2016-01-05

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Browne, MA

http://hdl.handle.net/10026.1/6607

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

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26   **Summary**

27   1.   Pollution is a global issue at the frontier between ecology, environmental science,

28   management, engineering and policy. Legislation requires experiments to determine

29   how much contamination an ecosystem can absorb before there are structural or

30   functional changes. Yet, existing methods cannot realistically simulate regimes of

31   chemical disturbance and determine impacts to assemblages in ecosystems. This is

32   because they lack ecologically relevant species and biotic interactions, are logistically

33   difficult to set-up, and lack environmentally relevant regimes of chemical and abiotic

34   disturbance that organisms experience in polluted areas.

35   2.   We solved these long-standing environmental, logistical and ecological problems by

36   developing a programmable dosing-system. This dosing-system simulates, *in situ*,

37   regimes of chemical disturbance to assemblages by manipulating the concentration,

38   duration, timing and frequency of pollutants to which they are exposed.

39   3.   Experiments with priority pollutants (the metal copper and the biocide Chlorpyrifos)

40   and mussel assemblages revealed consistent plumes of contamination within mussel

41   beds. Mussels at the sources of experimental plumes of copper created by the dosing-

42   system accumulated 670% more copper in their tissues compared to mussels 0.5-50 m

43   away. In addition, when mussels were exposed to increasing concentrations of metal

44   there was a concomitant increase in the amount of metals in the tissues of mussels.

45   Combining the dosing-system with an established hierarchy of ecotoxicological assays

46   revealed mussel assemblages exposed to copper and/or Chlorpyrifos (had 40-70%

47   fewer worms, whilst biocides caused 81% fewer amphipods and mussels to filter 48%

48   less water. Combinations of copper and/or Chlorpyrifos had no effects on the

49   abundance of crabs, the respiratory functions of assemblages or the viability of

50   molluscan haemocytes.

51   4.   As global contamination accelerates we discuss how this technological advance will

52   enable a diverse array of ecologists, mangers and policy-makers to understand and

53   reduce pollution.

54

55   **Key-words:** *In situ*, levels of biological organization, cell, assemblage, filtration, pollution,

56   multiple stressors, environmentally relevant, ecologically relevant.

57

58   **Introduction**

59   Global infiltration of chemical contaminants causes global reductions in biodiversity (Johnston

60   & Roberts 2009) that jeopardize the useful functions and services that ecosystems provide.

61   Mitigating these problems is difficult because humans use >99 million different chemicals

62   (with 15,000 new chemicals added each day) and <0.3% of these chemicals have any form of

63   regulation (CAS 2015). Legislation (EC 2006; EPA 1980) requires experiments to determine

64   the quantity of a pollutant (known in the US as a "*Total Maximum Daily Allowance*" or

65   elsewhere as "*Environmental Quality Standard*") an organism or its ecosystem can safely

66   absorb before there is a change in its structure and function. For 30 years, however,

67   experimental methods to determine these "critical loads" (Groffman *et al.* 2006) have been

68   criticised for (i) lacking ecologically relevant species and biotic interactions (Kimball & Levin

69   1985; Underwood 1995; Underwood & Peterson 1988), (ii) being logistically difficult to set-

70   up, and (iii) lacking environmentally relevant regimes of chemical, abiotic and biotic

71   disturbance that organisms experience in polluted areas. We explain these long-standing

72   global issues and provide a novel technological solution in the form of a programmable dosing-

73   system that simulates, *in situ*, regimes of chemical disturbance to assemblages.

74

75   *Ecological problems*

76   Laboratory experiments provide useful information about the uptake and the sensitivity of

77   organisms to contaminants; however, toxicological testing in laboratories is not the ecological

78   testing of toxicology (Underwood & Peterson 1988) that is required under commitments to

79   international legislation (EC 2006; EPA 1980) for three reasons. Firstly, experiments are

80   usually based on a single or few species that survive easily in the artificial conditions of the

81   laboratory and are thought to be sensitive to contaminants but are not always endemic in

82   polluted habitats. Secondly, experiments rarely study more than one species at a time and it is

83   well known that individual organisms often respond to contaminants in different ways to

4

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)

110   and impregnated plaster-blocks that dissolve with the movement of water (e.g. Morrisey et al.

