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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 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 mussel beds. 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 metal there was a concomitant increase in the amount of metals 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 biocides caused 81% fewer amphipods and mussels to filter 48% less 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: *In situ*, levels of biological organization, cell, assemblage, filtration, pollution, multiple stressors, environmentally relevant, ecologically relevant.

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 (EC 2006; EPA 1980) 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 function. For 30 years, however, experimental methods to determine these "critical loads" (Groffman *et al.* 2006) have been criticised for (i) lacking ecologically relevant species and biotic interactions (Kimball & Levin 1985; Underwood 1995; Underwood & Peterson 1988), (ii) being logistically difficult to set-up, and (iii) 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 (EC 2006; EPA 1980) for three reasons. Firstly, 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

84 populations and assemblages (Browne *et al.* 2015). Thirdly, important processes and
85 interactions that normally occur under natural conditions are lacking in the laboratory (e.g.
86 recruitment, competition, emigration; Underwood 1995; Underwood & Peterson 1998).
87
88 *Logistical problems*
89 It is impractical to do realistic experiments with assemblages in most laboratories so the
90 solution has been to use field experiments to determine whether effects actually arise over and
91 above the natural variation that is explicitly excluded in the laboratory (Kimball & Levin 1985).
92 In laboratory experiments (including micro/mesocosms) concentrations of contaminants are
93 manipulated and naturally varying confounding variables (e.g. temperature, salinity, light,
94 food) are kept artificially constant. In contrast, field experiments expose organisms to natural
95 variations in all environmental variables (e.g. tidal-cycle, spates, storms, droughts, predation,
96 competition) except the one of interest (e.g. dose of contaminant), which is manipulated *in situ.*
97 Where direct comparisons have been made, findings from field experiments have often been
98 inconsistent with those from laboratories (e.g. Thompson, Norton & Hawkins 1998). Closer
99 examination reveals that laboratory experiments can suffer from significant artefacts (e.g.
100 transferring organisms from habitats and caring for them in laboratories; Honkoop *et al.* 2003;
101 Dissanayake, Galloway & Jones 2008) that alter the performance and survival of organisms.
102 For instance, differences in their diet (Dissanayake et al., 2009), architecture of aquaria (e.g.
103 glass vs plastic tanks; Teuten et al. 2007), water-quality (e.g. salinity, pathogens, gradients of
104 temperature) can affect whether or not a contaminant transfers into their tissues and impacts
105 the functional well being of an organism (Camus et al., 2000; Fischer 1986; Parry & Pipe
106 2004; Roast et al., 2001). To overcome some of these ecological, physiological and logistical
107 problems researchers do field experiments, here assemblages are exposed to doses of
108 contaminants *in situ* using a range of dosing-systems. These include fences that trap
109 contaminants (e.g. oil; McGuinness 1990), paints (e.g. anti-foulants; Johnston & Webb 2000)

and impregnated plaster-blocks that dissolve with the movement of water (e.g. Morrisey et al. 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. Where necessary procedural controls are included, they can be costly and time-consuming. 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 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; Maekpeace, 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 (Bendedetti-Cecchi 2003) and existing field and laboratory technologies are not able to expose organisms to environmentally relevant regimes of chemical disturbance. Therefore, to fulfill legislative commitments (EC 2006; EPA 1980) 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. 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 & MaQuadid 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 (10 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, Wengzou Changzin Electronics, www.aquavolt.com.au), batteries (Banner, AccuPro 12v-7A) and charger as an electrical back-up in case the power-supply failed (Fig 1E-H). The dosing-system discharges 4.88 ± 0.5 L h^{-1} to each plot and neither coiling the tubes ($F_{1,8}$=1.22, P = 0.30) nor using 3 different chokes (plastic collars made from irrigation micro-sprayers that reduce the internal diameter of the tube; $F_{3,36}$=0.86, P=0.47) 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; Figure 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.

