Differential copper toxicity in invasive and native ascidians of New England provides support for enhanced invader tolerance

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ABSTRACT: Colonial ascidians are a group of prolific invaders found in urban marine environments. Despite often high levels of pollution in these areas, some species of ascidians not only tolerate but thrive in these conditions, while native species decline. The role of pollution tolerance in establishment success is often overlooked, and therefore the mechanisms employed by invaders are poorly understood. This research explores both acute and chronic copper toxicity of *Botrylloides violaceus*, a prominent invader in the Gulf of Maine, and a native counterpart, *Aplidium glabrum*. Acute toxicity tests revealed that *B. violaceus* larvae are significantly more tolerant to exposure than *A. glabrum* larvae (median effective concentration for settlement inhibition [EC_{50}] values = 107.9 and 46.7 µg l^{-1}, respectively). Further, the results indicate that *B. violaceus* is more tolerant than members of several other native benthic phyla using a species sensitivity distribution approach. Chronic toxicity tests determined that EC_{50} values impacting the number of zooids per colony and colony area for *A. glabrum* were achieved at environmentally relevant concentrations of copper, while *B. violaceus* values were over twice the concentration (approximately 8 and 20 µg l^{-1}, respectively). These findings support the hypothesis that invader pollution tolerance is indeed greater than that of native species, both within and beyond the same phyla, and likely plays a role in invader establishment and spread as described in the invader tolerance model.

KEY WORDS: Ascidians · Invasive · Ecotoxicology · Acute · Chronic · Copper · Pollution

INTRODUCTION

The invasion of non-native species into established ecosystems is a major threat to global biodiversity (Wilcove et al. 1998). The global ocean is home to an estimated 225 000 known species (Grosberg et al. 2012), many of which are threatened by the presence of marine invaders (Bax et al. 2003). Although natural range expansion of species has been documented due to climatic and geological events, human-mediated expansions occur at a much more rapid rate and span greater distances (Lockwood et al. 2007). Such invasions lead to a myriad of impacts on native communities. For example, invaders may outcompete native species for resources, including space and food,

leading to high mortality rates and a loss in native species biodiversity. The impacts on native communities can then result in losses to essential ecosystem services such as water filtration and control of other species' populations (Lockwood et al. 2007).

However, increased native community biodiversity may act as a natural barrier against invader success (biotic resistance hypothesis; Elton 1958). Several controlled, local-scale studies have illustrated that communities with high biodiversity utilize resources more efficiently, which makes it more difficult for introduced species to become established (Case 1990, Stachowicz & Byrnes 2006, Janiak et al. 2013). In contrast, as species richness decreases, invasive species become more successfully established (Sta-

chowicz et al. 1999). Therefore, as biodiversity becomes increasingly threatened by the presence and number of invasive species, a positive feedback will occur, leading to further loss of species richness and the potential homogenization of a system at an accelerated rate.

In addition to invasive species, marine communities are also challenged with marine pollution, particularly along industrialized coastlines and in urban harbors. Many native marine organisms are susceptible to chemical pollutants released or concentrated in coastal areas from anthropogenic activities (Crooks et al. 2011). Several studies have shown increased tolerance of invasive species to different environmental parameters, including pollution, and suggest that this is likely aiding in their success in urban harbors (Lambert & Lambert 2003, Zhan et al. 2015). Native organisms therefore experience dual challenges — competition with invasive species for space and food, and toxicity from marine pollution. As resistance toward marine invasions depends on high native biodiversity, the impact of marine pollution may go far beyond impacts to individuals. If marine pollution acts to diminish native biodiversity, it may act as a facilitator for marine invasions (Zhan et al. 2015). This possibility depends highly on the comparative tolerances of native and invasive marine species.

Marine invasive colonial ascidians are found within biofouling communities, which are also home to mollusks, crustaceans, echinoderms, macroalgae, cnidarians, and other native colonial ascidians. They are strong competitors for resources, and therefore the presence of these invaders can present new challenges for the native community members (Edwards & Stachowicz 2010). Their incredible success as invasive species (Ramsay et al. 2008) and high tolerances to a number of environmental stressors make them an attractive model for studying the impact of marine pollution on invasive success. *Botrylloides violaceus* Oka, 1927 is a particularly aggressive invasive colonial ascidian and was first documented in the Gulf of Maine in the 1980s (Carlton 1989). The success of this species throughout the North Atlantic coast has been attributed to rapid proliferation and higher temperature and salinity tolerances compared with native species (Dijkstra et al. 2008, 2011). In comparison, there are several native colonial ascidian species found in biofouling communities within the Gulf of Maine, including *Aplidium glabrum* (formerly *Amaroucium glabrum* Verrill, 1871). *A. glabrum*, a colonial Aplousobranch ascidian species native to the Northwest Atlantic Ocean, is distributed from the Bay of Fundy, Canada, down to Cape Hatteras off the

mid-Atlantic US coast (Vanden Berghe et al. 2005). Despite the ecological significance of ascidian invasions in marine environments, very little is known about their toxicological thresholds, and therefore the role pollution plays in their establishment and proliferation. Further, even less is known regarding the toxicity of marine pollutants to native ascidian species. Understanding the comparative sensitivities of invasive and native ascidians to common marine contaminants is critical for defining the role of marine pollution in ascidian invasions.

Copper is an essential metal for life, yet its introduction into the marine environment via antifouling agents and land-based industrial processes has led to negative impacts on exposed communities (Neira et al. 2011). The use of copper as the active ingredient in antifouling agents has increased considerably since the ban of tributyltin (Dafforn et al. 2011), resulting in acutely toxic concentrations of up to 80 µg l^{-1} in urban harbors (Hall et al. 1988, Manisseri & Menon 1995, Negri & Heyward 2001, Katranitsas et al. 2003, Bellas et al. 2004). These concentrations negatively impact marine organisms through oxidative stress and reduced lysosomal stability, which can ultimately lead to mortality (Svendsen & Weeks 1997, Neira et al. 2014, Oldham et al. 2014).