111   1995; Cartwright, Coleman & Browne 2006) or electronic pumps that deliver pulses of

112   contaminants (Roberts *et al.* 2008). These dosing-systems, however, also suffer from logistical

113   and financial problems. Fences cannot contain all contaminants and may alter biotic

114   interactions, the plaster itself can affect the physiology and behaviour of animals (Cartwright,

115   Coleman & Browne 2006) and existing pumps have to be operated manually. Where necessary

116   procedural controls are included, they can be costly and time-consuming. Furthermore,

117   running field experiments can be difficult due to tides and spates in aquatic habitats, poor

118   weather, a lack of electricity and running-water, and a lack of security can make the risk of

119   losing sophisticated and expensive equipment unacceptably large, especially in busy areas.

120   Therefore, there is a need for inexpensive, safe and autonomous technologies for manipulating

121   the dose of contaminants that can be deployed across aquatic and terrestrial habitats.

122

123   *Environmental problems*

124   Monitoring shows organisms in habitats experience complex regimes of chemical disturbance

125   with the concentrations, timing and frequency of toxicants to which these organisms are

126   exposed (e.g. transient contamination events through seasonal urban run-off; Maekpeace,

127   Smith & Stanley 1995; Church, Granato & Owens 1999). These regimes are expected to

128   become more complicated due to accelerated chemical production and climatic change

129   (Schiedek *et al.* 2007). This is problematic because variation (spatial and temporal) in

130   disturbances is known to cause variation in ecological impacts (Bendedetti-Cecchi 2003) and

131   existing field and laboratory technologies are not able to expose organisms to environmentally

132   relevant regimes of chemical disturbance. Therefore, to fulfill legislative commitments (EC

133   2006; EPA 1980) to determine whether or not the ecosystem can safely absorb one or more

134   contaminants, scientists need technologies that allow them to expose assemblages to

135   environmentally relevant treatments based on observations (from monitoring) about the

136   concentrations, timing and the frequency at which assemblages in habitats are exposed to

137   chemical toxicants.

138

139   *The technological solution to understanding pollution*

140   To overcome the ecological, logistical and environmental problems with the existing dosing-

141   systems described above we developed a novel - programmable and automated – dosing-

142   system that allows ecologically and environmentally relevant testing of single and multiple

143   pollutants on natural systems by being able to manipulate: (i) the number of chemicals, (ii)

144   their concentration and the (iii) duration, (iv) frequency and (v) timing of influxes over

145   extended periods. As metals and biocides make up over 50 % of the priority pollutants we

146   explored their single and combined impacts on the structure and functions of mussel

147   assemblages. These assemblages are ideal because mussels are important ecosystem engineers

148   with clear links between their "health" and ecological impacts (Browne *et al.* 2015). For

149   instance, their shells create habitat and their feeding transfers sediments, algae, nutrients and

150   energy from the water-column into the mussel bed allowing them to support diverse

151   assemblages (e.g. Cole & MaQuadid 2010). Separate laboratory studies have shown that

152   exposure to pollutants can damage their cells (i.e. fragmented plasma membranes, smaller

153   lysosomes) and organs (i.e. gills, gut) and this has been linked to reduced feeding, respiration,

154   growth and survival, and less diverse assemblages (Browne *et al.* 2015). No field experiment

155   has, however, tested these predictions by manipulating the presence and absence of metals

156   and/or biocide in an ecologically and environmentally relevant manner.

