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reliabletesting of low volume samples
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UCL Open Environment

Covering letter

Dear Prof. Osborn,

on behalf of the co-authors I would like to submit the following manuscript „Miniaturisation of the *Daphnia magna* immobilisation assay for the reliable testing of low volume samples” for consideration for publication as a research article in UCL Open: Environment.

We consider our manuscript as a valuable contribution to the journal and its readers because we propose an adaptation of the standardised *D. magna* assay by demonstrating its robustness for a broad range of anthropogenic pollutants.

In the context of hazard or risk assessment of anthropogenic substances (pesticides, pharmaceuticals, nano particles or microplastic), the testing of these substances and mixtures in a fast and efficient but still meaningful and robust way is a matter of discussion within regulatory bodies, scientists and industry. For several test items (e.g. nano particle samples, environmental river samples or extracts thereof) with limited availability of sample volume in conventional high volume formats the testing of a range of concentrations is not possible. To lessen this limitation we analysed literature and tested different methods and propose here a miniaturised format for the *Daphnia magna* acute immobilisation assay. By comparing 15 substances in the conventional and the miniaturised format we demonstrate that the sensitivity of the assay is not affected by the lower volume (→ higher density of animals). Overall this would decrease the effort in testing of precious samples without decreasing the significance of the results. Findings have been presented in part at the 32nd SETAC EU conference in Copenhague 2022 as a poster (1.07.P-We017 “Miniaturised Acute Daphnia magna Assay for the Testing of Low-Volume Samples in the Scope of Monitoring Environmental Contamination”). Beside that, we have not submitted our manuscript to any other journal. All authors declare that they have no conflicts of interest to disclose, and all authors have approved the final version of this manuscript for submission.

Thank you for your time,

Sincerely,

Eberhard Küster

Miniaturisation of the *Daphnia magna* immobilisation assay for the reliable testing of low volume samples

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Abstract

International standard test guidelines for the ecotoxicological characterisation of various substances use organisms like algae, daphnids and fish embryos. These guidelines use relatively high volumes of water for the process of testing. However, for various samples such as extracts from environmental monitoring or leachates from microplastic aging experiments, the amount of available sample volume is limited. Using the exposure volumes as recommended in test guidelines would not allow to test a range of different concentrations or to repeat tests. Lower media volumes would allow the testing of more samples (more concentrations per sample, more test repetitions for statistical robustness) but it may also decrease the possible number of organisms tested in the same volume. Here, we aimed at reducing the test volumes in the acute daphnia assay without impacting animals' sensitivity towards toxicants. A literature review on existing miniaturisation approaches was used as a starting point. Subsequently, assays employing conventional as well as reduced test volumes were compared for 15 selected test substances with a diverse spectrum of lipophilicity. Results showed that there are differences in EC_{50} between the two approaches, but that these differences were overall only within a range of a factor of two to three. Further, by retrieving EC50 values for the genus Daphnia and 15 test substances from the US EPA database, we demonstrated that our results are well inline with the general differences in sensitivities.

Keywords: miniaturisation, extract testing, leachate testing, microplastic, nano particle, environmental monitoring, groundwater, crustacea, pesticide, plankton testing

Introduction

Most guidelines for aquatic ecotoxicity testing were established for the testing of individual substances, usually not restricted regarding their availability. For example, the ISO (1) (6341:2012) and (2) OECD TG 202 recommend test volumes of 2 mL per neonate, adding up to a recommended 10 mL per test concentration when testing 5 neonates per technical replicate. This adds up to a total volume of 50 mL for the generation of a complete dose-response relationship with 5 test concentrations if only a single experiment is done and only one technical replicate is used (controls not included here). With usually using 4 technical replicates per test concentration or dilution, this further increases the needed volume to 200 mL in a single experiment. In consequence, volumes and replicates needed for the testing are normally quite large as restriction of volume or numbers of replicate tests is not an obstacle with single substance testing . These volumes as well as the number of neonates per replicate are seen as prerogative in terms of robustness of data sets and subsequent statistical reliability which is needed for the hazard evaluation of single substances. If one needs to do tests with other standard organisms such as the algae or fish embryo test, the required sample volumes would even increase further.

However, in several cases there is a restriction on sample volumes, for example in ecotoxicological monitoring of environmental water samples. Currently, European ground- and surface water monitoring focuses on chemical analyses (European water framework directory (WFD) and its daughter regulations). As usually not all substances in an environmental sample are analysed, but the mixture of all substances contributes to overall ecotoxicity, applying ecotoxicological tests is seen as a valuable supplemental method to chemical analysis, allowing to include the overall toxicity of all bioavailable substances in a mixture (3–5). At the same time, the sample volumes for the different tests are often restricted as obtaining and preparing water samples for monitoring is elaborate and costly. Chemical analysis usually requires sample volumes in the mL to µL range. In contrast to that, as demonstrated above, ecotoxicological tests with organisms often need sample volumes above 200 mL per sample. This may be one reason why ecotoxicological tests such as the acute Daphnia immobilisation assay is not as often used as it might be helpful. The same restrictions apply to other types of samples such as leachates prepared from microplastics (6,7), fractionated microplastics samples and other materials with limited sample availability (8), Kühnel et al. (under review).

Standard tests with daphnids carried out in our laboratory so far used 15 mL of medium for 5 neonates (1 neonate per 3 mL) and four technical replicates adding up to 60 mL for a single concentration e.g. (9–11). This is in the following referred to as the “conventional approach”. A dose response curve with a 1:2 dilution thus needs 120 mL of sample volume for a single experimental run.

This motivated this study, which aimed at developing a robust but sensitive *D. magna* immobilisation test requiring less sample volume than the conventional assay.

