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Simulating regimes of chemical disturbance and testing impacts in the ecosystem using a novel programmable dosing system

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Summary

1. Pollution is a global issue at the frontier between ecology, environmental science, management, engineering and policy. Legislation requires experiments to determine how much contamination an ecosystem can absorb before there are structural or functional changes. Yet, existing methods cannot realistically simulate regimes of chemical disturbance and determine impacts to assemblages in ecosystems. This is because they lack ecologically relevant species and biotic interactions, are logistically difficult to set up and lack environmentally relevant regimes of chemical and abiotic disturbance that organisms experience in polluted areas.

2. We solved these long-standing environmental, logistical, experimental and ecological problems by developing a programmable dosing system. This dosing system simulates, *in situ*, regimes of chemical disturbance to assemblages by manipulating the concentration, duration, timing and frequency of pollutants to which they are exposed.

3. Experiments with priority pollutants (the metal copper and the biocide chlorpyrifos) and mussel assemblages revealed consistent plumes of contamination within patches of mussel. Mussels at the sources of experimental plumes of copper created by the dosing system, accumulated 670% more copper in their tissues compared to mussels 0·5–50 m away. In addition, when mussels were exposed to increasing concentrations of copper, there was a concomitant increase in the amount of copper in the tissues of mussels. Combining the dosing system with an established hierarchy of ecotoxicological assays revealed mussel assemblages exposed to copper and/or chlorpyrifos had 40–70% fewer worms, whilst chlorpyrifos alone caused an 81% reduction in the number of amphipods and caused mussels to filter 48% fewer particles from the water. Combinations of copper and/or chlorpyrifos had no effects on the abundance of crabs, the respiratory functions of assemblages or the viability of molluscan haemocytes.

4. As global contamination accelerates, we discuss how this technological advance will enable a diverse array of ecologists, mangers and policy-makers to understand and reduce pollution.

Key-words: assemblage, cell, ecologically relevant, environmentally relevant, filtration, *in situ*, levels of biological organization, multiple stressors, pollution

Introduction

Global infiltration of chemical contaminants causes global reductions in biodiversity (Johnston & Roberts 2009) that jeopardize the useful functions and services that ecosystems provide. Mitigating these problems is difficult because humans use >99 million different chemicals (with 15 000 new chemicals added each day) and <0·3% of these chemicals have any form of regulation (CAS 2015). Legislation (EPA 1980; EC 2006, http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52006PC0397) requires experiments to determine the quantity of a pollutant (known in the US as a 'Total Maximum Daily Allowance' or elsewhere as 'Environmental Quality Standard') an organism or its ecosystem can safely absorb before there is a change in its structure and/or function. For 30 years, however, experimental methods to determine these 'critical loads' (Groffman *et al.* 2006) have been criticized for (i) lacking ecologically relevant species and biotic interactions (Kimball & Levin 1985; Underwood & Peterson 1988; Underwood 1995), (ii) being logistically difficult to set up and (iii)

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lacking environmentally relevant regimes of chemical, abiotic and biotic disturbance that organisms experience in polluted areas. We explain these long-standing global issues and provide a novel technological solution in the form of a programmable dosing system that simulates, *in situ*, regimes of chemical disturbance to assemblages.

ECOLOGICAL PROBLEMS

Laboratory experiments provide useful information about the uptake and the sensitivity of organisms to contaminants; however, toxicological testing in laboratories is not the ecological testing of toxicology (Underwood & Peterson 1988) that is required under commitments to international legislation (EPA 1980; EC 2006) for three reasons. First, experiments are usually based on a single or few species that survive easily in the artificial conditions of the laboratory and are thought to be sensitive to contaminants but are not always endemic in polluted habitats. Secondly, experiments rarely study more than one species at a time and it is well known that individual organisms often respond to contaminants in different ways to populations and assemblages (Browne *et al.* 2015). Thirdly, important processes and interactions that normally occur under natural conditions are lacking in the laboratory (e.g. recruitment, competition, emigration; Underwood 1995; Underwood & Peterson 1988).

LOGISTICAL PROBLEMS

It is impractical to do realistic experiments with assemblages in most laboratories so the solution has been to use field experiments to determine whether effects actually arise over and above the natural variation that is explicitly excluded in the laboratory (Kimball & Levin 1985). In laboratory experiments (including micro/mesocosms), concentrations of contaminants are manipulated and naturally varying confounding variables (e.g. temperature, salinity, light, food) are kept artificially constant. In contrast, field experiments expose organisms to natural variations in all environmental variables (e.g. abiotic: tidal cycle, spates, storms, droughts; biotic: predation, competition) except the one of interest (e.g. dose of contaminant), which is manipulated *in situ*. Where direct comparisons have been made, findings from field experiments have often been inconsistent with those from laboratories (e.g. Thompson, Norton & Hawkins 1998). Closer examination reveals that laboratory experiments can suffer from significant artefacts (e.g. transferring organisms from habitats and caring for them in laboratories; Honkoop *et al.* 2003; Dissanayake, Galloway & Jones 2008) that alter the performance and survival of organisms.

