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1 **Aquatic tri-trophic standardized microcosm TriCosm**

2

3 **Name** Verena Riedl

4 **Email** [verena.riedl@york.ac.uk](mailto:verena.riedl@york.ac.uk)

5

6 **A standardized tri-trophic small-scale system (TriCosm) for the assessment of stressor**  
7 **induced effects on aquatic community dynamics**

8

9 Verena Riedl,<sup>a,b,\*</sup> Annika Agatz,<sup>a</sup> Rachel Benstead,<sup>b</sup> and Roman Ashauer,<sup>a</sup>

10 <sup>a</sup>University of York, Environment Department, Heslington, York, UK

11 <sup>b</sup>Fera Science Ltd., Centre for Chemical Safety and Stewardship, Sand Hutton, York, UK

12 \* Address correspondence to [verena.riedl@york.ac.uk](mailto:verena.riedl@york.ac.uk)

13

14 **ABSTRACT**

15 Chemical impacts on the environment are routinely assessed in single-species tests.  
16 They are employed to measure direct effects on non-target organisms but indirect effects on  
17 ecological interactions can only be detected in multi-species tests. Micro- and mesocosms are  
18 more complex and environmentally realistic, yet, they are less frequently used for  
19 environmental risk assessment because resource demand is high while repeatability and  
20 statistical power are often low. Test systems fulfilling regulatory needs (i.e. standardization,  
21 repeatability and replication) and the assessment of impacts on species interactions and  
22 indirect effects are lacking. Here we describe the development of the TriCosm, a repeatable  
23 aquatic multi-species test with three trophic levels and increased statistical power. High  
24 repeatability of community dynamics of three interacting aquatic populations (algae,  
25 *Ceriodaphnia*, *Hydra*) was found with an average coefficient of variation of 19.5% and the

26 ability to determine small effect sizes. The TriCosm combines benefits of both single-species  
27 tests (fulfillment of regulatory requirements) and complex multi-species tests (ecological  
28 relevance) and can be used, for instance at an intermediate tier in environmental risk  
29 assessment. Furthermore, comparatively quickly generated population and community  
30 toxicity data can be useful for the development and testing of mechanistic effect models.

31

## 32 **KEYWORDS**

33 ecological risk assessment, aquatic invertebrates, population-level effects, food chain, aquatic  
34 microcosm, multi-species testing

35

36

## 37 **INTRODUCTION**

38 The thorough assessment of environmental risks is essential for chemicals that could  
39 potentially be released into the environment. Agricultural pesticides, for instance, are used to  
40 enhance crop production but due to their toxic nature they may have negative effects on  
41 organisms other than the targeted species (Benton et al. 2007; Rockström et al. 2009; Beketov  
42 et al. 2013; Stehle and Schulz 2015).

43 The risks of pesticides to non-target organisms are routinely assessed in i) simple single-  
44 species tests at lower tiers and, if lower tier assessments raise concern, in ii) complex  
45 microcosms or mesocosms (European Food and Safety Authority (EFSA) 2013). However,  
46 systems that bridge the gap between the two alternatives to an intermediate level of  
47 complexity are lacking. The former require less effort and rapidly deliver large amounts of  
48 highly repeatable data on the performance of individual non-target organisms. The  
49 information obtained is, however, often of low ecological relevance as it is not directly  
50 relevant at the population and community level (Fleeger et al. 2003; Liebig et al. 2008). In

51 contrast, micro-/mesocosms are environmentally more realistic, yet, they are less frequently  
52 used in environmental risk assessment. Unlike single-species tests, they are resource, time  
53 and effort demanding. A variety of ecologically interacting factors can rapidly lead to  
54 divergent system dynamics and increase the variance between replicates impeding the  
55 understanding of dose-response relationships (Landis et al. 1997).

56 The importance of integrating environmental complexity into testing approaches has been  
57 acknowledged as a priority for the assessment of chemical safety (Landis et al. 1997; Landis  
58 2003; Bednarska et al. 2013; Scientific Committee on Health and Environmental Risks  
59 (SCHER) et al. 2013). Chemical exposure could trigger indirect effects through interactions  
60 with the environmental context such as the hydrological regime (Stampfli et al. 2013),  
61 temperature (Moe et al. 2013), food quality (Campos et al. 2014) or other organisms (Del  
62 Arco et al. 2015; Viaene et al. 2015); indirect effects have important implications for the  
63 sensitivity of communities (Fleeger et al. 2003).

64 Organisms living in a contaminated environment may be pushed towards the boundaries  
65 of their ecological niche and become more susceptible to additional stressors (Van Straalen  
66 2003; Bednarska et al. 2013). Food chain processes, such as competition for food and altered  
67 predation were shown to be particularly relevant to determine the magnitude of toxic effects  
68 (Heugens et al. 2001; Bednarska et al. 2013; Kattwinkel et al. 2015). For instance, the no  
69 observed effect concentration (NOEC) of the herbicide prometryn to ciliates was found to be  
70 approximately 145 times lower in a bi-trophic microcosm compared to single-species tests.  
71 The lower threshold was likely caused in response to an indirect and toxicant induced  
72 reduction of food (Liebig et al. 2008). Intraspecific competition can also change the  
73 sensitivity to pesticides (Foit, Kaske, and Liess 2012; Viaene et al. 2015) and indirectly  
74 altered predation rates can lead to cascading effects on other trophic interactions and  
75 ecosystem functions (Englert et al. 2012; Agatz et al. 2014; Viaene et al. 2015). Multi-species

76 testing using environmentally more relevant approaches, i.e. at the population and community  
77 level is clearly needed to assess indirect toxicant effects such as shifts in ecological  
78 interactions (Fleeger et al. 2003; Benton et al. 2007).

79 The necessity towards an inclusion of ecological interactions in chemical impact  
80 testing was described 10 years ago when a review on 14 years of pesticide studies in  
81 freshwater test systems was published (Relyea and Hoverman 2006). At the time, the authors  
82 found only 133 studies with at least two potentially interacting species of which only 17  
83 studies focused on three trophic levels with producers, herbivores and carnivores.