We used the review by (12) as a starting point for our miniaturisation approach and complemented this database by additional approaches retrieved from the scientific literature (e.g. (13,14). This first literature screening indicated three basic approaches to achieve a reduction in sample volume for the miniaturisation of the daphnia assay: reduce the ratio volume-per-neonate (i.e., increase density), reduce the number of concentrations tested or reduce amount of neonates per replicate or concentration. In addition, the impact of miniaturisation on animal fitness and behaviour was studied. Based on this review a scheme for a miniaturised daphnia assay in a 24 well format was developed. In the following, this approach is referred to as "miniaturised approach" (14). As a goal we wanted to demonstrate that under miniaturised test conditions no changes in the overall results in terms of the respective substances EC_{50}s occurred, and that factors such as increased animal density would not impact the sensitivity of the test organisms. This was done by comparing the conventional approach to the miniaturised approach by testing 15 selected chemicals. Finally, our results were put into the context of general sensitivity differences by comparing them to results obtained with the genus Daphnia and the 15 test substances. This was done by retrieving respective EC50 values from the US EPA ECOTOX database.

Material and Methods

Literature review

A literature search for existing miniaturisation approaches for the daphnia immobilisation assay (keywords: daphnia AND miniatur*) in the Web of Science™ database was done, based on the PRISMA guidance paper (15) In addition, the so called Abstract Sifter (16) was used with the "query run: daphnia magna" and the follow up sifter terms "miniaturi", "volume" and "well" to scan the PubMed database. Bibliometric software Zotero (www.zotero.org) was used to find and delete the overlap of both databases. The different miniaturisation approaches were compared with the OECD or ISO standard guidelines especially in relation to sensitivity to positive controls and assay parameters such as used volume or density of neonates (summarised in Table 1). This guided in the development of the miniaturisation approach regarding medium volume and animal density.

Daphnia cultivation and biotesting

Cultivation medium was as described in (17). Adult daphnids were cultured singly in 80 mL of ADaM (Aachen Daphnia Medium, ADaM artificial freshwater) in 100 mL borosilicate Pyrex® glasses (Th. Geyer, Germany). Medium was exchanged completely on Mondays and Fridays. Feeding with microalgae (*S. vacuolatus*) (18) was adapted to the age of adults and done on Mondays, Wednesdays and Fridays (19). Daphnids at age of 1, 2 and 3-5 weeks were fed $1x10^9$, $2.3x10^9$ and $2.7 x10^9$ fL / animal on Mondays and Wednesdays and 1.5, 3.5 and 4.1 $x10^9$ fL of algae volume on Fridays, respectively. On Fridays, the daphnids were additionally fed with 250µL brewer yeast (SIGMA, Seelze, Germany) suspension in destilled water (1g/L). Details of the specific feeding regime are published in the dissertation of Knops, M. (20). The animals were fed with the equivalent of 0.07 mg carbon/ Daphnia/ day.

After the systematic assessment of existing approaches for miniaturisation (Table 1), we focused on increase in animal density i.e. volume reduction to allow the testing of low volume samples. As well, we adopted a multi-well-plate format for easier handling and microscopic observation of immobilisation. Based on this previous considerations, an approach using one-tenth of the regular standard volume of 60 mL was tested and compared to assays conducted with conventional volumes.

Accordingly, the testing was done in a) 15 mL pyrex borosilicate vials closed with a lid (i.e. the “conventional” approach), b) 24- well borosilicate glass well plates (Irlbacher company, Schönsee, Germany, 2 mL volume) (i.e. the “miniaturised” approach). The test substances were dissolved and diluted in ADaM and 15 mL or 1.5 mL was added to each vial or well respectively before adding 5 neonates per vial or well (<24 h of age) with a pipette in a fixed volume of 50 µL ADaM. The pyrex vials were closed with a lid made of PBT (polybutylen terephthalat) screwcaps with inert PTFE-lined rubber discs. The 24- well plate were covered with a self-made glass cover to decrease evaporation. The exposure was done in the dark and at room temperature for 48 hours. After 24 and 48 h, immobilized and dead neonates compared to controls served as effect parameter of toxicity. Immobilisation and any other effects were checked using a stereo microscope (Leica Wild MZ-8, Leica, Wetzlar, Germany) during the use of the well plates. Positive (potassiumdichromate, p.a., CAS RN 7778-50-9, Fluka analytics, Seelze, Germany) and negative controls (ADaM medium) were tested in parallel with each substance. For the positive control, usually two alternating concentrations (EC_{20} and EC_{50}) of a concentration response curve, which was build up over the last years, were used.

Substance selection and Data evaluation

For the comparison of conventional and miniaturised approach, 15 substances were selected (aldicarb, benzylcarbamate, chlorpyrifos, diazinon, dimethoate, erythromycin, methanol, metolcarb, N,N-Dimethylphenylcarbamat, pirimicarb, potassiumdichromate, SDS, tebuconazol, terbutylazine and tramadol; see Table 3). Selection criteria included lipohilicity as well availability of data for the conventional approach. For each substance, dose-response curves were modelled using SigmaPlot software (vers. 13) and EC_{50} values calculated. The EC_{50} values were compared and differences below a factor of 3 were considered to reflect comparable sensitivities of neonates towards the respective substance in both test approaches.

ECOTOX database data retrieval

Beyond the comparison of neonate sensitivities in the conventional and the miniaturised approached, also the general sensitivity of daphnids over various test formats, species as well as additional variations in tests for the 15 substances was assessed. For the calculation of the geometric mean of test results of daphnid exposed to the selected test substances, data from the US EPA Ecotox database were used. Geometric mean is the metric used to compute species specific average sensitivity when multiple data are available. Data retrieval from the Ecotox database was similar for all substances and the following selection criteria were used: Habitat: Aquatic, Chemicals: CAS RN, Effect measurements: Mortality groups → Mortality, Endpoints: Concentration based endpoints → LD_{50}, LC_{50}, EC_{50}, ED_{50}, Species: daphni*, Test conditions: observation duration (Num days)_ 2, Exposure media → water (salt & fresh), Exposure types → Only aquatic & static, Test location_ lab.