157

158   **Materials and methods**

159   *Dosing-system and location of study*

160   The dosing-system was built by attaching up to ten 640 L tanks (IBC;

161   www.smithsofthedean.co.uk), 100 separate lengths of 100 m (10 km in total) polyethylene

162   tubing (diameters: 4 mm internally, 6 mm externally) coiled on plywood reels (200 mm

163   flange/barrel-diameters; www.northeastreel.com) for dispensing, digital-timers (TM-619,

164   Wengzou Changzin Electronics, www.aquavolt.com.au), batteries (Banner, AccuPro 12v-7A)

165   and charger as an electrical back-up in case the power-supply failed (Fig 1E-H). The dosing-

166   system discharges 4.88 ± 0.5 L h^{-1} to each plot and neither coiling the tubes ($F_{1,8}$=1.22, P =

167   0.30) nor using 3 different chokes (plastic collars made from irrigation micro-sprayers that

168   reduce the internal diameter of the tube; $F_{3,36}$=0.86, P=0.47) significantly reduced the rate of

169   discharge. At full capacity, the system can deliver controlled doses of contaminants to either

170   (i) 200 individual experimental plots, with 25 different treatment-combinations, or (ii) by

171   combining smaller plots, an area the size of 100 m^2 could be dosed, and (iii) temporally, the

172   duration of dosing could be manipulated to the nearest minute, hour, day, week, month or

173   indefinitely. This is because the timer(s) can be programmed to automatically start and stop at

174   the pre-programmed times to simulate transient and more prolonged discharges of

175   contaminants without the need for persons to be at experimental sites to operate it.

176   With permission from Malahide Marina and Fingal County Council we completed a series of

177   experiments to determine the capacity of the dosing-system to manipulate levels of

178   bioaccumulation and test the ecotoxicological impacts of pollutants (metals and/or biocides) on

179   multiple levels of biological organization (cell to assemblage). The system was deployed on

180   the floating pontoons on the Northern and Southern pontoons of Malahide Marina (Ireland;

181   Figure 1) as the marina has running-water, electricity, and a security system that prevents

182   vandalism and theft. Here mussel-assemblages living on the vertical-side of the floating

183   pontoons were used as part of our experiments.

184

185   *Experiment 1: size of plume*

186   A copper solution (0.423 mg Cu L^{-1} seawater, CuSO$_4$.(H$_2$O)$_5$, CAS N° 7758-99-8) was

187   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

213   avoid cross-contamination, each replicate/treatment combination was >1.5 m apart, in this and

214   the remaining experiments.

215

216   *Experiment 3: impacts across levels of biological organization*

217   To determine whether the dosing-system can be combined with a suite of ecotoxicological

218   assays to determine changes in the structure and functions of assemblages we used our system

219   to discharge from five tanks (one tank with seawater; two tanks with 7.7 mg copper sulphate L^{-1}

220   ^{1} seawater; two tanks with 250 mg chlorpyrifos L^{-1} seawater) to five groups of experimental

221   mussel assemblages (control; +seawater; +metal; +biocide; +metal+biocide). One day later we

222   quantified changes in the structure (numbers of worms, amphipods and crabs) and functions

223   (viability of haemocytes from mussels, filtration and respiration) of mussel assemblages using

224   established techniques (see ecotoxicological assays). We chose one day for logistical reasons

225   and because previous ecotoxicological work that has used this duration (see Wu et al 2005;

226   Canty et al. 1997) to show metals, biocides and other pollutants causing these types of impacts

227   across levels of biological organization.

228

229

230   *Chemical analysis*

231   This was performed under ISO 9001:2008 protocols at Plymouth University. All glassware and

232   plastic containers were cleaned in a phosphate free degreasing soap-free detergent (10 %

233   solution of Decon 90), then rinsed in water (18 MW cm conductivity), followed by immersion

234   in a nitric acid (10 % Aristar grade by VWR) bath for 24 hr using 18M Ω cm water and rinsed

235   again in 18 MΩ cm water. All items were dried in a particle-free environment and stored in

236   sealed and cleaned polyethylene bags until required. To preserve dissolved Cu and to prevent

237   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 °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 °C; 10 mL) (Sigma-Aldrich) for 4 d. The samples were then transferred to boiling

245   tubes for digestion at 100 °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

264   Moreira et al. 2013). Previous research has shown that exposure to pollutants, however,

265   degrades these functions by damaging the plasma membrane and/or shrinking their lysosome.