Because of its prevalence and potency to many marine species, we chose copper as a model pollutant and conducted experiments to determine whether chemical pollutants selectively inhibit the growth and settlement of native species, while allowing invasive species to thrive. *B. violaceus* and *A. glabrum* were selected to represent invasive and native species of the biofouling community. Our experiments were designed to identify sensitive toxicological endpoints and uncover the acute and chronic toxicity thresholds for both species. These threshold toxicity values were compared with those of other native fouling community members using a species sensitivity distribution (SSD) approach, which provided a broader view of the potential impacts of copper pollution to the community. Finally, our results were contextualized within the invader tolerance model (ITM) to predict how pollution may act to facilitate invasion success of marine communities.

MATERIALS AND METHODS

Expt 1: acute copper toxicity

We conducted short-term or acute toxicity tests to determine how copper exposure affected the survival

and settlement of larval ascidians. Both lethal (LC_{50}; median concentration causing a 50% reduction in survival) and sublethal (EC_{50}; median concentration causing a 50% reduction in settlement) endpoints were recorded during Expt 1. Initial settlement studies revealed that 100% (total n = 100) of *Botrylloides violaceus* and 92% (total n = 50; reduced number based on larvae availability) of *Aplidium glabrum* larvae settled within 48 h, and therefore 48 h was a sufficient test duration. The range of concentrations (8–4800 µg l^{-1}) used in the acute toxicity tests were initially derived from published toxicity values for the invasive ascidian *Ciona intestinalis* (Bellas et al. 2001), but were then adjusted to include the higher concentrations necessary to reach toxicity values of interest.

Larvae collection

Intact colonies of *B. violaceus* and *A. glabrum* were gently removed and collected from the Massachusetts Maritime Academy floating dock (41° 44′ 19.58″ N, 70° 37′ 36.44″ W) using a paint scraper. Colonies were placed in seawater filtered to 5 µm at 33 ppt ± 1 ppt salinity and ambient laboratory temperature (18°C ± 1°C). Larvae were immediately released in response to disturbance from the removal process. Released larvae were collected with a larvae collector system based on the design of Watanabe & Lambert (1973) and is further described in the Supplement (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m595p135_supp.pdf). Larvae were held for up to 10 min in the Nytex catchment until test initiation.

Experimental units

Sterile 60 × 15 mm polystyrene Petri dishes were used as the experimental units for the 48 h acute larval toxicity tests. Seven nominal concentrations of total copper ions were used to capture both targeted LC_{50} (mortality) and EC_{50} (settlement inhibition) values: 37, 75, 150, 300, 600, 1200, 2400, and 4800 µg l^{-1}. Stock solutions were prepared using cupric chloride dihydrate ($CuCl_2 \cdot 2H_2O$; MP Biomedicals) and deionized water; Stock A (10 mg per 100 ml) and Stock B (1 mg per 100 ml; prepared using Stock A). Experimental concentrations were prepared by adding the appropriate stock to seawater filtered to 5 µm at 33 ppt ± 1 ppt salinity and ambient laboratory temperature (18°C ± 1°C). Following collection, 5 larvae of either *B. violaceus* of *A. glabrum* were placed in a

Petri dish containing 20 ml of the appropriate copper treatment in filtered seawater (or for the control, only filtered seawater). For each concentration tested, 5 replicate Petri dishes were set up, except for one test iteration of *B. violaceus* that had 3 replicates per concentration due to insufficient larvae availability.

Water samples were collected at test initiation and were analyzed for total copper at the University of Massachusetts Boston Environmental Analytical Facility. Samples were acidified (pH < 1, ultrapure HNO_3) (~1.0 ml) on collection, then spiked with indium (internal standard to correct for instrument drift) to a final concentration of 20 µg l^{-1}. Samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Perkin Elmer DRC II) under dynamic reaction cell (DRC; NH_3(g)) to reduce detection interferences on ^{63}Cu and ^{65}Cu. Calibration standards (7) were made in trace element-free seawater across a range from 1 µg l^{-1} to 10 mg l^{-1} total Cu. Quality control standards were made at 3 µg l^{-1} and 3 mg l^{-1} Cu. All standards were also spiked with indium, as above. Copper concentrations were quantified by external calibration with internal standardization, with each sample analyzed in triplicate and calibration and check standards analyzed between each 5 samples. Analytical uncertainties expressed as relative standard deviation and relative error were quantified as in United States Environmental Protection Agency (US EPA) method 200.8 (US EPA 1994). Copper calibration curves consistently had a R^2 > 0.9995.

Acute toxicity endpoints

At the conclusion of the test, each larva was examined under a microscope to determine survival and settlement success. Settlement inhibition was defined as the inability to metamorphose from the pelagic larval stage to the sessile adult form. Individuals were considered 'inhibited' if they remained as larvae until the 48 h benchmark. If not moving, larvae were examined using a light microscope to determine whether blood circulation and heart rate were detectable. If not, the individuals were considered dead (D). Individuals with an overall healthy (H) appearance were feeding, as indicated by their siphons being open (SO), and had plump ampullae and blood circulating. If an individual did not have these characteristics, it was recorded as not healthy (NH). Irregular siphon response was recorded in 3 ways. If siphons exhibited a delayed response of closing when probed (>1 s), those organisms were classi-

fied as SS. Siphons closed entirely on inspection indicated that no feeding was occurring (SC). Finally, siphons that did not respond to stimuli at all were labeled as NSR. In addition to siphon response, a small zooid size that differed visually from 'healthy' individuals was also recorded, as indicated by a shrunken or shriveled appearance and with a surface area measuring <1 mm^2 (SM). Further, affected ampullae were recorded as either shrunken (SA) or if very few were present (<6 count) or none at all, NAM. Individuals that did not exhibit either external (stimuli-induced) or internal (heart rate) response were considered dead (D). Significance was recognized for differences between treatments for each endpoint at p < 0.05.

To better understand both invasive and native colonial ascidian sensitivity to copper pollution in the context of the broader fouling community, we employed a SSD approach. A SSD provides an exposure–response relationship for a given chemical that is based on the response of different species (Suter 2006). In this way, related toxicology endpoints (EC$_{50}$ values) were compiled and summarized for multiple species and then modeled using US EPA CADDIS software (US EPA 2010a). This approach employs a log-probit model to plot concentrations at which different species exhibit a standard response to a pollutant exposure (US EPA 2010a). CADDIS ranked the toxicity values of each species from the input list. The ranks were then plotted to indicate species sensitivity relative to one another.