Results

Literature search

Eleven studies on miniaturization could be identified with the above-mentioned keywords in the literature database(s) and using the Abstract Sifter (16) specifically using small volumes or microtiter- or wellplates of different sizes. Results are summarized in Table 1. The original abstract sifter file included 3801 publications dating back to the year 1926 (keyword query run “daphnia magna”). Usage of three sifter terms gave ten publications with a frequency count similar and above 4. A screen shot of the first 42 publications found with the abstract sifter can be seen in the appendix (Table_A2). Data show that in comparison to the OECD and ISO guidelines (conventional approach) the range of the different parameters sometimes cover three orders of magnitude i.e. the volume needed per replicate ranges from 200 µL to 200 mL (mean of 9 mL). The animal density (neonates/ mL) covers a little more than one order of magnitude (ranges from 1 neonate per 0.1 to 6 mL and a mean of 1.5), as does the number of neonates needed per sample (ranging from 10 to 80 animals, mean 18). Regarding daphnia sensitivity, no major differences were observed, and no minimal requirements regarding volume or water column height were made. Accordingly, the approach for miniaturisation tested here would be in the lower range of volume/ replicate, neonates and volume per sample needed but would be in the upper range of the animal density (3.3 animals/ mL). From the density point of view it is equal to a single neonate/ 0.3 mL as it was used by (12) in a 96-well plate. Irrespective of the different volumes, neonate numbers and densities, the material of the testing containers was borosilicate glass and polystyrene material.

Table 1: Overview of miniaturisation approaches reported in the literature and the respective volumes and formats that were adopted or compared.

Volume in technical replicate (mL)	Neonates / technical replicate	Density (neonates/mL)	technical replicates/ sample*	Neonates/ sample* (or concentration replicate)	Total vol. needed/ sample*	Format & material of test container	Reference
10	5	0.5	4	20	40	Chemically inert material (no specific format recommended)	(2) & (1)
15	5	0.33	4	20	60	15 mL pyrex glass vial	(9–11)
1.5	5	3.33	2-4	10-20	3-6	24 well (Glass)	This study
10	5	0.5	4	20	40	Glass beaker,	(14)
10	5	0.5	4	20	40	6 well (PS),	
2	1	0.5	10	10	20	24 well (PS)	
10	5	0.5	4	20	40	6 well (PS)	(21)
2	1	0.5	10	10	20	24 well (PS)	
200	20	0.1	4	80	800	Glass beaker	(12)
12	10	0.83	4	40	48	Glass beaker	
12	10	0.83	4	40	48	Petri dish	
8- 12	8-20	0.66-2.5	4	32-80	48	6 well (PS)	
6	10	1.66	4	40	24	12 well (PS)	
3	18	6	4	72	12	24 well (PS)	
1	3	3	4	12	4	48 well (PS)	
0.3	1	3.3	4		1.2	96 well (PS)	
1	1	1	20	20	20	48-well titer plates (PS)	(13)

10	5	0.5	2	10	>20	12 mL plastic (PS?) cell wells	(22)
1	5	5	4	20	4	24 well (PS)[(3)]	(23)
10	5	0.5	>1[(2)]	20	40	24 well (PS)	DaphtoxKit F Benchprotocol (Microbiotests Inc.)
0.2	1	5[(3)]	3-4			24 well[(1)] (PS)	(24)
10	5[?]	0.5[(3)]	3-4	20	200	Glass tubes	
20	10[?]	0.5[(3)]	3-4	40	80	Glass beakers	

all volumes: mL, PS: polystyrene, *sample = single concentration in a dose response relationship or replicate of env. sample, [(1)] taken from SI Table 3 of Di Paolo et al. 2016, [(2)] as deduced from the benchprotocol (downloaded at www.microbiotests.com, March 2022) by the company Microbiotests Inc. [(3)] deduced from the paper

Comparison of miniaturised acute daphnia bioassay with the standard bioassay based on literature data

The studies listed in Table 1 used a variety of control substances to compare their datasets for large volume versus low volume daphnid tests. These assays were of course adapted for different reasons but will be used here as a standard of comparison for our own results. In Table 2, we hence summarized the respective EC_{50} values and general conclusions that have been made by the authors on the miniaturisation approach. Overall, none of the EC_{50} values differed more than a factor of two between the conventional and the miniaturised tests and all authors of the studies thus did assume a good comparability of the two methodological approaches.

Table 2: Overview of reference chemicals that have been used to compare daphnia sensitivities between test set-ups of miniaturised daphnia assays and standard guideline volume test set-ups

Substance (CAS RN)	Miniaturised Test EC_{50} (mg/L) *(all concentrations nominal)*	Standard Test (mg/L) or *literature data*	Conclusions (copied from references)	Reference
- Kepone (143-50-0)	1.6	1.6	"Toxicity values, as well as the variation among tests, using the miniaturised test system were very similar to those values using the standard U.S. EPA methods. Therefore, it appears that the miniaturised test system can be used to conduct toxicity tests and provide accurate results."	(13)
-Linear alkyl benzene sulfonate (LAS), (-)	8.4	7.66		
- Pentachlorophenol (87-86-5)	2.23	2.73		
- Sodium lauryl sulfate, (151-21-3)	21.8	12.7		
- Synthetic effluent composed of 12 chemicals (each 1 mg/L)				
Triclosan (3380-34-5), (dosing via spiking of extracts of pristine creek water with triclosan)	Modelled EC_{50} range of three independent test labs: 0.351-0.516	*Geometric mean from reported literature in DiPaolo et al: 0.403*	"EC_{50} values obtained with the different test set-ups in different laboratories are in good accordance, tests show comparable sensitivity"	(24)
Acridine (260-94-6), (dosing via spiking of extracts of pristine creek water with triclosan)	Modelled EC_{50} range of four independent tests: 3-5.1	*Geometric mean from reported literature: 3.76*	See above	(24)
Cadmium chloride	0.98-1.4	1.4	"Although from our toxicity	(12)

(10108-64-2)		1.4-1.91	measurements for cadmium chloride … we observe that the % mortality induced … may vary slightly across different experiments, in all cases there were no significant differences between the different conditions tested.”	
Nickel chloride	9.1-14.3			
Formamide	~0.8		The same observation was made for nickel chloride and formamide.	
K2Cr2O$_7$	0.518	0.557	“The sensitivity of daphnids towards $K_2Cr_2O_7$ was comparable (based on EC_{50} values) between test set ups”	(14)
$AgNO_3$	0.0031	not analysed/ analysable	“Comparable $AgNO_3$ toxicity was also reported by others (Allen et al. 2010; Asghari et al. 2012; Karen et al. 1999)”	(14)

Comparison of our miniaturised acute daphnia bioassay with the conventional bioassay by testing 15 selected substances

Fifteen substances with existing data for the conventional approach from the UFZ laboratory, as well as with increasing logKow were tested in the miniaturised assay to evaluate the possible differences in the sensitivities due to laboratory-specific handling, cultivation etc.(Fig. 1 & Table 3). Problems with daphnia swimming behaviour or deviation from normal behaviour due to reduction of height of the water columns was not observed. The exact physico-chemical and other information about the substances are collected in Table 3.

