however, effects of indirect exposure to PST on metamorphosed juveniles are not known.

Sheepshead minnows and mummichogs are both common in estuaries and salt marshes along the eastern seaboard of the U.S. (Talbot & Able, 1984; Hettler, 1989; Robineau *et al.*, 1991*a*, *b*). For both of these species, larval emergence and development coincide with toxic *Alexandrium* spp. bloom events in the northern portions of their range, providing an opportunity for larval exposure to PST. Sheepshead minnow larvae are *c*. 3 mm total length ($L_T$) at hatching and are planktivorous (Warlen, 1963), making them susceptible to direct exposure to PST through consumption of *Alexandrium* spp. cells. At *c*. 6 mm $L_T$, mummichog larvae are too large to consume *Alexandrium* spp. cells directly, but could be susceptible to vector-mediated PST exposure. Both sheepshead minnows and mummichogs are common prey for birds and commercially important fish species (Kneib & Stiven, 1978; Roundtree & Able, 1992). Thus, the sheepshead minnow and mummichog are central to the estuarine food web and their potential as both a victim and vector of PST make them interesting subjects in the attempt to understand the impact of toxic *Alexandrium* spp. blooms on aquatic ecosystems. The recent National Plan (Ramsdell *et al.*, 2005) for harmful algal blooms (HAB) identified the transfer and pervasiveness of toxins in food webs and the impacts on trophic structure as priority areas for future research. There are currently few studies that have provided a food web approach to the study of such impacts.

 

Behavioural responses have proven to be sensitive indicators of sublethal exposure in previous research on anthropogenic toxicants (Weis *et al.*, 2001; Sloman *et al.*, 2002; Alvarez & Fuiman, 2005). Behavioural abnormalities that reduce prey capture or predator avoidance can have obvious effects on larval survival, which could affect populations and facilitate the transmission of PST through the food web.

In the present study, copepods were allowed to feed on toxic *A. fundyense* and were then fed to fishes to simulate natural exposure of fish larvae to PST. Prey capture, swimming performance and predator avoidance behaviours were investigated in winter flounder, sheepshead minnows and mummichogs following exposure to PST *via* a copepod vector. These three behaviours, plus cell selection, were also examined in newly hatched sheepshead minnow larvae that were exposed directly to toxic *A. fundyense* cells in order to better understand the impacts of PST exposure on larval fishes.

## METHODS

### DINOFLAGELLATE CULTURES

Toxic strains of *A. fundyense* (CCMP 1846) and non-toxic strains of *A. tamarense* (CCMP 115) were obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, U.S.A. Cultures were maintained in f/2 media (Guillard & Ryther, 1962) in 1 l flasks in 0·45 μm filtered, autoclaved sea water (salinity 30). Stock cultures were kept in an incubator at 16° C with a 14L:10D photoperiod. The dinoflagellates were subcultured *via* transfer to new containers with f/2 media every 3–4 days. The original culture (200 ml in 1 l flask) was transferred into 800 ml of fresh, autoclaved f/2 media using sterile transfer techniques.

Cell densities were determined using a Sedgewick Rafter counting chamber (Guillard & Ryther, 1962; McAlice, 1971). Cells were used while in exponential growth phase and experimental concentrations were obtained through dilution of the original suspension with filtered sea water.

### MAINTENANCE AND EXPOSURE OF COPEPOD VECTORS

Natural zooplankton assemblages were collected from Sandy Hook Bay, New Jersey, U.S.A., using a 63 μm mesh plankton net, filtered through a 200 μm mesh. Samples were concentrated and sorted to remove all non-copepod zooplankton. Remaining copepods were equally distributed into two 1 l flasks with 500 ml of autoclaved sea water (salinity 30) and allowed to acclimate overnight in the incubator at 16° C. One of the two containers was exposed to a toxic strain of *A. fundyense* for 24 h, as a mixture of 50:50 by volume, *A. fundyense* (300 cells $ml^{-1}$) and green algae [a combination of *Isochrysis galbana* (Parke) and *Thalassiosira pseudonana* (Hasle & Heimdal)] at *c.* $2{\cdot}5 \times 10^5$ cells $ml^{-1}$. Copepods were seen actively consuming the dinoflagellates. In the second container, control copepods were given the green algal mixture only.

Laboratory cultures of the harpacticoid copepod *C. canadensis* obtained from SUNY (Stony Brook, NY, U.S.A.) were maintained on the green algal mixture described above ($>2{\cdot}5 \times 10^5$ cells $ml^{-1}$), which was added three times weekly, and exposed as described above for the mixed copepods. Copepod cultures were maintained at 16° C in 30 salinity autoclaved sea water under a 14L:10D photoperiod. Prior to experiments, adult, non-gravid copepods were exposed to a toxic strain of *A. fundyense* for four consecutive days as a mixture of 50:50 by volume, *A. fundyense* (300 cells $ml^{-1}$) and green algal mixture. Control copepods (1 l culture) were given the green algal mixture and

non-toxic *A. tamerense*. Copepods actively consumed the toxic dinoflagellates with little or no mortality over this time period. Following exposure, toxic (exposed) or control (unexposed) copepods were fed to the fishes.

## LARVAL FISH MAINTENANCE AND EXPOSURE

Larval fishes were maintained and exposures were conducted in an incubator at 16° C in filtered sea water of salinity 30, under a 14L:10D photoperiod, unless otherwise noted. Fishes were fed newly hatched *Artemia* spp. nauplii to satiation daily. Feeding was halted 24 h prior to running experiments.

