Miniaturising acute toxicity and feeding rate measurements in Daphnia magna

Grintzalis, Konstantinos; Dai, Wenkui; Panagiotidis, Konstantinos; Belavgeni, Alexia; Viant, Mark R.

DOI:
10.1016/j.ecoenv.2017.02.002

Document Version
Peer reviewed version

Citation for published version (Harvard):
Grintzalis, K, Dai, W, Panagiotidis, K, Belavgeni, A & Viant, MR 2017, 'Miniaturising acute toxicity and feeding rate measurements in Daphnia magna', *Ecotoxicology and Environmental Safety*, vol. 139, pp. 352-357. https://doi.org/10.1016/j.ecoenv.2017.02.002

Miniaturising acute toxicity and feeding rate measurements in *Daphnia magna*

Konstantinos Grintzalis[1], Wenkui Dai[1], Konstantinos Panagiotidis[1,2], Alexia Belavgeni[1,2], Mark R. Viant[1*]

[1]School of Biosciences, University of Birmingham, Birmingham, B15 2TT, United Kingdom
[2]Department of Biology, Section of Animal Biology, School of Natural Sciences, University of Patras, Patras, 26500, Greece

[*]Corresponding author: Mark R. Viant, Email: m.viant@bham.ac.uk

Keywords: OECD, immobilisation, algae, multiwell plate, automation, metal

Abstract

Phenotypic markers of animal health form an essential component of regulatory toxicology. Immobilisation of neonate water fleas - *Daphnia magna* - as a surrogate measure of their mortality following exposure to a chemical for 24-48 hours forms the basis of the internationally utilised OECD acute toxicity test 202. A second important marker of animal physiology and health is feeding rate, which in *Daphnia* is determined by measuring the algae feeding rate.

Given the widespread use of OECD test 202 for acute toxicity as well as the quantification of feeding rate in toxicological studies of daphniids, significant benefits could result from miniaturising this assay. In particular, miniaturisation would use fewer animals, less media and chemicals, less laboratory space and make the tests more compatible with automation, and therefore could result in considerable time savings. Furthermore, miniaturising phenotypic markers to the ultimate level of a single animal per well would facilitate multiple measurements of other phenotypic markers, such as behavioural responses, which could be integrated at the individual level.

In this study we used a wide range of exposure vessels to evaluate the impacts of systematically varying total media volume, surface to volume ratio and animal density for the acute toxicity testing of cadmium. We demonstrate that *Daphnia* acute toxicity tests using single animals within 24- or 48-well plates produce equivalent results as for traditional test configurations, for different chemicals. Considering algae feeding rates by *Daphnia*, we studied the impacts of varying algae concentration, total volume and animal density. After having demonstrated that multiwell plates can again yield equivalent test results as traditional experimental setups, we used miniaturised test vessels to show the impact of metals on the feeding activity on daphniids for both neonates and adult animals. Overall we confirm the feasibility of a multiwell approach for *Daphnia* toxicity testing that requires less time and materials than a traditional assay and can provide phenotypic characterisation at a single animal level.

Highlights

- Acute toxicity is studied as function of exposure vessel size and animal density
- No significant change in toxicity occurs with miniaturisation
- *Daphnia* feeding rate test is also miniaturised in volume and animals used
- Both endpoints provide automation compatible single animal phenotypic markers

1. Introduction

Aquatic organisms are used extensively for toxicity testing, i.e. of water samples from the environment or of single or mixtures of chemicals. Among those organisms, *Daphnia magna* is widely employed because of its geographical distribution, central role in freshwater food webs, adaptation to a range of habitats and sensitivity to anthropogenic chemicals. As such, toxicity testing protocols have been reported by the U.S. Environmental Protection Agency and the international Organisation of Economic Cooperation and Development (OECD) (Dodson and Hanazato, 1995; Shaw *et al*. 2008; Lampert 2011). Furthermore, *Daphnia* are relatively easy to culture and manipulate in the laboratory.

Phenotypic markers of animal health are central components of regulatory toxicology. Specifically, in *Daphnia magna*, immobilisation of neonates to estimate acute mortality following exposure to a chemical for 24 to 48 hours forms the basis of the internationally utilised OECD acute toxicity test 202 (OECD, 2004). A typical experimental setup comprises of a relatively high volume of media (e.g. 200 ml) and a low animal density to avoid additional stress of the daphniids due to crowding (OECD, 2004). Employment of different media volumes and alternative exposure vessels has been reported for *Daphnia magna* although not as a systematic variation of a wide range of test vessel parameters (Bahrndorff *et al*. 2015; Baumann *et al*. 2014; Powell *et al.* 1996).

Given the widespread international use of OECD test 202, which in part explains why as much as 8% of all experimental data for aquatic animals in toxicological databases is derived from *Daphnia* (Denslow *et al.* 2007), significant benefits could result from miniaturising this assay. In particular, miniaturisation would use fewer animals, lower volumes of media, less test chemical and less laboratory space, and therefore could result in considerable time and cost savings. Furthermore, miniaturising to the ultimate level of a single animal per exposure vessel would facilitate multiple measurements of other phenotypic markers, such as behavioural responses, which could be integrated at the individual level. However, attempting to miniaturise such a test would require taking into account the impact of several different parameters that define the exposure setup such as the total volume (V), the surface to volume ratio (S:V), which describes the shallowness or depth of the exposure vessel, and the animal density.

Another marker of animal physiology and health is feeding rate, which in *Daphnia* is determined by measuring algae feeding over a defined time period. For this assessment of feeding rate, several approaches can be used to indirectly count the algae, including absorbance, chlorophyll fluorescence or radio-labelling of the algae (Agatz *et al.* 2013; Furuhagen *et al*. 2014; Reynaldi *et al*. 2006). In order to achieve sufficient sensitivity, traditional test setups require high numbers of *Daphnia*, focus exclusively on adults that are more than a few days old, and require high volumes of algae. Furthermore, the duration of the test is usually 24 hours, which may influence the results; e.g. sedimentation of the algae during the test. To date there has been no study reported into how several test setup parameters such as media volume, animal density or concentration of algae may affect the test performance. Therefore, miniaturising this test to use less media, fewer animals, and require less time, would provide toxicity test data more cost effectively.

In this study we systematically varied a range of test parameters for both the *Daphnia* acute immobilisation test and algae feeding rate test with the objective to miniaturise these assays, including using multiwell plates. Such miniaturisation should improve the compatibility of these tests with laboratory automation that ultimately could improve the throughput of regulatory toxicity testing. Specifically, for the acute toxicity test that uses neonates, we evaluated several exposure vessels of differing total volume, S:V ratio and animal density, utilising cadmium chloride as a model toxicant. For measurements of algae feeding by *Daphnia*, we studied the impacts of total volume, algae concentration and animal density. Furthermore we sought to demonstrate the applicability of the feeding rate test for animals of different ages.

2. Materials and Methods

2.1. Culturing of *Daphnia magna*.

Daphniids were cultured in conformity with the OECD guidelines in 4 litre beakers containing OECD media, using a 16h:8h light:dark photoperiod at 20^{o}C, and a total of 80 adults. Media was renewed every four days and cultures were fed with an algal suspension (*Chlorella vulgaris*, 0.2 mg carbon per animal per day) supplemented daily with dried baker's yeast (0.2 mg yeast per day) and an organic seaweed extract. We used neonates (<24 hours old, from the third brood from 14 days old adults) for both acute toxicity and algae feeding rate tests, and separately used a range of *Daphnia* ages up to adults for algae feeding rate studies..

2.2. Acute toxicity testing.

For acute toxicity testing, 20 neonates were used per replicate of each chemical concentration, and the exposure conducted for 48 hours in OECD media in the absence of food, using multiple different exposure vessels. The reference condition was set to 200 ml volume of media in a glass beaker, and was compared with all other volumes and exposure vessels (glass beakers, multiwell plates and Petri dishes). Initially we focused on cadmium chloride to study the impacts of total volume (V), surface to volume

ratio (S:V) and animal density on the acute toxicity of *Daphnia magna* neonates in different types of exposure vessels (Supplementary Figure 1). Having measured the acute toxicity under the reference conditions and calculated several effective concentration (EC) values (Supplementary Figure 2), we selected three test concentrations that correspond to three levels of toxicity under the reference conditions (EC25, EC50, EC75) to assess the effects of differing test configurations. Subsequently, we used our recommended miniaturised test setups to study the single animal toxicity of cadmium chloride, nickel chloride and formamide.