For instance, differences in their diet (Dissanayake, Galloway & Jones 2008), architecture of aquaria (e.g. glass vs. plastic tanks; Teuten *et al.* 2007) and water quality (e.g. salinity, pathogens, gradients of temperature) can affect whether a contaminant transfers into their tissues and impacts the functional well-being of an organism (e.g. Camus *et al.* 2000; Roast, Widdows & Jones 2001; Parry & Pipe 2004). To overcome some of these ecological, physiological and logistical problems, researchers do field experiments, where assemblages are exposed to doses of contaminants *in situ* using a range of dosing systems. These include fences that trap contaminants (e.g. oil; McGuinness 1990), paints (e.g. antifoulants; Johnston & Webb 2000) and impregnated plaster blocks that dissolve with the movement of water (e.g. Morrisey, Underwood & Howitt 1995; Cartwright, Coleman & Browne 2006) or electronic pumps that deliver pulses of contaminants (Roberts *et al.* 2008). These dosing systems, however, also suffer from logistical and financial problems. Fences cannot contain all contaminants and may alter biotic interactions, the plaster itself can affect the physiology and behaviour of animals (Cartwright, Coleman & Browne 2006), and existing pumps have to be operated manually. Although, some of these problems can be overcome by including procedural controls in the experiments, these can be costly in terms of time, effort and finances. Furthermore, running field experiments can be difficult due to tides and spates in aquatic habitats, poor weather, a lack of electricity and running water, and a lack of security can make the risk of losing sophisticated and expensive equipment unacceptably large, especially in busy areas. Therefore, there is a need for reusable, relatively inexpensive, safe and autonomous technologies for manipulating the dose of contaminants that can be deployed across aquatic and terrestrial habitats.

ENVIRONMENTAL PROBLEMS

Monitoring shows organisms in habitats experience complex regimes of chemical disturbance with the concentrations, timing and frequency of toxicants to which these organisms are exposed (e.g. transient contamination events through seasonal urban run-off; Makepeace, Smith & Stanley 1995; Church, Granato & Owens 1999). These regimes are expected to become more complicated due to accelerated chemical production and climatic change (Schiedek *et al.* 2007). This is problematic because variation (spatial and temporal) in disturbances is known to cause variation in ecological impacts (Benedetti-Cecchi 2003) and existing field and laboratory technologies are not able to expose organisms to environmentally relevant regimes of chemical disturbance (Underwood 1995). Therefore, to fulfil legislative commitments (EPA 1980; EC 2000) to determine whether or not the ecosystem can safely absorb one or more contaminants, scientists need technologies that allow them to expose assemblages to environmentally relevant treatments based on observations (from monitoring) about the concentrations, timing and the frequency at which assemblages in habitats are exposed to chemical toxicants.

THE TECHNOLOGICAL SOLUTION TO UNDERSTANDING POLLUTION

To overcome the ecological, logistical and environmental problems with the existing dosing systems described above, we developed a novel – programmable and automated – dosing system that allows ecologically and environmentally relevant testing of single and multiple pollutants on natural systems by being able to manipulate: (i) the number of chemicals, (ii) their

concentration and the (iii) duration, (iv) frequency and (v) timing of influxes over extended periods. The system devised is relatively inexpensive, as it is less time-consuming to maintain the appropriate treatments than the methodologies described above (see Table 1) and it can be re-used indefinitely. As metals and biocides make up over 50% of the priority pollutants, we explored their single and combined impacts on the structure and functions of mussel assemblages. These assemblages are ideal because mussels are important ecosystem engineers with clear links between their 'health' and ecological impacts (Browne *et al.* 2015). For instance, their shells create habitat and their feeding transfers sediments, algae, nutrients and energy from the water column into the mussel bed allowing them to support diverse assemblages (e.g. Cole & McQuaid 2010). Separate laboratory studies have shown that exposure to pollutants can damage their cells (i.e. fragmented plasma membranes, smaller lysosomes) and organs (i.e. gills, gut) and this has been linked to reduced feeding, respiration, growth and survival, and less diverse assemblages (Browne *et al.* 2015). No field experiment has, however, tested these predictions by manipulating the presence and absence of metals and/or biocide in an ecologically and environmentally relevant manner.

Materials and methods

DOSING SYSTEM AND LOCATION OF STUDY

The dosing system was built by attaching up to ten 640-L tanks (IBC; www.smithsofthedean.co.uk), 100 separate lengths of 100 m (12 km in total) polyethylene tubing (diameters: 4 mm internally, 6 mm externally) coiled on plywood reels (200 mm flange/barrel-diameters; www.northeastreel.com) for dispensing, digital timers (TM-619, Wenzhou Changxin Electronics, www.aquavolt.com.au), batteries (Banner, AccuPro 12v-7A) and charger as an electrical backup in case the power supply failed (Fig. 1e–h). The total cost was estimated to be around €11K (based on 2010–11 figures) and this included 12 km tubing (€2500), 10 tanks (640 L; 1500), 18 bilge pumps (€475), 10 manifolds (€500), control panels with timers (€1400), two batteries with protective boxes (€450), two chargers for batteries (€700), 1·5 km of electrical wiring (€100), 10 wooden reels to coil tubing (€750) and additional expenses (electrician, fuses, PVC tubing, drill bits, paint for reels and covers for tanks; €1825).

The dosing system discharges $4{\cdot}88 \pm 0{\cdot}5$ L h^{-1} to each plot and neither coiling the tubes ($F_{1,8} = 1{\cdot}22$, $P = 0{\cdot}30$) nor using three different chokes (plastic collars made from irrigation microsprayers that reduce the internal diameter of the tube; $F_{3,36} = 0{\cdot}86$, $P = 0{\cdot}47$) significantly reduced the rate of discharge.