SSDs were originally developed to determine concentrations of a chemical that affect a particular percentage of species (e.g. predicted low effect concentration at the 5th percentile), but they can also be used for determining the relative tolerance of a particular species, as demonstrated in the present study. Acute EC$_{50}$ values for settlement inhibition along with morphological deformation EC$_{50}$ values for other larval benthic species retrieved from the EPA ECOTOX database (https://cfpub.epa.gov/ecotox/) were entered into CADDIS. Toxicity values for several phyla commonly found in benthic communities were included in our ranked list: annelids, chordates, molluscs, echinoderms, and arthropods.

All statistical analyses were performed using R version 3.3.0. Dose–response curves were constructed for larval settlement following the methods described by Hasenbein et al. (2017) to determine copper toxicity thresholds (EC$_{50}$ and LC$_{50}$ values). Settlement inhibition in acute tests was representative of successful zooid metamorphosis and was therefore used as an indicator for establishment success or failure.

No observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC) were identified by comparing acute endpoint measurements (zooid size, ampullae appearance, siphon response, mortality). Data for each endpoint were checked for normality using the Shapiro test and for homogeneity using the Fligner-Killeen test. The Kruskal-Wallis test with a Dunn's test for multiple comparisons was employed for data with homogeneous variance and the Wilcoxon-Mann Whitney test with multiple comparisons was used for data with heterogeneous variance. A p-value < 0.05 was used to identify statistical differences between endpoint measures.

Expt 2: chronic copper toxicity

We designed chronic toxicity assays to determine how copper impacted the growth of ascidians following settlement. No protocols were available in the ascidian ecotoxicology literature; therefore, standards were developed using knowledge of ascidian biology and personal observation. Expt 2 primarily focused on sublethal endpoints including surface area as a measurement of growth, number of zooids or cloning events, and heart rate. Colonial ascidians undergo numerous rounds of asexual reproduction, or cloning, prior to sexual reproduction (Grosberg 1988). This allows colonies to grow to an adequate size for spawning, but also represents an important competitive strategy for species within the biofouling community. We initiated toxicity tests in August due to the availability of adult ascidian colonies and continued the tests for 11 wk. After this time, water temperatures were too low and variable and so the experiments were terminated.

Larvae collection and initial settlement

Larvae were collected as previously described. Once collected, larvae were forced to settle on the desired substrate using the freshwater shock method described by Epelbaum et al. (2009). Briefly, larvae were placed in freshwater for 30–60 s, transferred to 33 ppt ± 1 ppt seawater filtered to 5 μm, and then immediately transferred in a single droplet of water and placed on the target substrate. Larvae begin to metamorphose immediately.

Once larvae metamorphosed and were securely fastened to their Petri dish substratum, the Petri dishes were suspended in holding tanks with clean

33 ppt ± 1 ppt seawater filtered to 5 µm for approximately 10 d. Single Petri dishes containing 10 to 11 d old juveniles were then suspended in 750 ml of treatment solution with 0, 1, 10, 20, 40, or 80 µg l^{-1} copper concentration. The containers were not aerated, and the water was manually renewed twice weekly for the 11 wk duration of the experiment. Stock solutions were prepared using cupric chloride dihydrate (CuCl$_2$·2H$_2$O; MP Biomedicals) as described above. Experimental concentrations were prepared by adding the appropriate stock to seawater filtered to 5 µm at 33 ppt ± 1 ppt salinity and ambient laboratory temperature (18°C ± 1°C).

Experimental units were fed 20 µl of an alternating diet of Microvert (Kent Marine) and Liquifry No. 1. (Interpret) based on previously published data (Epelbaum et al. 2009). Mortality was recorded twice weekly, while sublethal endpoints (i.e. colony surface area, number of zooids per colony, and heart rate) were measured once weekly. Detailed notes and observations of morphological abnormalities and mortality were also recorded. Photos of colonies were captured at 4 wk and 10 wk and were used to document long-term changes reflected in surface area and zooid count fluctuations.

Chronic toxicity endpoints

Colony surface area was measured using Image J photo processing software (Schneider et al. 2012). The numbers of zooids per colony were manually counted using a dissecting microscope under low power (×4 magnification). Heart rate was recorded by observing 3 individuals per colony for 30 s each and averaging their calculated beats per minute (BPM). Observations were recorded only for 30 s because ascidian hearts change the direction in which they pump abruptly (every 45–90 s; Mukai et al. 1987), and therefore a time duration was chosen that was not affected by treatment.

Chronic endpoints were used as indicators of sublethal, organismal-level stress. Dose–response curves were constructed to generate EC$_{50}$ values according to the same procedure as those for acute values. The number of zooids and colony area were representative of impacts on asexual reproduction. Variations in heart rate were indicative of metabolic changes. Data for each endpoint were checked for normality and homogeneity, and comparisons were made according to the same procedures as those employed for acute endpoint analysis.

RESULTS

Acute copper toxicity

During acute exposures, the invasive *Botrylloides violaceus* was more tolerant of Cu compared with the native *Aplidium glabrum*. As all measured concentrations were within 10% of the nominal values (Table S1 in the Supplement); nominal values were used in subsequent analyses. Dose–response curves were constructed for both *B. violaceus* and *A. glabrum* to determine the EC$_{50}$ concentration for settlement inhibition and LC$_{50}$ values for mortality (Table 1, Fig. S2 in the Supplement). Settlement is a critical endpoint for subsequent metamorphosis, growth, and reproduction; therefore, the inability to settle would ultimately lead to death. Mean settlement was significantly impacted at 75 µg l^{-1} (p = 0.009) in *B. violaceus* and all higher concentrations tested, and thus represented the LOEC. The comparative LOEC for *A. glabrum* was 37 µg l^{-1} (p = 0.008). While *B. violaceus* toxicity tests revealed an average EC$_{50}$ for settlement at 107.9 µg l^{-1}, *A. glabrum* was over twice as sensitive. Mortality represented by the LC$_{50}$ occurred at much higher concentrations for both species, but again *A. glabrum* showed a greater sensitivity compared with the invasive ascidian, *B. violaceus* (Table 1).