2.3. Algae feeding rate.

For algae feeding, we investigated the test configurations using daphniids from neonates to adults. The test was performed in various well plates (6, 12, 24 and 48 multiwell plates) to determine the impacts of total volume, algae concentration and adult *Daphnia* density. Daphniids were incubated for 1 hour in OECD media and the feeding of algae (*Chlorella vulgaris*) was measured using a cell counter (CASY, Roche). The CASY counter requires 0.2 ml from the media appropriately diluted in the linear measuring range of the instrument. In addition, the feeding rate was assessed as a function of animal age (1, 2, 3, 5, 7, 10, 14, 21 and 28 days), and also after exposing daphniids of different ages to metals (Cd, Cu, Ni) to estimate the impact of stress on their feeding performance.

2.3. Statistical analysis.

Statistical analysis was performed using one-way analysis of variance (ANOVA) by means of the GraphPad Prism program. Results were expressed as average±SD and considered statistically significant for $P<0.05$. Following the collection of data as described above, which provided us with experimental measurements of % mortality, we conducted a power analysis (Minitab version 17.1.0, Lead Technologies) to determine the power of our statistical tests. Selecting an effect size of 20%, for n=5 biological replicates and with a standard deviation derived from our experimental data, we determined that our *Daphnia* acute toxicity studies have a power of ca. 0.7. While a power of 0.8 is generally regarded as ideal, this result confirms that n=5 biological replicates is sufficient for us to make robust inferences from our sample data.

3. Results and Discussion

3.1. Miniaturisation of acute toxicity testing.

In this study we investigated the impacts of total volume (V), surface to volume ratio (S:V) and animal density on the acute toxicity of cadmium chloride using *Daphnia magna* neonates in several different types of exposure vessels (Supplementary Figure 1) and our hypothesis was that as we miniaturise the test environment the acute toxicity of the *Daphnia* would change significantly. First, the maximum number of neonates that could survive in each type of exposure vessel using different volumes of OECD media (in the absence of cadmium) was determined (Supplementary Table 1); none of the following experiments exceeded this animal density.

An acute toxicity curve for cadmium (Supplementary Figure 2) was generated using 20 animals per beaker, each containing 200 ml OECD media, which was considered as the reference condition. Using cadmium chloride concentrations corresponding to three levels of mortality (EC25=0,98 mg/l, EC50=1.43 mg/l and EC75=1.91 mg/l, for this reference condition), we measured the differences in % of mortality occurring at these concentrations across a range of exposure vessels (glass beakers, multiwell plates and Petri dishes).

Surface to volume ratio describes an important part of the shape of the exposure vessels, e.g. the shallowness or depth of that exposure vessel. Aquatic organisms have evolved to have an increased surface area in their body plan to increase their drag in the water. This reduces their rate of sinking and allows them to remain near the surface with less energy expenditure. An increased S:V (e.g. a Petri dish) corresponds to a shallow exposure vessel, with less space available for vertical migration. Table 1 shows the % of mortality in high volume exposure vessels at the three test concentrations of cadmium chloride (from 40-200 ml). In terms of observed mortality, no significant deviation from the reference conditions was observed (ANOVA, $p>0.05$) for all of the exposure vessels (for each Cd concentration), indicating no detectable impact of total volume, S:V or animal density for the beakers and Petri dishes (Table 1). Therefore, we conclude that reducing the total volume for the acute toxicity test is feasible.

Table 1. Effect of total volume and S:V on the toxicity of cadmium chloride.

Vessel	Total volume (ml)	Surface:Volume (S:V) cm^2ml^{-1}	Animals per ml	% mortality after $CdCl_2$ exposure		
				0.98 mg/l $CdCl_2$	1.43 mg/l $CdCl_2$	1.91 mg/l $CdCl_2$
Beaker	**200**	**0.17**	**0.1**	**25±7.8**	**46±10.8**	**83±5.7**
Beaker	40	0.85	0.5	19±6.5	49 ±12.5	80±5
Beaker	40	0.12	0.5	20±9.4	46.3±12.5	85±7.9
Beaker	40	0.31	0.5	17±7.6	48±7.6	82±4.5
Beaker	100	0.66	0.2	18±4.5	41.3±8.5	90±13.2
Petri dish	40	1.5	0.5	23±5.7	53.8±13.2	86.3±8.5
Petri dish	100	1.4	0.2	27±4.5	52±9.1	81±4.2

Toxicity is expressed as % of mortality compared to the absence of cadmium chloride in different exposure vessels. Data represent average±SD (n=5) of values expressed as a percentage of mortality. The reference condition is in bold. No statistically significant changes were detected (ANOVA, $p>0.05$).

Next we used well plates to examine the toxicity in smaller volumes. Keeping the animal density constant at one animal per ml in the well plates, with the exception of the 96-well plate for which there was a restriction on the volume used, we compared the toxicity of cadmium chloride in different types and sized multiwell plates (Table 2). Again, no statistically significant changes in % mortality were detected (ANOVA, $p>0.05$) at each of the three concentrations of $CdCl_2$. Therefore, transforming the acute toxicity test into a miniaturised version appears possible. To our knowledge this is the first attempt to perform this test in the most confined condition using a 96 well plate, which shows no significant difference from the other plate designs. Finally, to examine the potential impact of the material of the exposure vessels, we compared plastic multiwell plates with equivalent glass vessels, which were again shown to induce no difference on *Daphnia* mortality (Supplementary Table 2).

Table 2. Performance of different multiwell vessels at the same animal density and their impact on cadmium toxicity.

Vessel	Total volume (ml)	Surface:Volume (S:V) cm^2ml^{-1}	Animals per ml	% mortality after $CdCl_2$ exposure		
				0.98 mg/l $CdCl_2$	1.43 mg/l $CdCl_2$	1.91 mg/l $CdCl_2$
Beaker	**200**	**0.17**	**0.1**	**34.5±4.3**	**64±6.5**	**79±4.2**
6 well plate	12	0.9	1	37.9±4.9	65.7±7.3	76.7±5.2
12 well plate	6	0.63	1	31±6.5	63±5.7	81±4.2
24 well plate	3	0.76	1	31±7	57±3.4	79.2±3.7
48 well plate	1	0.95	1	32.3±4	63.3±3.5	78.3±3.5
96 well plate	0.3	1.23	3.3	36.3±4.8	66.3±4.8	80±7.9

Toxicity is expressed as % of mortality compared to the absence of cadmium chloride in different exposure vessels. Data represent average±SD (n=5) of values expressed as a percentage of mortality. The reference condition is in bold. No statistically significant changes were detected (ANOVA, $p>0.05$).

Animal density represents a potentially important factor in determining toxicity and could be limiting in a small volume environment. We therefore assessed the impact of animal density by maintaining all other vessel characteristics for two different exposure vessels (Table 3). Again, no statistically significant changes in % mortality were detected (ANOVA, $p>0.05$) at each of the three concentrations of $CdCl_2$. This indicated that we could perform the toxicity testing in low volume vessels while concentrating more animals per well in order to save materials (e.g. media) and increase the level of replication.

Table 3. Impact of animal density on cadmium toxicity using multiwell plates and beakers.

Vessel	Total volume (ml)	Surface:Volume (S:V) cm^2ml^{-1}	Animals per ml	% mortality after $CdCl_2$ exposure		
				0.98 mg/l $CdCl_2$	1.43 mg/l $CdCl_2$	1.91 mg/l $CdCl_2$

Beaker	**200**	**0.17**	**0.1**	**27.5±8.2**	**53±8.37**	**79±8.2**
Beaker	40	0.12	0.42	20 ± 9.4	50±11.7	81±5.5
Beaker	40	0.12	0.83	26 ± 8.2	54±6.5	84±6.5
Beaker	**200**	**0.17**	**0.1**	**16.7± 2.6**	**38.3±5.2**	**66.4±7.5**
6 well plate	12	0.90	1.25	16.1± 2.5	43.9±4.9	61.1±6.9
6 well plate	12	0.90	0.83	15±3.2	40±4.5	63.3±6.1
6 well plate	12	0.90	0.43	19±6.5	39.2±4.9	65±4.1

Data represent average±SD (n=5) of values expressed as a percentage of mortality. The reference condition is in bold. No statistically significant changes were detected (ANOVA, p>0.05).

To prove that our miniaturisation approach can be performed using single animal monitoring in the acute toxicity test, we investigated the two multiwell systems (24- and 48-well plates) further. Specifically we measured the % mortality of cadmium chloride, nickel chloride and formamide, each at three concentrations (low, medium and high; Table 4). Relative to the corresponding reference condition (standard glass beaker), we showed that there were no significant differences between the observed toxicities, for each chemical at each concentration. This result strengthens our conclusion that miniaturised exposure vessels using multiwell plates is a suitable system for *Daphnia* acute toxicity testing.