For the comparison of both test approaches, only EC_{50} immobilisation (48h exposure) values were used. Parameters of all concentration-effect curves are shown in Appendix (Table A_1).

Key results, as also presented in Fig. 1, show a toxicity range in terms of EC_{50} for all test substances of roughly between 1 and 100 µmol/L. Two substances – Chlorpyrifos and Dimethoate- were specifically more toxic than the rest (data ranging from 0.0001 – 0.001 µmol/L). The EC_{50} values of the miniaturised toxicity tests, as performed in our lab, indicate a general trend of a slightly higher sensitivity, with two deviations between conventional and miniaturised assay for erythromycin and potassiumdichromate. An exception was SDS, which showed less toxicity in the miniaturised assay. The negative controls did not show any difference in immobilisation between the two test approaches.

Ecotox database retrieval for comparing miniaturised with conventional daphnid tests

Table 3 also shows the literature data retrieved from the US EPA ECOTOX database for the 15 selected test substances (https://cfpub.epa.gov/ecotox/) (see also: (25)). Data are depicted as the geometric mean of all retrieved data (see Material & Methods for exact search parameters). Figure 1 is the graphical presentation of Table 3. Key results show that lipophilicity (as $logK_{OW}$) ranged from -0.77 to 4.96 (Methanol & Chlorpyrifos, respectively) with an equal number of substances from $logK_{OW}$ <1-2 and >2. Baseline toxicity, -as calculated with the formula published in the ECOSAR software (Version 1.11), varied over 5 orders of magnitude with predominance of substances with a baseline toxicity of between 0.1 and 2 mmol/L. Chlorpyrifos was the substance with highest baseline toxicity (0.0011 mmol/L) while MeOH had the lowest (588 mmol/L). Comparison of EC_{50} values retrieved from the ECOTOX database, the conventional approach (analysed in glass) and the miniaturised test (also analysed in glass) actually did show differences between the EC_{50}. But these were not greater than a factor of 2-3.

Table 3: Toxicity data of the 15 single substances tested in our lab under both the conventional and the miniaturised test protocol (all concentrations are nominal) with 48 hours exposure and immobilisation as endpoint

Testsubstances (alphabetical order)	CAS RN (MW) *logKow*	Watersolubility [1] (chem-dashboard) µmol/L exp. or predicted median	Baseline tox _Daphnids _48h, (µmol/L) [4]	Geometric mean of daphnid tests collected from the ECOTOX database [3] (µmol/L) n= number of found & used data	OECD202 standard (this study) EC_{50} mg/L [2] µmol/L	miniaturised test (this study) EC_{50} mg/L [2] µmol/L
Aldicarb	116-06-3 (190.26) *1.13*	31,600	1654	1.341 n= 10	0.7 3.679	0.3546 1.864
Benzyl-carbamate	621-84-1 (151.165) *1.20*	447,000	2276	- (no data in ECOTOX db) n=0	80-90 562.3	64.17 424.5
Chlorpyrifos	2921-88-2 (350.58) *4.96*	3.19	1.004	0.000697	- (not tested)	0.0001301 0.0003711

				n= 28		
Diazinon	333-41-5 (304.35) *3.81*	153	11.7	0.0023 n=37	0.0003 – 0.0008 0.0051	0.0003645 0.001198
Dimethoate	60-51-5 (229.2) *0.78*	142,000	5871.7	7.7353 n= 12	1.5941 6.955	0.2107 0.9193
Erythromycin	114-07-8 (733.93) *2.83*	355	181.3	32.700 n=1	240 327	29.05 39.58
Methanol	67-56-1 (32.04) *-0.77*	31,200,000	84596	- (no data in ECOTOX db) - n=0	18.26/ 3.29 569.9/ 102.7	13.96 435.7
Metolcarb	1129-41-5 (165.079) *1.70*	158,000	812.1	- (no data in ECOTOX db) - n=0	0.06 0.363	0.0343 0.208
N,N-Dimethyl phenyl carbamat	6969-90-0 (165.079) *1.56*	27,300 (predicted median)	1065.9	- (no data in ECOTOX db) - n=0	4 24.23	1.464 8.868
Pirimicarb	23103-98-2 (238.29) *1.70*	11,300	1528	0.080 n=1	0.01013 0.0425	0.005736 0.02407
Potassiumdichromate	7778-50-9 (294.19)	390,910,000	-	1.019	1.36 4.623	1.32 4.487

				n= 27		
Sodium dodecyl sulfate (SDS)	151-21-3 (288.4)	4,610	-, calculated as surfactant: 187.2	33.588 n=82	5.55 19.244	9.64 33.425
Tebuconazole	107534-96-3 (307.82) *3.70*	117	11.1	20.52 n=4 (data from enantiomers included)	7.2798 23.65	13.92 45.22
Terbutylazine	5915-41-3 (229.710) *3.21*	37.2	37.8	- (no data in ECOTOX db) - n=0	3.9365 17.137	11.08 48.24
Tramadol	27203-92-5 (263.381) *2.63*	1,260	63.2	- (no data in ECOTOX db) n=0	97.8675 371.58	46.99 178.40

(1) data from Chemistry dashboard (https://comptox.epa.gov/dashboard), experimental data or predicted median

(2) complete concentration-effect relationship parameters in the Appendix Table_A1

(3) method of data retrieval: see methods section

(4) ECOSAR tool (Version 1.11) used within the Epi Suite software (neutral organic SAR)

logKow: from Epi Suite (experimental data used, if existing) via www.chemspider.com homepage

Data from the ECOTOX database usually covered a range of at least 3 orders of magnitude. The EC_{50} of the miniaturised toxicity tests usually were within a factor of 2-3 to the geomean of the literature data with the three exceptions dimethoate, pirimicarb and potassiumdichromate. Dimethoate and pirimicarb data from the miniaturised assay did show lower EC_{50} than the published ECOTOX database data (roughly a factor of 8 and 4 with dimethoate and pirimicarb, respectively - pointing to a slightly higher sensitivity. In contrast, the sensitivity of the miniaturised but also from the conventional assay to potassium dichromate was about 4 times less sensitive than the geomean of published data. For a few substances, only a very low number of data/ or no data at all could be retrieved from the ECOTOX database. Here, no comparison with our data was possible.