84 Yet, microcosms that describe impacts on populations and/or communities in systems  
85 smaller than 10 L are rare (Metcalf et al. 1971; Daam and Van Den Brink 2007; Liebig et al.  
86 2008; Englert et al. 2012; Foit, Kaske, and Liess 2012; Dolciotti et al. 2014; Del Arco et al.  
87 2015; Viaene et al. 2015). Mostly they were used to focus on impacts on intra- or  
88 interspecific competition (one trophic level) (Foit, Kaske, and Liess 2012; Dolciotti et al.  
89 2014; Del Arco et al. 2015; Viaene et al. 2015) or on consumer-resource relationships (two  
90 trophic levels) with herbivore-producer (Daam and Van Den Brink 2007) or predator-prey  
91 interactions (Barry and Davies 2004; Liebig et al. 2008; Englert et al. 2012). Tri-trophic  
92 systems are frequently used in terrestrial research, for example in plant-herbivore-parasite  
93 systems (Bredeson et al. 2015; Uhl et al. 2015) but few small test systems exist to assess  
94 direct and indirect impacts at the population and community level in the aquatic environment.  
95 Test formats include simulations of microbial detritus food chains (producer-consumer-  
96 decomposer (Fuma et al. 2000; Dawoud et al. 2017)) and producer-consumer communities  
97 with either invertebrate predator (Barry and Davies 2004) or vertebrate predator (Metcalf et  
98 al. 1971). Microbial tests were often conducted in culture flasks (250 ml) (Fuma et al. 2000;  
99 Dawoud et al. 2017) and small macroinvertebrate community tests were performed in

100 systems of few litres, for example in 10 L (Barry and Davies 2004) and 7 L systems (Metcalf  
101 et al. 1971).

102 Still, single-species systems appear convenient because they fulfill the regulatory needs  
103 for international standardization of test procedures, comparability of effect data, repeatability  
104 and replication (Liebig et al. 2008). Standardized and repeatable multispecies systems of  
105 intermediate complexity that bridge the simplicity of single species tests and the complexity  
106 of microcosms, yet fulfill regulatory requirements, are rare. To our knowledge, there is only  
107 one standardized microcosm (Taub 1989) available that falls into this category. The aquatic  
108 system was registered for pesticide testing (American Society for Testing of Materials  
109 (ASTM) 2011) and effects on two trophic levels covering ten primary producer and five  
110 primary consumer species can be assessed. It is, however, rarely used for standardized effect  
111 assessment, perhaps due to its relative complexity and the lack of mechanistic understanding  
112 of the interactions between species involved.

113 We developed a new test system with species interacting across three trophic levels  
114 and increased statistical power (i.e. standardization and low replicate variability). The system  
115 was designed to be cost-effective, rapid, repeatable with well understood population  
116 dynamics to i) allow the detection of small changes in population dynamics due to direct and  
117 indirect interactions, and ii) link observed effects to known system processes. Here we  
118 describe the standardized aquatic tri-trophic microcosm (hereafter TriCosm) focusing on  
119 system design and variability in the control treatment.

120

## 121 **MATERIALS AND METHODS**

### 122 *Test organisms*

123 The TriCosm comprises populations of the green alga *Pseudokirchneriella*  
124 *subcapitata*, the cladoceran *Ceriodaphnia dubia* and the cnidarian *Hydra viridissima*. This

125 dynamic food-chain is subject to fluctuating but predictable changes in food supply and  
126 intraspecific competition and is interconnected through consumer-resource relationships. The  
127 species were chosen based on their rapid life cycles and their sensitivity to toxicants. The  
128 green alga *P. subcapitata* and the cladoceran *C. dubia* are routinely used for tests in the  
129 regulatory risk assessment framework (Organization for Economic Co-operation and  
130 Development (OECD) 2004; OECD 2006; OECD 2012).

131 *P. subcapitata* stock cultures were obtained from the Culture Collection of Algae and  
132 Protozoa (CCAP, Scotland, UK) and used to initiate a culture line prior to each study and  
133 cultured in OECD media (OECD 2006). *C. dubia* were obtained from Unilever (Safety and  
134 Environmental Assurance Centre, Bedford, UK) and cultured as age specific cultures in  
135 moderately hard, synthetic freshwater (United States Environmental Protection Agency  
136 2002). They were fed five times per week with a suspension of yeast, cerophyl<sup>®</sup> and trout-  
137 chow (~3.5 ml) and *P. subcapitata* (~11 x 10<sup>7</sup> cells/day) (United States Environmental  
138 Protection Agency 2002). *H. viridissima* were obtained from the Department of Evolutionary  
139 Zoology (University of Debrecen, Hungary), cultured in modified T82MV medium (modified  
140 after ASTM E1366-11 2011, [Table SI 1](#), [Table SI 2](#)) and fed with newly hatched *Artemia*  
141 *salina* three times per week ad libitum. Both animal cultures were kept at 25 ± 1 °C and  
142 12/12h light/dark cycle.

143

#### 144 *The TriCosm*

145 TriCosms consist of Pyrex<sup>®</sup> crystallizing dishes (Sigma-Aldrich, UK) filled with 500  
146 ml of T82MV medium (ASTM E1366-11 2011, [Table SI 1](#), [Table SI 2](#)) that was determined  
147 as suitable for each species. The systems were covered with transparent watch glasses  
148 (diameter 125 mm; Sigma-Aldrich, UK) and positioned on an orbital laboratory shaker  
149 (Adolf Kuehner AG Switzerland, Type LS-W) set at 65 rpm throughout the test. The

150 experiments were set up for 21 days at  $25 \pm 1$  °C, 12/12h light/dark, 1100 lux at the water  
151 surface with cool white fluorescent light tubes 58.5 W (approx. 1.3 m above the test vessels).  
152 TriCosms were started with *P. subcapitata* cells from a culture in logarithmic growth phase,  
153 *C. dubia* neonates (< 24h age) from the third or fourth brood of cultured mothers and *H.*  
154 *viridissima* without visible buds ( $\leq 2$  d age). Water parameters (pH, dissolved O<sub>2</sub>) and animal  
155 and algal populations were monitored throughout the test duration two, three and five times  
156 per week, respectively.