Experiment 1: size of plume

A copper solution (0.423 mg Cu L^{-1} seawater, $CuSO_4.(H_2O)_5$, CAS N^o 7758-99-8) was continuously added to single patches (each >1m x 0.5m) of mussels in two sites for 14 d

188 (northern and southern pontoons). Mussels at these sites live on the floating pontoons where
189 they form larger patches that cover the entire length of the floating pontoons. Copper was used
190 as a tracer in this study because it is recognized globally as a priority pollutant (EC 2006; EPA
191 1980), with inputs from run-off, discharges (e.g. sewage, storm-water), corrosion of
192 infrastructure and anti-fouling paint on boat-hulls (Makepeace, Smith & Stanley 1995).
193 Mussels (*Mytilus edulis*) growing on the floating pontoons of the marina allowed us to measure
194 bioaccumulation of pollutants discharged from the dosing-system. These organisms are
195 relatively resistant to many pollutants that accumulate in their tissues, thereby increasing
196 concentrations to levels more easily detected than those in the environment (Widdows &
197 Donkin 1992). This has allowed researchers to use the species complex as 'sentinel' organisms
198 to investigate global levels of contamination (Widdows & Donkin 1992). Mussels (after 14 d)
199 and water samples (100 mL; after 7 and 14 d) were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 10, 50 m
200 in both directions along the pontoons from experimental point-sources (Figure 4). Mussels
201 were left in seawater (Instant Ocean; 18MΩ cm water with <0.015 ppm Cu) for 24 hr so that
202 they evacuated their guts and were then frozen in pre-cleaned 100 mL polyethylene bags.
203 Copper concentrations in tissues of mussel and seawater were determined using an Inductively
204 Coupled Mass Spectrometer (see chemical analysis).
205
206 *Experiment 2: manipulating concentrations of pollutant*
207 The dosing-system was deployed on the Northern Pontoon with three treatments (0, 50 and
208 1269 µg mg Cu L^{-1} seawater) housed in separate tanks. The second treatment was
209 representative of concentrations from anti-fouling paints and the third treatment is the largest
210 mean concentration found in European stormwater (Makepeace, Smith & Stanley 1995).
211 Solutions from each tank were added to randomly chosen mussel patches (110 mm in
212 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 one day for logistical reasons and because previous ecotoxicological work that has used this duration (see Wu et al 2005; Canty et al. 1997) to show metals, biocides and other pollutants causing these types of impacts across levels of biological organization.

Chemical analysis

This was performed under ISO 9001:2008 protocols at Plymouth University. 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 hr using 18M Ω 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

238 acid (Sigma-Aldrich, AR grade) was added and samples were stored at <5 ^{o}C in the dark. The
239 frozen mussels were freeze-dried (Labconco, Freezone 6) in their storage containers for 3 d.
240 The length, width and height of the shell-valves were measured to the nearest mm using
241 callipers. Mussels were then shucked by removing the mussel from its shell and the mass of
242 the freeze-dried mussel measured (Scalehouse, ALD114CM) and recorded. Each freeze-dried
243 mussel was then transferred to a polyethylene container for digestion with concentrated nitric
244 acid (20-25 $^{\circ}$C; 10 mL) (Sigma-Aldrich) for 4 d. The samples were then transferred to boiling
245 tubes for digestion at 100 $^{\circ}$C for 48 hr (Skalar, Tecator 1016 Digester Heat-Block). Finally, the
246 acidic solutions containing the digested mussels were transferred to pre-cleaned polyethylene
247 screw-top 100 mL containers (Linnux, UK) and diluted to 10 mL with 18 MΩ cm water for
248 storage prior to analysis. The concentration of copper in the solutions containing the digested
249 mussel was determined using an Inductively Coupled Plasma Mass Spectrometer (X Series 2,
250 Thermo Fisher Scientific, Hemel Hempstead, U.K.). This instrument was operated in
251 'collision/reaction cell mode', with 7 % H_2 in He as the collision/reaction gas, to negate the
252 effect of polyatomic interferences, e.g. ^{23}Na, ^{40}Ar and ^{25}Mg, ^{40}Ar on ^{63}Cu and ^{65}Cu respectively.
253 All mussel digests were diluted hundred-fold prior to analyses and In and Ir, added to give a
254 concentration of 10 mg per L in the diluted digests, were used as internal standards to account
255 for instrumental drift.
256
257 *Ecotoxicological assays*
258 The viability of haemocytes (cells within the haemolymph that function in the immune system
259 of invertebrates) from mussels was assessed as in Browne *et al.* (2008). This measured the
260 ability of their haemocytes to accumulate a red dye, with 'healthier' well-functioning cells
261 accumulating more dye than cells exposed to pollutants. Haemocytes were used because they
262 can be easily collected and play a major role removing harmful waste, supplying tissues with
263 nutrients and energy, healing wounds, degrading pathogens and adding minerals to their shell