266   Haemolymph (50 μL) withdrawn from abductor muscles of three mussels was placed into

267   duplicate wells of a 96-well microtitre plates (pre-treated with Poly-Lysine). The plate was

268   then agitated (1400 rpm for 60 s) and then left for 50 min for the cells to adhere. Excess cells

269   were then discarded and the wells rinsed with phosphate buffer (pH 7.4). Neutral red dye

270   (0.4%) was then added and cells were incubated in the dark for 3 hr to prevent photolysis.

271   Wells were then washed with phosphate buffer again before a solution of 1% acetic acid/20%

272   ethanol was added to precipitate the dye. Absorbance was read at 550 nm using a

273   spectrophotometer, protein was quantified and results were presented as optical density per

274   gram protein.

275   Respiration transfers oxygen from seawater to the tissues of organisms and carbon dioxide in

276   the opposite direction. To measure this, mussel-patches were incubated *in situ* and in the dark

277   using an opaque chamber. Changes in dissolved oxygen (mg O^2 L^{-1}) were measured using a

278   probe (HQ20 Hach Lange Ltd portable LDO™, Loveland, USA). To ensure that

279   measurements were taken at the correct time points, a linearity test was undertaken to test how

280   long it took the volume of water to be depleted of oxygen. Concentrations of oxygen in the

281   water were measured every two minutes over an hour to identify the period during which there

282   was a linear decrease of oxygen in the water. On the basis of this test, measurements of

283   oxygen were taken after 10 and 20 minutes. The initial ten minutes as defined by the linearity

284   test has been shown to also allow for acclimatisation of the assemblages and to ensure

285   photosynthesis had ceased after covering with an opaque chamber (Noël *et al.* 2010).   The

286   chamber was also fitted with a pump to ensure water was circulated and that concentrations of

287   oxygen were homogenous and representative. Rates of oxygen uptake by the assemblages were

288   estimated using the equation; Δ [O_2] dark/ Δt dark, Δ [O_2] dark is the difference in dissolved

289   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

14

342   mussels across experimental treatments, it seems plausible that the capacity of mussel-

343   assemblages to respire (by consuming oxygen and releasing carbon dioxide) is probably

344   largely regulated by mussels, rather than the other organisms. Given that short-term exposure

345   to the pollutants elicited behavioural and ecological changes in the assemblages with no

346   reductions in respiration and the viability of molluscan haemocytes (an established biomarker

347   used in programmes of monitoring) work is clearly needed to test the capacity of sub-lethal

348   biomarkers of pollution to forecast these ecological impacts (Forbes, Palmqvist & Bach 2006;

349   Browne *et al.* 2015). Our dosing-system would allow scientists for the first time to

350   experimentally determine whether environmental assessments made at lower levels of

351   biological organisation indicate the progress of recovery processes at higher levels.

352   This is important because the use of biomarkers is largely based on single-compound

353   exposures carried out in the laboratory and compounds are assumed to have additive or

354   independent effects in habitats with mixtures of contaminants (Forbes, Palmqvist & Bach

355   2006). In our experiment, combinations of pollutants had no effects on the abundance of crabs,

356   the respiratory functions of assemblages or the viability of molluscan haemocytes. Exposure to

357   Chlorpyrifos, however, caused assemblages to filter 48% less water. A number of models

358   could explain this observation (e.g. mussels could be sensitive to Chlorpyrifos, in terms of

359   chemoreception or paralysis, so do not filter during its presence; whilst copper could affect the

360   bioavailability and toxicity of Chlorpyrifos to mussels) but they require further experiments

361   with detailed chemical analyses. Experiments are also needed to determine the ecological

362   and/or toxicological mechanisms causing the observed reductions in the numbers of worms and

363   amphipods in our experiment.