157

### 158 *Monitoring of the populations*

159 The systems were placed on an orbital shaker and slow shaking kept the algal cells  
160 suspended. Only suspended algae were measured and no stirring was necessary before  
161 sampling as preliminary studies showed significant correlation between suspended and total  
162 algal concentrations (cells/ml) ( $r_s = 0.98$ ,  $p < 0.01$ ,  $n = 90$ , **Figure SI 1**). In-vivo fluorescence  
163 activity of water subsamples (5 x 200  $\mu$ l) was measured with a plate reader (Tecan<sup>®</sup> Infinite  
164 200 PRO, settings **Table SI 3**) to determine the algal concentration (cells/ml).

165 *C. dubia* and *H. viridissima* were monitored with non-invasive methods to avoid  
166 impacts on population dynamics and counted by eye three times per week. *C. dubia* were  
167 visually grouped in two age-classes, juveniles and adults based on their dimensional  
168 similarity with individuals in cultures aged younger or older than 4 d. All manual counts were  
169 repeated until count differences did not exceed 20% of the lower value.

170

### 171 *Assessing a suitable community composition*

172 Tests with different setups were performed to optimize replicate variability, test  
173 duration, addition times and densities for each species. A full factorial design for density and  
174 timing was not feasible due to a too high number of possible combinations. Hence,



175 preliminary tests were performed to determine which algae-grazer combination in terms of  
176 organism abundance would prevent both algal blooms and the death of grazers due to  
177 starvation. No preliminary tests were done to determine the impact of *Hydra* predation on *C.*  
178 *dubia* numbers prior to the test outlined in Table 1. Two organism densities and different  
179 addition times were chosen based on preliminary testing and four different setups were  
180 conducted simultaneously (Table 1). *C. dubia* were added on the same day as the green algae  
181 in all experiments, except for setup 1 where grazers were added 1 day later to allow short  
182 acclimation of the algae to test conditions. Dependent on food concentrations, *C. dubia*  
183 matured later in setups 1, 2 than in setups 3, 4 hence *H. viridissima* were introduced to the  
184 systems 5 and 4 days, respectively, after *C. dubia* were added. The predators were added only  
185 once the grazers started reproducing to prevent variable numbers of *C. dubia* reproducers and  
186 neonates early on in the systems that could lead to noticeable impacts on community  
187 dynamics and replicate variability. Replicate numbers differed between setups 1, 2 and 3, 4  
188 due to space constraints on the shaker platform.

189

#### 190 *Validation of an optimal experimental setup*

191 The coefficient of variation (CV) was calculated as a standardized measure of  
192 variance between replicates. It was expressed as a percentage and indicates the magnitude of  
193 the standard deviation in comparison to the mean. Thus, if the inherent variability between  
194 replicates is large compared to the size of the measured endpoint (e.g. animal abundance) a  
195 significant treatment effect could only be detected if the response was very large (Sanderson  
196 2002).

197 We computed the CVs for the algal concentration (cells/ml) and the total number of  
198 *C. dubia* and *H. viridissima* on every sampling day. The values were then compared within  
199 and between setups to monitor replicate variation over time and to determine an optimal setup

200 in terms of low variance. The experimental setup with the lowest replicate variability  
201 (Experiment 1) was determined and repeated (Experiment 2) to assess the reproducibility of  
202 system dynamics and replicate variability.

203

#### 204 *Population dynamics and interactions*

205 In the interacting system, the intermediate trophic layer is directly affected by both  
206 variations of food availability and predation strength, while indirect effects between the  
207 bottom and top trophic level regulate a bottom up or top down controlled system. A  
208 comparison of algal and grazer dynamics between systems where grazers are subject to i)  
209 variations of food but not to predation and ii) a combination of food limitation and predation  
210 can thus yield information on species interaction strength and whether the system is  
211 controlled by bottom up or top-down effects. Hence, we performed additional tests with i)  
212 only algae (Experiment 2A, n = 8) and ii) algae and grazers (Experiment 2B, n = 8). The  
213 experiments were carried out simultaneously to Experiment 2 and according to the  
214 experimental conditions and test setup used as for Experiments 1 and 2 (see Table 1 for  
215 details). Experiments 2A and 2B were then compared to determine direct impacts of grazers  
216 on algal dynamics and Experiments 2 and 2B were compared to assess i) indirect effects of  
217 predators on algal growth and ii) direct effects of predators on *C. dubia* population  
218 trajectories.

219

#### 220 *Statistical analyses*

221 An a priori power analysis was performed to estimate minimum detectable response  
222 sizes between control and treated TriCosm populations and increase the reliability and  
223 transparency of the derived endpoints (EFSA 2013). The minimum detectable difference  
224 (MDD), i.e. the size of a variation between sample averages required to be detected as

225 significantly different, is dependent on the chosen Type I error value  $\alpha$ , the number of  
226 replicates employed and on the inherent variance such as replicate variability and/or sampling  
227 error (Brock et al. 2015). Here, we computed the MDD using the CVs assuming similar  
228 variance among controls and treatments. We hypothesized the use of 8 replicates and  
229 estimated the sensitivity of the TriCosm to reveal chemical effects for each population and at  
230 each sampling point. The MDD was calculated as described by Brain et al. (2005) from Sokal  
231 and Rohlf (1995):

$$\text{MDD} = \frac{\sqrt{2} (t_{\alpha,v} + t_{\beta,v}) \times \text{CV}}{\sqrt{n}}$$

233  
234 where  $t_{\alpha,v}$  and  $t_{\beta,v}$  are the t-values for  $\alpha$  and  $\beta$  set to 0.05 and 0.2, respectively, for a  
235 confidence level of 95% and a power of 80% at  $v$  degrees of freedom. CV is the coefficient of  
236 variation and  $n$  is the number of replicates used. The degrees of freedom were computed as  $v$   
237  $= k(n-1)$  and the number of groups  $k$  was set to 2, e.g. to compare each treatment to the  
238 control. The calculated MDDs were compared to MDD classes as proposed by the European  
239 Food Safety Authority (EFSA) that grouped MDD sizes into five classes and described the  
240 likely ability of effect detection (Class 0: MDD > 100% = no effect detection, Class I: MDD  
241 90 – 100% = only large effects, Class II: MDD 70 – 90% = large to medium effects, Class III:  
242 MDDs 50 – 70% = medium effects, Class IV: MDD < 50% small effects) (EFSA 2013).