Moreira et al. 2013). Previous research has shown that exposure to pollutants, however, degrades these functions by damaging the plasma membrane and/or shrinking their lysosome. Haemolymph (50 µL) withdrawn from abductor muscles of three mussels was placed into duplicate wells of a 96-well microtitre plates (pre-treated with Poly-Lysine). 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 hr 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.

Respiration transfers oxygen from seawater to the tissues of organisms and carbon dioxide in the opposite direction. To measure this, mussel-patches 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 LDO™, Loveland, 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 two minutes 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 minutes. The initial ten minutes as defined by the linearity test has been shown to also allow for acclimatisation of the assemblages and to ensure photosynthesis had ceased after covering with an opaque chamber (Noël *et al.* 2010). The chamber was also fitted with a pump to ensure water was circulated and that concentrations of oxygen were homogenous and 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

290 the dark period and Δt dark is the time difference between these measurements. Respiration
291 calculated for each individual plot every hour and expressed as $mg.O_2.L^{-1}.hr^{-1}$ (Noël *et al.*
292 2010).
293 We then tested the capacity of assemblages to clear particulate matter from seawater *in-situ*
294 using the same purpose-built chamber. 5 mL of a solution containing microalgae (*Isochrysis*
295 *galbana*) was injected into the chamber. The solution of microalgae was prepared such that it
296 gave a concentration of algal cells of 12 -15,000 per 0.5 mL in the chamber. To ensure that
297 microalgae remained suspended in solution, the chamber was fitted with a circulation pump.
298 After initial introduction of algal cells, 20 mL samples of seawater from within the chamber
299 were taken at three time intervals: 0 (T0), 15 (T1) and 30 (T2) min respectively. The numbers
300 of particles retained in samples were counted using flow-cytometry and clearance rates
301 calculated as the change in concentration per unit time using the following equation; clearance
302 rate = V (loge C1 − loge C2)/t, where V is the volume of water in the chamber and C1 and C2
303 are the algal concentration at the beginning and end of the time interval (t) (Noël *et al.* 2010).
304 To determine whether the structure of assemblages was affected by the experimental
305 treatments, the numbers of worms, amphipods and crabs found in each patch of mussel were
306 counted across the experimental treatments. Formal comparisons across the experimental
307 treatments were made using total numbers in these broad taxonomic groups and were not based
308 on the numbers of species present.
309
310 **Results**
311 The dosing-system consistently produced small concentrated plumes of contamination within
312 mussel beds on the outsides of both pontoons (Experiment 1) and the plume could not be
313 detected in water-samples from outside the mussel bed (Figure 2b; Table 2). Mussels at the
314 sources of experimental plumes created by the dosing-system accumulated 670% more copper
315 in their tissues compared to mussels 0.5-50 m away (Figure 2a; Table 1). This shows