At the conclusion of the 48 h toxicity tests, several observational endpoints were recorded including not

Table 1. Median (±SE) effective concentration causing a 50% reduction in settlement (EC$_{50}$) or in survival (LC$_{50}$) from each copper toxicity test for both *Botrylloides violaceus* and *Aplidium glabrum*. Discrete tests had varying numbers of individuals per replicate and number of replicates per treatment based on larvae availability. Discrete tests are referred to as AGI, AGII, BVI, and BVII to indicate the species used in the test (AG: *A. glabrum*; BV: *B. violaceus*) and the iteration of the test (I: first; II: second). Averages are highlighted in **bold**

Iteration	No. of replicates	No. of ind replicate^{-1}	EC$_{50}$ (µg l^{-1})	LC$_{50}$ (µg l^{-1})
BVI	3	5	112.1 ± 17.7	2434.6 ± 325
BVII	5	5	90.4 ± 16.9	2294.9 ± 407.3
Average BV	–	–	**107.9 ± 17.3**	**2363.5 ± 174.1**
AGI	5	5	38.9 ± 2.9	656.4 ± 122.5
AGII	5	5	49.5 ± 3.3	640.9 ± 175
Average AG	–	–	**46.7 ± 3.6**	**656.8 ± 92.0**

healthy (NH) appearance, siphon condition (SS, SC, NSR), zooid size (SM), and ampullae condition (SA, NAM) at the conclusion of the 48 h toxicity tests. These endpoints acted as a useful analog indicator of health by elucidating physical traits that were visibly impacted. Overall, *B. violaceus* tolerated concentrations higher than *A. glabrum* based on all observational responses recorded (Table 2). *B. violaceus* zooids presented as healthy in both controls and the 37 µg l^{-1} treatment, while *A. glabrum* was clearly impacted at 37 µg l^{-1} due to their small size and the shrunken appearance of their ampullae. Conversely, *B. violaceus* zooid health, size, and ampullae were not significantly impacted until the 75 µg l^{-1} treatment. *A. glabrum* and *B. violaceus* experienced extreme ampullae shrinking or absence (NAM) at 150 and 300 µg l^{-1} copper, respectively.

To visually compare the impacts of acute copper exposure on each species, we combined the observational metrics to more easily differentiate between healthy and unhealthy colonies. We grouped the observational endpoints into 4 distinct classes: (1) normal size based on zooid size (SM), (2) functioning normally based on healthy appearance and siphon condition (H + SA + SO), (3) unhealthy based on siphon condition (SS + SC + NSR), or (4) not living if there was no visible heart beat (D) (Fig. 1). Decreases in colony size and health, and increases in unhealthy colonies and mortality were evident with increasing concentration. This pattern was apparent for both *A. glabrum* and *B. violaceus*, yet normal size and healthy colonies were observed at higher concentrations in *B. violaceus* than in *A. glabrum*. Approximately 50% of *B. violaceus* colonies exhibited normal function at 300 µg l^{-1}, compared with *A. glabrum* at 75 µg l^{-1}.

The copper exposure SSD combined the colonial ascidian toxicity dataset presented here with other benthic species to identify relative tolerances (Fig. 2). A complete table of EC$_{50}$ values for the selected species is provided in Table S2 in the Supplement. The SSD offers several key findings. First, *B. violaceus* is more tolerant of pollution relative to every other species included in the dataset (100th percentile), with an EC$_{50}$ value

Table 2. Threshold copper concentrations (p-values ≤ 0.05) which indicate the exposure level resulting in significant effects on each endpoint (lowest observable effect concentration; LOEC)

Endpoint	Threshold concentration (µg l^{-1})	
	Botrylloides violaceus	*Aplidium glabrum*
Overall condition		
Not healthy appearance (NH)	75	37
Reduced surface area (SM)	75	37
Mortality (D)	4800	600
Ampullae condition		
Shrunken ampullae (SA)	75	37
Few present (NAM)	300	150
Siphon response		
Delayed response (SS)	300	75
Closed siphons (SC)	1200	150
Unresponsive and closed (NSR)	1200	600

of 107.9 µg l^{-1}. The non-native tubeworm *Hydroides elegans* (phylum Annelida) follows closely behind with an EC$_{50}$ of 100 µg l^{-1}. Relative toxicity concentrations drop off significantly following *H. elegans*,
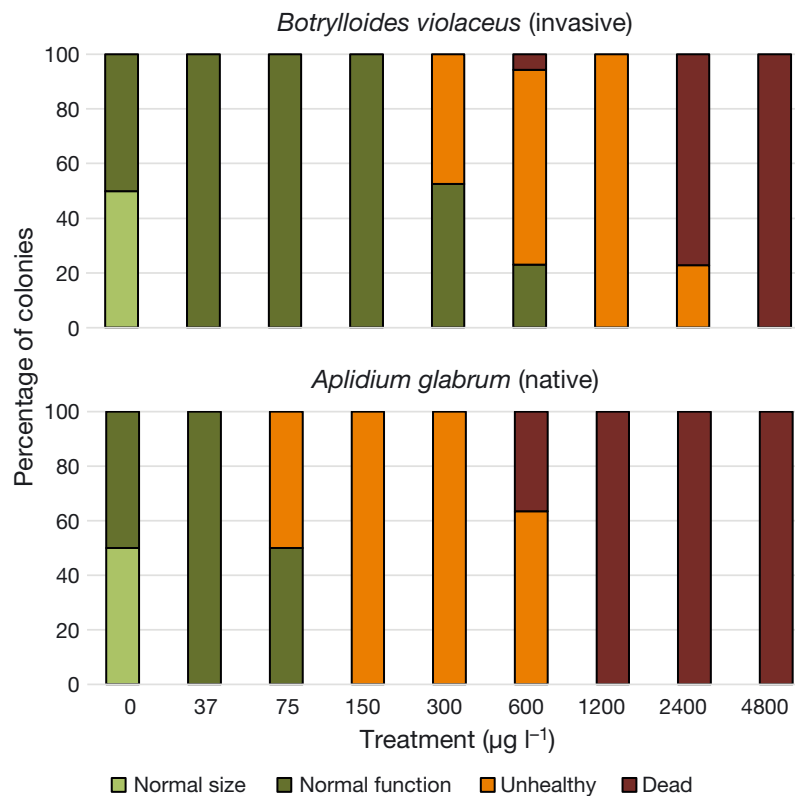