Table 4. Comparison of single animal toxicity testing in 24- and 48-well plates with the reference condition for Cd, Ni and formamide.

Vessel (V; S:V; Animals per ml)		**Beaker (200; 0.17; 0.1)**	24-well plate (3; 0.76; 0.33)	48-well plate (1; 0.95; 1)
Cd (mg/l)	**0.98**	**36.6±4.1**	36.3±4.8	44±5.9
	1.43	**70.7±4.5**	66.3±4.8	63.3±10
	1.91	**86±6**	80±7.9	78.3±3.3
Ni (mg/l)	**6.1**	**14±2.6**	16±2.5	16.3±4.8
	9.1	**39±6.3**	44.2±5.9	42.8±8.3
	14.3	**70±6.3**	67.6±6.5	71.5±6.6
Formamide (%)	**0.5**	**20.3±6.8**	20.8±7	22.2±6.2
	0.85	**59±4**	55±4	56.5±5
	1.15	**65.6±9**	67±6.6	68±6

Toxicity is expressed as % of mortality compared to the absence of metal in different exposure vessels. Data from independent experiments for each metal represent average±SD (n=5) of values expressed as a percentage of mortality. Toxicity was compared for each chemical with the reference condition which is in bold. No statistically significant changes were detected (ANOVA, p>0.05).

Although from our toxicity measurements for cadmium chloride (Tables 1-4) we observe that the % mortality induced by a specific concentration (low, medium, high) may vary slightly across different experiments, in all cases there were no significant differences between the different conditions tested. This subtle variation could also be explained by age-related toxicity as recently reported by Traudt *et al.* (2017a, 2017b) in neonates of *Daphnia magna* who observed a 10-fold difference between their tests in neonates and batch experiment effects. For our study we spot checked the alterations in pH and oxygen and found there were no differences between miniaturised and higher volume vessels (Supplementary Table 3). To our knowledge there has been no study to date describing in such a systematic manner the effects of miniaturisation on *Daphnia* acute toxicity testing.

3.2. Miniaturisation of algae feeding rate

In this study we present a miniaturised approach for algae feeding studies in daphniids. For the initial tests we used both neonates (<24 hours old) and adult *Daphnia* (up to 28 days old) as the minimum and maximum ages, respectively. Initially, various numbers of neonates and adults were tested for performing the test for up to one hour. Neonates, due to their small size, consume a relatively small amount of algae and consequently the number of algae consumed per animal per hour is constant for

different numbers of neonates tested (Supplementary Figure 3A and 3C). In contrast, for adults (28 days), the test is linear for up to one hour (Supplementary Figure 3B and 3C) because adults consume higher amounts of algae, therefore, decreasing significantly the excess of algae in their media more rapidly.

Next we examined the performance of the test for adults. Specifically, we explored the impact of algae concentration, absolute number of algae, total volume of media and the number of adult *Daphnia* (i.e. animal density) on the feeding rate. Daphniids constantly filter their media and remove algae from their environment. Therefore, the concentration of algae in media is crucial to their feeding rate. In addition, the total volume and the absolute amount of algae are also important. The number of daphniids per ml of media will also impact the available amount of algae per animal. Although this concept has been introduced previously in the literature (Shashkova and Grigor'ev, 2013), it has not been studied in *Daphnia* feeding tests.

Keeping the concentration of algae constant and increasing the volume of the well plate results in a proportional increase of the absolute amount of algae. Initially, with constant animal density (one adult daphniid per 1.2 ml), we determined that there was no difference in the algae feeding rate per animal by increasing the volume of the test vessel with a constant concentration of algae (Figure 1A). This proved the reproducibility of our test from smaller to larger volumes as well as repeatability when the vessel parameters are maintained proportionally, i.e. when doubling the volume (for a fixed concentration of algae) the algae feeding rate per individual remained the same when the animal density is held constant.

In contrast, with a constant animal density and constant total volume, and varying the concentration (and therefore the absolute amount) of algae, we concluded that the concentration of algae could significantly alter the test results (Figure 1B). This is explained by the fact that daphniids filter their media to remove the algae constantly.

Finally, by increasing the volume and decreasing the concentration of algae proportionally to achieve the same absolute amount of algae, and keeping the animal density constant (one adult daphniid per 1.2 ml or alternatively to one animal for each test vessel size) we verified again that the more concentrated algae suspension resulted in a higher algae feeding rate in the lowest volume (Figure 1C). Therefore, it is not the absolute number of algae but their concentration that played a crucial role in the test. In addition, particularly for the case of small volumes, daphniid density becomes restricting, e.g. 4 daphniids in 4.8 ml underestimates the individual algae feeding because the animals compete for the limited supply of algae (Figure 1C).

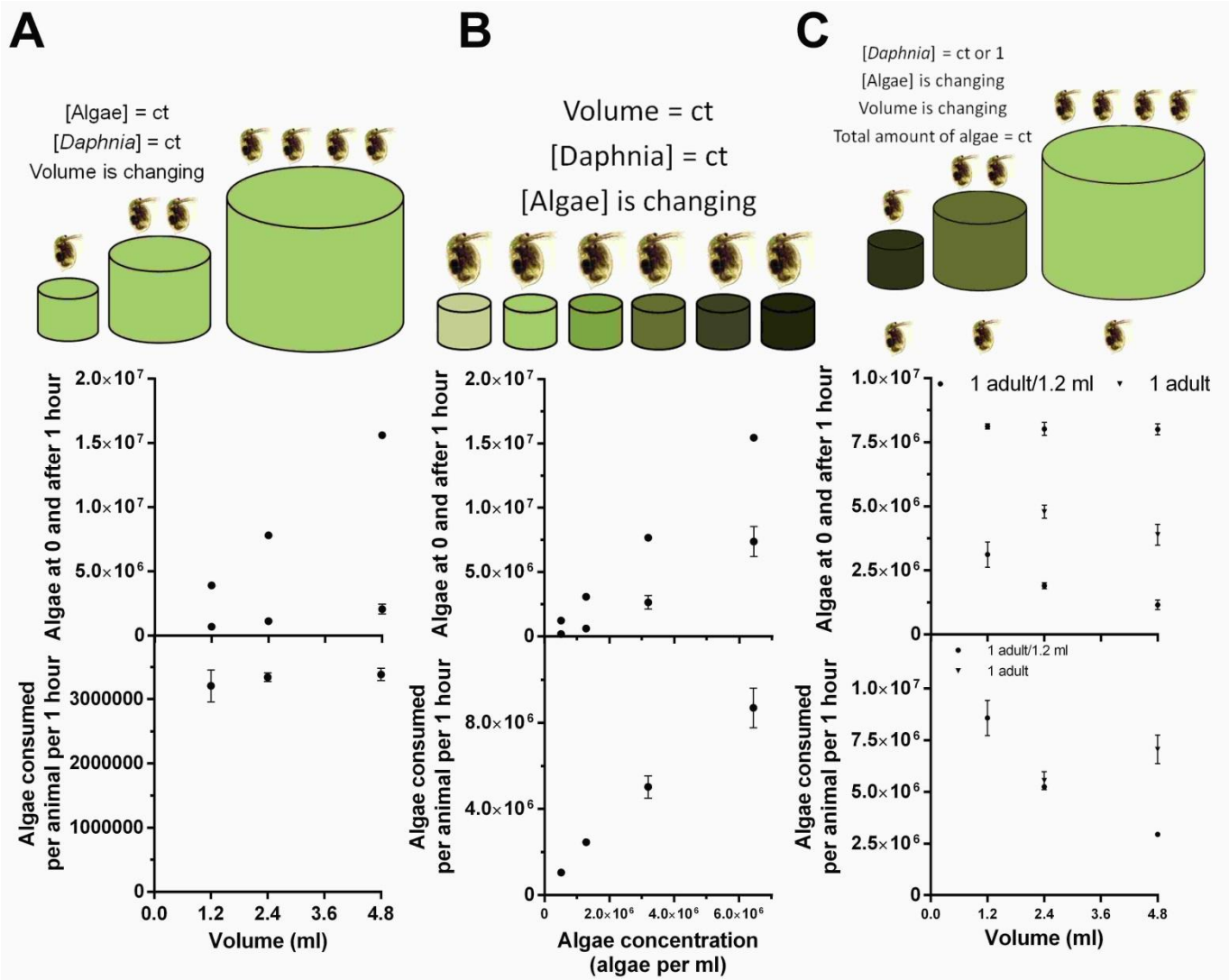


Figure 1. Impact of (A) total volume, (B) concentration of algae and (C) total volume and concentration of algae, on the feeding rate of adults. (A) The density of adult daphniids and the concentration of algae were constant and the consumption of algae was tested in varying volumes. (B) The volume (2.4 ml) and the density of adult daphniids (one adult per 2.4 ml) were constant and the consumption of algae was tested in varying algae concentrations. (C) The animal density was explored at one adult per test vessel and constant at one adult per 1.2 ml with varying volumes and concentration of algae in order to achieve the same absolute algae amount. Data represent average±SD (n=5).