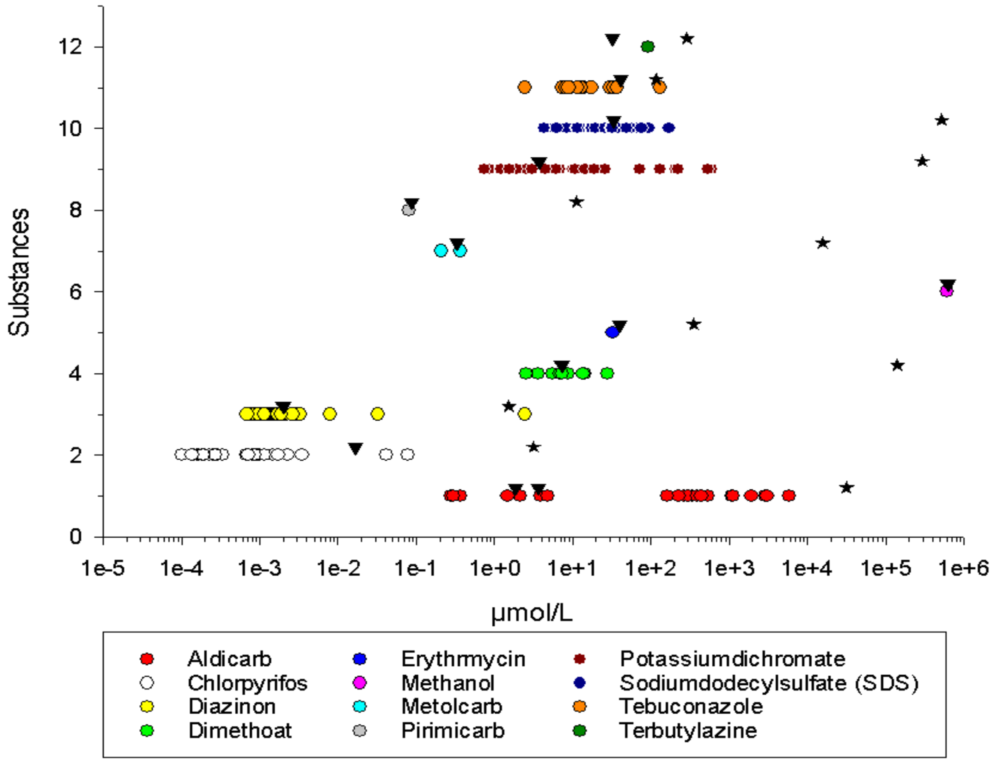

Fig. 1: retrieved US EPA ECOTOX EC_{50} data (acute daphnia immobilisation after 48h) of the 15 test substances in comparison to UFZ Biotox data (miniaturised approach_black triangles). In addition, the water solubility limits are shown (star symbols). Two independent miniaturised tests were done with Aldicarb (thus two triangles are depicted in the fig.).

Discussion

In recent aquatic monitoring and assessments, the sample volume sizes decreased steadily. For example, in the frame of the German national action plan (NAP) to evaluate the pesticide contamination of creeks close to agricultural used land ("Small Stream Monitoring Project" financed by the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU), research code 3717634030), https://www.ufz.de/kgm/index.php?en=44480. Within this project, the pesticide contamination of small streams in Germany was assessed with diverse methods. One assessment approach included the rain event sampling ("agricultural run-off") of water and the concentration, extraction and finally, quantification of the water contaminants such as pesticides. These extracts were then supposed to be analysed via different bioassays to evaluate their ecotoxicological activity in parallel to the known pesticide contamination. As many bioassays needed to be tested, the sample volume for each bioassay was restricted. One main motivation in this special monitoring project was hence the need to deal with low sample volumes (e.g. water extracts as are used in surface water monitoring, (26,27) and still enable a reliable biological effect analysis with standard biotests. These biotests did include *in vitro* tests with different cell lines (28) and organismic biotests such as microalgae, daphnia and zebrafish embryos. In addition to monitoring of pesticide contamination, the miniaturised Daphnia assay might be also used for other purposes. Small volume testing could be used for leachate analysis (6), the testing of nanomaterials (14) or the analysis of microplastic effects. For all these above mentioned purposes, only small amounts of test volume can be produced and thus used in the bioassays. The main goal of this work was to evaluate existing research data and verify a reliable *D. magna* immobilisation assay in a miniaturised format specifically for the testing of samples with limited volume. The hypothesis we followed was based on the theory that a decrease in the test volume i.e. increase of density of the acute daphnia test would not have negative effects on sensitivity. Thus, for verification, data obtained in the conventional acute daphnia test as described in the (OECD202) (2) as well as the miniaturised format were systematically compared for 15 selected substances.