At full capacity, the system can deliver controlled doses of contaminants to either (i) 200 individual experimental plots, with 25 different treatment combinations, or (ii) by combining smaller plots, an area the size of 100 m^2 could be dosed, and (iii) temporally, the duration of dosing could be manipulated to the nearest minute, hour, day, week, month or indefinitely. This is because the timer(s) can be programmed to automatically start and stop at the pre-programmed times to simulate transient and more prolonged discharges of contaminants without the need for persons to be at experimental sites to operate it.

With permission from Malahide Marina and Fingal County Council, we completed a series of experiments to determine the capacity of the dosing system to manipulate levels of bioaccumulation and test the ecotoxicological impacts of pollutants (metals and/or biocides) on multiple levels of biological organization (cell to assemblage). The system was deployed on the floating pontoons on the northern and southern pontoons of Malahide Marina (Ireland; Fig. 1) as the marina has running water, electricity and a security system that prevents vandalism and theft. Here, mussel assemblages living on the vertical side of the floating pontoons were used as part of our experiments.

Table 1. The relative advantages of existing methods used in laboratory and field experiments. Shading refers to potential challenges that can cause persistent (grey shading), frequent (blue) or no problems (green) in different set-ups

Advantages of different types of experimental set-ups		Laboratory			Field				
		Static	Flow through	Microcosm	Fences	Plaster blocks	Paint	Watering cans, pumps	Our dosing system
Ecotoxicological relevance (biological scales where impacts can be tested)	Suborganismal								
	Organismal								
	Population								
	Assemblage								
	Ecosystem								
Logistical and environmental advantages	Includes natural environmental variation								
	Can test for stress(es) due to translocation of organisms								
	No water quality problems								
	No husbandry required								
Capacity to simulate and manipulate chemical regime	Concentration								
	Number								
	Timing								
	Frequency								
	Automatic dosing								

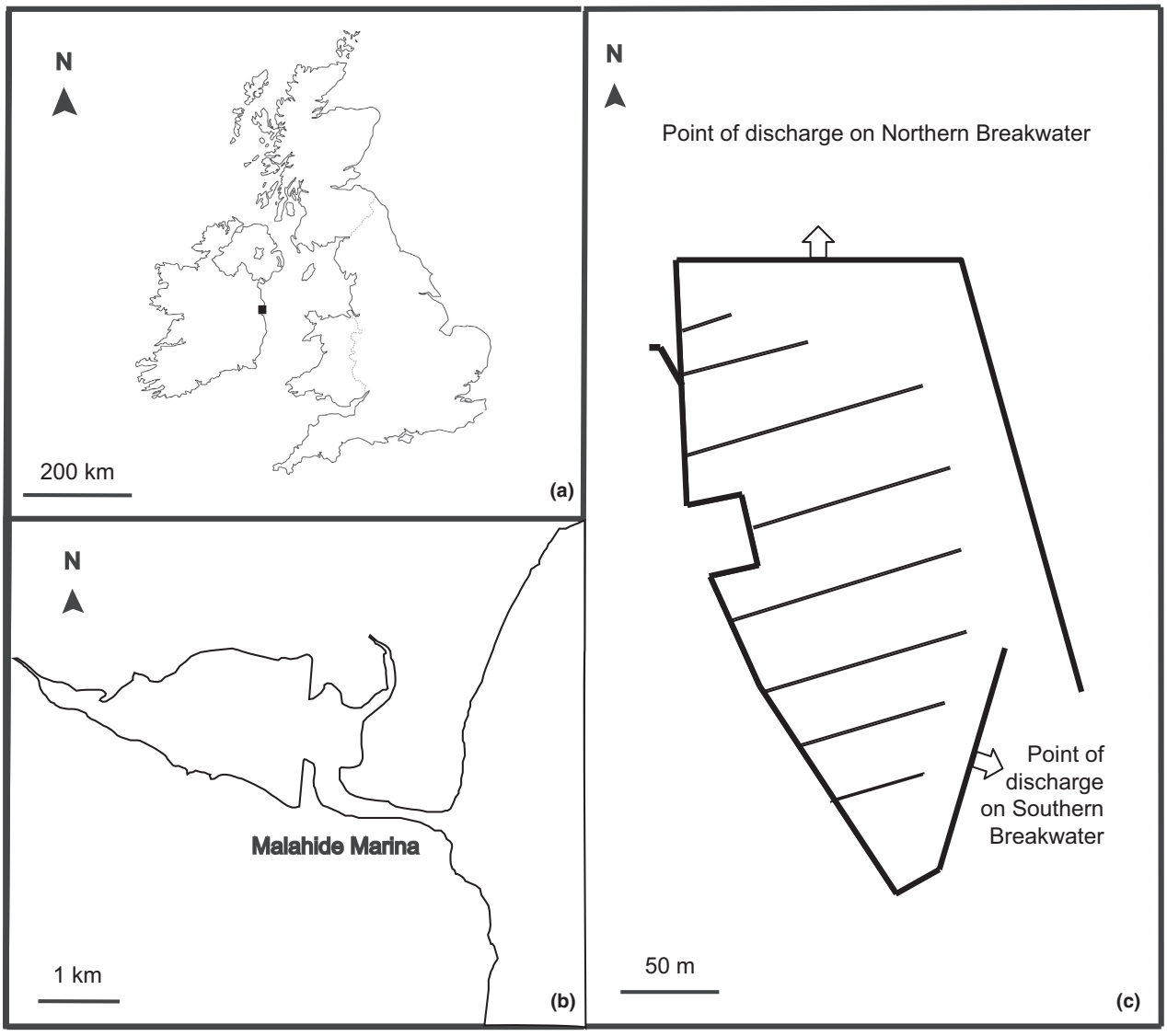

Fig. 1. Location of experiments within the British Isles (a, b) and Malahide Marina (c; 53N 27′ 17·5; 6E 9′ 12·46).