The number of toxic copepods that could be consumed by each species without causing death was determined based on results of initial acute exposures in which individual larvae were fed one toxic *C. canadensis* copepod every 15 min until mortality occurred ($n = 10$). Feeding was halted when fishes began to swim erratically and lose equilibrium, which was followed by death. The number of copepods consumed was noted (Table I). Since the primary focus of the study was on sublethal effects, it was necessary to determine initially the number of copepods (dose) that would be lethal to the larvae so as to use lower numbers in the subsequent experiments on behavioural effects. Other endpoints would not have been useful in establishing the sublethal dose. The Rutgers University Animal Care and Facilities Committee reviewed and approved the protocol, and the numbers of larvae used in the lethal experiments was kept to a minimum (10). Larvae exposed to lethal doses died almost instantly, with no apparent distress.

### *Winter flounder exposures*

Newly settled juveniles (8–12 mm total length, $L_T$) originated from broodstock from Raritan Bay, New Jersey, or western Gulf of Maine (GOM). Fish were maintained at 16° C in 30 salinity filtered sea water under a 14L:10D photoperiod. Daily rations of *Artemia* spp. nauplii were added to tanks; feeding was halted 24 h before running experiments. Since preliminary experiments showed that six toxic copepods were lethal, sublethal exposure to PST involved individual fish placed into 80 mm diameter finger bowls with either five toxic *C. canadensis* copepods (exposed to toxic strain of *A. fundyense*) or five control copepods (exposed to non-toxic strain). Larger winter flounder (18–22 mm $L_T$) that were used in predator-avoidance experiments were given 10 copepods for sublethal exposure, since 12 was the lethal dose.

### *Sheepshead minnow exposures*

Fertilized eggs were obtained from the EPA laboratory in Gulf Breeze, Florida, and hatching occurred *c.* 7–10 days post-fertilization. Eggs and larvae were maintained either in an incubator (16° C) or on the bench top (22° C) in filtered sea water at 30 salinity under a 14L:10D photoperiod. Daily rations of *Artemia* spp. nauplii were added

TABLE I. Results of acute exposures where fish larvae ($n = 10$ per species) were fed one exposed *Coullana canadensis* copepod every 15 min until mortality occurred

| Species | Mean ± S.E. number of copepods eaten to cause mortality |
|---|---|
| *Pseudopleuronectes americanus* (8–12 mm $L_T$) | 6·1 ± 0·5 |
| *P. americanus* (18–22 mm $L_T$) | 11·9 ± 0·7 |
| *Cyprinodon variegatus* (8–10 mm $L_T$) | 8·9 ± 0·5 |
| *Fundulus heteroclitus* (8–10 mm $L_T$) | 12·3 ± 0·6 |

$L_T$, total length.

to tanks, except when feeding was halted 24 h before running experiments. Larvae were *c*. 3 mm $L_T$ at hatching and able to consume *Alexandrium* spp. cells directly. Therefore, both direct (consuming *Alexandrium* spp.) and indirect (*via* copepod vector) exposures were performed with this species. Direct exposure to *Alexandrium* spp. cells began at yolk supply depletion (*c*. 2 days post-hatch). Groups of five first-feeding larvae were transferred to 80 mm diameter glass finger bowls containing filtered sea water with *c*. 300 cells $ml^{-1}$ of either the non-toxic (control) or toxic strain of *Alexandrium* spp. There were six replicates for each treatment group. Exposures were run for 48 h at 16° C or 72 h at 22° C. Concentrations of algae were replenished every 24 h using stock solutions of *Alexandrium* spp. Indirect exposures *via* toxified copepods began when larvae reached 8–10 mm $L_T$. Individual larvae were transferred into individual 55 mm diameter watch glasses and either seven (based on preliminary trials) toxic or control *C. canadensis* copepods were added, resulting in two treatment groups.

*Mummichog exposures*

Gravid adults were collected from Belmar, New Jersey, and strip spawned in the field. Fertilized eggs were returned to the laboratory, cleaned, sorted and placed into glass finger bowls (*c*. 30 eggs per bowl) at 16° C in filtered sea water under a 14L:10D photoperiod. Hatching began within 2 weeks of fertilization and larvae were transferred into larger glass bowls. Daily rations of *Artemia* spp. nauplii were added to tanks; feeding was halted 24 h before running experiments. Newly hatched larvae would not consume *Alexandrium* directly, so larval exposures began at *c*. 5–7 days post-hatch (8–10 mm $L_T$) with copepod vectors. Both single species (*C. canadensis*) and mixed copepod assemblages (largely *Acartia* spp.) were used as vectors.

In larval exposures with mixed copepods, four groups of 12 larvae each were placed in 100 mm diameter glass finger bowls containing *c*. 500 copepods (or *c*. 40 per larva). Two groups were given toxic copepods (exposed to toxic *A. fundyense*) and two were given control copepods. Containers were placed in a 16° C incubator and left overnight (*c*. 12 h) to allow larvae to consume copepods. Behavioural experiments were begun by 0700 hours the following morning, by which time all copepods had been consumed. In exposures with only *C. canadensis* as vectors, individual larvae were transferred into small glass finger bowls and either 10 toxic or control copepods were added (since 12 had been found to be lethal).

## PREY-CAPTURE EXPERIMENTS

Behavioural assays were performed to determine if exposure to PST affected the ability of fish larvae to capture prey. Time limits set for the different feeding experiments were based on preliminary observations of the feeding rate of each species on the particular prey item, with the goal that some, but not all of the prey would be consumed.

*Winter flounder*

Two hours after exposure (feeding on exposed or control copepods), fish were given five non-toxic *C. canadensis*. With the New Jersey population, these trials were run in 55 mm diameter watch glasses with 15 ml sea water at 16° C, while with the larger GOM fish they were conducted in larger 80 mm diameter finger bowls with 50 ml sea water at 16 ° C. Attempts and captures over a 5 min period were recorded on video-tape during the trials ($n = 20$ per treatment), which was also used to analyse the swimming activity. Data were analysed using a two-sample *t*-test.