With the literature search, a rather smaller number of 7 publications was found which directly had the purpose of also using a miniaturised assay in one way or another. All publications showed more or less that a miniaturisation with a decrease in volume and increase in density of daphnids did not change the single substance EC_{50} results by usually more than a factor of 2-3. (12) compared a variety of different parameters for the testing and observed no significant difference in sensitivity to cadmium- & nickel chloride and formamide. Our adapted daphnia test in 24-well glass titer plates was very much comparable to the identified publications with the one exception that most of the studies used plastic material (i.e. polystyrene) micro titer plates. In conclusion, the differences seen with the selected substances were too small to infer that the miniaturisation would completely misguide an assessment under the test conditions used. Further, no obstacles regarding animal behaviour were reported. Our comparison of conventional and miniaturised toxicity values for 15 selected substances was well in line with these observations. As well, data for both approaches fit into the dataset retrieved from the US EPA ECOTOX database, with EC_{50} values clearly being within the range of observed toxicity values. Overall, the highest variation of published toxicity data was observed for aldicarb (5 orders of magnitude). Here it needs to be pointed out that no information on the test format was retrieved and we assume a variation of approaches was used. Beside that high range of EC_{50} only data from two different daphna species were used (*D. magna* and *D. laevis*). Still, out of necessity, the hypothesis was that a) usage of different clones of the same species and b) the sensitivity of the different daphnid species would be comparable (at least for the first hypothesis (29) showed that this might not be the case). This comparability was not checked for all 15 substances though and thus it can not be excluded, that some of the variances of the EC_{50} data observed are due to a possibly higher or lower sensitivity of the different daphnid species compared to *D. magna*. Data of the other substances (beside aldicarb) came from tests with 13 other species (*D. carinata, D. laevis, D. longispina, D. ambigua, D. pulex, D. similis, D. obtusa, Ceriodaphnia dubia, Ceriodaphnia reticulata, Ceriodaphnia rigaudi, Ceriodaphnia cornuta, Moinodaphnia macleayi, Moina macrocopa*). But even with this comparable high number of daphnid species, the majority of published daphnid tox data were generated with only four species (*D. magna*, D. *laevis*, *D. pulex* and *C. dubia*). Such a high variability in toxicity data might also be due to the different sensitivities of the daphnid species. In contradiction to that, a review by (30) showed that *Daphnia magna* is among the most sensitive species (referring to organic substances) and that more sensitive species do not differ from *D. magna* by more than a factor of 10. In addition, a recent literature study (by the Procter and Gamble Company together with the US EPA) did not find any differences between the species sensitivity of *D. magna* and *D.pulex* in acute and chronic tests (31). Nevertheless, some substances did show differences of one to two orders of magnitude between the two species. Here, also a possible effect of nutrition of adult daphnids on sensitivity of the offspring might also explain some of the differences as was shown by (32) for cadmium. Neonates of well-fed adults were 2-3 times less sensitive than the less-fed adult offspring. This difference was explained by possible energy limitation for detoxification of cadmium. Barata et al. (33) found that differences in tolerance to certain metals were influenced by water hardness among *D. magna* clones, that genetic variations influence sensitivity to toxins ((34) and that phenotypic plasticity ((35) and citations therein) further increases the complexity to control sensitivity in toxicity tests, as a whole suite of parameters may “disturb” a controlled experimental setup. Although the above observations by (32) may only be transferred to other metals, it seems plausible that energy limitation due to detoxification could also be a factor in other toxicity tests. In addition, (36), (37) showed that the test water composition may also influence sensitivity of neonates.

15 test substances were selected, covering a wide range of lipophilicity, to also analyse potential substance loss due to sorption processes to the walls of the glass well plate. This possible loss is also predominantly covered in the OECD standard test guidance #23 (38) “Testing of difficult substances” remarking that an estimated loss of more than 20 % of the starting concentration over time of testing should be paralleled by chemical quantification. As this is an even bigger challenge with test vial material made from plastic (the most often used test vessel material), quite a few papers covered different test systems, organisms, cell lines and tried to pin down the various parameters which might disturb a more realistic toxicity assessment of tests done in small volumes especially in polystyrene titer plates. The parameters reviewed included the definition of thresholds for physico-chemical parameters such as lipophilicity and resulting sorption to test well material, sorption to test medium and else (39–46). Others (47) introduced passive dosing for testing hydrophobic organic substances. e.g. PDMS for testing the effects of PAH with a logKow of above 3.5 and could show the better reproducibility of tests done with these silicone-based material.

The results cited above were published with the assumption that sorption of lipophilic substances to plastic-based well plate material might be substantial and may also disturb testing even in glass material if surface to volume ratio is high. The loss of bioavailable test substances would increase the risk of underestimation of toxicity and thus misguide hazard/ risk assessors. All the papers cited above gave limits, thresholds or work-arounds to deal with a possible loss of the bioavailable fraction. These included $\log K_{OW}$ limits in microalgae and fish embryo testing (43,44), but also solutions for calculation or minimisation of possible loss (46) (39). To sum up, a $\log K_{OW}$ of around or above 3 may pose a risk of loss larger than 20 %. So, a loss of substances due to sorption or volatilization can be expected in the miniaturised test (44) & (43). Goal of this work however, was not to show the differences between titer plate material (glass versus plastic or open versus closed exposure systems) but to see whether the volume decrease (i.e. density increase) might pose a risk for underestimation of toxicity. Still, in other publications, a miniaturised assay was used for risk assessment of water extracts (8,23,48,49) without any obvious problems in terms of higher effects in the negative controls. As a quantification of substance concentration was not done by us, data were compared to literature data, in which mostly no quantification was done either (50). Comparison was on the level of EC or LC_{50} results. Data of the miniaturised daphnia assay most often were close to the mean or geometric mean of the literature data and thus seemed to be of similar quality. This is in concordance with other publications and meets our expectations of a similar sensitivity. With the 15 substances tested and the 13 which could be directly compared, no effects could be seen which might be explained by the higher density of daphnids per volume of test well. Density and also intraspecific competition is seen critical in sub-chronic and chronic daphnia tests ((51) and may have significant effects on sensitivity to toxic substances as was shown in studies by (52,53). But here with the acute tests, density did not seem to be a problem for sensitivity at least to the chemicals tested.