EXPERIMENT 1: SIZE OF PLUME

A copper solution (0·423 mg Cu L^{-1} seawater, $CuSO_4.(H_2O)_5$, CAS No 7758-99-8) was continuously added to single patches of mussels (each mussel patch was <1 m × 0·5 m size) in two sites for 14 days (northern and southern pontoons; Figs 1 and 2). Mussels at these sites live on the floating pontoons where they form larger patches that can cover the entire length of the floating pontoons. Copper was used as a tracer in this study because it is recognized globally as a priority pollutant (EPA 1980; EC 2006), with inputs from run-off, discharges (e.g. sewage, storm water), corrosion of infrastructure and antifouling paint on boat hulls (Makepeace, Smith & Stanley 1995). Mussels (*Mytilus edulis*) growing on the floating pontoons of the marina allowed us to measure bioaccumulation of pollutants discharged from the dosing system. These organisms are relatively resistant to many pollutants that accumulate in their tissues, thereby increasing concentrations to levels more easily detected than those in the environment (Widdows & Donkin 1992). This has allowed researchers to use the species complex as 'sentinel' organisms to investigate global levels of contamination (Widdows & Donkin 1992). Mussels (after 14 days) and water samples (100 mL; after 7 and 14 days) were collected at 0, 0·25, 0·5, 1, 2, 4, 6, 10, 50 m in both directions along the pontoons from experimental point sources (Fig. 2). Mussels were left in seawater (Instant Ocean; 18 MΩ cm water with <0·015 ppm Cu) for 24 h so that they evacuated their guts and were then frozen in pre-cleaned 100-mL polyethylene bags. Concentrations of copper in the tissues of mussel and seawater were determined using an inductively coupled mass spectrometer (see 'Chemical Analysis').

EXPERIMENT 2: MANIPULATING CONCENTRATIONS OF POLLUTANT

The dosing system was deployed on the northern pontoon (Figs 1 and 2) with three treatments (0, 50 and 1269 μg mg Cu L^{-1} seawater) housed in separate tanks. The second treatment was representative of concentrations from antifouling paints and the third treatment is the largest mean concentration found in European storm water (Makepeace, Smith & Stanley 1995). Solutions from each tank were added to randomly chosen mussel patches (110 mm

Fig. 2. Dosing system (a) set-up at Malahide Marina with the white box containing the programmable timers (b) each connected to bilge pumps which themselves are connected to the blue tubing via the grey manifolds (c). Through this, mussel assemblages on the vertical side of the pontoon received controlled doses of pollutants (d, e).

in diameter) for 6 weeks and concentrations of copper quantified as previously mentioned. To avoid cross-contamination, each replicate/treatment combination was >1·5 m apart, in this and the remaining experiments.

EXPERIMENT 3: IMPACTS ACROSS LEVELS OF BIOLOGICAL ORGANIZATION

To determine whether the dosing system can be combined with a suite of ecotoxicological assays to determine changes in the structure and functions of assemblages, we used our system to discharge from five tanks (one tank with seawater; two tanks with 7·7 mg copper sulphate L^{-1} seawater; two tanks with 250 mg chlorpyrifos L^{-1} seawater) to five groups of experimental mussel assemblages (control; +seawater; +metal; +biocide; +metal +biocide). One day later, we quantified changes in the structure (numbers of worms, amphipods and crabs) and functions (viability of haemocytes from mussels, filtration and respiration) of mussel assemblages using established techniques (see 'Ecotoxicological Assays'). We chose a 1-day duration to simulate a realistic, short pollution event and to reproduce, in an ecologically and environmentally realistic manner, previous ecotoxicological work that has used this duration in laboratories to document the impacts of metals, biocides and other pollutants across levels of biological organization (see Wu, Siu & Shin 2005; Canty *et al.* 2007).

CHEMICAL ANALYSIS

This was performed under ISO 9001:2008 protocols at Plymouth University, UK. All glassware and plastic containers were cleaned in a phosphate-free degreasing soap-free detergent (10% solution of Decon 90), then rinsed in water (18 MW cm conductivity), followed by immersion in a nitric acid (10% Aristar grade by VWR) bath for 24 h using 18 MΩ cm water and rinsed again in 18 MΩ cm water. All items were dried in a particle-free environment and stored in sealed and cleaned polyethylene bags until required. To preserve dissolved Cu and to prevent bacterial/microalgae growth in the samples of seawater, 100 µL of concentrated hydrochloric acid (Sigma-Aldrich, AR grade, St. Louis, MO, USA) was added and samples were stored at <5 °C in the dark. The frozen mussels were freeze-dried (Labconco, Freezone 6) in their storage containers for 3 days. The length, width and height of the shell valves were measured to the nearest mm using callipers. Mussels were then shucked by removing the mussel from its shell and the mass of the freeze-dried mussel measured (Scalehouse, ALD114CM) and recorded. Each freeze-dried mussel was then transferred to a polyethylene container for digestion with concentrated nitric acid (20–25 °C; 10 mL) (Sigma-Aldrich) for 4 days. The samples were then transferred to boiling tubes for digestion at 100 °C for 48 h (Skalar, Tecator 1016 Digester Heat-Block, Skalar Analytical B.V., Breda, The Netherlands). Finally, the acidic solutions containing the digested mussels were transferred to pre-