364   Pollution is often a transboundary problem and given our dosing-system's flexibility and

365   environmental realism (Table 3) it could be used across continents and habitats (i.e. freshwater,

366   marine and terrestrial habitats), as part of coordinated and distributed experiments (Fraser *et al.*

367   2012), to simulate and test the impacts of current (Church, Granato & Owens 1999) or

368   predicted regimes of chemical disturbance. Experiments in terrestrial habitats would be

369   straightforward providing there was sufficient access to electricity and water, and the spatial

370   extent of the plume in soil, air and organisms was quantified to ensure adequate spacing of

371   experimental plots. Our dosing-system therefore has practical applications and implications

372   for scientists, managers and policy-makers, in relation to international environmental trends,

373   risk assessment and the evaluation and design of environmental regulations to prevent

374   pollution. For instance, by identifying the location, nature and extent of ecotoxicological

375   impacts caused by particular pollutants at a global scale, it will reduce the considerable

376   uncertainty about the types and concentrations of pollutants that can be safely absorbed by

377   ecosystems. This will enable managers to prioritize resources for cleaning-up degraded

378   habitats and allow policy-makers to develop legislation that eliminates the production of

379   problematic chemicals in favour of safer alternatives (Rochman et al. 2013). Consequently,

380   our dosing-system will allow a broad-range of scientists (ecologists, industrial ecologists,

381   ecotoxicologists, conservation biologists, environmental scientists), managers and policy-

382   makers to fulfil legislative requirements to understand, forecast and reduce pollution in a

383   changing world.

384

393

394   **References**
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499   FIGURES
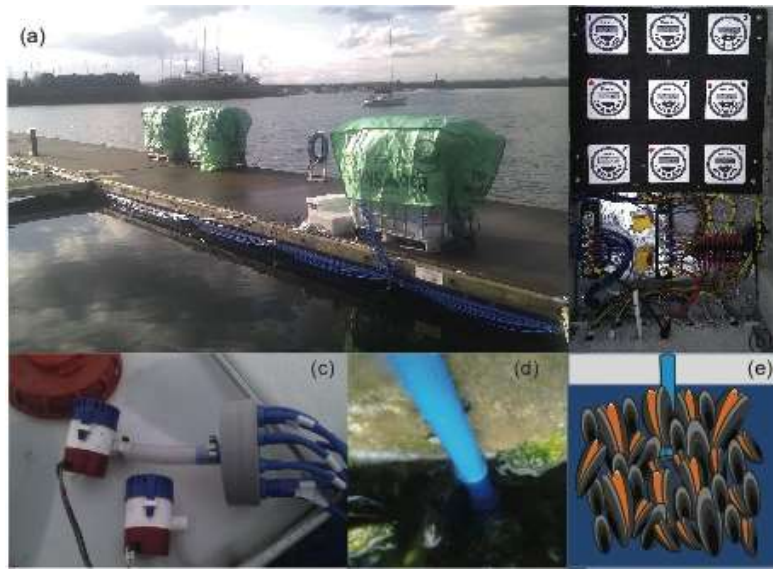
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501   Figure 1. Dosing system (a) set-up at Malahide Marina (53N 27' 17.5; 6E 9' 12.46) with the