316 experimental units in ecotoxicological experiments with this dosing-system need to be
317 separated by more than 0.5 m to avoid cross-contamination. There were no detectable
318 increases in the concentrations of copper in seawater outside the exposed mussel patches,
319 indicating that the copper was either taken in by the mussels, transported away from the
320 pontoon or precipitated out. In addition, when mussels were exposed to increasing
321 concentrations of copper, there was a concomitant increase in the amount of copper in the
322 tissues of mussels (Experiment 2; Figure 2c). Combining the dosing-system with an
323 established hierarchy of ecotoxicological assays (Experiment 3; Figure 3) revealed mussel
324 assemblages exposed to metals and/or biocides having 40-70% fewer worms ($F_{1,12} = 8.11$, $P <$
325 0.05; including the species *Pomatoceros lamarcki, Cirratulus cirratus, Nereis pelagica,*
326 *Neanthes irrorata, Capitella capitata and Manayukia aesturina*) and those exposed to biocides
327 having 81% fewer amphipods ($F_{1,12} = 6.51$, $P < 0.05$; including the species *Ampithoe*
328 *gammaroides, A. rubricata, Corophium volutator, Leucothoe spinicarpa, Gammarus chevreuxi,*
329 *G. zaddachi, G. duebeni, G. salinus, Hyale nilssoni, Melita palmata, M. obtusata* and
330 *Sunamphitoe pelagica*) with mussels that filtered 48% less water ($F_{1,12} = 8.11$, $P < 0.05$).
331 Combinations of pollutants had no effects on the abundance of crabs, the respiratory functions
332 of assemblages or the viability of molluscan haemocytes.
333
334 **Discussion**
335 The dosing-system allows the number of chemicals, their concentrations and the duration,
336 frequency, timing and spatial scale of influxes to be manipulated *in situ*. By combining the
337 dosing-system with a hierarchy of ecotoxicological assays we showed the practical value of our
338 system for generating environmentally and ecologically relevant data about the capacity of
339 pollutants to affect the structure and functions of assemblages. In our experiments the
340 respiratory functions of mussel-assemblages did not change despite reductions in the numbers
341 of organisms contributing to the respiration. Because there was no observed mortality in the

mussels across experimental treatments, it seems plausible that the capacity of mussel-assemblages to respire (by consuming oxygen and releasing carbon dioxide) is probably largely regulated by mussels, rather than the other organisms. Given that 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) work is clearly needed to test the capacity of sub-lethal biomarkers of pollution to forecast these ecological impacts (Forbes, Palmqvist & Bach 2006; Browne *et al.* 2015). Our dosing-system would allow scientists for the first time to experimentally determine whether environmental assessments made at lower levels of biological organisation indicate the progress of recovery processes at higher levels.

This is important because the use of biomarkers is largely based on single-compound exposures carried out 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% less water. A number of models could explain this observation (e.g. mussels could be sensitive to Chlorpyrifos, in terms of chemoreception or paralysis, so do not filter during its presence; whilst copper could affect the bioavailability and toxicity of Chlorpyrifos to mussels) but they require further experiments with detailed chemical analyses. 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 ecosystems. This 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). Consequently, our dosing-system will allow a broad-range of scientists (ecologists, industrial ecologists, ecotoxicologists, conservation biologists, environmental scientists), managers and policy-makers to fulfil legislative requirements to understand, forecast and reduce pollution in a changing world.

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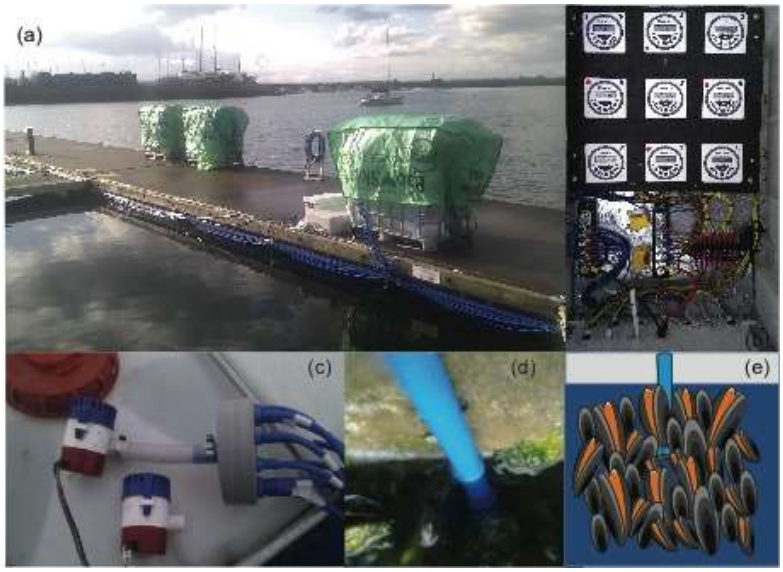
499 FIGURES



500

501 Figure 1. Dosing system (a) set-up at Malahide Marina (53N 27' 17.5; 6E 9' 12.46) with the
502 white box containing the programmabled timers (b) each connected to bilge-pumps which
503 themselves are connected to the blue-tubing via the grey manifolds (c). Through this, mussel
504 assemblages on the vertical-side of the pontoon received controlled doses of pollutants (d, e).