cleaned polyethylene screw-top 100-mL containers (Linux, UK) and diluted to 10 mL with 18 MΩ cm water for storage prior to analysis. The concentration of copper in the solutions containing the digested mussel was determined using an Inductively Coupled Plasma Mass Spectrometer (X Series 2; Thermo Fisher Scientific, Hemel Hempstead, UK). This instrument was operated in 'collision/reaction cell mode', with 7% H_2 in He as the collision/reaction gas, to negate the effect of polyatomic interferences, for example $^{23}Na^{40}Ar$ and $^{25}Mg^{40}Ar$ on ^{63}Cu and ^{65}Cu, respectively. All mussel digests were diluted hundred-fold prior to analyses and In and Ir, added to give a concentration of 10 mg L^{-1} in the diluted digests, were used as internal standards to account for instrumental drift.

ECOTOXICOLOGICAL ASSAYS

Haemocytes are cells within the hemolymph that function in the immune system of invertebrates. Their viability in mussels was assessed as in Browne *et al.* (2008), using an assay which measured the ability of haemocytes to accumulate a red dye, with 'healthier' well-functioning cells accumulating more dye than cells exposed to pollutants. Haemocytes were used because they can be easily collected and play a major role removing harmful waste, supplying tissues with nutrients and energy, healing wounds, degrading pathogens and adding minerals to their shell (see brief review in Moreira, Browne & Coleman 2013). Previous research has shown that exposure to pollutants, however, degrades these functions by damaging the plasma membrane and/or shrinking their lysosome. After measuring rates of respiration and filtering of the mussels assemblages, hemolymph (50 µL) was withdrawn from abductor muscles of three mussels and was placed separately into duplicate wells of a 96-well microtitre plates (pre-treated with polylysine). The plate was then agitated (1400 rpm for 60 s) and then left for 50 min for the cells to adhere. Excess cells were then discarded and the wells rinsed with phosphate buffer (pH 7·4). Neutral red dye (0·4%) was then added and cells were incubated in the dark for 3 h to prevent photolysis. Wells were then washed with phosphate buffer again before a solution of 1% acetic acid/20% ethanol was added to precipitate the dye. Absorbance was read at 550 nm using a spectrophotometer, protein was quantified, and results were presented as optical density per gram protein.