*Sheepshead minnow (small larvae after direct exposures to* Alexandrium *spp.)*

Individual larvae were placed in 15 mm diameter straight-walled glass depression slides with 5 ml sea water containing five *Artemia* spp. nauplii and observed under a

dissecting microscope. The number of attempts and successful captures in 1 min was noted ($n = 20$ per treatment). Data were analysed using a two-sample *t*-test.

*Sheepshead minnow (larger larvae after indirect exposure via copepods)*

Two hours after feeding on *C. canadensis* copepods (toxic or non-toxic), individual larvae ($n = 20$ per treatment) were given 5 min to consume five non-toxic *C. canadensis*. Experiments were conducted in 55 mm diameter watch glasses with 15 ml sea water at 16° C. Data were analysed using a two-sample *t*-test.

*Mummichog (mixed copepods as vectors)*

A four-way exposure design was used to investigate effects of PST on both the mummichog larvae and the copepods. Two hours after exposure, exposed and control fish larvae were given either toxic or non-toxic copepods during prey-capture trials, resulting in four groups (control fish and control copepods; control fish and toxic copepods; exposed fish and control copepods; exposed fish and toxic copepods). Individual larvae were placed into a 55 mm diameter watch glass with 30 copepods. The number of copepods eaten in 2 min was recorded. Two independent trials (each with $n = 10$ per group) were run. Data were analysed separately for each trial using one-way ANOVA or Kruskal–Wallis ANOVA when data were not normally distributed, followed by an appropriate *post hoc* test.

*Mummichog (*C. canadensis *as vector)*

Two hours after exposure to toxic or non-toxic copepods, individual larvae were placed in 55 mm diameter watch glasses with five non-toxic *C. canadensis*. The number of attempts and successful captures of exposed and control larvae ($n = 20$ per treatment) over a 3 min period were recorded. Data were analysed using a two-sample *t*-test.

## SWIMMING BEHAVIOUR EXPERIMENTS

Behavioural assays for swimming activity were performed to determine if sublethal exposure to PST negatively impacts swimming performance in larval fishes. The sedentary nature of winter flounder made it necessary to include copepod prey in the container in order to induce activity; this was not needed for sheepshead minnows or mummichogs. For winter flounder, time spent in motion and distance travelled were determined from video recordings during the prey-capture experiments. A VCR connected to a computer was used to analyse the videotaped exchanges with the computer package, 'NIH Image' (NIH, Bethesda, MD, U.S.A.). The movement of the fishes was traced on the screen and the software calculated distance travelled. Time spent in motion was also determined through videotape observation ($n = 20$ per treatment).

Swimming performance was tested in sheepshead minnow and mummichog larvae using a 'racetrack' experiment described by Heath *et al.* (1993). The racetrack design consists of a Petri plate with a small watch glass in the centre, placed over eight radiating lines. Individual larvae ($n = 20$ per treatment) were placed in the racetrack and probed to maintain constant motion. To maintain uniformity, the same person conducted all trials, probing was done using a standardized method, and testing was blind. The number of lines crossed in 1 min was recorded. Data were analysed using a two-sample *t*-test.

## PREDATOR-AVOIDANCE EXPERIMENTS

Behavioural assays were also performed to determine if exposure to PST affects the ability of fish larvae to avoid predators, following either direct or indirect exposures described previously. Larval fishes are both predator and prey, and it is essential to

understand how exposure to a toxic *A. fundyense* bloom might impact either of these ecologically important roles. Therefore, in some of the experiments, effects of the exposure on predator avoidance were studied. Just like in the estuary, these experiments involved some fish larvae being consumed by a predator. Since the study focused on food webs, using a food web approach in the behavioural studies is the most appropriate and ecologically relevant endpoint. Other endpoints would not have provided comparably suitable and ecologically relevant information. The Rutgers University Animal Care and Facilities Committee reviewed and approved the protocol, and the number of larvae used was kept to a minimum. These experiments did not cause severe distress for the larvae as there were no stressful chase sequences and they did not see the predator coming. The larvae were consumed instantly in one unanticipated motion by the predator.

*Winter flounder*

Predator avoidance experiments were run on winter flounder juveniles (18–22 mm $L_T$, obtained from Chatham Aquafarms, MA, U.S.A., GOM broodstock) using summer flounder *Paralichthys dentatus* (L.) (150–170 mm $L_T$) as predators. The winter flounder were initially given 10 toxic or non-toxic *C. canadensis*. Then one exposed and one control winter flounder were added together to the experimental chamber (clear plastic 510 mm diameter with 4 mm depth of sand and 50 mm of 30 salinity sea water at 20° C) containing one summer flounder that had acclimated to the container for 1 h. The two winter flounder (exposed and control) were added together on the opposite side of the tank from the predator, and were differentiated by size, colouration or other distinguishing characteristics. Size differences between the two were <3 mm and were alternated between the two treatment groups (*i.e.* some trials with the experimental fish smaller, some with the control fish smaller). Trials were videotaped and the first fish eaten (*i.e.* control or exposed) was recorded ($n = 20$ trials).

*Sheepshead minnow (direct exposure of newly hatched larvae)*

Groups of larvae were placed in a Nalgene container (280 × 175 × 120 mm), with 25 mm deep sea water (salinity 30), and one adult grass shrimp *Palaemonetes pugio* (Holthuis) 20 mm total length. Experiments run at 22° C had four larvae per container, with six replicates per treatment. Experiments run at 16° C had six larvae per container, with five replicates per treatment. The duration of both experiments was 3 h and number of larvae eaten every 30 min was recorded. Data were analysed using a $\chi^2$-test.

*Sheepshead minnow (older larvae) and mummichogs (exposed via toxic copepods)*

Predator avoidance experiments were run using juvenile winter flounder (30–35 mm $L_T$) as predators. One exposed and one control larva were added together to the experimental chamber (280 × 175 × 120 mm) containing one winter flounder. The two larvae (exposed and control) were differentiated by size. Size differences were <3 mm and treatment groups alternated smaller larvae between trials. Which of the two larvae was consumed first ($n = 20$ trials) was recorded. Data were analysed using $\chi^2$.