Having standardised our approach, we employed the test system to analyse the impacts of metals on feeding rate as well as study the feeding rate as a function of daphniid growth using the 24 well plate (Figure 2). As daphniids grow, there is an increase in their individual feeding rates (Figure 2A) which is related to their size; this feeding rate is decreased when the animals are starved for 24 or 48 hours (Figure 2A insert). This observation is consistent with the prevailing knowledge that feeding rate is related to the size of the animal since as the animal grows it can filter more media, and explains why adults filter their media more rapidly.

During growth, the response of daphniids to a 24-hour metal exposure is different for different ages (Figure 2B). Finally, exposure of adult daphniids to Cu, Cd or Ni for 24 hours, or exposure for 24 hours followed by a 24-hour recovery, or exposure for 48 hours, results in a decrease to the individual feeding rate both in a concentration-dependent and time-dependent manner (Figure 2C). This decrease does not recover, even when the animals are transferred to clean OECD media for 24 hours.

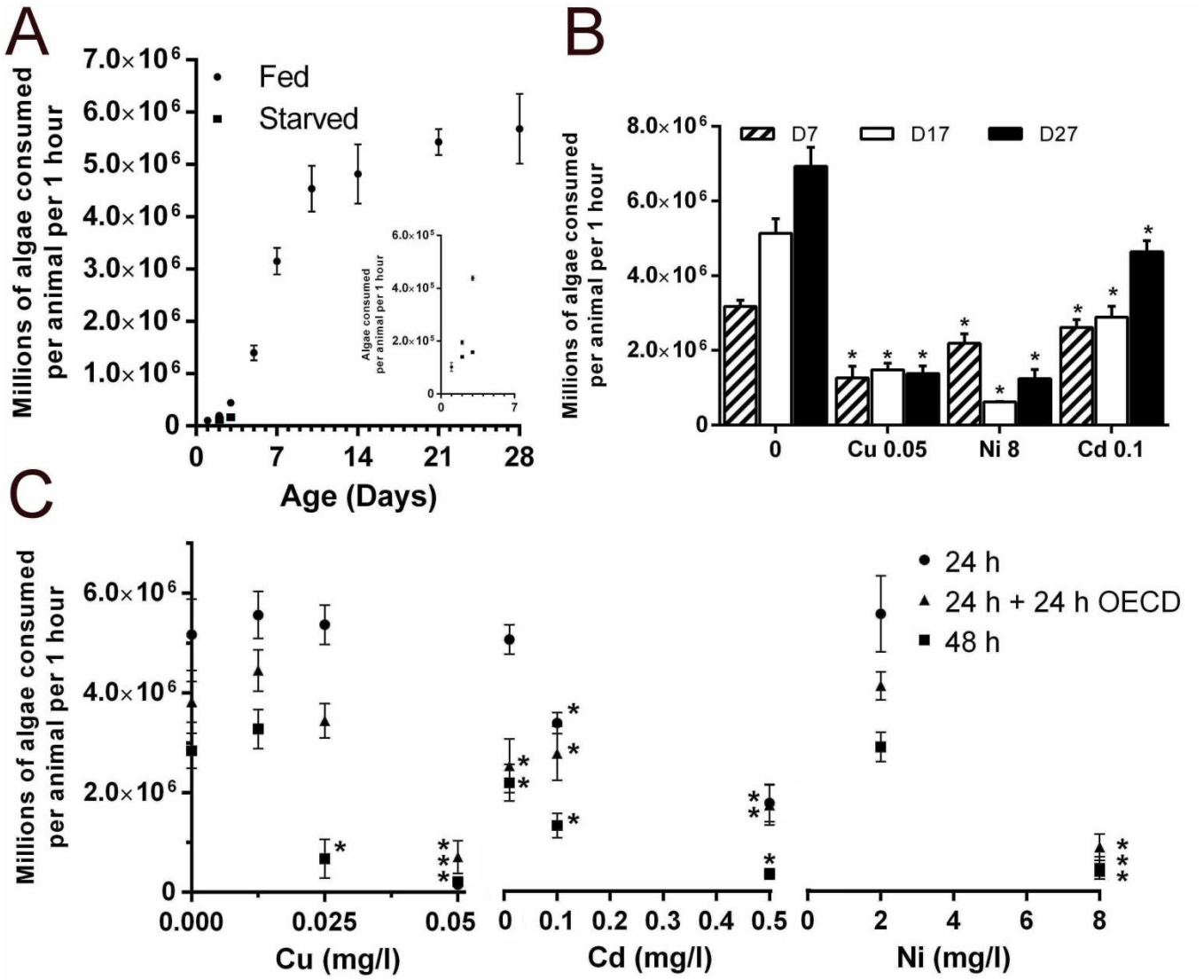


Figure 2. Feeding rate at different growth stage and after exposure to metals. (A) Feeding rate was determined during *Daphnia* growth during feeding (circles) and starvation (squares). (B) Daphniids of different ages were exposed for 24 hours to metal stress and the impact on algae consumption was assessed. (C) The impact of exposure to Cu, Cd, Ni for 24 or 48 hours, or exposure for 24 hours followed by a 24-hour recovery, was assessed for algae consumption using adult daphniids. Data represent average±SD (n=5). *Statistically significant effect of metal treatment relative to unexposed controls, conducted for each age group (ANOVA, $p<0.05$).

In the literature, algae feeding rates are mainly measured using either the fluorescence of chlorophyll or using radio-labelled algae (Furuhagen *et al*. 2014). Shashkova and Grigor'ev (2013) measured algae feeding in a period of 17 hours using several daphniids in culture flasks, while Agra *et al*. (2010) employed five 4-day old juveniles of *Daphnia longispina* in 20 ml algae suspensions. Such approaches require a significant number of animals, and therefore, cannot measure single animal feeding rates, and increased algae volume and testing time for testing, which in turn increases the testing time for several experimental conditions as reported elsewhere (Agra *et al.* 2010). Moreover, increasing the time of an experiment may potentially result in sedimentation of the algae which can introduce measurement variation, also it introduces the confounding factor of daphniid age which potentially affects feeding rates during the test. Our approach is more rapid, providing consistent feeding rates at specific animal ages, is reproducible, and can measure single animal algae feeding allowing multiple measurements of other phenotypic markers on individuals, simultaneously.

4. Conclusions

In this study we present miniaturised test systems for acute toxicity and algae feeding measurements in daphniids. Acute toxicity testing is widely used in ecotoxicological studies in

cladocerans. Using multiple types of vessel, we systematically and extensively studied a complete set of test design parameters (V, S:V, animal density) and optimised the acute toxicity performance for single animals per vessel, which could be combined, for example, with live animal tracking using cameras to automate the monitoring of mobility (Ekvall *et al.* 2013; Chevalier *et al.* 2014). Feeding rate is an important parameter for estimating the allocation of energy supplies in cladocerans and this test could aid in toxicological studies. Our studies show how several parameters, i.e. algae concentration, animal density, and total volume, affect the feeding rate performance of daphniids. We validated the approach for daphniids during growth and applied it to estimate the impact of metal stress on the feeding physiology of daphniids. Importantly, both assays have been miniaturised to enable phenotypic measurements on single animals, opening the door to multi-endpoint, automated testing platforms. With the on-going international shift away from vertebrate animal testing in toxicology, we envision testing in lower animal species will increase substantially in importance. For example, state of the art omics approaches (Bluemel 2012) are beginning to gain support by industry and regulators; such studies conducted on aquatic invertebrates such as *Daphnia* could utilise a miniaturised high throughput toxicity range finding experiment to first select appropriate exposure concentrations for the subsequent omics investigations.

5. Acknowledgements

This study was supported by an Erasmus+ mobility grant to Konstantinos Panagiotidis and Alexia Belavgeni. The authors would like to thank the UK Natural Environment Research Council for funding (NE/I028246/1) and the EU FP7 project FutureNanoNeeds - Framework to respond to regulatory needs of future nanomaterials and markets (FP7-NMP-2013-LARGE-7).