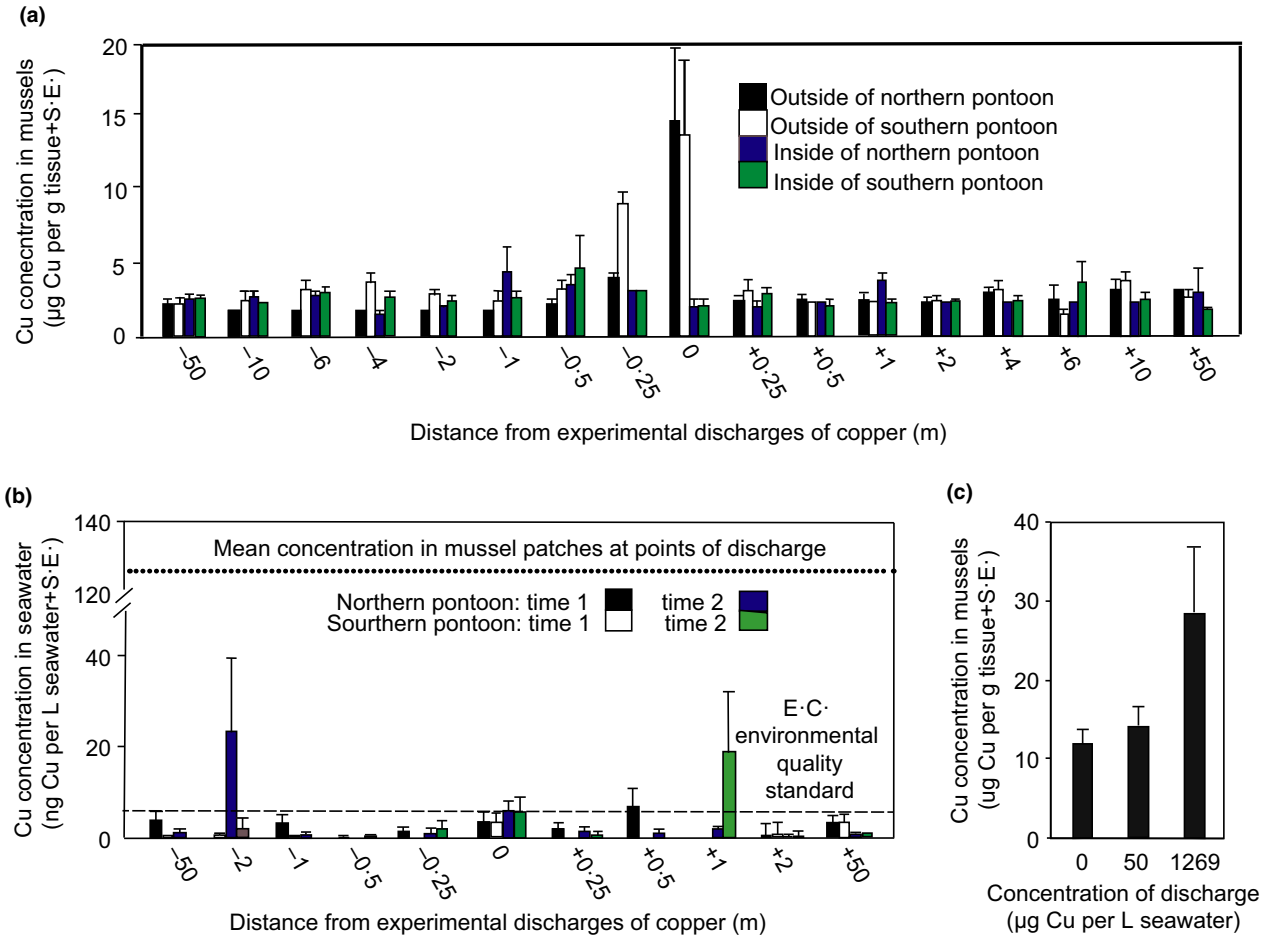

Fig. 3. Spatial distribution of copper in mussels (a) and seawater (b) at different distances from experimental discharges (Experiment 1). Not all water samples were analysed because the plume could not be detected in water samples outside of the mussel bed in either direction (Table 3). In contrast, mussels within the plume had over 670% more copper in their tissues compared to mussels 0·5–50 m away ($F_{16,136} = 3{\cdot}34$, $P < 0{\cdot}001$; Table 2). (c) Experimental discharges with increasing concentrations of copper caused increasing concentrations of copper in the tissues of mussels (Experiment 2). There were larger concentrations in seawater at −2 and +1 m distance from the discharge on the northern and southern pontoon, respectively, at time 2 that were not present at time 1 (Fig. 3b). These provide evidence for potential spatial and temporal variation in the distances travelled by the copper in the water, perhaps associated with differences in tides or wave action causing different localized eddies at different times. It should, however, be noted that the accumulation of copper in mussels showed no such variation, suggesting that there was negligible total spread to plots >0·25 m from the discharge point over the duration of the study.

Respiration transfers oxygen from seawater to the tissues of organisms and carbon dioxide in the opposite direction. To measure this, experimental patches of mussels were incubated *in situ* and in the dark using an opaque chamber. Changes in dissolved oxygen (mg O^2 L^{-1}) were measured using a probe (HQ20 Hach Lange Ltd portable LDOtm, Loveland, CO, USA). To ensure that measurements were taken at the correct time points, a linearity test was undertaken to test how long it took the volume of water to be depleted of oxygen. Concentrations of oxygen in the water were measured every 2 min over an hour to identify the period during which there was a linear decrease of oxygen in the water. On the basis of this test, measurements of oxygen were taken after 10 and 20 min. The initial 10 min as defined by the linearity test has been shown to also allow for acclimatization of the assemblages and to ensure photosynthesis had ceased after covering with an opaque chamber (Noel *et al.* 2010). The chamber was also fitted with a pump to ensure water was circulated and that concentrations of oxygen were homogenous and therefore representative. Rates of oxygen uptake by the assemblages were estimated using the equation: Δ [O_2] dark/Δt dark, Δ [O_2] dark is the difference in dissolved oxygen concentration between measurements taken, respectively, at the beginning and end of the dark period, and Δt dark is the time difference between these measurements. Respiration calculated for each individual plot and expressed as mg O_2 L^{-1} h^{-1} (Noel *et al.* 2010).

We then tested the capacity of assemblages to remove particles from seawater *in situ* using the same purpose-built chamber. Five millilitre of a solution containing microalgae (*Isochrysis galbana*) was injected into the chamber. The solution of microalgae was prepared such that it gave a concentration of algal cells of 12–15 000 per 0·5 mL in the chamber. To ensure that microalgae remained suspended in solution, the chamber was fitted with a circulation pump. After initial introduction of algal cells, 20 mL samples of seawater from within the chamber were taken at three time intervals: 0 (T0), 15 (T1) and 30 (T2) min, respectively. The numbers of particles retained in samples were counted using flow cytometry and clearance rates calculated as the change in concentration per unit time using the following equation: clearance rate = V (loge C1 − loge C2)/t, where V is the volume of water in the chamber and C1 and C2 are the algal concentrations at the beginning and end of the time interval (t) (Canty *et al.* 2007).

To determine whether the structure of assemblages was affected by the experimental treatments, the numbers of worms, amphipods and crabs found in each patch of mussel were counted across the experimental treatments. For simplicity, formal comparisons across the experimental treatments were made using total numbers in these broad taxonomic groups and were not based on the numbers of species present.

Results

The dosing system consistently produced small concentrated plumes of contamination within the patches of mussel on the outsides of both pontoons (Experiment 1) and the plume could not be detected in samples of water from outside of the patches of mussel (Figure 3b; Table 3). Mussels at the sources of experimental plumes created by the dosing system accumulated 670% more copper in their tissues compared to mussels 0·5–50 m away (Fig. 3a; Table 2). These results show that any experimental units used in ecotoxicological experiments with this dosing system need to be separated by more than 0·5 m to avoid cross-contamination. There were no detectable increases in the concentrations of copper in seawater outside the exposed mussel patches, indicating that the copper was either, taken in by the mussels, transported away from the pontoon or precipitated out. In addition, when mussels were exposed to increasing concentrations of copper, there was a concomitant increase in the amount of copper in the tissues of mussels (Experiment 2; Fig. 3c). Combining the dosing system with an established hierarchy of ecotoxicological assays (Experiment 3; Fig. 4) revealed mussel assemblages exposed to metals and/or biocides having 40–70% fewer worms ($F_{1,12}$ = 8·11, P < 0·05; including the species *Pomatoceros lamarcki, Cirratulus cirratus, Nereis pelagica, Neanthes irrorata*, *Capitella capitata* and *Manayukia aesturina*) and those exposed to biocides having 81% fewer amphipods ($F_{1,12}$ = 6·51, P < 0·05; including the species *Ampithoe gammaroides, A. rubricata, Corophium volutator*, *Leucothoe spinicarpa*, *Gammarus chevreuxi*, *G. zaddachi*, *G. duebeni, G. salinus*, *Hyale nilssoni*, *Melita palmata, M. obtusata* and *Sunamphitoe pelagica*) with mussels that filtered 48% fewer particles ($F_{1,12}$ = 8·11, P < 0·05). Combinations of pollutants had no effects on the abundance of crabs, the respiratory functions of assemblages or the viability of molluscan haemocytes.