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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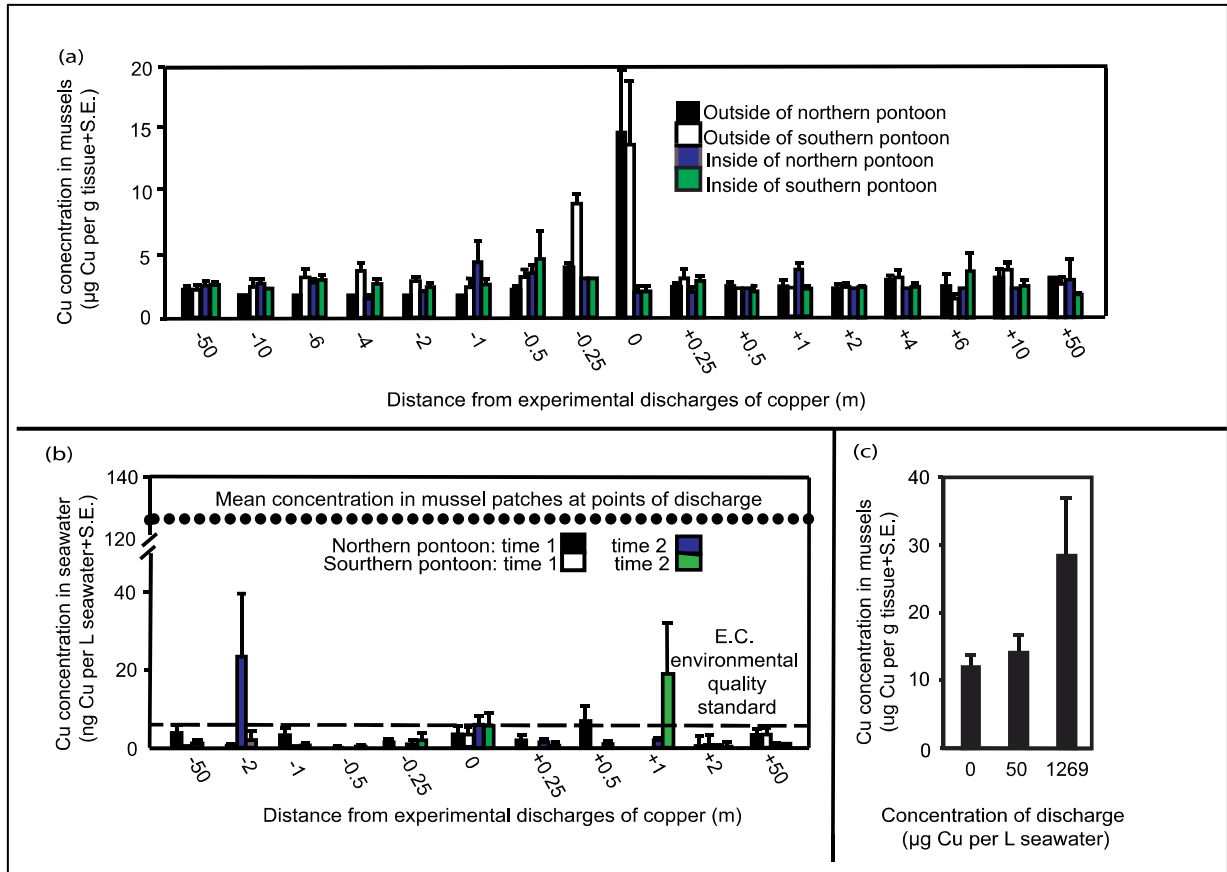
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508   Figure 2. Spatial distribution of copper in mussels (a) and seawater (b) at different distances

509   from experimental discharges (Experiment 1). Not all water samples were analyzed because

510   the plume could not be detected in water-samples outside of the mussel bed in either direction

511   (Table 1). In contrast, mussels within the plume had over 670% more copper in their tissues

512   compared to mussels 0.5-50 m away ($F_{16,136}=3.34$, $P<0.001$; Table 2). (c) Experimental

513   discharges with increasing concentrations of copper caused increasing concentrations of copper

514   in the tissues of mussels (Experiment 2).