6. References

Agra A.R., Guilhermino L., Soares A.M.V.M., Barata C. 2010. Genetic costs of tolerance to metals in *Daphnia longispina* populations historically exposed to a copper mine drainage. Environ Toxicol Chem. 29, 939-946.

Agatz A., Cole T.A., Preuss T.G., Zimmer E., Brown C.D. 2013. Feeding inhibition explains effects of imidacloprid on the growth, maturation, reproduction, and survival of *Daphnia magna*. Environ Sci Technol. 47, 2909-2917.

Bahrndorff S., Michaelsen T.Y., Jensen A., Marcussen L.F., Nielsen M.E., Roslev P., 2015. Automated swimming activity monitor for examining temporal patterns of toxicant effects on individual *Daphnia magna*. J Appl Toxicol. 36, 896-902.

Baumann J., Sakka Y., Bertrand C., Köser J., Filser J., 2014. Adaptation of the Daphnia sp. acute toxicity test: miniaturization and prolongation for the testing of nanomaterials. Environ Sci Pollut R. 21, 2201-2213.

Bluemel J. 2012. Toxicity testing in the 21st century: Challenges and perspecives. J Dru Metab Toxicol. 3, e112.

Chevalier J., Grote M., Keller M., Pandard P., Cachot J., 2014. A new multi-cell exposure system for continuous tracking of *Daphnia* behavior for toxicity assessments. J Environ Anal Toxicol. 5, 1.

Denslow N., Colbourne J.K., Dix D., Freedman J.H., Helbing C.C., Kennedy S., Williams P.L., 2007. Selection of surrogate animal species for comparative toxicogenomics. In: DiGiulio R., Benson, W.H. (Eds.), Taylor and Francis, Genomic Approaches for Cross-Species Extrapolation in Toxicology, Washington, DC.

Dodson S.I., Hanazato T., 1995. Commentary on effects of anthropogenic and natural organic chemicals on development, swimming behavior, and reproduction of *Daphnia*, a key member of aquatic ecosystems. Environ Health Persp. 103, 7-11.

Ekvall M.T., Bianco G., Linse S., Linke H., Bäckman J., Hansson L.-A., 2013. Three-dimensional tracking of small aquatic organisms using fluorescent nanoparticles. PLoS ONE 8, e78498.

Furuhagen S., Liewenborg B., Breitholtz M., Gorokhova E. 2014. Feeding activity and xenobiotics modulate oxidative status in *Daphnia* magna: implications for ecotoxicological testing. Environ Sci Technol. 48, 12886-128892.

Lampert W. 2011. *Daphnia*: development of model organism in ecology and evolution, In: Kinne O. (Ed.), Excellence in Ecology Series. International Ecology Institute, Oldendorf/Luhe, Germany. 2011.

Organization for Economic Cooperation and Development (OECD). 2004. OECD Guidelines for Testing of Chemicals, No. 202, Daphnia sp. Acute Immobilisation Test, p. 12. Organisation for Economic Cooperation and Development, Paris, France.

Powell R.L., Moser E.M., Kimerle R.A., Mckenzie D.E., Mckee M,. 1996. Use of a miniaturized test system for determining acute toxicity of toxicity identification evaluation fractions. Ecotox Environ Safe. 35, 1-6.

Reynaldi S., Duquesne S., Jung K., Liess M. 2006. Linking feeding activity and maturation of *Daphnia magna* following short-term exposure to fenvalerate. Environ Toxicol Chem. 25, 1826-1830.

Shaw J.R., Pfrender M.E., Eads B.D., Klaper R., Callaghan A., Colson I., Jansen B., Gilbert D., Colbourne J.K. 2008. *Daphnia* as an emerging model for toxicological genomics, In: C Hogstrand, P Kille, (Eds.), Advances in Experimental Biology, Comparative Toxicogenomics, Elvesier Science 2, 165-219.

Shashkova T.L., Grigor'ev Yu.S. 2013. Impact of heavy metals on the trophic activity of *Daphnia* depending on feeding conditions and age of crustaceans. Contemp Prob Ecol., 6, 662-666.

Traudt E.M., Ranville J.F., Meyer J.S. 2017a. Effect of age on acute toxicity of Cd, Cu, Ni, and Zn in individual-metal exposures to *Daphnia magna* neonates. Environ Toxicol Chem. 25, 1-7.

Traudt E.M., Ranville J.F., Meyer J.S. 2017b. Age-related differences in sensitivity to metals can matter for *Daphnia magna* neonates. Integr Environ Assess Manag. 13, 208-210.

Wickramarathna L.N., Noss C., Lorke A. 2014. Hydrodynamic trails produced by *Daphnia*: Size and energetics. PLoS ONE 8, e78498.

Supplementary Material

Supplementary Table 1. Maximum number of animal concentration of neonates surviving in the multiwell plates tested with different volumes.

Vessel	Total volume (ml)	Animals	Animals/ml	% Mortality
6 well plate	8	20	2.5	10±4.5
6 well plate	10	20	2	9.2±3.8
6 well plate	12	20	1.66	10.8±6.7
6 well plate	12	18	1.5	12±7
6 well plate	12	15	1.25	0
6 well plate	12	12	1	0
6 well plate	12	10	0.8	0
6 well plate	12	8	0.66	0
12 well plate	5	8	1.6	0
12 well plate	5	10	2	0
24 well plate	2	5	2.5	0
48 well plate	1	2	2	0
48 well plate	1	4	4	5±7.07
48 well plate	1.5	2	1.33	5
48 well plate	1.5	4	2.67	0

Data represent average±SD (n=5) of values expressed as a percentage of mortality.

Supplementary Table 2. Impact of exposure vessels material.

Vessel	V (ml)	S:V	Animals per ml	% mortality after $CdCl_2$ exposure		
				0.98 mg/l $CdCl_2$	0.98 mg/l $CdCl_2$	0.98 mg/l $CdCl_2$
Beaker	**200**	**0.17**	**0.1**	**13.00 ± 2.7**	**35.83 ± 3.8**	**67.00 ± 4.5**
Beaker	12	1.03	0.83	12.50 ± 2.9	33.00 ± 7.6	62.00 ± 4.5
Petri dish	12	1.08	0.83	13.00 ± 2.6	30.83 ± 5.9	63.33 ± 7.5

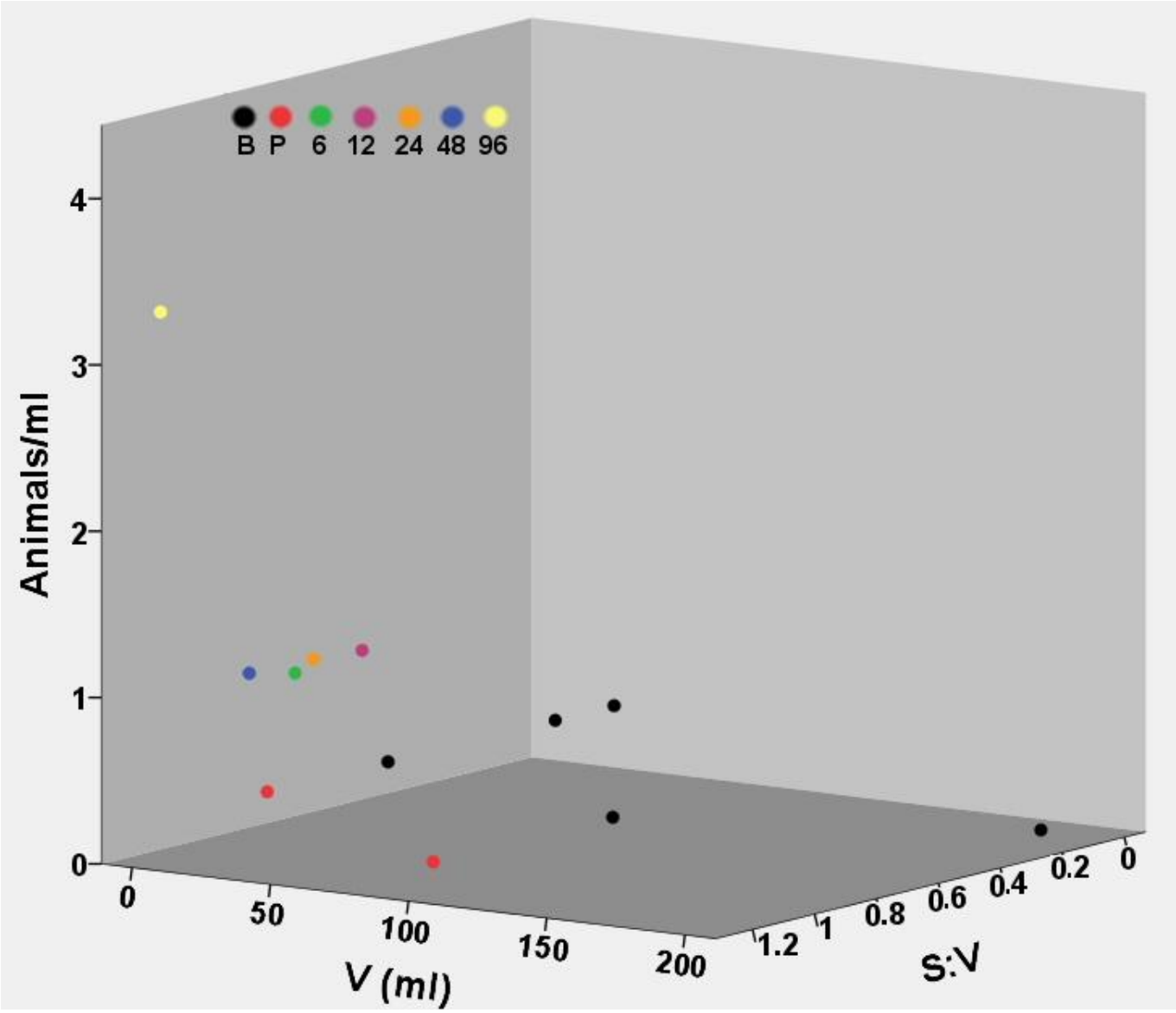
Data represent average±SD (n=5) of values expressed as a percentage of mortality. The reference condition is in bold. Statistical significant values were determined with ANOVA (p>0.05).