Discussion

The dosing system allows the number of chemicals, their concentrations and the duration, frequency, timing and spatial scale of influxes to be manipulated *in situ*. By combining the dosing system with a hierarchy of ecotoxicological assays, we showed the practical value of our system for generating environmentally and ecologically relevant data about the capacity of pollutants to affect the structure and functions of

Table 2. Spatial distribution of copper in the tissues of mussels collected at different distances from the two experimental discharges from the dosing system. Analysis of variance with three factors, including 'Pontoon' (Po) was fixed with two levels (North, South); 'Side' (Si) was fixed and orthogonal with two levels (Inner, Outer) and 'Distance' (Di) from experimental discharge was fixed and orthogonal with 17 levels (+50, +10, +6, +4, +2, +1, +0·5, +0·25, 0, −0·25, −0·5, −1, −2, −4, −6, −10, −50). There were three replicates of each combination

Source	d.f.	MS	F
Pontoon = Po	1	1·94	0·58
Side = Si	1	24·21	7·25**
Distance = Di	16	25·32	7·58***
Br × Si	1	5·10	1·53
Br × Di	16	3·12	0·93
Si × Di	16	28·78	8·62***
Br × Si × Di	16	1·84	0·55
Res	136	3·34	
Cochran's test	C = 0·33**		
SNK tests	Outside of pontoon: 0 m > +0·25 m > all other distances 0 m: Outside of pontoon > Inside of pontoon +0·25 m: Outside of pontoon > Inside of pontoon		

Statistical significance is denoted by P < 0·01** and 0·001***.

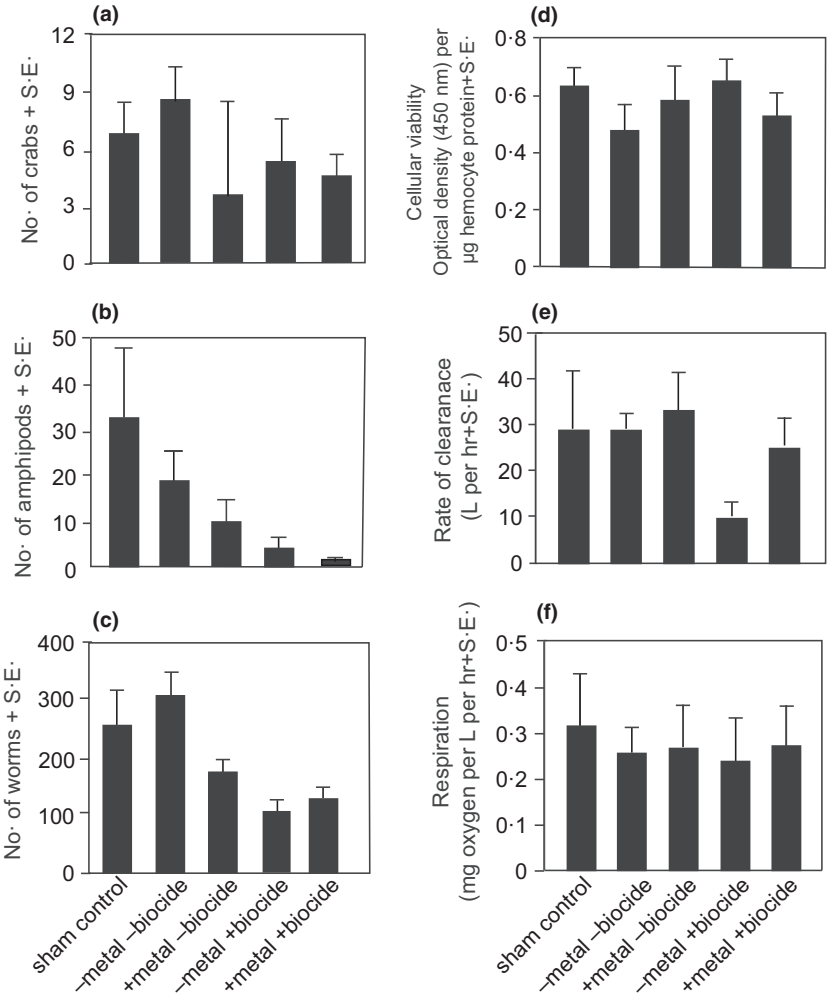

Fig. 4. Impacts of metal and/or biocide on the structure (numbers of crabs, amphipods, worms; a–c) and functions of mussel assemblages (viability of molluscan haemocytes, capacity of assemblage to filter the water and respire; d–f).

