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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, managers 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
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 1998).

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. tidal-cycle, spates, storms, droughts, 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 et al., 2009), architecture of aquaria (e.g. glass vs plastic tanks; Teuten et al. 2007), water-quality (e.g. salinity, pathogens, gradients of temperature) can affect whether or not a contaminant transfers into their tissues and impacts the functional well being of an organism (Camus et al., 2000; Fischer 1986; Parry & Pipe 2004; Roast et al., 2001). To overcome some of these ecological, physiological and logistical problems researchers do field experiments, here 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. 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 \(4.88 \pm 0.5 \text{ L h}^{-1}\) 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\(_2\)O)\(_5\), CAS No 7758-99-8) was
continuously added to single patches (each >1m x 0.5m) of mussels in two sites for 14 d
Browne et al. Running title: *In situ* testing of pollutants in the ecosystem

(northern and southern pontoons). Mussels at these sites live on the floating pontoons where they form larger patches that 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 (EC 2006; EPA 1980), with inputs from run-off, discharges (e.g. sewage, storm-water), corrosion of infrastructure and anti-fouling 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 d) 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 in both directions along the pontoons from experimental point-sources (Figure 4). Mussels were left in seawater (Instant Ocean; 18MΩ cm water with <0.015 ppm Cu) for 24 hr so that they evacuated their guts and were then frozen in pre-cleaned 100 mL polyethylene bags.

Copper concentrations in 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 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 anti-fouling paints and the third treatment is the largest mean concentration found in European stormwater (Makepeace, Smith & Stanley 1995).

Solutions from each tank were added to randomly chosen mussel patches (110 mm 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 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 18MΩ 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) 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 d. 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 d. The samples were then transferred to boiling tubes for digestion at 100 °C for 48 hr (Skalar, Tecator 1016 Digester Heat-Block). Finally, the acidic solutions containing the digested mussels were transferred to pre-cleaned polyethylene screw-top 100 mL containers (Linnux, 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, U.K.). This instrument was operated in ‘collision/reaction cell mode’, with 7 % H₂ in He as the collision/reaction gas, to negate the effect of polyatomic interferences, e.g. ²³Na, ⁴⁰Ar and ²⁵Mg, ⁴⁰Ar on ⁶³Cu and ⁶⁵Cu respectively. All mussel digests were diluted hundred-fold prior to analyses and In and Ir, added to give a concentration of 10 mg per L in the diluted digests, were used as internal standards to account for instrumental drift.

Ecotoxicological assays

The viability of haemocytes (cells within the haemolymph that function in the immune system of invertebrates) from mussels was assessed as in Browne et al. (2008). This measured the ability of their 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
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; $\Delta [O_2]_{\text{dark}}/ \Delta t_{\text{dark}}, \Delta [O_2]_{\text{dark}}$ is the difference in dissolved oxygen concentration between measurements taken respectively at the beginning and end of
the dark period and $\Delta t_{\text{dark}}$ is the time difference between these measurements. Respiration calculated for each individual plot every hour and expressed as $\text{mg.O}_2\text{L}^{-1}\text{hr}^{-1}$ (Noël et al. 2010).

We then tested the capacity of assemblages to clear particulate matter from seawater in-situ using the same purpose-built chamber. 5 mL 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 $= V \left(\log C_1 - \log C_2\right)/t$, where V is the volume of water in the chamber and C1 and C2 are the algal concentration at the beginning and end of the time interval (t) (Noël et al. 2010).

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. 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 mussel beds on the outsides of both pontoons (Experiment 1) and the plume could not be detected in water-samples from outside the mussel bed (Figure 2b; Table 2). 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 (Figure 2a; Table 1). This shows
experimental units 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; Figure 2c). Combining the dosing-system with an established hierarchy of ecotoxicological assays (Experiment 3; Figure 3) 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 nilssonii*, *Melita palmata*, *M. obtusata* and *Sunamphitoe pelagica*) with mussels that filtered 48% less water (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 assemblages. In our experiments the respiratory functions of mussel-assemblages did not change despite reductions in the numbers 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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Highway Runoff Data and Methodology Synthesis, USGS, Northborough, M.A. URL.


Figure 1. Dosing system (a) set-up at Malahide Marina (53N 27' 17.5; 6E 9' 12.46) 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).
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).
Figure 3. 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).
Figure 4. Location of experiments within the British Isles and Malahide Marina.
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 $0.001^{***}$.

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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, -0.25, -0.5, -1, -2, -4, -6, -10, -50). There were 5
replicates of each treatment and statistical significance is denoted by $P<0.001$***.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time = Ti</td>
<td>1</td>
<td>93.64</td>
<td>2.61</td>
</tr>
<tr>
<td>Pontoon = Po</td>
<td>1</td>
<td>60.07</td>
<td>5.69</td>
</tr>
<tr>
<td>Distance = Di (Di)</td>
<td>10</td>
<td>81.48</td>
<td>0.58</td>
</tr>
<tr>
<td>Ti x Po</td>
<td>1</td>
<td>10.55</td>
<td>0.29</td>
</tr>
<tr>
<td>Ti x Di</td>
<td>10</td>
<td>140.97</td>
<td>3.93***</td>
</tr>
<tr>
<td>Po x Di</td>
<td>10</td>
<td>97.02</td>
<td>0.94</td>
</tr>
<tr>
<td>Ti x Si x Di</td>
<td>10</td>
<td>102.83</td>
<td>2.87***</td>
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<tr>
<td>Res</td>
<td>176</td>
<td>35.88</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Advantages of different types of experimental set-ups</th>
<th>Laboratory</th>
<th>Field</th>
<th>Our dosing-system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecotoxicological relevance (biological scales where impacts can be tested)</td>
<td>Static</td>
<td>Flow-through</td>
<td>Microcosm</td>
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<tr>
<td>Suborganismal</td>
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<tr>
<td>Organismal</td>
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<td></td>
<td></td>
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<tr>
<td>Population</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Assemblage</td>
<td></td>
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<td></td>
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<tr>
<td>Ecosystem</td>
<td></td>
<td></td>
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<tr>
<td>Logistical and environmental advantages</td>
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<tr>
<td>Includes natural environmental variation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Can test for stress(es) due to translocation of organisms</td>
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<tr>
<td>No water-quality problems</td>
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<td></td>
</tr>
<tr>
<td>No husbandry required</td>
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<td></td>
</tr>
<tr>
<td>Capacity to simulate and manipulate chemical regime</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
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<td>Number</td>
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<td>Timing</td>
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<td>Frequency</td>
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<td>Automatic dosing</td>
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