243 To assess species interactions between system components, population dynamics were  
244 compared graphically between experiments and significant differences were assumed where  
245 95% confidence intervals did not overlap.

246

## 247 **RESULTS**

### 248 *Optimizing the experimental setup*

249 The community dynamics (  
250 **Figure 1**) and the coefficients of variation differed among the four setups (**Figure 2**)  
251 and over time (**Figure SI 2**). In general, the algal concentration (cells/ml) peaks were  
252 followed by *C. dubia* abundance peaks and a constant increase of *H. viridissima* populations.  
253 The highest *C. dubia* peak 172 ( $\pm 10$ ) individuals (mean  $\pm$  95% confidence interval range)  
254 was found in Setup 1 on day 14 following an algal peak on day 6 when an average of 6.86 ( $\pm$   
255 0.64)  $\times 10^5$  cells/ml was measured (  
256 **Figure 1A**). The highest algal peak of 11.51 ( $\pm 5.59$ )  $\times 10^5$  cells/ml appeared in Setup  
257 3 on day 11 with increasing variance in terms of organism numbers between replicates over  
258 time (  
259 **Figure 1C**). *H. viridissima* populations showed steady growth during the test duration  
260 and increased in numbers by an average of 42 ( $\pm 6$ ), 45 ( $\pm 8$ ), 72 ( $\pm 18$ ) and 54 ( $\pm 15$ )  
261 individuals in Setup 1, 2, 3 and 4, respectively (**Figure 1A - D**). Final counts differed due to  
262 different addition numbers, timings and food availability (*C. dubia* abundances), however,  
263 *Hydra* populations showed the smallest replicate variance when compared to algae and *C.*  
264 *dubia* (**Figure 2**). The CVs of all test variables in each setup increased over time (**Figure SI 2**)  
265 with setup 3 showing the highest replicate variability (with the exception of *H. viridissima*)  
266 and setup 1 showing the lowest average CV (with the exception of *H. viridissima*) (**Figure 2**).  
267 High CVs observed in setup 2 - 4 indicated reduced ability to detect treatment related system  
268 alterations. Therefore we selected setup 1 (Experiment 1) as most appropriate setup  
269 procedure (**Protocol SI 4**) and repeated the test (Experiment 2) to evaluate the repeatability of  
270 the system.  
271  
272 *Validation of the test setup*

273 The population dynamics of experiment 1 and the repeated experiment 2 were similar  
274 (Figure 3). The algal populations peaked on day 6 and day 5 with average algal  
275 concentrations of  $6.86 (\pm 0.64) \times 10^5$  and  $8.83 (\pm 0.90) \times 10^5$  cells/ml in experiments 1 and 2,  
276 respectively (Figure 3A). The highest *C. dubia* abundance was measured 8 days after the  
277 algal peaks in both cases. Grazer numbers declined 1 day earlier in experiment 2 and  
278 individual counts were lower due to smaller juvenile numbers of  $147 (\pm 10)$  and  $97 (\pm 24)$   
279 juveniles in experiment 1 and 2, respectively (Figure 3B). The dynamics of *H. viridissima*  
280 populations were similar between experiments (Figure 3C) but experiment 1 showed a  
281 slightly steeper population increase with a larger final population of  $45 (\pm 6)$  and  $33 (\pm 9)$   
282 individuals in experiment 1 and 2, respectively. Due to slightly different sampling  
283 frequencies, we computed 15, 11 and 8 CV values in experiment 1 and 14, 9 and 7 CVs in  
284 experiment 2 for algal concentrations (cells/ml), *C. dubia* and *H. viridissima*, respectively. As  
285 observed for population dynamics, replicate variance was similar between populations in both  
286 experiments (Figure SI 3). The CVs of algal concentrations (cells/ml) increased by day 7, 14  
287 and 21 to an average of 9, 26 and 26% in experiment 1 and 10, 25 and 47% in experiment 2,  
288 respectively. The CVs calculated for *C. dubia* populations increased from 9 to 15 and 33% in  
289 experiment 1 and from 4 to 23 and 24% in experiment 2. *H. viridissima* were added on day 6,  
290 so the replicate variability was 18, 18% and 17, 29% by day 14 and 21 in experiment 1 and 2,  
291 respectively.

292

### 293 *Population dynamics and interactions*

294 Significant reductions of algal concentrations (cells/ml) by 33.4% were found on the  
295 first sampling day after *C. dubia* addition, on day 4. An average algal concentration of  $9.18$   
296  $(\pm 0.48) \times 10^5$  cells/ml was found in Experiment 2A (only algae), while grazed algae in  
297 Experiment 2B (algae and grazers) reached an abundance of  $6.11 (\pm 0.43) \times 10^5$  cells/ml by

298 day 4 and stayed significantly lower throughout the experimental duration (Figure 4A).  
299 Grazed algal concentrations in systems with and without predator (Experiments 2 and 2B,  
300 respectively) showed similar trajectories until day 14. After day 14, algal concentrations in  
301 Experiment 2B stayed moderately constant with an average of  $2.12 (\pm 0.21) \times 10^5$  cells/ml  
302 until day 20. On the contrary, algal concentrations in Experiment 2 (grazers and predators)  
303 increased to  $7.63 (\pm 0.37) \times 10^5$  cells/ml by day 20 exceeding average algal abundances of  
304 Experiment 2B by 75.0% (Figure 4B).