Supplementary Table 3. Oxygen and pH were measured in beaker and the 24-multiwell plates to check for differences during miniaturisation after 48 hours exposure.

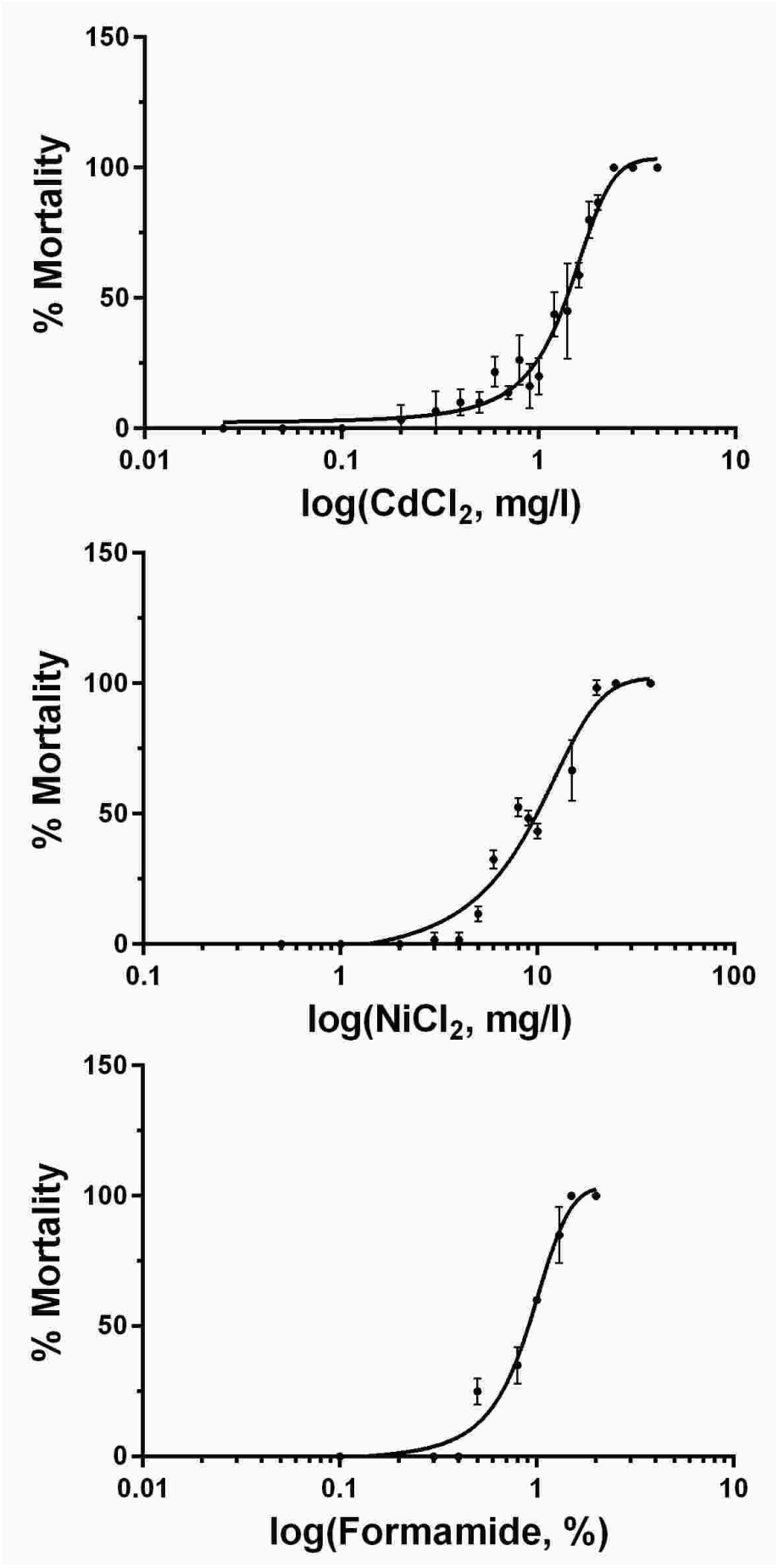
Vessel	Total volume (ml)	Animals	Animals/ml	Oxygen µmol/ml	pH
Beaker	200	0.17	0.1	259.2±5.2	7.364±0.005
24 well plate	3	0.76	1	285.9±5.3	7.366±0.005

Data represent average±SD (n=5).

Supplementary Figure 1. Different vessels are represented with V, S:V, and animal density. Beakers (B) and Petri dishes (P) are indicated by letters, and multiwell plates by their number of wells.



Supplementary Figure 2. Toxicity curves of cadmium chloride, nickel chloride and formamide. Toxicity is expressed as % of mortality compared to the absence of each chemical in the reference condition tested (200 ml volume in glass beakers). Data represent average±SD (n=4) of values expressed as a percentage of mortality.



Supplementary Figure 3. Feeding rate of different number of (A) neonates or (B) adults in 1 hour and for (C) neonates and adults for 1 and up to 2 hours. Data represent average±SD (n=5).