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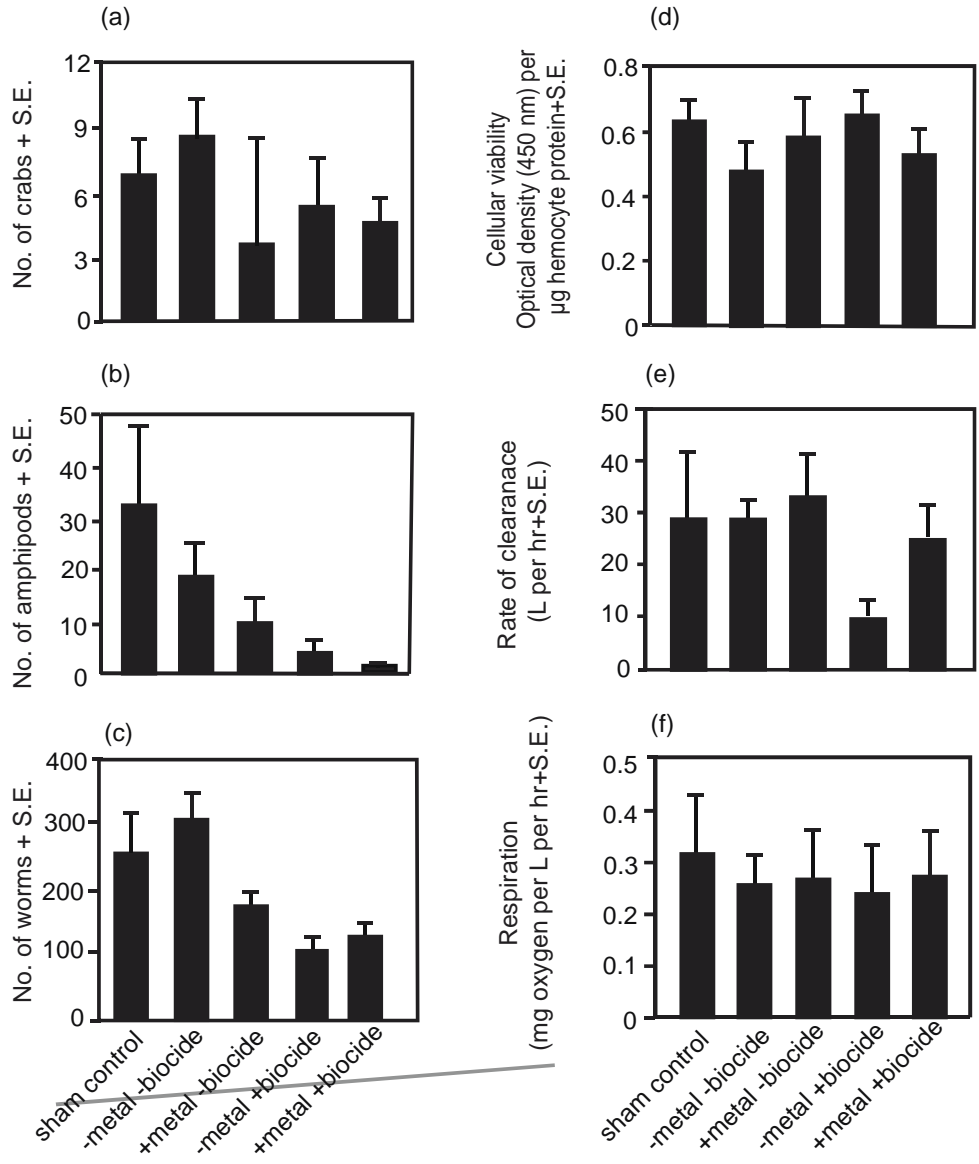
Figure 3. Impacts of metal and/or biocide on the structure (numbers of crabs, amphipods,

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

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

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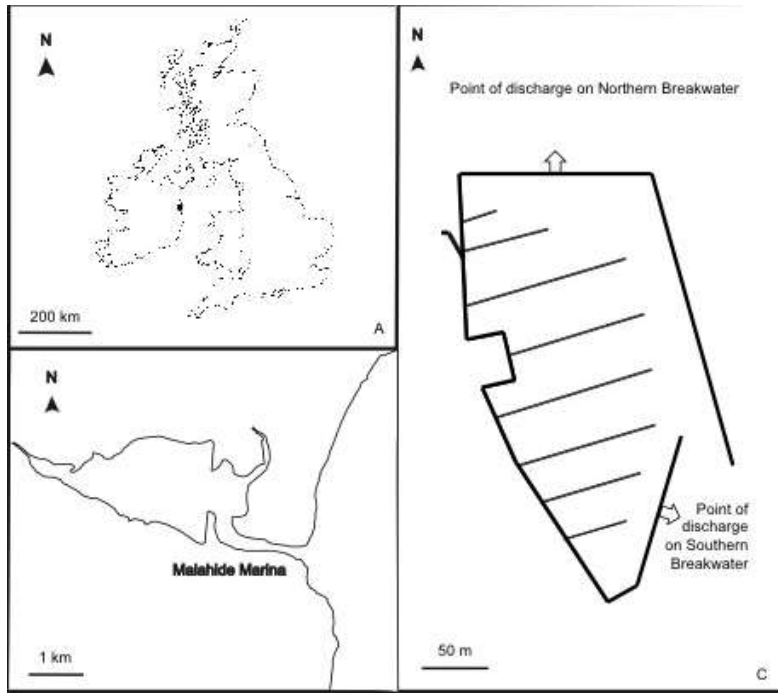
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524   Figure 4. Location of experiments within the British Isles and Malahide Marina.