305 Grazer population dynamics were similar in Experiment 2 and 2B until day 6 when  
306 predators were added to Experiment 2 (Figure 4C). Population numbers peaked in both  
307 experiments on day 13 but steeper population growth curves in Experiment 2B lead to an on  
308 average 39.1% larger peaking population of  $210 (\pm 21)$  individuals when compared to  $128 (\pm$   
309  $25)$  individuals in Experiment 2. A similarly steeper population decrease in experiment 2B  
310 resulted in similar total *C. dubia* counts of  $36 (\pm 9)$  and  $37 (\pm 10)$  individuals in systems  
311 without and with predators, respectively, by day 20. Population dynamics of total grazer  
312 numbers largely reflected the trajectories of *C. dubia* juveniles that rapidly increased until  
313 day 13 to  $175 (\pm 23)$  and  $107 (\pm 23)$  individuals constituting 88.3% and 90.1% of the total *C.*  
314 *dubia* population in experiments 2B and 2, respectively. By day 20, juvenile numbers  
315 dropped to  $6 (\pm 4)$  and  $15 (\pm 6)$  individuals while adult grazers showed a moderate but  
316 constant increase throughout the test and constituted 84.9% and 59.7% of the total *C. dubia*  
317 populations in experiments without and with predators, respectively (Figure 4D).

318

### 319 *Statistical analyses*

320 Minimum detectable differences (MDDs) of hypothetical TriCosm exposures were  
321 calculated according to control variance and were similar between experiments 1 and 2. The  
322 TriCosm became less sensitive over time as replicate variation and MDDs increased. When

323 variances between controls and treatments are similar, the TriCosm is estimated to be  
324 sufficiently sensitive to identify differences of 12% ( $\pm 4$ ), 36% ( $\pm 7$ ) and 50% ( $\pm 17$ ) for *P.*  
325 *subcapitata* and 9% ( $\pm 7$ ), 31% ( $\pm 4$ ) and 38% ( $\pm 19$ ) for *C. dubia* populations in week 1  
326 (day 1 – 6), week 2 (day 7 – 13) and week 3 (day 14 – 21), respectively (averaged MDDs  
327 between Experiment 1 and 2). Averaged MDDs for *H. viridissima* were 25% ( $\pm 14$ ) and 35%  
328 ( $\pm 7$ ) in week 2 and 3, respectively (Figure 3). The MDDs for critical endpoints in the  
329 TriCosm can be assigned to MDD classes III (50 – 70%) and IV (< 50%), indicating the  
330 ability to determine small and medium sized effects, respectively (EFSA 2013).

331

## 332 **DISCUSSION**

333 The assessment of chemical effects with single-species tests fulfills regulatory  
334 requirements; however, primary goals of protecting populations and ecosystems might not be  
335 adequately addressed. That is because information obtained at the individual level is often not  
336 ecologically relevant since there are neither directly proportionate relationships between  
337 direct and indirect effects nor amongst responses at the individual, population and community  
338 level. An understanding of impacts on interactions in ecologically relevant test settings is thus  
339 critical and a priority for chemical safety assessment as unexpected shifts in community  
340 profiles cannot be predicted in single-species tests (Fleeger et al. 2003; Benton et al. 2007;  
341 Liebig et al. 2008; SCHER et al. 2013).

342 We designed the TriCosm as a rapidly cycling, tri-trophic system with a producer-  
343 herbivore-carnivore community of small size for the purpose of quick detection of chemical  
344 impacts on species interactions. Our system is comparatively smaller (0.5 L) than many other  
345 multi-trophic systems (Metcalf et al. 1971; Daam and Van Den Brink 2007; Foit, Kaske,  
346 Wahrenndorf, et al. 2012; Dolciotti et al. 2014; Del Arco et al. 2015) and all system  
347 components exhibit rapid generation times so that treatment effects can be measured on

348 several generations and at different life stages during short test durations (21 days compared  
349 to 80 days (Metcalf et al. 1971) and 33 days (Barry and Davies 2004) in other tritrophic  
350 macroinvertebrate communities). Also the predator *Hydra* is a rapid reproducer with  
351 generation times of only three days under favourable conditions (Habetha et al. 2003).  
352 Chemical impacts on population dynamics can thus be detected not only at the producer-  
353 consumer level but also at a higher trophic level. The choice of a small and rapidly  
354 reproducing predator has further the advantage that it can be added at an early experimental  
355 stage (day 6) when compared to vertebrate predators that are often introduced shortly before  
356 test termination as they quickly consume remaining invertebrate preys (Metcalf et al. 1971;  
357 Harrass and Taub 1985).

358 All multi-species systems have ecologically interacting components that are not  
359 independent in statistical terms as they constantly adapt to changing conditions in a dynamic  
360 environment. In fact, it has been frequently reported that even though communities are set up  
361 identically as replicates, minor variations at the beginning and/or throughout the experiments  
362 (e.g. starting conditions or uneven sample removal) can quickly lead to the development of  
363 unique properties in each replicate (Landis et al. 1997; Sanderson 2002; Van Straalen 2003).  
364 Indeed, different population dynamics and replicate variability were observed in four  
365 different TriCosm setups and indicated strong sensitivity to starting conditions and  
366 interaction strength. The statistical quality (in terms of interpretability, reproducibility and  
367 replicability) of environmentally more realistic data obtained in multi-species tests is thus  
368 often reason for concern in the registration procedure of pesticides (Sanderson 2002).

369 The repeatability and reproducibility of the TriCosm were thus given major  
370 consideration during test development. Initial properties and sampling techniques were  
371 adjusted and confirmed as optimized when experiments conducted at different times showed  
372 low coefficients of variation (CVs) and high reproducibility of system dynamics.



373 Desynchronized population dynamics were observed between experiments that can be  
374 attributed to random fluctuations in test conditions (e.g. quality of the animals) and could  
375 occur even if procedures are standardized. For these reasons we assessed the repeatability by  
376 comparing CVs and not the total organism abundances. Nonetheless, a comparison of total  
377 abundances or derived variables (e.g. population growth rates) is also appropriate when  
378 chemical effects are assessed since differences between population trajectories are most  
379 likely and primarily due to chemical impacts rather than fluctuations of test conditions.