Fig. 1. Percentage of colonies at each concentration falling into different observational categories. Observational endpoints were combined to assess colony size (SM), if colonies appear to be functioning normally (H + SA + SO) or appear unhealthy (SS + SC + NSR), and mortality (D). See 'Materials and methods–Acute toxicity endpoints' for abbreviations
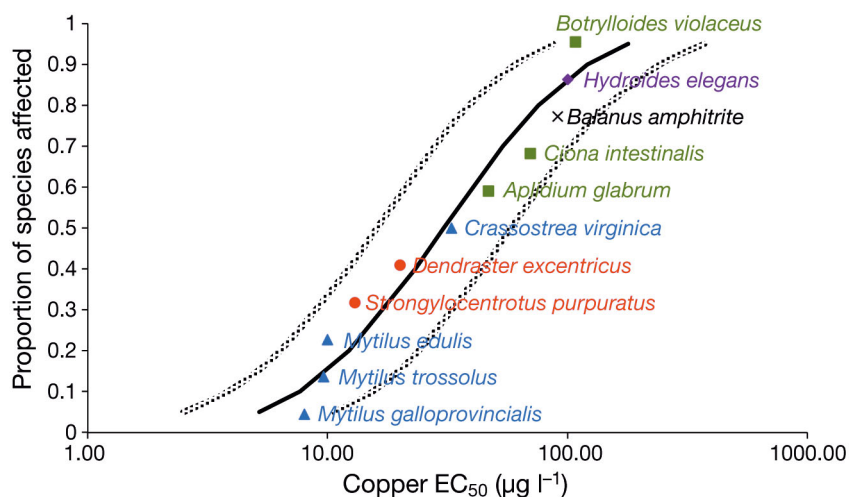
Fig. 2. Species sensitivity distribution for benthic species found in New England, including both the native *Aplidium glabrum* and the invasive *Botrylloides violaceus* acute median effective concentration for settlement inhibition (EC$_{50}$) values for morphological effects due to copper exposure. Species are grouped by phyla accordingly: Annelida = purple diamond (Gopalakrishnan et al. 2007); Chordata = green squares (Bellas et al. 2005a); Mollusca = blue triangles (Calabrese et al. 1977, Rivera-Duarte et al. 2005, Arnold et al. 2009, Nadella et al. 2009); Echinodermata = pink circles (Rivera-Duarte et al. 2005); Arthropoda = black cross (Qiu et al. 2005). The black curve represents the central tendency and the dotted lines represent the prediction intervals

with the next highest concentration being 70 µg l^{-1} for *Ciona intestinalis*. *A. glabrum* is in approximately the 60th percentile. Echinoderms and arthropods exhibit intermediate sensitivity relative to the other phyla. Members of the phylum Mollusca appear to be most sensitive to copper pollution, representing the lowest percentiles of the group (all values included in the SSD are from larval studies).

Chronic copper toxicity

Chronic copper toxicity for *B. violaceus* and *A. glabrum* was assessed in an 11 wk study by recording growth rate (number of zooids per colony), colony area, and heart rate. The numbers of zooids are directly indicative of cloning events that have occurred. Colony area may suggest whether cloning events have occurred, although cloning is not necessary for an expanding colony to experience positive growth. In addition, heart rate was monitored for each replicate by recording mean BPM in 3 randomly selected individuals per colony. Chronic EC$_{50}$ values were calculated for each of the 3 endpoints measured (Table 3).

During the 11 wk copper exposure, the number of zooids increased in both *B. violaceus* and *A. glabrum* colonies; however, the rate and frequency at which cloning occurred differed between the species. *B. violaceus* underwent rapid cloning relative to *A. glabrum*. At the conclusion of the assay, *B. violaceus* control colonies had a mean of 61.2 zooids (SE = 7.3), while *A. glabrum* controls had a mean of only 21.6 zooids (SE = 1.8) (Fig. 3A,D, respectively). For *B. violaceus*, 40 µg l^{-1} was the lowest concentration that resulted in a significant decrease of colony size (p = 0.01), number of zooids (p = 0.009), and heart rate (p = 0.007). However, impacts were noted in *A. glabrum* at much lower concentrations. Both colony size (p = 0.0002) and number of zooids (p = 0.0003) were significantly affected at 10 µg l^{-1}. Heart rate was affected at 20 µg l^{-1} (p = 0.0001). This suggests that heart rate was a less-sensitive metric to observe effects between treatment groups.

Temporal trends for each of the 3 endpoints were monitored weekly throughout the duration of the chronic copper toxicity test (Fig. 3) and compared at the final timepoint. *B. violaceus* zooid count and colony area were continuously greater than the controls at 1 µg l^{-1}, although this effect was not statistically significant. At higher concentrations, growth rates in zooid count and colony area were significantly lower throughout the 11 wk test. In general, as the colonies grew, the heart rate steadily declined, including in the controls. The trends observed in *A. glabrum* samples followed relatively the same growth rate pattern as their invasive counterparts, but were significantly lower in the measurements of number of zooids and colony area. Heart rate gener-

Table 3. Chronic median (±SE) effective concentration for settlement inhibition (EC$_{50}$) values for the number of zooids per colony, colony surface area, and heart rate (BPM: beats per minute). This value represents the median copper concentration at which 50% of test organisms exhibited significantly impacted growth (zooid count and area) and physiological function (heart rate)