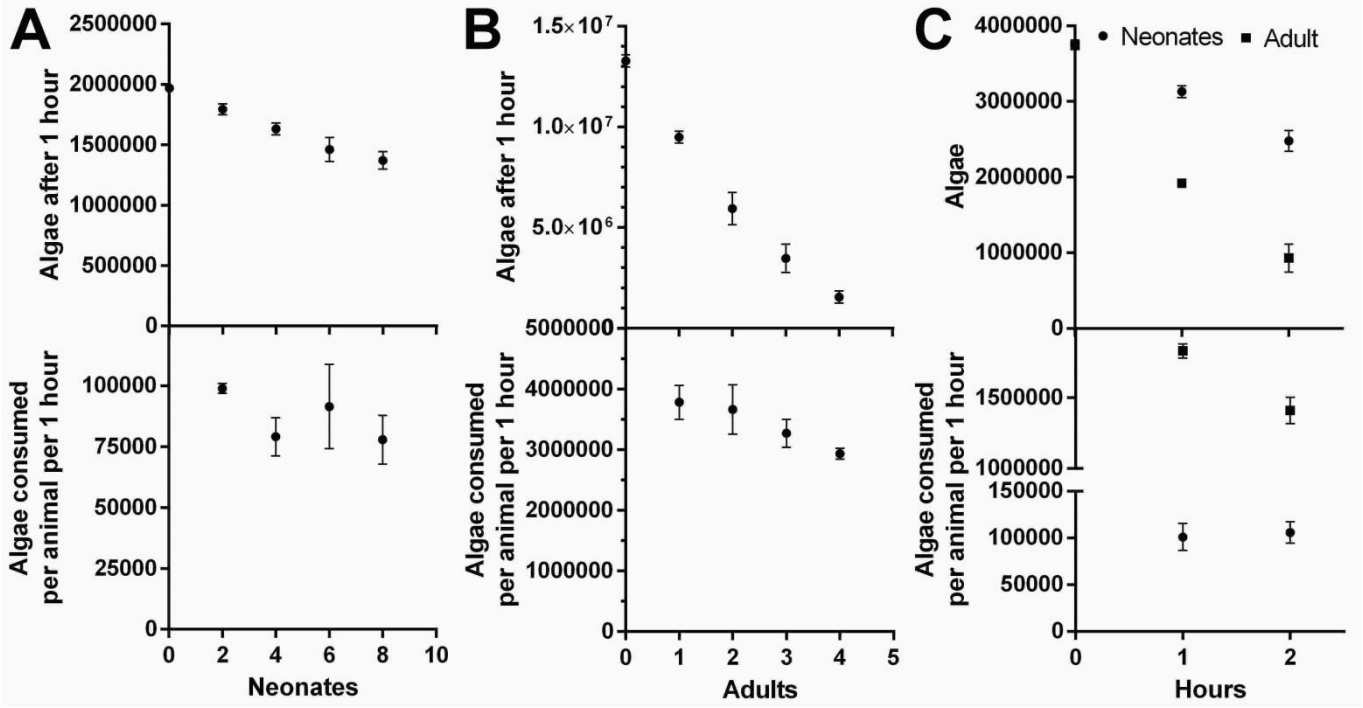


Figure 1 High resolution

Click here to download high resolution image

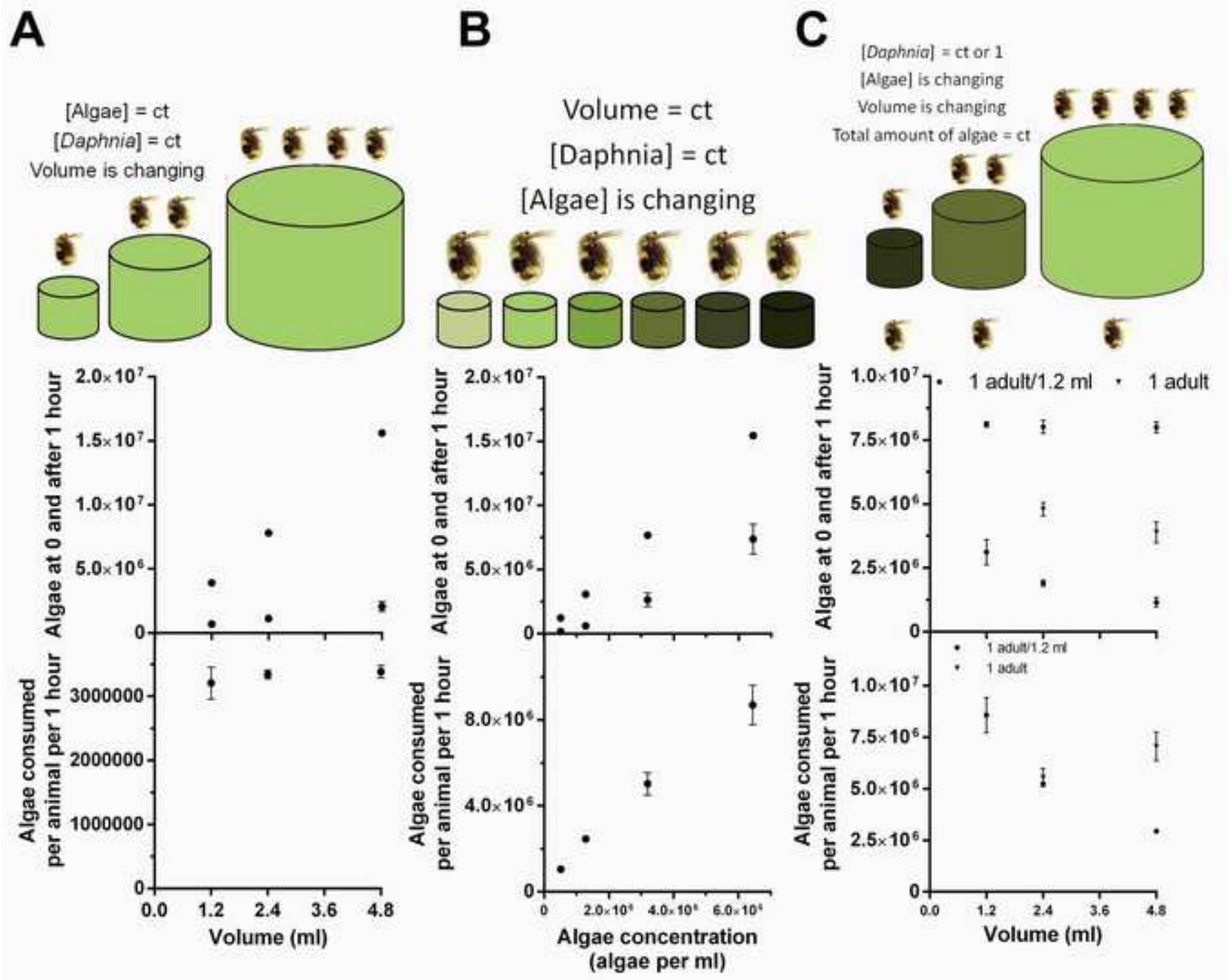
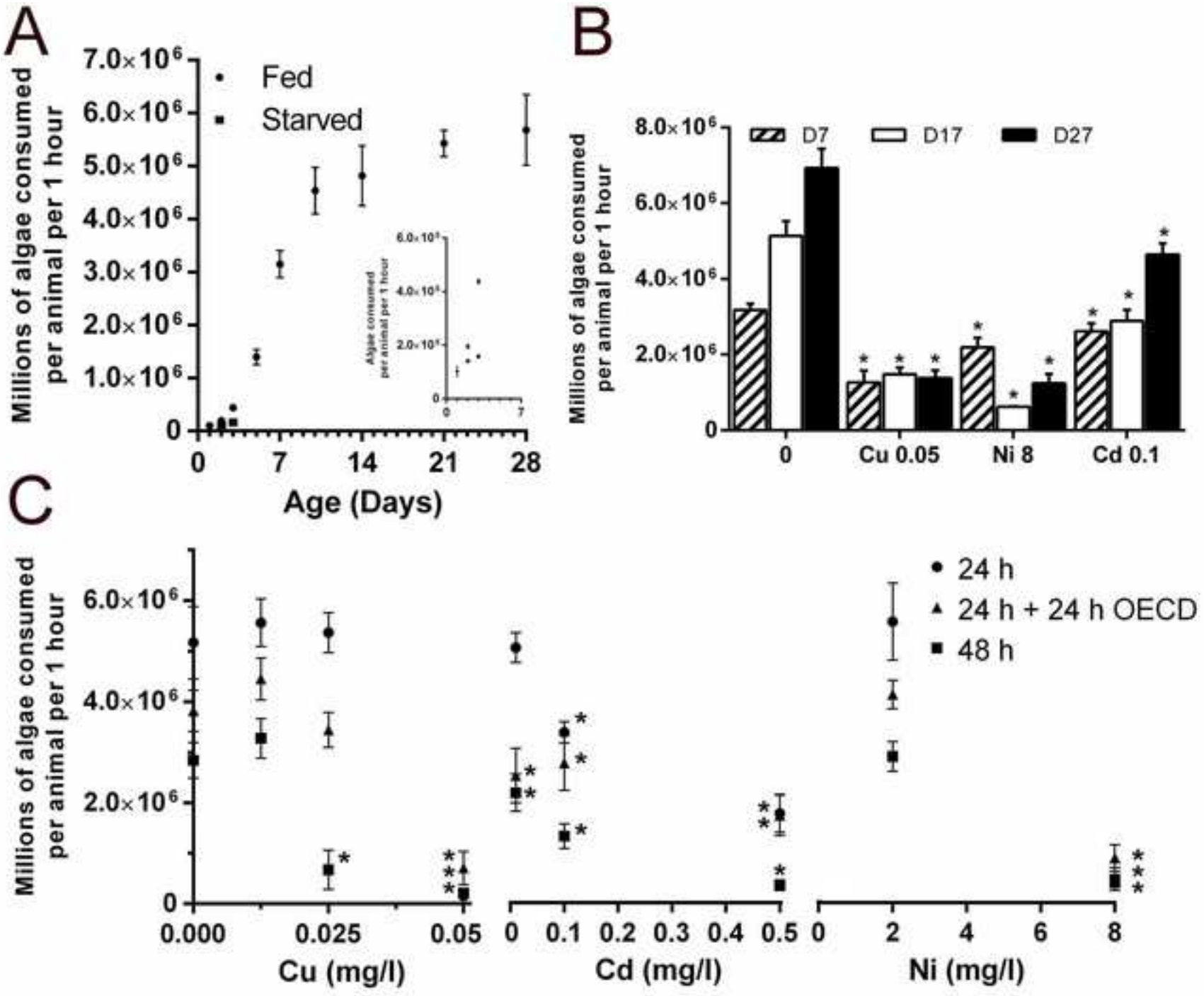