380         When the TriCosm is used for chemical effect assessment, two factors of major  
381 importance are i) the presence of interactions rather than the exact timing when these occur  
382 and ii) low CVs so that treatment responses can be interpreted with greater certainty and  
383 distinguished from unexplained sample variability (Sanderson 2002).

384         The ability to detect significant effects does depend on the magnitude of an effect but  
385 also on the ability of the test system to detect responses and that is in turn determined by the  
386 inherent variance among replicates. Test variables with coefficients of variation (CV) in the  
387 range of up to 30% have been theorized as acceptable and manageable in terms of practicality  
388 and costs (Kraufvelin 1998). According to a review (Sanderson 2002) that analyzed two  
389 decades of pesticide studies with micro/mesocosms, the values of CVs appear to be generally  
390 higher. The author reported an average of 45% (32% in smaller and less realistic indoor  
391 systems) with larger values in studies where animals were involved and an average use of 3.5  
392 replicates. The average CV of 19.5% measured in the tri-trophic system on the contrary  
393 showed smaller variance and was determined with a higher number of replicates (n = 8).

394         The CVs were further used for the calculation of theoretically detectable minimum  
395 differences (MDDs) between controls and treatments under the assumption of similar  
396 variances. It is to be mentioned, however, that the variance could increase, decrease and/or  
397 remain similar in treated systems (Kraufvelin 1998; Sanderson 2002). A modification of the

398 number of replicates, groups or treatments, though, can decrease MDDs and allow the  
399 detection of desired effect sizes. Due to often large variability in micro-/mesocosms, EFSA  
400 may still regard endpoints with MDD classes I and II (70 – 100%) relevant but considers the  
401 exceeding of class II ideal (i.e. MDDs < 70%) (EFSA 2013). Most projected MDDs of  
402 critical endpoints in the aquatic system correspond to effect class IV (i.e. < 50%) (with  
403 exception of algae and grazers in week 3) and confirm the ability to reveal small toxicant  
404 induced effect sizes (EFSA 2013), distinguishing the TriCosm from other multi-trophic  
405 systems.

406 As expected, variations of population trajectories were observed as a result of  
407 interactions with other system components. Algal concentrations (cells/ml) and predation  
408 both directly impacted on the middle trophic layer while they indirectly impacted on the top  
409 and the bottom level, respectively. An initially small grazing pressure of juvenile *C. dubia*  
410 allowed algal populations to grow exponentially which in turn favored the development of  
411 grazer populations. As a consequence of an increasing grazing pressure by maturing and  
412 reproducing *C. dubia*, the algal concentrations dropped, yet the grazer population numbers  
413 further increased for approximately one week after food availability became limiting. The  
414 continuing population growth is attributable to a rise of juvenile numbers as adult *C. dubia*  
415 most likely matured eggs and stored energy before algal concentrations decreased. Peaking *C.*  
416 *dubia* populations thus coincided with lows of food availability and caused the decrease of  
417 grazer numbers. Algae stabilized and remained at relatively constant levels as concentrations  
418 were most likely too low to be further reduced if maximum grazer filtering rates were  
419 reached. Grazer population numbers decreased due to food shortage and independently of  
420 whether predators were present or not. While predation did not cause *C. dubia* populations to  
421 crash, it directly reduced grazer numbers, intraspecific competition among them and  
422 indirectly favored algal populations to recover. An increase of algal concentrations after

423 grazing release was, however, not observed in Experiment 1 where grazer populations  
424 reached larger abundances but decreased later and might be due to a different quality of  
425 neonates used to start the experiments. Algal populations in Experiment 1 were thus subject  
426 to a higher and prolonged grazing pressure impeding the recovery of algal abundances within  
427 the experimental duration. An indirect effect after grazing release by *Hydra* could, however,  
428 likely be expected if the test duration was prolonged. Bottom up and top down processes are  
429 thus both likely regulating population dynamics in the TriCosm. When the system is exposed  
430 to chemicals it will thus depend on the mode of action of the toxicant impacting on one or  
431 more trophic levels leading to direct, indirect or both effects on the trajectories of interacting  
432 populations.

433         The focus during system development was not on achieving a steady state community  
434 and impacts on resilience cannot be assessed, neither can system shifts be detected.  
435 Nonetheless, it can indicate the recovery potential of species after stressor removal and detect  
436 small changes in interactions as the system moves through a single cycle of the middle  
437 trophic layer. Ecological impacts of toxicants rapidly propagate in an interacting system and  
438 the grazer level is directly influenced by variations in food availability and predation.  
439 Toxicant impacts on the population dynamics of this critical and key trophic layer will  
440 therefore yield important information on the ecological relevance and protectiveness of data  
441 obtained in single-species tests. Population responses to combined stressor exposures, e.g. to  
442 toxicants, predation and/or food fluctuations, could be used to facilitate both the development  
443 and the testing of mechanistic effect models. Measured community responses in terms of  
444 individual abundance changes and population trajectories could be employed for the  
445 calibration and parameter fitting of ecological models. In turn, chemical effects on  
446 interactions within a simple freshwater community can be measured and quantified in the

447 TriCosm and provide empirical benchmarking to estimate and test model prediction accuracy  
448 and power.

449         There is no doubt that the complexity of the TriCosm community is low when  
450 compared to natural systems. But besides offering higher statistical power when compared to  
451 larger and / or more complex microcosms, the impacts on system processes can be quantified  
452 as interactions change. This makes it possible to assess the effects of environmental  
453 contaminants on i) species interactions, ii) indirect effects and iii) at the population and  
454 community level. An understanding of which and to what extent processes are affected may  
455 also give insights into responses of more complex systems (Benton et al. 2007; Daam and  
456 Van Den Brink 2007; Boonstra et al. 2011) .