assemblages. In our experiments, the respiratory functions of assemblages dominated by mussels were not reduced despite reductions in the numbers of organisms contributing to the respiration. Because there was no observed mortality in the mussels across experimental treatments (Experiment 3), it seems plausible that the capacity of mussel-dominated assemblages to respire (by consuming oxygen and releasing carbon dioxide) is probably largely regulated by the mussels, rather than organisms associated with the mussels. The short-term exposure to the pollutants elicited behavioural and ecological changes in the assemblages with no reductions in respiration and the viability of molluscan haemocytes (an established biomarker used in programmes of monitoring). Indeed, we expected some of these biological and ecological impacts because of observations from previous ecotoxicological work (Serrano *et al.* 1997; Pipe *et al.* 1999; Wu, Siu & Shin 2005; Ashauer, Boxall & Brown 2006). Therefore, our results suggest that work is clearly needed to test further the capacity of sublethal biomarkers of pollution to forecast these ecological impacts (Forbes, Palmqvist & Bach 2006; Browne *et al.* (2015). Using our dosing system allows scientists, for the first time, to experimentally determine whether environmental assessments made at lower levels of biological organization

Table 3. Spatial distribution of copper in seawater outside of the mussel patches collected at different distances from the two experimental discharges from the dosing system. Analysis of variance with three factors, including 'Time' (Ti) was random and orthogonal with two levels (9th and 14th September 2010), 'Pontoon' (Po) was fixed and orthogonal with two levels (North, South); 'Distance' (Di) from experimental discharge was fixed and orthogonal with 11 levels (+50, +10, +6, +4, +2, +1, +0·5, +0·25, 0, −0·25, −0·5, −1, −2, −4, −6, −10, −50)

Source	d.f.	MS	*F*
Time = Ti	1	93·64	2·61
Pontoon = Po	1	60·07	5·69
Distance = Di	10	81·48	0·58
Ti × Po	1	10·55	0·29
Ti × Di	10	140·97	3·93***
Po × Di	10	97·02	0·94
Ti × Si × Di	10	102·83	2·87***
Res	176	35·88	
Cochran's test	$C = 0{\cdot}50$**		
SNK tests:	Time 2 on Northern Breakwater: +2 m > all other distances Time 2 on Southern Breakwater: −1 m > all other distances		

There were five replicates of each treatment and statistical significance is denoted by $P < 001$** and $P < 0001$***.

indicate the progress of recovery processes at higher levels (populations and assemblages). This is important because the use of biomarkers in expensive programmes of monitoring is largely based on single-compound exposures done in the laboratory and compounds are assumed to have additive or independent effects in habitats with mixtures of contaminants (Forbes, Palmqvist & Bach 2006). In our experiment, combinations of pollutants had no effects on the abundance of crabs, the respiratory functions of assemblages or the viability of molluscan haemocytes. Exposure to chlorpyrifos, however, caused assemblages to filter 48% fewer particles from the water. A number of models could explain this outcome (e.g. mussels could be sensitive to chlorpyrifos, in terms of chemoreception or paralysis, so do not filter in its presence, whilst copper could affect the bioavailability and toxicity of chlorpyrifos to mussels), but further work would be required to clarify these mechanisms. Experiments are also needed to determine the ecological and/or toxicological mechanisms causing the observed reductions in the numbers of worms and amphipods in our experiment.

Pollution is often a transboundary problem, and given our dosing system's flexibility and environmental realism (Table 3), it could be used across continents and habitats (i.e. freshwater, marine and terrestrial habitats), as part of coordinated and distributed experiments (Fraser *et al.* 2012), to simulate and test the impacts of current (Church, Granato & Owens 1999) or predicted regimes of chemical disturbance. Experiments in terrestrial habitats would be straightforward providing there was sufficient access to electricity and water, and the spatial extent of the plume in soil, air and organisms was quantified to ensure adequate spacing of experimental plots. Our dosing system therefore has practical applications and implications for scientists, managers and policy-makers, in relation to international environmental trends, risk assessment and the evaluation and design of environmental regulations to prevent pollution. For instance, by identifying the location, nature and extent of ecotoxicological impacts caused by particular pollutants at a global scale, it will reduce the considerable uncertainty about the types and concentrations of pollutants that can be safely absorbed by the ecosystem. In comparison with previous methods (Table 1), our fully programmable dosing system has the ability to expose whole assemblages (across different levels of biological organization) to varying levels of contaminants, without the need for translocating organisms or having to maintain their husbandry. In financial terms, our dosing system is cheaper to assemble than equipping a laboratory for ecotoxicological testing. Although in the short term, our system is more expensive than other field-based methods (e.g. plaster blocks that also include ecologically relevant species, and biotic and abiotic interactions), in the longer term the dosing system can be continually re-used without additional expenditure. The largest methodological advance is that our dosing system has the capacity to simulate complex regimes of influx of multiple contaminants, something that is not possible with previous methods (see Table 1 for comparison with other established methods). This is important because it will enable managers to prioritize resources for cleaning up degraded habitats and allow policy-makers to develop legislation that eliminates the production of problematic chemicals in favour of safer alternatives (Rochman *et al.* 2013; Browne *et al.* 2015). Consequently, we believe our dosing system will allow a broad range of scientists (ecologists, industrial ecologists, ecotoxicologists, conservation biologists, environmental scientists), managers and policy-makers to cost-effectively fulfil legislative requirements to understand, forecast and reduce pollution in a changing world.

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Data accessibility

All data were produced using the methods outlined in the article and these data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.b5g97.

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