## CELL SELECTION EXPERIMENTS WITH SHEEPSHEAD MINNOW LARVAE

Newly-hatched sheepshead minnow larvae were small enough to consume toxic *Alexandrium* spp. directly. To determine whether they could actively select between the two different strains of *Alexandrium* spp., live *Alexandrium* spp. cells were treated with the vital stain, 5-chloromethylfluorescein diacetate (CMFDA) that emits green fluorescence at 516 nm (purchased from Molecular Probes®, Eugene, OR, U.S.A.). Unstained *Alexandrium* spp. naturally emits red at 630 nm. Staining one of the two strains in a 50:50 mixture of *Alexandrium* spp. (toxic and non-toxic) allowed exposure

 

of larvae to both strains simultaneously. Cell staining methods were based on Teegarden (1999). Two different mixtures containing 300 cells $ml^{-1}$ of each strain were produced: mixture A: toxic *A. fundyense* was stained and mixture B: non-toxic *A. tamarense* was stained. Mixtures were dispensed equally into individual containers and larvae (seven per container) were allowed to feed on cells for 3 h at 16° C. Three collections were made: initial no fish (immediately after dispensing), final with fish and final no fish (control). Upon collection, similar samples were pooled, concentrated, preserved with 1% cold glutaraldehyde and analysed on a Becton–Dickinson Fluorescence Activated Cell Sorter (FACSCaliber, Franklin Lakes, NJ, U.S.A.) to determine the ratio of stained to unstained cells. Results were analysed using goodness of fit $\chi^2$ analysis, where initial ratio of stained to unstained cells was used to determine the expected ratio following cell consumption.

### SAXITOXIN ANALYSIS

Samples of *Alexandrium* spp. culture, exposed copepods (*C. canadensis* and the mixed copepods), and exposed fish larvae or juveniles were frozen at −20° C and sent to the NOAA/NMFS Laboratory in Charleston, SC, U.S.A., for determination of PST concentrations using a receptor-binding assay (Doucette *et al.*, 1997). This assay utilizes the specific interactions of PSP toxins with their biological receptor (voltage-dependent sodium channel) and is performed on a microtitre filter plate with results quantified by standard liquid scintillation counting. It provides an estimate of the integrated toxic potency of all the PSP toxins since each toxin present in a sample is bound by the receptor with an affinity proportional to its toxic potency. Results are expressed as saxitoxin (STX) equivalents and were found to be in good agreement with mouse bioassays and high performance liquid chromatography (HPLC) results (Doucette *et al.*, 1997).

## RESULTS

Winter flounder, sheepshead minnows and mummichogs were all vulnerable to vector-mediated *Alexandrium* spp. toxins with mortality occurring after consuming as few as six to 12 toxic copepods (Table I). Eating fewer copepods resulted in sublethal effects on behaviours, including prey-capture ability, predator avoidance and swimming performance (Table II and Figs 2–6). Sheepshead minnow larvae that consumed toxic *A. fundyense* directly suffered mortality in 48 h (11%) and 72 h (47%).

### PREY-CAPTURE EXPERIMENTS

Juvenile winter flounder from both New Jersey and GOM showed no significant changes in prey-capture ability after exposure to PST *via* copepod vectors.

Sheepshead minnow larvae directly exposed to toxic *A. fundyense* cells at 16° C exhibited a significant reduction in both attempts and captures of *Artemia* spp. nauplii compared with controls (attempts: *t*-test, $n = 20$, $P < 0{\cdot}001$; captures: *t*-test, $n = 20$, $P < 0{\cdot}001$; Fig. 2). These exposed larvae tended to be quiescent on the bottom, while controls swam more actively, capturing more prey; however, prey-capture ability was not significantly affected by exposure to PST at 22° C, or when *C. canadensis* were used as vectors, regardless of fish species tested (Table II). *Coullana canadensis* exhibited a strong predator avoidance response, whereby they escaped from the mouths of the mummichog larvae after capture. The escape behaviour was observed repeatedly during larval exposure, with the copepod being repeatedly captured and released from the

TABLE II. Effects on three larval fish species exposed to paralytic shellfish toxins through a *Coullana canadensis* copepod vector. Swimming performance of winter flounder (WF) was time (mean ± S.E.) and distance travelled (mean ± S.E.) during prey-capture experiments, and for mummichogs (M) and sheepshead minnows (SM) was mean ± S.E. number of lines crossed in racetrack. Bold values are when controls and exposed are statistically significantly different from each other ($P < 0{\cdot}05$)

| | Prey capture | | | | Swimming performance | | | | | | Predator avoidance | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Attempts | | Captures | | Lines crossed | | Time in motion (s) | | Distance travelled (mm) | | Captured (%) | |
| | C | E | C | E | C | E | C | E | C | E | C | E |
| Species | | | | | | | | | | | | |
| WF (NJ) | 6·2 ± 1·1 | 4·8 ± 0·6 | 3·5 ± 0·4 | 2·8 ± 0·4 | | | **12·41 ± 1·05** | **5·87 ± 0·55** | **161·4 ± 30·3** | **89·5 ± 18·6** | | |
| WF (GOM) | 4·2 ± 0·4 | 4·4 ± 0·5 | 3·4 ± 0·3 | 2·9 ± 0·3 | | | 31·05 ± 6·23 | 29·33 ± 7·22 | 220·7 ± 39·6 | 189·1 ± 27·2 | **85** | **15** |
| SM | 7·0 ± 0·6 | 6·8 ± 0·5 | 3·3 ± 0·3 | 2·8 ± 0·3 | 21·4 ± 1·4 | 21·0 ± 1·7 | | | | | 65 | 35 |
| M | 5·7 ± 0·6 | 4·8 ± 0·6 | 3·0 ± 0·3 | 2·3 ± 0·3 | **34·5 ± 1·4** | **23·3 ± 1·4** | | | | | 55 | 45 |

C, control; E, exposed; GOM, Gulf of Maine; NJ, New Jersey.