457

## 458 **CONCLUSION**

459         The TriCosm is a novel aquatic test system and could be a tool to address shifts in  
460 ecological interactions. It suggests that a cost-effective approach of chemical environmental  
461 safety testing with more ecological relevance whilst being statistically powerful is feasible. It  
462 can provide important insights into chemical safety in multi-trophic systems and facilitate the  
463 development and testing of mechanistic effect models for environmental risk assessment.

464 Even so, a careful examination of the replicability of the TriCosm both within and between  
465 laboratories with and without chemical exposure is needed.

466

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469

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475

476 *Data availability* — Data and calculation tools are available from the corresponding author  
477 ([Verena.riedl@york.ac.uk](mailto:Verena.riedl@york.ac.uk)).

478

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610

611

612 **TABLES AND FIGURES**

613 Table 1. The TriCosm community composition at the beginning of four test setups

|         | <i>P. subcapitata</i> |     | <i>C. dubia</i> |     | <i>H. viridissima</i> |     | Replicates |
|---------|-----------------------|-----|-----------------|-----|-----------------------|-----|------------|
|         | Cells/ml              | Day | Individuals     | Day | Individuals           | Day |            |
| Setup 1 | 2 x 10 <sup>4</sup>   | 0   | 10              | 1   | 3                     | 6   | 8          |
| Setup 2 | 2 x 10 <sup>4</sup>   | 0   | 10              | 0   | 6                     | 5   | 8          |
| Setup 3 | 4 x 10 <sup>4</sup>   | 0   | 10              | 0   | 6                     | 4   | 7          |
| Setup 4 | 4 x 10 <sup>4</sup>   | 0   | 20              | 0   | 6                     | 4   | 7          |

614

615

616 Figure 1 Algal concentrations (cells/ml) and total number of *C. dubia* and *H. viridissima* over  
 617 21 days. Shown are means ± 95 % confidence intervals in four test setups (A – D) (see Table  
 618 1 for details).

619

620 Figure 2 Coefficients of variation (%) of algal concentrations (cells/ml), total abundance of  
 621 *C. dubia* and *H. viridissima* at each sampling event. Black horizontal lines indicate 95 %  
 622 confidence intervals in setups 1 - 4.

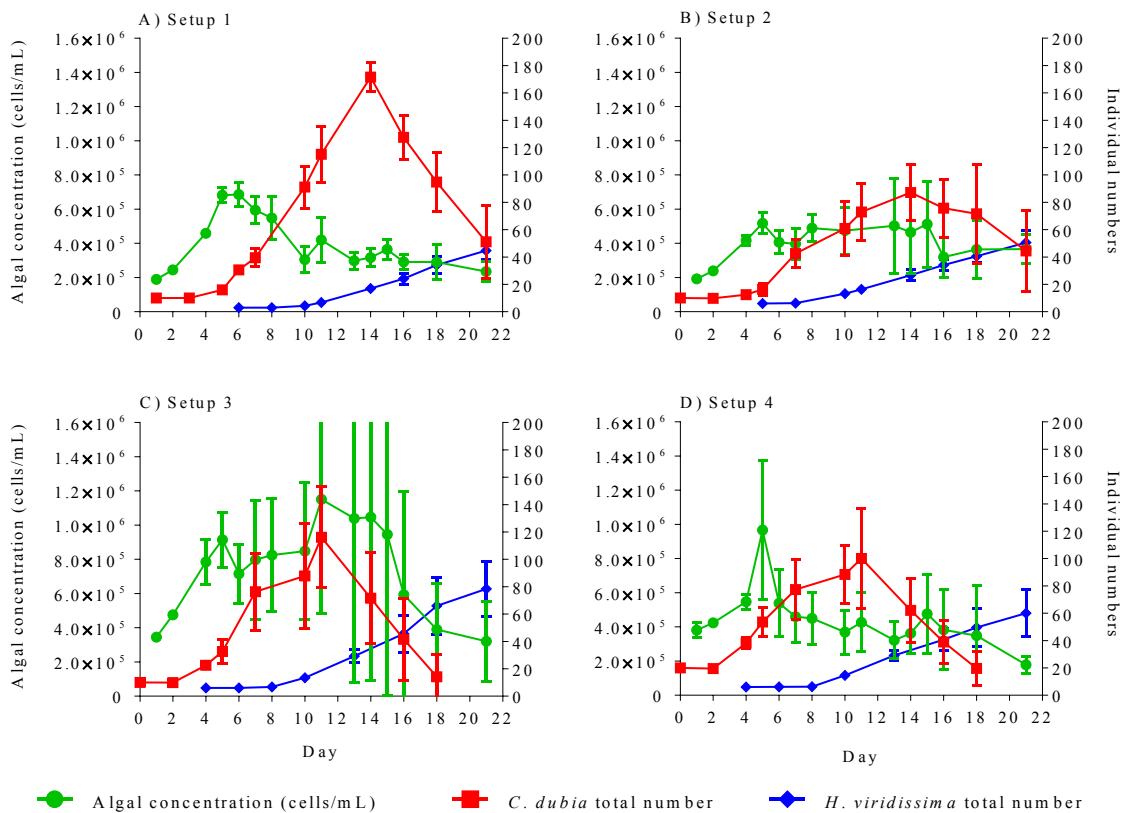
623

624 Figure 3 Abundance of (A) *P. subcapitata*, (B) *C. dubia* and (C) *H. viridissima* at each  
 625 sampling point over 21 days. Shown are means  $\pm$  95% confidence intervals and minimum  
 626 detectable differences (% MDD) below the x- axis of experiment 1 and experiment 2 (Exp 1,  
 627 Exp 2).

628

629 Figure 4 Algal population trajectories compared between (A) ungrazed (green circles) and  
 630 grazed (without predation, blue diamonds) systems and (B) grazed systems with (red stars)  
 631 and without predation (blue diamonds). Population dynamics of *C.dubia* with (red stars) and  
 632 without (blue diamonds) predation as (C) total individual number and (D) juveniles  
 633 (continuous line) and adults (dotted line).