Conclusion and outlook

For *D. magna* immobilisation assays for test materials with limited sample availability, e.g. water extracts, leachates and nano materials, there was a need to strongly reduce the volume of the medium (as in (13)). Hence, the volume of medium used per animal was reduced. The volume to neonate-ratio reported in (12) to the 24-well format by using 5 neonates in a volume of 1.5 ml medium was adopted. Advantage is that mobility and mortality are quick and easily assessible using a microscope, because one well with 5 neonates can be observed at once. Further, the test is more economical in terms of time for preparation, substances required and amount of toxic waste that is generated. It is anticipated to further develop this set-up for a behavioural assay involving live-tracking of animals with a camera where using multi well plates is a favourable approach (12,54). This requires the use of one neonate per well and hence, a further reduction of volume may be anticipated.

As the testing in 24-well glass microtiter plates did not show great differences in terms of sensitivity to the substances tested in this study might also be useful for the analysis of nano particles or microplastic or the ecotoxicological monitoring of environmental samples. The approach established here is transferable to many other types of samples with limited availability.

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Authorship Contribution

Küster, E.: conception and design of the work, idea, organization of laboratory work, first versions of the manuscript, Addo G.G.: Acquisition and testing and analysis of samples, discussion of manuscript, Aulhorn S.: data acquistion & analysis, Kühnel D.: revision and draft of nano particle data, analysis and final approval discussion, correction nano particle work. All co- authors gave final approval of the manuscript and ensure the accuracy and integrity of their part of work.

Data availability statement

(see below)

Competing and Conflicts of Interest

The authors declare that they have no known competing or other conflicts of interest in conjunction with this manuscript and any parts of it which could have influenced the work reported.

Ethics Approval

Ethical approval for the above research was not needed as tests with invertebrates e.g. daphnids do not fall under the EU regulation Directive 2010/63/EU for the protection of animals for scientific purposes.

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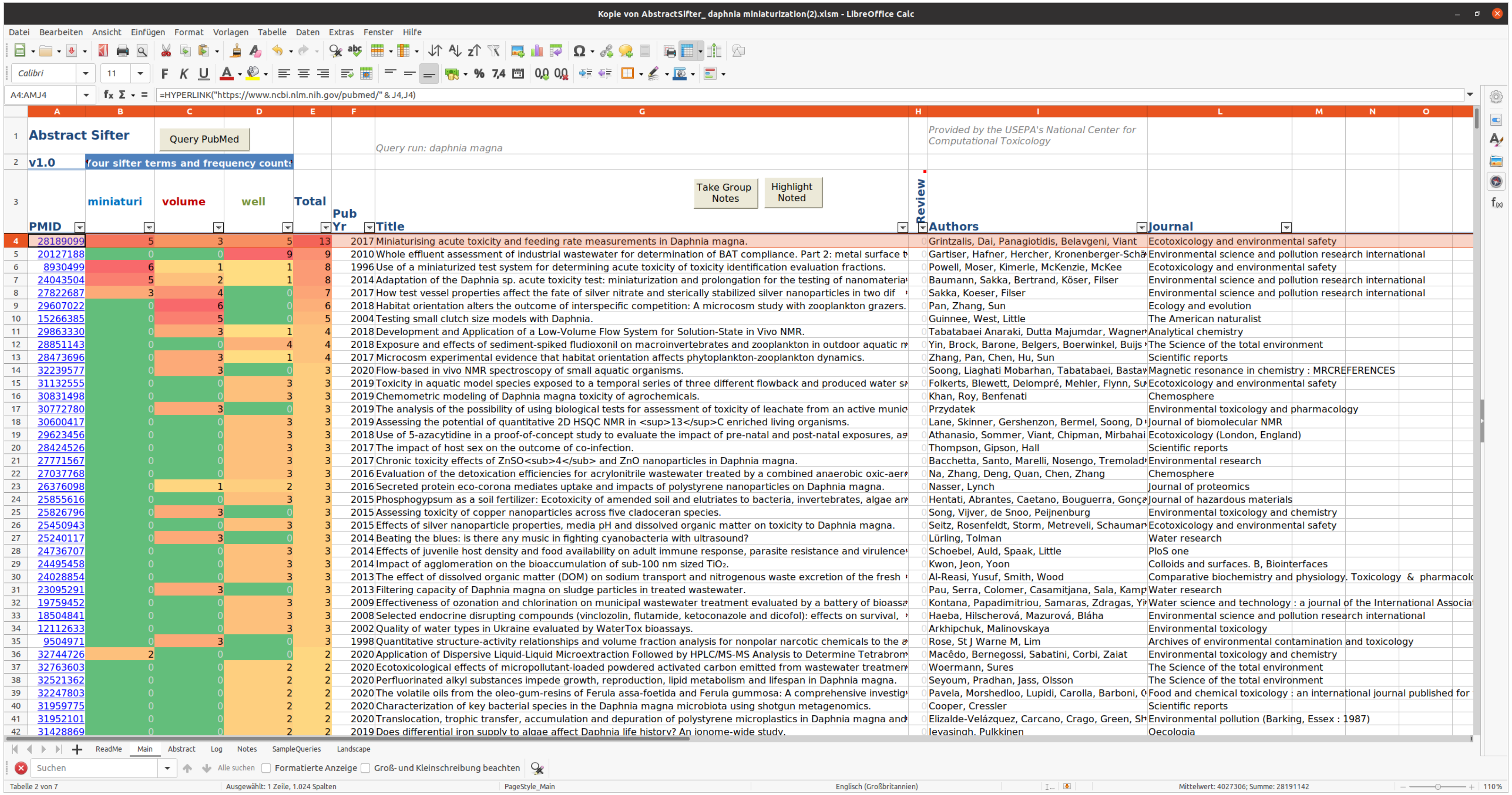
Appendix

Table A_1. Conc. -effect relationships curve parameters, sigmoidal hill model, 4 parameters, f = y0+a*x^b/(c^b+x^b) with y0= min, a=max, b=p= slope, c= EC_{50}) of all 15 test substances