 

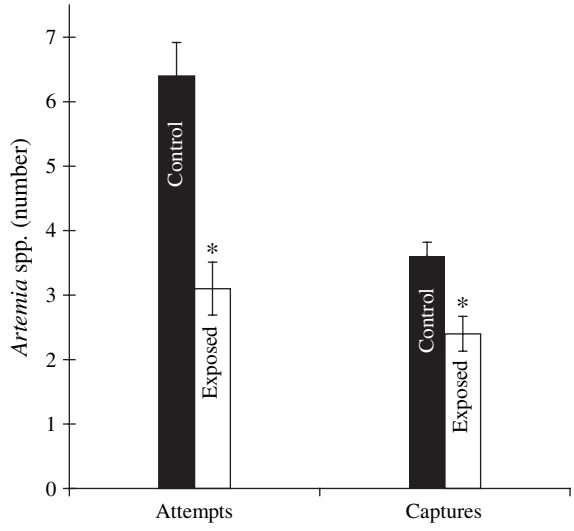


FIG. 2. Mean ± S.E. prey capture by *Cyprinodon variegatus* after direct exposure to toxic *Alexandrium fundyense* at 16° C *v.* unexposed controls, showing attempts and captures of *Artemia* spp. nauplii within 1 min [*, $P < 0{\cdot}001$ ($n = 20$)] for both attempts and captures.

same larva. Further investigation revealed no differences in escapes and recaptures between toxic and control copepods.

Mummichog trials using the mixed-copepod assemblage revealed a significant reduction in prey-capture ability for exposed mummichog larvae consuming control copepods (trial 1: $F_{3,36}$, $P < 0{\cdot}001$; Fig. 3) but not for those consuming exposed copepods, suggesting that the exposed copepods were easier for the impaired fish to capture. In the second trial, both exposed and control mummichog larvae captured more exposed copepods than control copepods, again indicating an effect of the PSTs on the copepods themselves ($F_{3,36}$, $P < 0{\cdot}001$; Fig. 3). In contrast, mummichog larvae that had been exposed to PST *via C. canadensis* showed no change in prey-capture ability.

## SWIMMING PERFORMANCE AND ACTIVITY EXPERIMENTS

Swimming activity of winter flounder from New Jersey was significantly reduced after PST exposure. Both time spent in motion (*t*-test, $n = 20$, $P < 0{\cdot}05$) and distance travelled (*t*-test, $n = 20$, $P \leq 0{\cdot}05$) were reduced, whereas no differences were observed in flounder from the GOM (Table II and Fig. 4). These results were unexpected since the larger container used for the GOM experiments was expected to amplify the impacts of lower activity and reduce incidental prey encounters. There may be population differences in susceptibility, as noted in other species.

After consuming toxic copepods, mummichog larvae had reduced swimming performance compared with control larvae regardless of the copepod species used as a vector (mixed copepod vector: *t*-test, $n = 20$, $P < 0{\cdot}001$; *C. canadensis* vector: *t*-test, $n = 20$, $P < 0{\cdot}001$; Table II and Fig. 5). Sheepshead minnows were the only species in which swimming performance was unaffected by

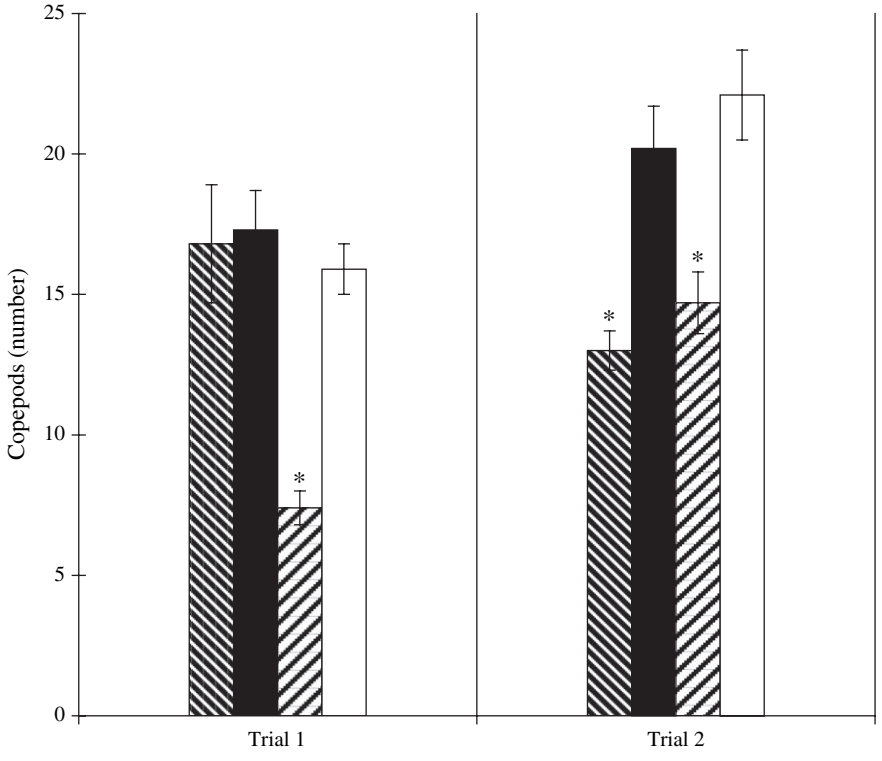


FIG. 3. Mean ± S.E. prey capture by *Fundulus heteroclitus* as the number of captures of mixed copepods within 2 min. A four-way exposure design involved control fish and non-toxic copepods (▧), control fish and toxic copepods (■), exposed fish and non-toxic copepods (▨) and exposed fish and toxic copepods (□). Two identical trials were run [*, significantly lower than other groups, $P < 0{\cdot}001$ ($n = 10$)].

exposure to PST, whether it was direct *via* consuming *Alexandrium* spp. or indirect *via* consuming toxic copepods.