634



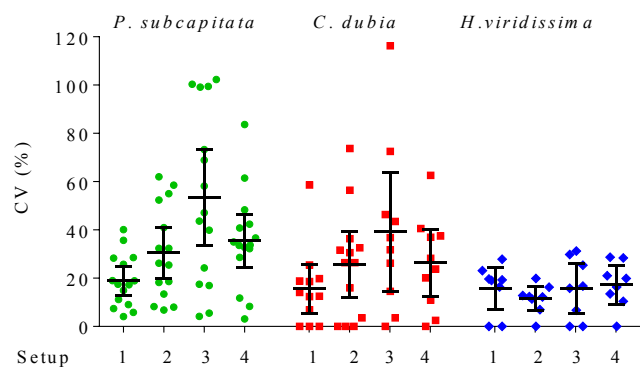
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636 Figure 5

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638

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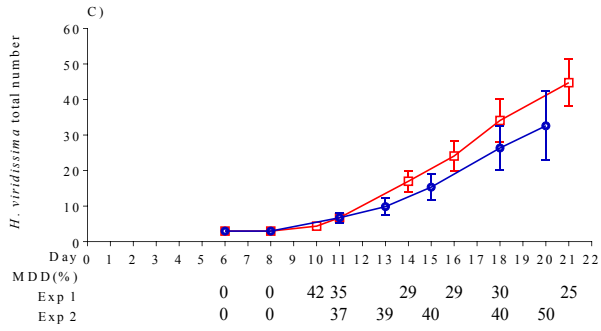
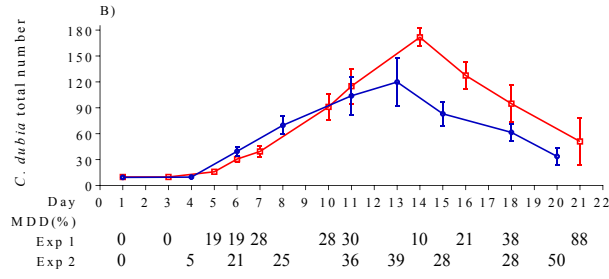
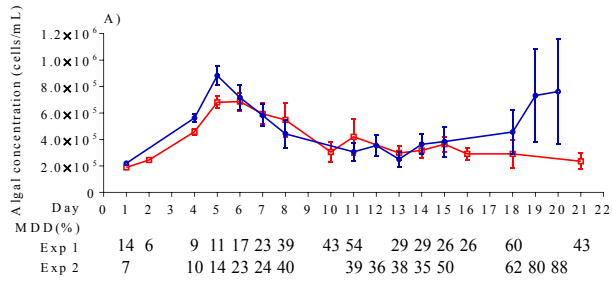


640

641 Figure 2

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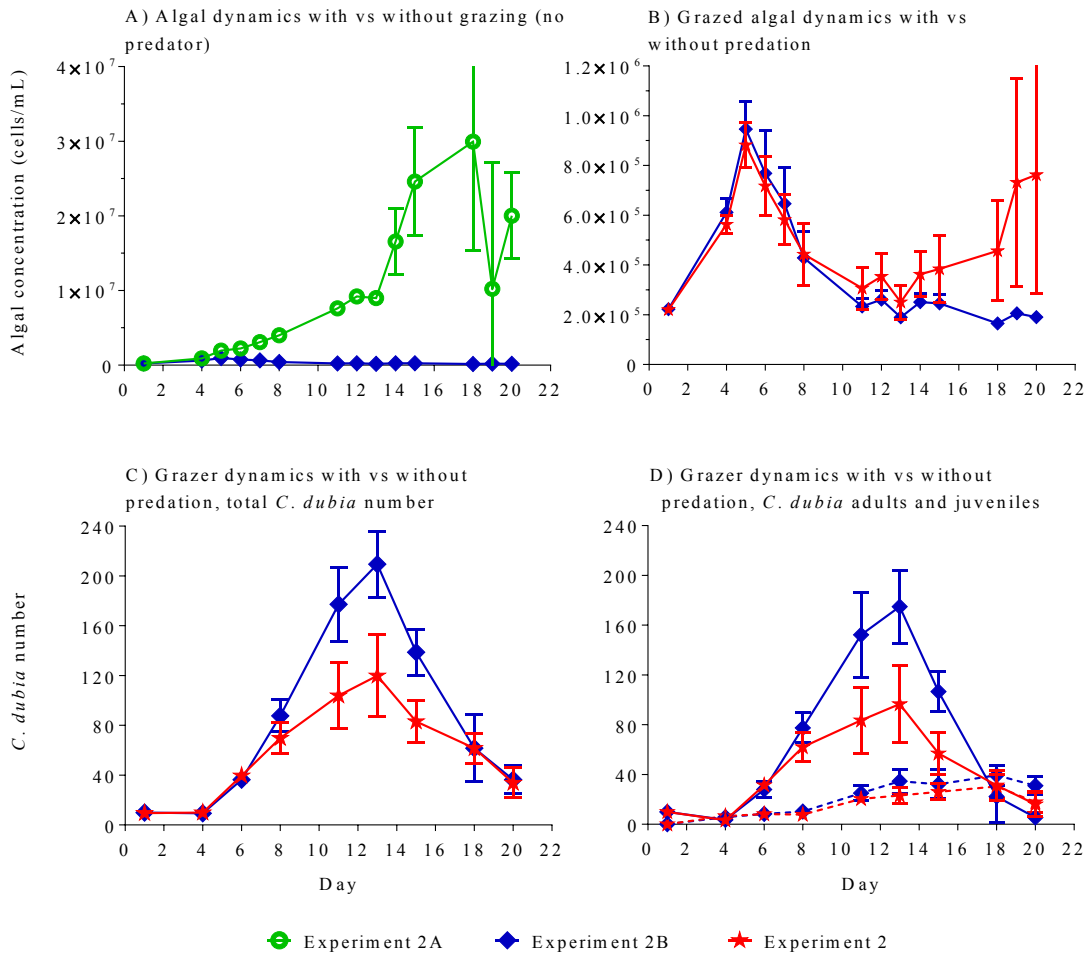
Experiment 1      Experiment 2

644

645 Figure 3

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649 Figure 4

650