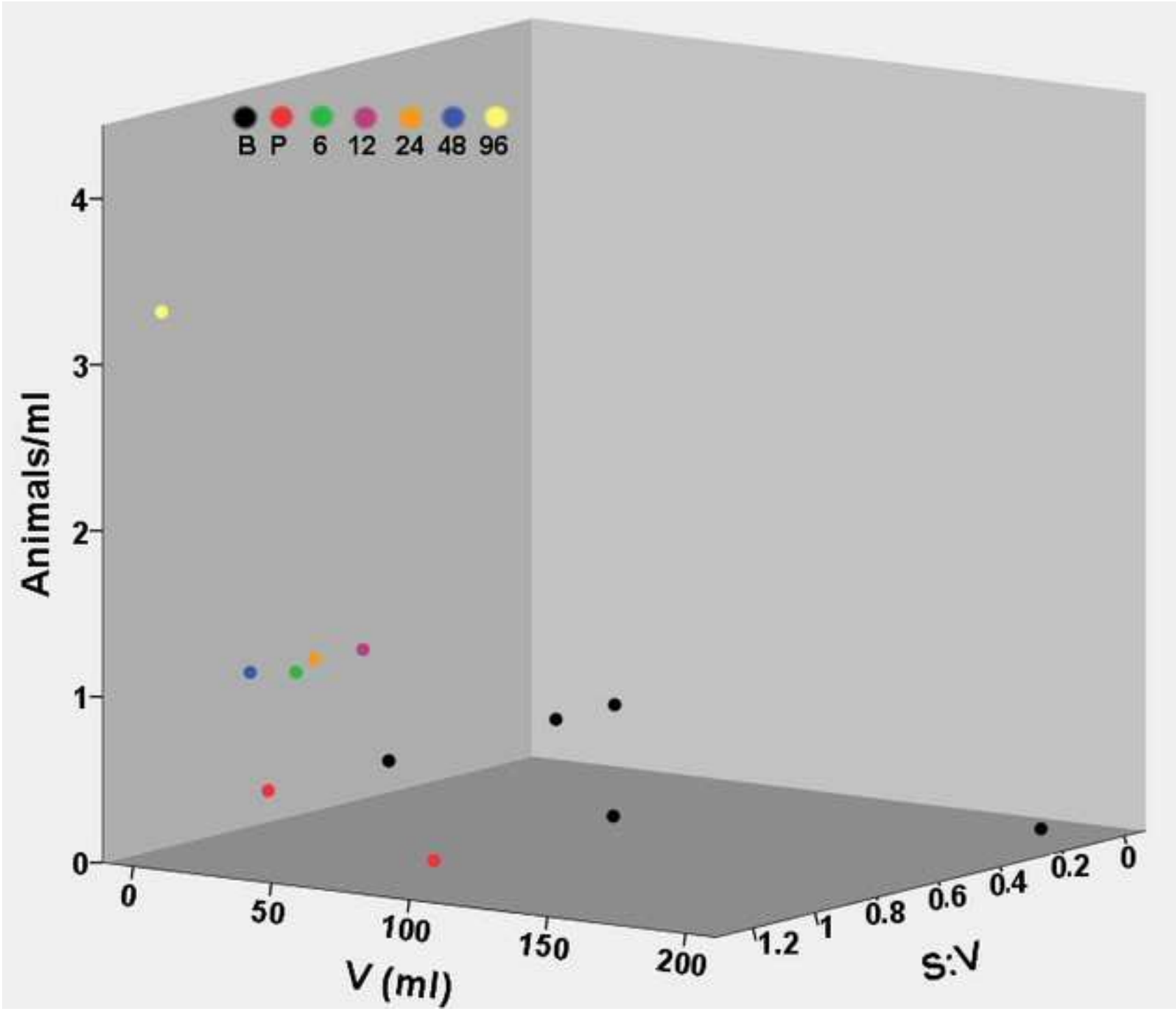
Figure 2 High resolution

Click here to download high resolution image



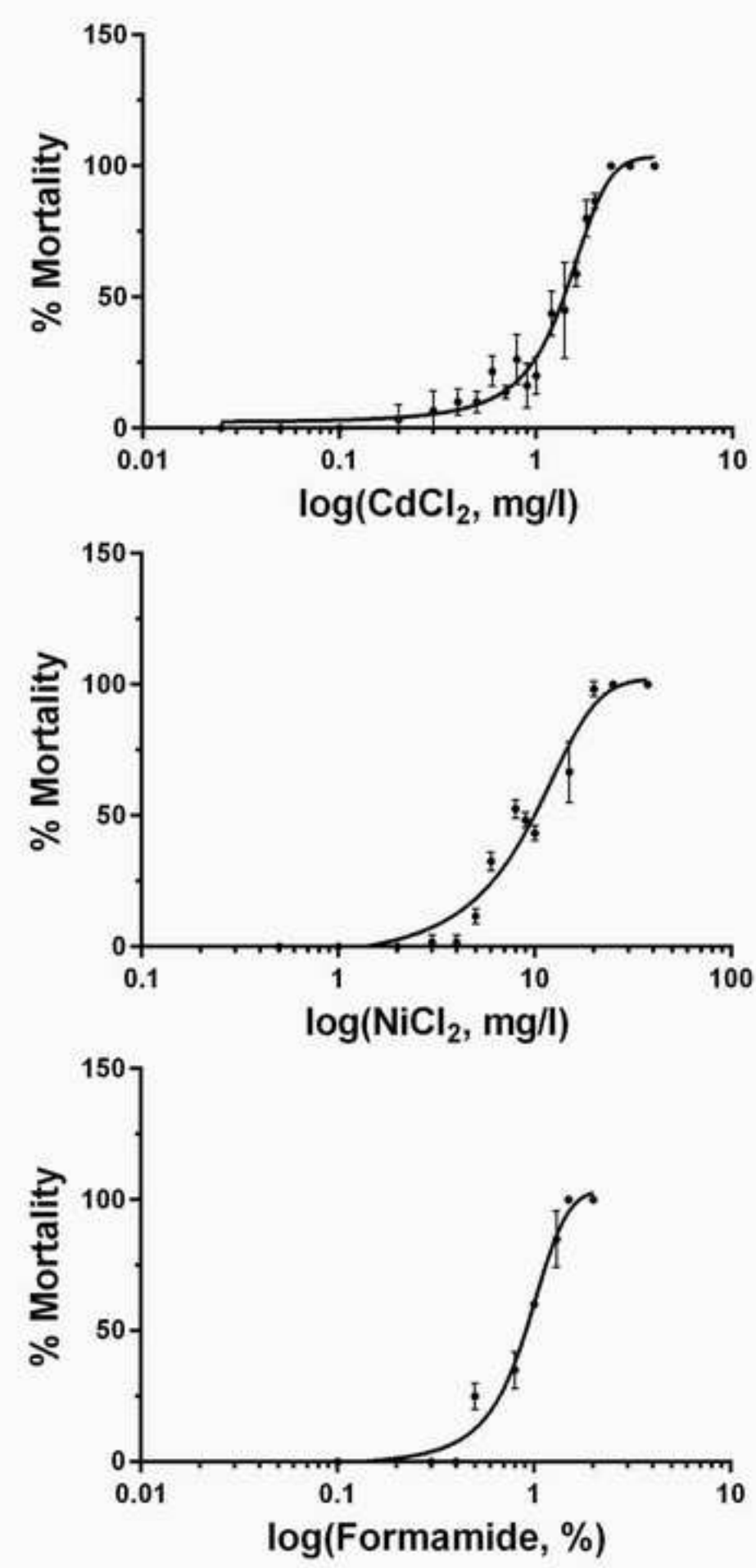
Supplementary Figure 1 High resolution

Click here to download high resolution image



Supplementary Figure 2 High resolution

Click here to download high resolution image



Supplementary Figure 3 High resolution

Click here to download high resolution image