## PREDATOR-AVOIDANCE EXPERIMENTS

Predator avoidance was impacted by exposure to PST in several of the experiments, but the feeding mode of the predator appeared to be an important factor in the susceptibility of the larvae. Summer flounder predators consumed control winter flounder first 85% of the time, when both a PST-exposed and a control winter flounder were provided ($\chi^2$, $n = 20$, $P < 0{\cdot}05$). Videotape analysis showed that the PST-exposed winter flounder tended to bury themselves quickly in the substratum, while the more active control fish swam more and were therefore more likely to attract the attention of the predator, *i.e.* the reduced activity of the exposed fish protected them from this predator.

When winter flounder were used as the predator on sheepshead minnow larvae, control larvae were captured first in 13 out of 20 trials, but the difference was not significant ($\chi^2$, $P > 0{\cdot}05$). When the active grass shrimp were used as

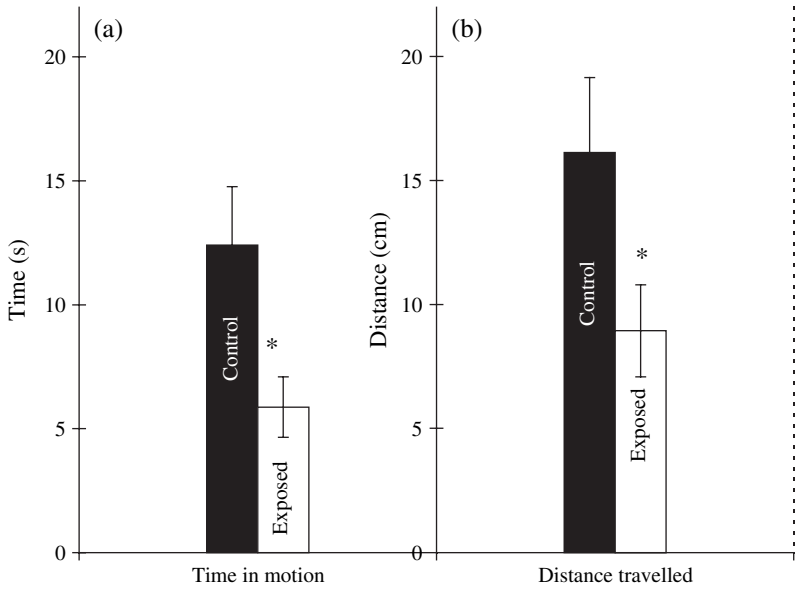


FIG. 4. Swimming activity of *Pseudopleuronectes americanus* from Raritan Bay, New Jersey, as (a) time spent in motion and (b) distance travelled in 5 min while feeding on *Coullana canadensis* (values are means ± S.E.). Treatment groups include controls (■) and paralytic shellfish toxins-exposed fish (□) [*, $P < 0{\cdot}05$ ($n = 20$) for both time and distance].

predators, the PST-exposed sheepshead minnow larvae (from both temperatures) were significantly more susceptible to capture than controls (Fig. 6). In contrast, PST-exposed mummichog larvae were no different in predator avoidance than controls.

## CELL-SELECTION EXPERIMENTS BY SHEEPSHEAD MINNOW LARVAE

First-feeding sheepshead minnow larvae were observed consuming *Alexandrium* spp. cells. After 3 h feeding, there was no difference in the ratio of stained to unstained cells, when compared with controls (cells without fish). Results were similar regardless of whether stained cells were the toxic species ($\chi^2$, $P > 0{\cdot}05$) or the non-toxic species ($\chi^2$, $P > 0{\cdot}05$), indicating that these larvae did not select for cells based on toxicity, stain or algal species.

## SAXITOXIN ANALYSIS

The *A. fundyense* culture (4600 cells $ml^{-1}$) contained 1·02 pg STXeq per cell. Exposed *C. canadensis* contained 34·9–35·8 μg STXeq per animal, while the mixed copepods accumulated only 5·1–6·3 μg STXeq per animal. Exposed winter flounder, sheepshead minnows and mummichogs contained 4·1–5·8, 0·9–1·2 and 1·7–2·5 μg STXeq $g^{-1}$, respectively. Thus, the winter flounder accumulated the highest concentration, despite having eaten the fewest copepods, and the

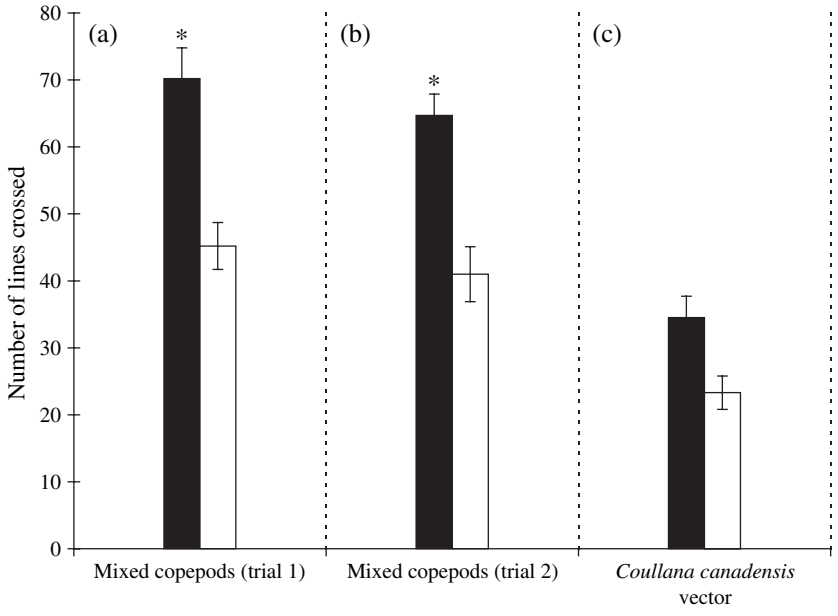


FIG. 5. Swimming performance of larval *Fundulus heteroclitus* as the mean ± S.E. number of lines crossed in the racetrack by larvae in (a) and (b) 1 min using mixed copepod vectors [*, $P < 0{\cdot}01$ ($n = 20$)] or (c) in 30 s using *Coullana canadensis* as vectors ($P > 0{\cdot}05$).

sheepshead minnow the lowest concentration of toxin from consuming the *C. canadensis*.