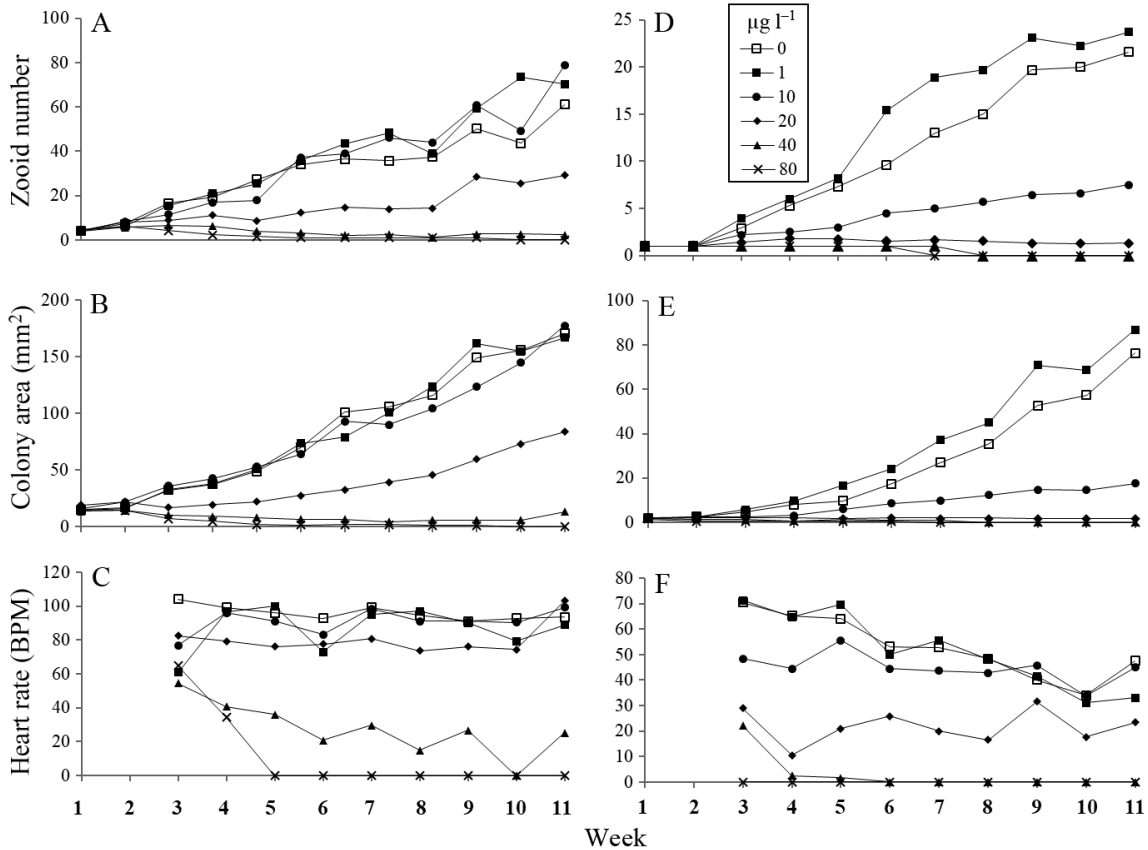| Species	Zooid count	Area (mm^2)	Heart rate (BPM)
Botrylloides violaceus	19.2 ± 4.6 µg l^{-1}	20.0 ± 3.5 µg l^{-1}	29.5 ± 2.3 µg l^{-1}
Aplidium glabrum	8.7 ± 2.2 µg l^{-1}	8.2 ± 4.02 µg l^{-1}	18.7 ± 10.2 µg l^{-1}

Fig. 3. Temporal trends of zooid count, colony area, and heart rate (BPM: beats per minute) during the chronic copper toxicity test for *Botrylloides violaceus* (A–C) and *Aplidium glabrum* (D–F)

ally declined for *A. glabrum*, but at a slower rate than that of *B. violaceus*.

DISCUSSION

Acute copper toxicity

Acute toxicity tests provided strong support for the hypothesis that invasive colonial ascidians are more tolerant of marine pollution than their native counterparts. *Botrylloides violaceus*, a prolific invasive colonial ascidian, was able to withstand greater concentrations of copper than the native *Aplidium glabrum*. *B. violaceus* survived concentrations 8 times greater than *A. glabrum*, while inhibition of settlement and metamorphosis of *B. violaceus* occurred at concentrations greater than twice that of *A. glabrum*.

The observational endpoints measured further supported these findings. Colony size and ampullae size or abundance were sensitive indicators for both invasive and native colonial ascidians. Both endpoints for *B. violaceus* were impacted at a higher concentration

($75 \ \mu g \ l^{-1}$) than *A. glabrum* ($37 \ \mu g \ l^{-1}$). Siphon activity is an indicator of regular feeding and defecation. Copper exposure caused negative impacts on siphon activity, including prolonged closing of the siphon and a lack of response to stimuli, but as with the other endpoints measured, *A. glabrum* showed a higher sensitivity. Delayed siphon response occurred at a concentration 4 times higher in *B. violaceus* than that of *A. glabrum* (300 and $75 \ \mu g \ l^{-1}$, respectively). At $1200 \ \mu g$ l^{-1} for *B. violaceus* and $600 \ \mu g \ l^{-1}$ for *A. glabrum*, siphons did not respond to stimuli at all, suggesting severe physiological fitness costs at these concentrations. *B. violaceus* continued to keep their siphons open and responsive at much higher concentrations than *A. glabrum*, suggesting that tolerance mechanisms rely on a trade-off of colony and ampullae size to allow for their continued function.

Others have investigated acute copper toxicity in the solitary cryptogenic ascidian *Ciona intestinalis* (Bellas et al. 2004), a major fouling species of US New England coastal waters (Pederson et al. 2005, McIntyre et al. 2013, Wells et al. 2014). Our SSD revealed that *C. intestinalis* has a relatively high tolerance to

copper (Fig. 2) compared with other benthic organisms. However, the SSD also demonstrated that *B. violaceus* has the highest copper tolerance of the other ascidian species *C. intestinalis* and *A. glabrum*. The relative copper sensitivity of ascidians compared with the sensitivities of other biofouling community members indicates that members of the Tunicata are generally more tolerant than most other phyla included in the SSD analysis. Further, this tolerance is often several times greater than native species present in the Gulf of Maine. For example, larvae from the phylum Mollusca were the most-sensitive group tested, with EC_{50} concentrations ranging from 8 to 32 µg l^{-1}, while the ascidians (including both colonial and solitary variants) EC_{50} values ranged from 46 to 107 µg l^{-1}. Despite the greater general tolerance of ascidians, significant differences remain amongst members of this phyla, including between the native and invasive species.