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526 **Tables**

527 Table 1. Spatial distribution of copper in the tissues of mussels collected at different distances
528 from the 2 experimental discharges from the dosing-system. Analysis of variance with 3
529 factors, including 'Pontoon' (Po) was fixed with 2 levels (North, South); 'Side' (Si) was fixed
530 and orthogonal with 2 levels (Inner, Outer) and 'Distance' (Di) from experimental discharge
531 was fixed and orthogonal with 17 levels (+50, +10, +6, +4, +2, +1, +0.5, +0.25, 0, -0.25, -0.5, -
532 1, -2, -4, -6, -10, -50). There were 3 replicates of each combination. Statistical significance is
533 denoted by $P<0.01^{**}$ and 0.001^{***}

534

535	**Source**	**df**	**MS**	**F**
536	Potoon = Po	1	1.94	0.58
537	Side = Si	1	24.21	7.25**
538	Distance = Di	16	25.32	7.58***
539	Br x Si	1	5.10	1.53
540	Br x Di	16	3.12	0.93
541	Si x Di	16	28.78	8.62***
542	Br x Si x Di	16	1.84	0.55
543	Res	136	3.34	

544
545 Cochran's test   C = 0.33**
546 SNK tests   Outside of pontoon: 0 m > + 0.25 m > all other distances
547   0 m: Outside of pontoon > Inside of pontoon
548   + 0.25 m: Outside of pontoon > Inside of pontoon
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563   Table 2. Spatial distribution of copper in seawater outside of the mussel patches collected at

564   different distances from the 2 experimental discharges from the dosing-system. Analysis of

565   variance with 3 factors, including 'Time' (Ti) was random and orthogonal with 2 levels (9th

566   and 14th September 2010), 'Pontoon' (Po) was fixed and orthogonal with 2 levels (North,

567   South); 'Distance' (Di) from experimental discharge was fixed and orthogonal with 11 levels

568   (+50, +10, +6, +4, +2, +1, +0.5, +0.25, 0, -0.25, -0.5, -1, -2, -4, -6, -10, -50). There were 5

569   replicates of each treatment and statistical significance is denoted by $P<0.001$***.

570
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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	

580
581   Cochran's test   C = 0.50**
582   SNK tests   Time 2 on Northern Breakwater: +2 m> all other distances
583   Time 2 on Southern Breakwater: -1 m> all other distances
584
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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*	green	green	green	green	green	green	green	green
	Organismal	green	green	green	green	green	green	green	green
	Population	blue	blue	blue	green	green	green	green	green
	Assemblage	blue	blue	blue	green	green	green	green	green
	Ecosystem	grey	grey	grey	green	green	green	green	green
Logistical and environmental advantages	*Includes natural environmental variation*	grey	grey	grey	green	green	green	green	green
	Can test for stress(es) due to translocation of organisms	grey	grey	grey	green	green	green	green	green
	No water-quality problems	grey	grey	grey	green	green	green	green	green
	No husbandry required	grey	grey	grey	green	green	green	green	green
Capacity to simulate and manipulate chemical regime	*Concentration*	green	green	green	blue	blue	blue	green	green
	Number	green	green	green	green	green	green	green	green
	Timing	blue	blue	blue	blue	blue	blue	blue	green
	Frequency	blue	blue	blue	blue	blue	blue	blue	green
	Automatic dosing	grey	grey	grey	green	green	green	grey	green

590