## DISCUSSION

The results clearly demonstrate that not only are larval fish highly susceptible to PST but exposure can occur through both direct consumption of cells and consumption of toxic prey items. The toxic algal concentrations (300 cells $ml^{-1}$) used for both copepod and larval fish exposures were comparable to average natural densities during bloom events (Therriault *et al.*, 1985); however, the actual toxicity (1·02 pg STXeq per cell) was on the low end of the general range for *A. fundyense* (Bricelj *et al.*, 1990; Teegarden & Cembella, 1996). Dinoflagellates can represent a substantial portion of first-feeding larval fish diets (Last, 1980). Results of the sheepshead minnow cell selection experiments suggested no discrimination between toxic and non-toxic cells, making it probable that a larva will consume toxic cells during a bloom event. All zooplankton vectors used in these studies, sequestered enough PSTs to kill fishes that ate as few as six to 12 *C. canadensis* and to induce sublethal behavioural effects in fishes that consumed even less. Although copepod grazing rates were not measured directly, copepods were observed consuming toxic cells. Field studies have confirmed some portion of the zooplankton community consumes toxic cells and bioaccumulates PST during bloom events (Turner *et al.*, 2000), and these toxic vectors have been linked to significant fish kills (White, 1980).

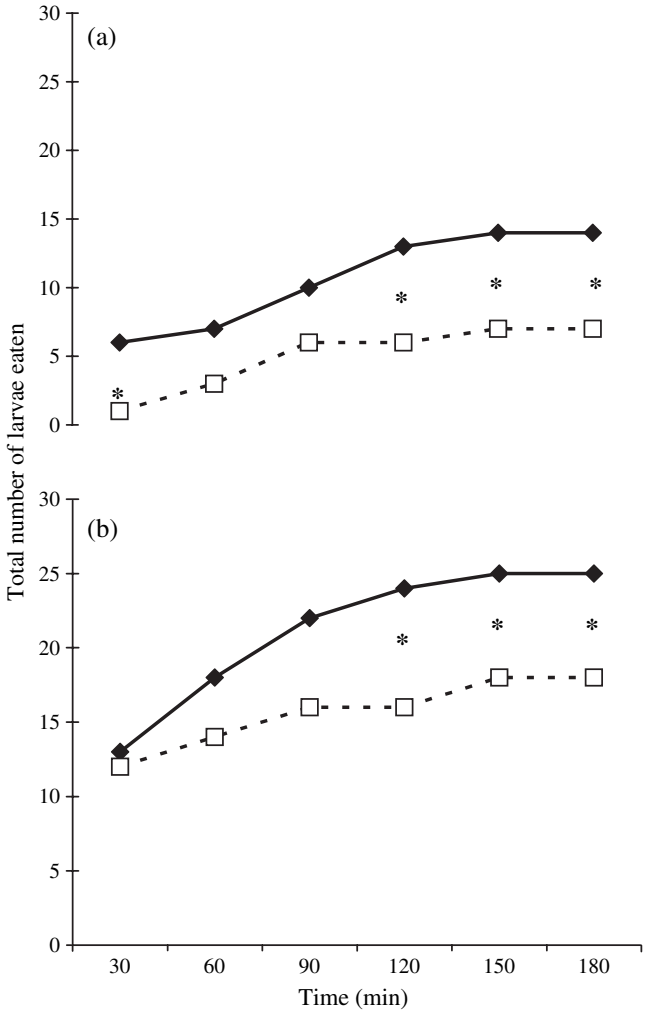


FIG. 6. Total predation on larval *Cyprinodon variegatus* by adult *Palaemonetes pugio*. *Cyprinodon variegatus* larvae had been exposed (◆) directly to *Alexandrium* spp. cells as follows: (a) 72 h exposure at 22° C (four larvae per shrimp) [*, significant ($P < 0{\cdot}05$) at 30, 120, 150 and 180 min] and (b) 48 h exposure at 16° C (six larvae per shrimp) [*, significant ($P < 0{\cdot}05$) at 120, 150 and 180 min]. □, unexposed controls.

Copepod vectors also exhibited sublethal effects of toxin exposure, as mummichog larvae captured toxic copepods (natural assemblage) at a significantly higher rate than control copepods (Fig. 3). These experiments exposed fishes to mixed copepod assemblages representative of ecologically relevant conditions of exposure. Behavioural effects of PST exposure reported in zooplankton include reduced naupliar activity, loss of motor control and abnormal backward swimming (Huntley *et al.*, 1986; Sykes & Huntley, 1987; Hansen *et al.*, 1992; Bagoien *et al.*, 1996). As was observed in the present study, these behaviours could make the exposed organisms easier to capture and promote the transfer of PST to the next trophic level. Consumption of the toxified copepod

assemblage induced significant sublethal effects in mummichog larvae, suggesting that some portion of the copepod population was grazing on cells and accumulating toxin.