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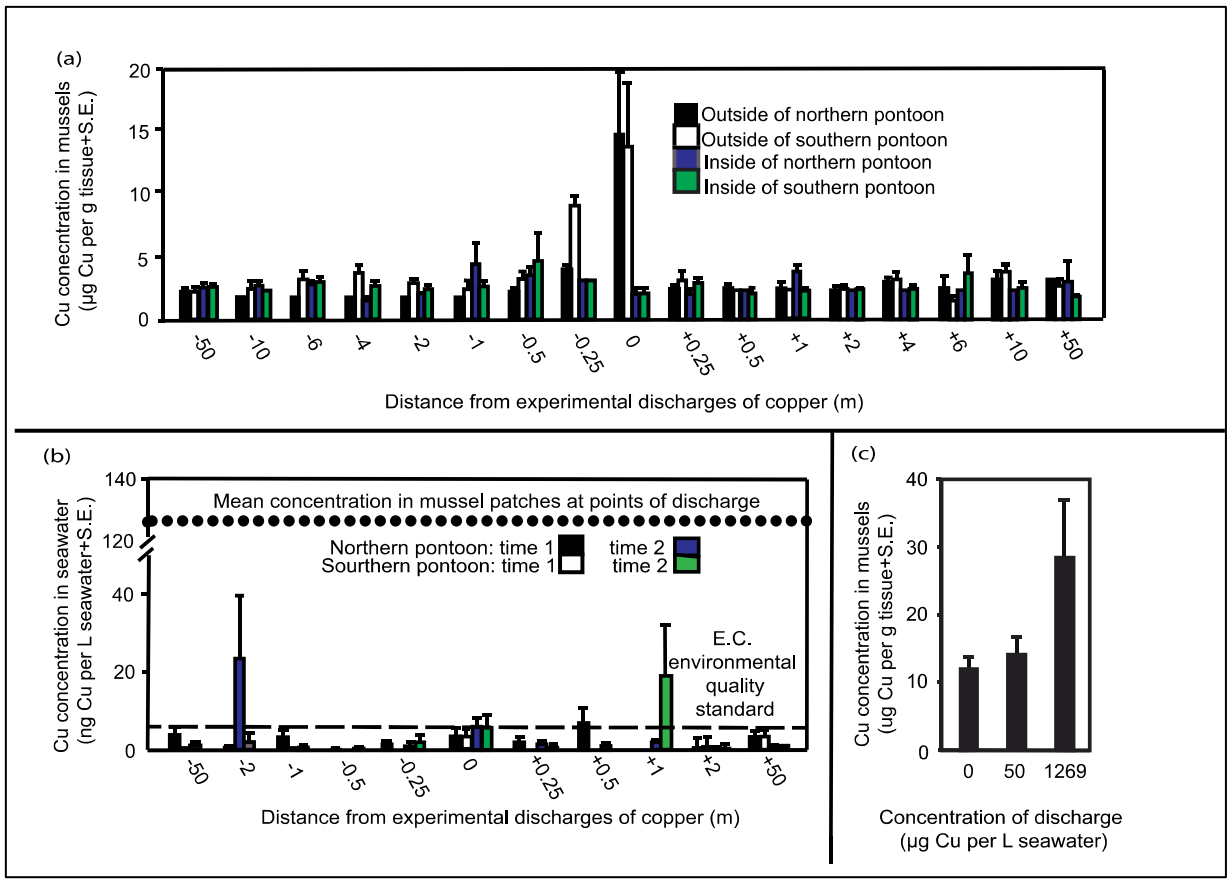
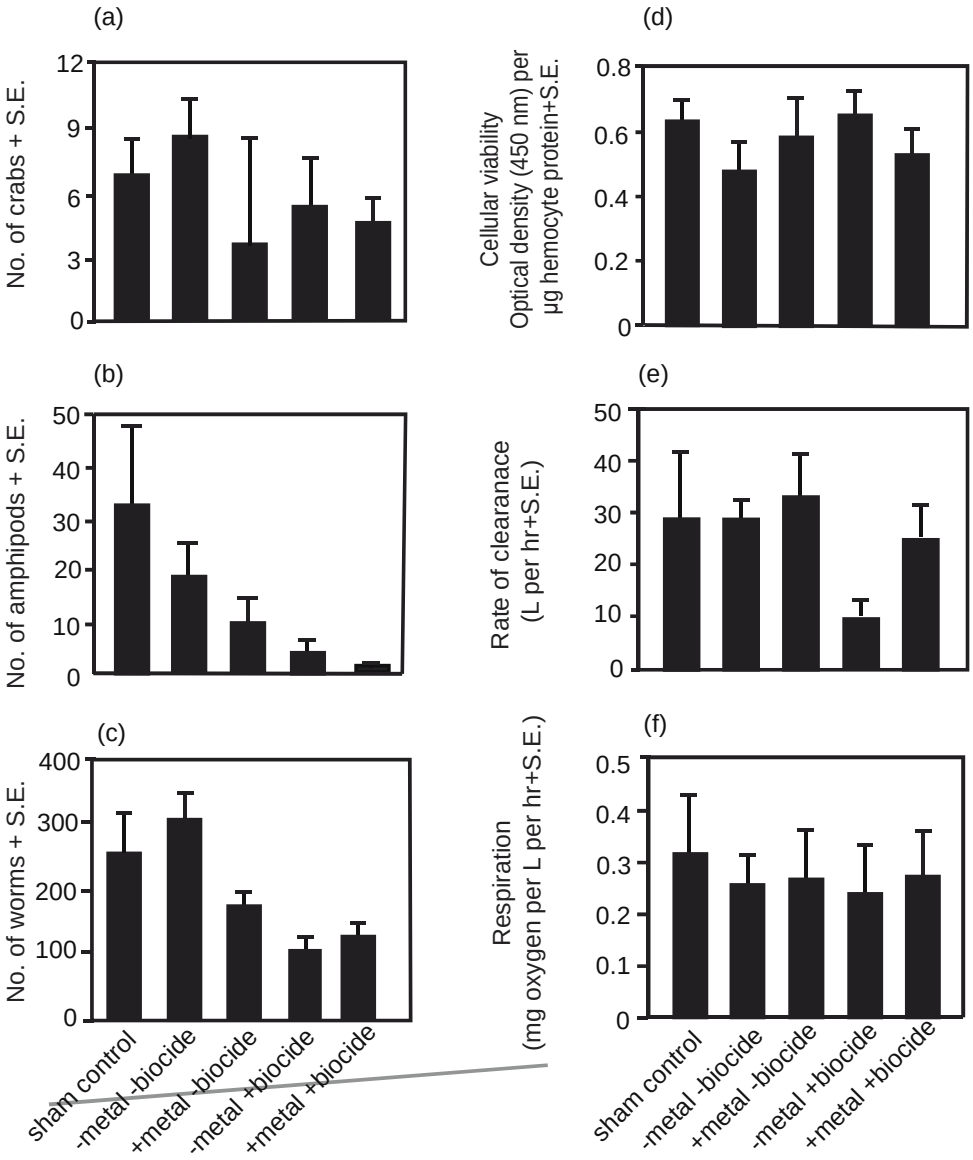