Chronic copper toxicity

The results of the chronic copper toxicity tests further support the hypothesis that invasive species are more tolerant of marine pollution. *B. violaceus* colonies exhibited an increased growth and cloning rate compared with *A. glabrum*, even in the controls, implying that *B. violaceus* can outcompete native ascidians in the wild. In addition, it was less susceptible to copper exposure. The growth of the ascidian colonies declined with increasing copper concentrations, measured both as colony area and number of zooids; however, growth of *A. glabrum* colonies was inhibited at lower concentrations.

In the 40 µg l^{-1} treatments, 100% mortality was recorded in *A. glabrum* at Week 8, while 60% of the *B. violaceus* colonies were able to tolerate this concentration. *B. violaceus* was also able to tolerate high copper concentrations for longer. At 80 µg l^{-1} Cu, a concentration that caused complete mortality to colonies of both *A. glabrum* and *B. violaceus*, *B. violaceus* colonies persisted for 3 wk longer than *A. glabrum.*

Finally, 3 endpoints (colony area, number of zooids, and heart rate) were compared during chronic toxicity tests to determine the most-sensitive indicators of sublethal toxicity. We determined that colony area and number of zooids were the most sensitive endpoints, while heart rate was a less-responsive metric. *B. violaceus* had a significantly greater mean surface area and number of zooids than *A. glabrum* in control samples. Further, the native species was impacted at lower concentrations relative to the invader, corro-

borating the results of the acute toxicity tests. In general, heart rates were less affected than other growth metrics measured in both species; although, the pattern of higher sensitivity in *A. glabrum* was consistent with other endpoints. One point to note is that the heart rate of *B. violaceus* is relatively higher than that of *A. glabrum*, even in the controls, but the role this plays, if any, in their tolerance to copper is not clear.

Our results are in agreement with field studies that have demonstrated the significant sensitivity of native biofouling species to copper exposure relative to invasive species present in the same ecosystems. Piola & Johnston (2009) confirmed that non-native bryozoans tolerated copper exposure at significantly greater concentrations than native species in both laboratory and field experiments. Toxicity tests were conducted in the laboratory using 2 native species and 2 invaders. Positive non-native growth was measured at the highest concentration tested (100 µg l^{-1} copper) while native species tested exhibited negative growth. In field experiments, adult invaders had reduced mortality in copper exposures relative to controls. In addition, non-native larvae had significantly greater recruitment than native species in copper treatments. The authors concluded that non-native species recruited, grew, fed, and recovered more efficiently than their native counterparts.

Additional studies of the impact of copper exposures on native species diversity in Australia (Piola & Johnston 2008) and California, USA, (Crooks et al. 2011) revealed not only that native species are more sensitive to copper pollution, but also that copper exposure can facilitate community-level shifts. In Australia, copper pollution significantly increased the spatial dominance of non-natives, which resulted in dramatic changes in community composition (Piola & Johnston 2008). Crooks et al. (2011) reported a similar finding in a California fouling community. Native species richness decreased by 40% with increasing copper concentrations, while non-native species richness was not negatively impacted. In addition, invasive species were tolerant to copper concentrations higher than those measured in the field, while native species were impacted at environmentally relevant concentrations (Crooks et al. 2011).

Within this context, our study provides mechanistic details, describing how copper decreases settlement success and reduces the growth of native species at low, environmentally relevant concentrations that do not impact the invasive species. These effects experienced at the individual level are likely responsible for the reduced biodiversity and community shifts observed in the field manipulation studies.

The mechanism of metal toxicity in ascidians is oxidative stress resulting from the formation of excess reactive oxygen species (Ferro et al. 2013, Franchi et al. 2017). Manifestations of oxidative stress in invertebrates range from cellular responses including protein and enzyme induction to changes in behavioral responses, such as altered feeding and reproduction (Hebel et al. 1997). An increase in the copy number of metallothionein protein genes, which are involved in detoxification and transport of metals, has been shown to enhance copper resistance in yeast (Hull et al. 2017), fruit flies (Maroni et al. 1987), and cyanobacteria (Gupta et al. 1992). Metallothioneins and related genes have been previously identified in ascidians (Franchi et al. 2011, 2017). It is therefore possible that copy number variation in these detoxification and transport genes may play a role in the observed high tolerance of the invader *B. violaceus*. Additional studies are needed to elucidate physiological processes responsible for the growth differences observed between native and invasive ascidians.

Although community-scale experiments support a greater tolerance of invasive species to copper pollution (Piola & Johnston 2008, Crooks et al. 2011), few studies have explored the underlying physiological mechanisms responsible for the observed tolerance differences between them. Research suggests that there could be growth costs associated with increased tolerance, even among different populations of exposed and unexposed invaders depending on genotype (Piola & Johnston 2006). This mechanism, however, would not account for the relative differences observed here between native and invasive populations living within the same community.

Ascidian invasions have become a worldwide concern. Some species may serve as a new food source, which could in turn increase predator abundance and result in community-wide impacts (Dijkstra et al. 2007). Further, the presence of colonial invaders such as *B. violaceus* inhibits the recruitment of native species including *A. glabrum* and potentially shift community species composition (Stachowicz et al. 1999, Dijkstra et al. 2007). Little is known about the specific ecological role of *A. glabrum* in New England; however, it is one of only a few native colonial ascidians (Wells et al. 2014) and is annually more abundant than other species such as *A. constellatum* Verrill, 1871 at the present study site (K. Osborne pers. obs.). This suggests its presence is important for buffering against invaders, providing community stability, and maintaining temporal succession alongside other benthic species.

Relevance to invasion success

In summary, both acute and chronic toxicity tests support the hypothesis that the invasive colonial ascidian *B. violaceus* is more tolerant to copper pollution than the native *A. glabrum*. In particular, tested copper concentrations of 10 µg l^{-1} impact native species growth and development and represent a concentration commonly found along urban coasts of the United States (US EPA 2010b), and yet this concentration does not have significant impacts on the invaders. Invader tolerance thresholds therefore enable invasion, which can alter community structure and function of native benthic communities (e.g. Dijkstra et al. 2007).