All three fish species were exposed to the vector, *C. canadensis*, thus allowing comparisons to be made among the different fish species (Table II). Sublethal exposures to PST were shown to substantially impact several critical behaviours. Winter flounder juveniles and mummichog larvae both exhibited reduced swimming performance following sublethal exposure to toxic *C. canadensis* copepods (Table II and Figs 4 and 5). The PSP toxins have been shown to reduce activity levels in several different species of invertebrates (Marsden & Shumway, 1992; Bagoien *et al.*, 1996). Prey-capture success was also reduced in mummichog larvae exposed to toxic mixed copepods (Fig. 5) and in sheepshead minnow larvae after direct exposure to *A. fundyense*.

Predator avoidance ability of winter flounder and sheepshead minnow larvae were both affected by PST exposure (Table II and Fig. 6), but the feeding mode of the predator plays an important role on the impact of reduced activity. When a sit-and-wait predator (summer or winter flounder) was used, the reduced activity of PST-exposed prey reduced detection and subsequent capture, but when an active predator (grass shrimp) was used, the reduced activity of exposed prey increased their susceptibility to capture. Consequently, the more active control winter flounder were consumed before exposed winter flounder 85% of the time, yet the delayed response of exposed sheepshead minnow larvae to the active foraging and quick attack of the grass shrimp led to significantly greater predation on exposed larvae than on controls (Fig. 6). Experiments examining physiological responses to PST exposure did not detect changes in respiration rate or gut retention time in either winter flounder juveniles or sheepshead minnow larvae (Samson, 2002). All of the fishes in these studies actively consumed both toxic and non-toxic copepods, and the copepods frequently escaped from the fishes' mouths after capture. The discovery of a gustatory receptor system stimulated by aqueous saxitoxin in both rainbow trout *Oncorhynchus mykiss* (Walbaum) and Arctic charr *Salvelinus alpinus* (L.) (Yamamori & Nakamura, 1988) suggests a mechanism for detection of PST exists, but it is unclear whether a fish could detect toxins that are sequestered in the tissues of a prey organism.

Overall, winter flounder juveniles appear most sensitive to vector-mediated PST exposure. They ate the fewest copepods and accumulated higher concentrations of the PST compared with sheepshead minnow and mummichog larvae. The fact that winter flounder form distinct, localized populations (Klein-MacPhee, 1978; Phelan, 1992; R. Schnuck & S. Saila, unpubl. data) raises legitimate concern for those populations residing in bays and estuaries that experience regular toxic bloom events. The present data comparing responses of fishes from GOM and New Jersey suggest differences in tolerance, which has also been observed in other species (Shumway *et al.*, 1987; Bricelj *et al.*, 2005). The GOM population was somewhat less affected by exposure (in terms of effects on swimming activity) than the New Jersey population. This may reflect the greater historical exposure of the GOM flounder to *Alexandrium* spp., which seldom blooms as far south as New Jersey. Some fish populations chronically exposed to anthropogenic chemicals become more tolerant to them

 

(Weis, 2002), and populations chronically exposed to *Alexandrium* spp. may also evolve greater tolerance to their toxins. An accurate assessment of winter flounder stock sustainability requires more information on impacts of toxic *Alexandrium* spp. blooms on year-class strength, juvenile recruitment and the contribution of particular areas to overall winter flounder stocks.

Mummichog larvae were also impacted by vector-mediated exposure, and first-feeding sheepshead minnow larvae were sensitive to direct exposure to toxic *A. fundyense*. Both species play critical roles in the trophic structure and function of estuaries that nurture commercially important species. Studies of several different Atlantic coast estuaries considered mummichogs among the most important fish species, based on per cent frequency, mean abundance and mean biomass (Clymer, 1978; Talbot & Able, 1984). These abundant fish are an important source of energy transformation in the estuaries, providing a constant supply of food to foraging fishes and birds (Valiela *et al.*, 1977; Meredith & Lotrich, 1979). Sublethal exposures of mummichogs and sheepshead minnow could produce additional PST vectors at a critical link in the estuarine food web.

This study was designed to address some basic questions regarding the vulnerability and sensitivity of larval and juvenile fishes, and provide initial baseline information on sublethal effects of PST exposure. It is important to understand the potential range of responses to PST exposure in order to better understand how a bloom impacts estuarine organisms. There has been very little research conducted on the sublethal responses of larval fishes to PST (Lefebvre *et al.*, 2004, 2005) with most of the previous investigations focused exclusively on lethal endpoints (White, 1981*a*, *b*; White *et al.*, 1989; Robineau *et al.*, 1991*a*, *b*; Bruslé, 1995). In these studies, mortality resulted from consuming as few as six copepods, suggesting bloom conditions will have lethal consequences. Subtle behavioural effects could also occur on the outskirts of the bloom or at times when blooms are not dense, and larvae that survive and accumulated PST could serve as vectors to transmit the toxins to higher trophic levels.

Behavioural changes such as reduced prey capture and predator avoidance that were observed in this study can also have obvious impacts on larval growth and survival. Larval fishes need to become proficient predators quickly to achieve critical daily ration requirements and maintain high growth rates. Growth rates can also impact predator avoidance, as susceptibility to predation has been shown to be a size-dependent relationship, wherein mortality rates decrease with increased size (Sogard, 1997; Sheaves, 2001). Reduced larval survival could be expressed at the population level in the form of reduced year-class strength and recruitment success. Heavy mortality or behavioural impairment of larval and juvenile fishes in the estuaries would probably go undetected during a bloom but might have population consequences in areas subject to recurring bloom events.

We thank D. Lonsdale of Stony Brook University, C. Chambers of NOAA Fisheries, H. Howell of UNH, B. Hemner of EPA Gulf Breeze and T. Cucci of Bigelow Laboratory for providing experimental organisms, and S. Morton of NOAA Fisheries for the PSP toxin analysis. We appreciate the technical assistance of A. Reduta, T. Robertson and C. Reina, and the advice of T. Glover.

 

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