Figure 2. Spatial distribution of copper in mussels (a) and seawater (b) at different distances from experimental discharges (Experiment 1). Not all water samples were analyzed because the plume could not be detected in water-samples outside of the mussel bed in either direction (Table 1). In contrast, mussels within the plume had over 670% more copper in their tissues compared to mussels 0.5-50 m away ($F_{16,136}$=3.34, P<0.001; Table 2). (c) Experimental discharges with increasing concentrations of copper caused increasing concentrations of copper in the tissues of mussels (Experiment 2).



516

517 Figure 3. Impacts of metal and/or biocide on the structure (numbers of crabs, amphipods,

518 worms; a-c) and functions of mussel assemblages (viability of molluscan haemocytes, capacity

519 of assemblage to filter the water and respire; d-f).

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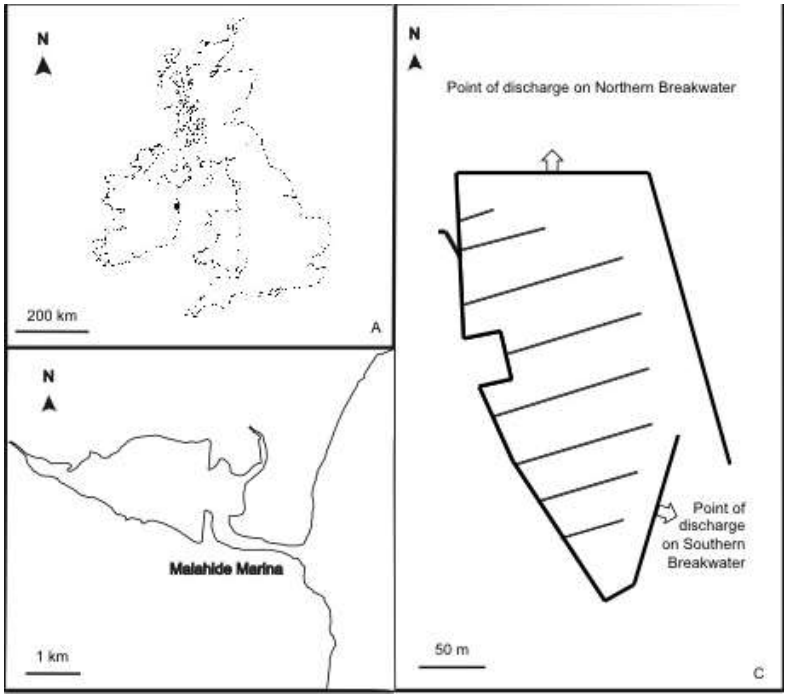

Figure 4. Location of experiments within the British Isles and Malahide Marina.

Tables

Table 1. Spatial distribution of copper in the tissues of mussels collected at different distances from the 2 experimental discharges from the dosing-system. Analysis of variance with 3 factors, including 'Pontoon' (Po) was fixed with 2 levels (North, South); 'Side' (Si) was fixed and orthogonal with 2 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 3 replicates of each combination. Statistical significance is denoted by $P<0.01$** and 0.001***

Source	**df**	**MS**	**F**
Potoon = Po	1	1.94	0.58
Side = Si	1	24.21	7.25**
Distance = Di	16	25.32	7.58***
Br x Si	1	5.10	1.53
Br x Di	16	3.12	0.93
Si x Di	16	28.78	8.62***
Br x Si x 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

Table 2. Spatial distribution of copper in seawater outside of the mussel patches collected at different distances from the 2 experimental discharges from the dosing-system. Analysis of variance with 3 factors, including 'Time' (Ti) was random and orthogonal with 2 levels (9th and 14th September 2010), 'Pontoon' (Po) was fixed and orthogonal with 2 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). There were 5 replicates of each treatment and statistical significance is denoted by $P<0.001$***.

Source	**df**	**MS**	**F**
Time = Ti	1	93.64	2.61
Pontoon = Po	1	60.07	5.69
Distance = Di	10	81.48	0.58
Ti x Po	1	10.55	0.29
Ti x Di	10	140.97	3.93***
Po x Di	10	97.02	0.94
Ti x Si x Di	10	102.83	2.87***
Res	176	35.88	

Cochran's test C = 0.50**

SNK tests Time 2 on Northern Breakwater: +2 m> all other distances

Time 2 on Southern Breakwater: -1 m> all other distances

586

587 Table 3. The relative advantages of existing methods used in laboratory and field experiments. Shading refers to potential challenges
588 within set-ups that can cause persistent (grey shading), frequent (blue) or no problems (green).
589

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								

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