Although this study focused on only 2 species of colonial ascidians, they can act as a proxy for other species commonly found together in biofouling communities on the northeast coast of the United States. In addition, the comparison of toxicity thresholds for these 2 species to other members of the biofouling community found that the invasive ascidian species is the most-tolerant species within this community. Recent field and laboratory studies conducted in Australia also provide support for the greater tolerance of invasive species over native species (Piola & Johnston 2009), while others have suggested that the broad tolerance of invasive species to a variety of environmental stressors can be applied to pollution exposure as well (Crooks et al. 2011). Taken together, these studies suggest that the greater tolerance of invasive species is likely generalizable beyond the 2 species compared in the present study and poses concern regarding the role pollution may play in facilitating the establishment and spread of invasive species.

Species richness is an important factor that determines a community's ability to resist the establishment of invasive species; thus, factors affecting native species biodiversity can act to enable more invasions. Marine pollution, including metals, organic pollutants, nutrients, and sewage, has been shown to negatively affect marine biodiversity (Johnston & Roberts 2009). Piola & Johnston (2008) further revealed that pollution can reduce native species diversity without impacting the growth of invasive species. Therefore, if native species are susceptible to pollutant concentrations commonly found in urban harbors, as shown in the present study, biotic resistance will become depleted, allowing for the establishment of invasive species.

To further conceptualize how marine pollution may facilitate the establishment and spread of invasive
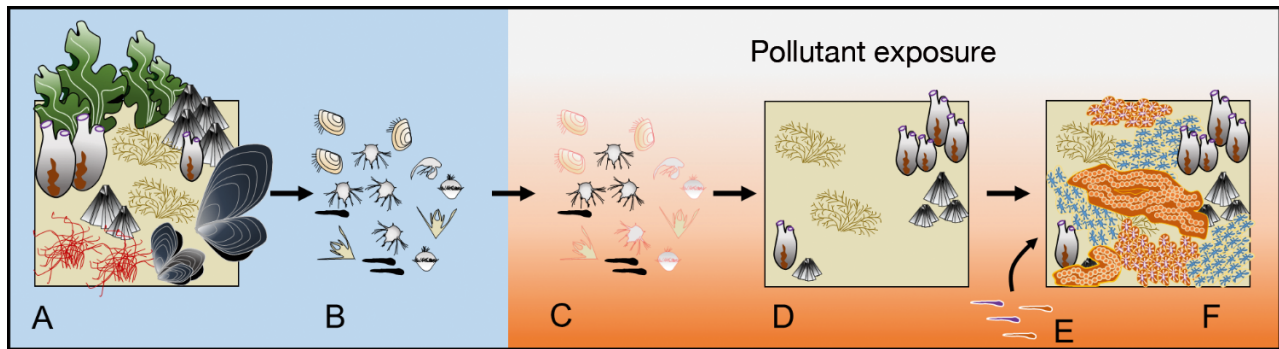
Fig. 4. Invader tolerance model of decreased biotic resistance and increased invasibility of polluted urban harbor fouling communities. (A) Adult biofouling assemblage with high diversity prior to the introduction of pollution and invaders. (B) Adult community reproduces and releases larvae. (C) Released larvae come in contact with marine pollution, causing mortality to some species (outlined in red). (D) Species richness and thus biotic resistance is decreased in the newly established community due to sensitive organism larval mortality. (E) Successful introduction of pollutant-tolerant invasive larvae via external propagule pressure. (F) Vulnerable community established by invasive species due to decreased biotic resistance and native organisms eliminated from dual stressors of pollution and competition from invaders

ascidian species, the invader tolerance model (ITM) was devised as part of this study. The ITM (Fig. 4) illustrates how marine pollution may act as a mechanism for colonial ascidians to invade and successfully establish in urban harbors. Using concepts from the previously described ascidian invasion process (Zhan et al. 2015), the ITM combines the notion that increased species richness reduces invader establishment success (Elton 1958) with the knowledge that marine pollution reduces native biodiversity (Johnston & Roberts 2009). The ITM identifies how to test these 2 independent factors and their possible interaction. This conceptual model highlights the importance of protecting native biodiversity in reducing colonial ascidian invasion, establishment, and spread within benthic communities.

The ITM is comprises 5 discrete events that demonstrate how marine pollution will theoretically play an integral role in decreasing the biotic resistance of a native biofouling community, and thus increase invasion success (Fig. 4). The ITM builds from the biotic resistance hypothesis, which states that invader success increases with decreasing native species richness, and provides a possible mechanism by which biodiversity first becomes depleted. In Part A of the ITM, a diverse assemblage of native biofouling species is present prior to the introduction of invasive species and marine pollution. Part B depicts adult community members spawning and releasing free-swimming larvae into the water column; many benthic fauna undergo a pelagic early life cycle phase (Strathmann 1974, Pechenik 1999). In Part C, free-swimming native larvae are exposed to marine pollutants via an episodic event. When such an event

occurs, sensitive species will experience mortality, thus decreasing biodiversity. As the remaining larvae settle and colonize a new community, the biotic resistance will be lower as a result of fewer species' present. In Part E, invasive species introductions impart propagule pressure on the recipient community. With heightened native community vulnerability resulting from pollution exposure, invasive propagules are presented with an opportunistic advantage to colonize. Finally, in Part F, the vulnerable native community has been successfully established by invaders due to the dual pressures of marine pollution and invasive species competition.

The findings from this research directly test and provide support for predictions of the ITM, including greater pollution tolerance in invasive species. This study addresses the ITM's question of whether sensitivity differences exist between native and invasive species at multiple life stages and also different concentrations of the marine pollutant copper. These differences were then compared with the sensitivities of several other phyla to address the question at the community level, although other variables remain to be tested. Further studies exploring the role of competition and biodiversity indices (e.g. species richness, evenness) will be needed to elucidate the complex relationship between invader success and pollution tolerance.

CONCLUSIONS

This research demonstrates that invasive and native colonial ascidians have differential tolerances

to copper exposure. The invader exhibited significantly higher tolerance relative to the native species. Invaders were also more robust to acute copper stress at sensitive life stages when compared with several other benthic species. Chronic assays revealed that the native ascidian was negatively impacted at environmentally relevant levels, while invader survival and proliferation continued at concentrations far exceeding such concentrations. These findings provide evidence to support that invasive species are more tolerant of marine pollution than native species in varying exposure length and concentration level scenarios.

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