Testsubstances (alphabetical order)	CAS RN	Conc. Effect Parameters, (24h exposure), (min=0, max=100)	Conc. Effect Parameters, (48h exposure), (min=0, max=100)	(µg or mg/L)
Aldicarb	116-06-3	EC_{50}= 1.28 p= 4.50	EC_{50}= 0.36 p= 2.62	mg
Benzyl-carbamate	621-84-1	EC_{50}= 95.31 p= 5.23	EC_{50}= 64.17 p= 2.66	mg
Chlorpyrifos	2921-88-2	EC_{50}= 1.29 p= 0.79	EC_{50}= 0.13 p= 2.02	µg
Diazinon	333-41-5	EC_{50}= 0.91 p= 1.78	EC_{50}= 0.37 p= 2.23	µg
Dimethoate	60-51-5	EC_{50}= 1.60 p= 1.09	EC_{50}= 0.21 p= 1.92	mg
Erythromycin	114-07-8	EC_{50}= 184.09 p= 17.86	EC_{50}= 29.05 p= 2.70	mg
Methanol	67-56-1	EC_{50}= 3.69 p= 3.81	EC_{50}= 1.76 p= 2.97	**%**
Metolcarb	1129-41-5	EC_{50}= 0.12 p= 2.75	EC_{50}= 0.034 p= 1.89	mg
N,N-Dimethylphenylcarbamat	6969-90-0	EC_{50}= 6.86 p= 2.65	EC_{50}= 1.46 p= 1.68	mg
Pirimicarb	23103-98-2	EC_{50}= 18.70 p= 3.62	EC_{50}= 5.74 p= 1.30	µg
Potassiumdichromate	7778-50-9	EC_{50}= 1.72 p= 4.55	EC_{50}= 1.32 p= 8.61	mg
Sodium dodecyl sulfate (SDS)	151-21-3	EC_{50}= 41.76 p= 19.62	EC_{50}= 9.64 p= 1.79	mg
Tebuconazole	107534-96-3	EC_{50}= 17.81 p= 153.15	EC_{50}= 13.19 p= 4.32	mg
Terbutylazine	5915-41-3	EC_{50}= 18.70 p= 1.63	EC_{50}= 11.08 p= 1.79	mg
Tramadol	27203-92-5	EC_{50}= 219.99 p= 3.36	EC_{50}= 46.98 p= 1.77	mg

Table A2_Abstract Sifter Results table (screenshot of query results of first 42 selected publications)



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=HYPERLINK("https://www.ncbi.nlm.nih.gov/pubmed/" & J4,J4)

Abstract Sifter v1.0 — Query PubMed — Your sifter terms and frequency counts — Query run: daphnia magna — Provided by the USEPA's National Center for Computational Toxicology

	PMID	miniaturi	volume	well	Total	Pub Yr	Title	Review	Authors	Journal
4	28189099	5	3	5	13	2017	Miniaturising acute toxicity and feeding rate measurements in Daphnia magna.	0	Grintzalis, Dai, Panagiotidis, Belavgeni, Viant	Ecotoxicology and environmental safety
5	20127188	0	0	9	9	2010	Whole effluent assessment of industrial wastewater for determination of BAT compliance. Part 2: metal surface t	0	Gartiser, Hafner, Hercher, Kronenberger-Schä	Environmental science and pollution research international
6	8930499	6	1	1	8	1996	Use of a miniaturized test system for determining acute toxicity of toxicity identification evaluation fractions.	0	Powell, Moser, Kimerle, McKenzie, McKee	Ecotoxicology and environmental safety
7	24043504	5	2	1	8	2014	Adaptation of the Daphnia sp. acute toxicity test: miniaturization and prolongation for the testing of nanomateria	0	Baumann, Sakka, Bertrand, Köser, Filser	Environmental science and pollution research international
8	27822687	3	4	0	7	2017	How test vessel properties affect the fate of silver nitrate and sterically stabilized silver nanoparticles in two dif	0	Sakka, Koeser, Filser	Environmental science and pollution research international
9	29607022	0	6	0	6	2018	Habitat orientation alters the outcome of interspecific competition: A microcosm study with zooplankton grazers.	0	Pan, Zhang, Sun	Ecology and evolution
10	15266385	0	5	0	5	2004	Testing small clutch size models with Daphnia.	0	Guinnee, West, Little	The American naturalist
11	29863330	0	3	1	4	2018	Development and Application of a Low-Volume Flow System for Solution-State in Vivo NMR.	0	Tabatabaei Anaraki, Dutta Majumdar, Wagner	Analytical chemistry
12	28851143	0	0	4	4	2018	Exposure and effects of sediment-spiked fludioxonil on macroinvertebrates and zooplankton in outdoor aquatic n	0	Yin, Brock, Barone, Belgers, Boerwinkel, Buijs	The Science of the total environment
13	28473696	0	3	1	4	2017	Microcosm experimental evidence that habitat orientation affects phytoplankton-zooplankton dynamics.	0	Zhang, Pan, Chen, Hu, Sun	Scientific reports
14	32239577	0	3	0	3	2020	Flow-based in vivo NMR spectroscopy of small aquatic organisms.	0	Soong, Liaghati Mobarhan, Tabatabaei, Bastaw	Magnetic resonance in chemistry : MRCREFERENCES
15	31132555	0	0	3	3	2019	Toxicity in aquatic model species exposed to a temporal series of three different flowback and produced water sa	0	Folkerts, Blewett, Delompré, Mehler, Flynn, Su	Ecotoxicology and environmental safety
16	30831498	0	0	3	3	2019	Chemometric modeling of Daphnia magna toxicity of agrochemicals.	0	Khan, Roy, Benfenati	Chemosphere
17	30772780	0	3	0	3	2019	The analysis of the possibility of using biological tests for assessment of toxicity of leachate from an active munic	0	Przydatek	Environmental toxicology and pharmacology
18	30600417	0	0	3	3	2019	Assessing the potential of quantitative 2D HSQC NMR in ¹³C enriched living organisms.	0	Lane, Skinner, Gershenzon, Bermel, Soong, D	Journal of biomolecular NMR
19	29623456	0	0	3	3	2018	Use of 5-azacytidine in a proof-of-concept study to evaluate the impact of pre-natal and post-natal exposures, as	0	Athanasio, Sommer, Viant, Chipman, Mirbahai	Ecotoxicology (London, England)
20	28424526	0	0	3	3	2017	The impact of host sex on the outcome of co-infection.	0	Thompson, Gipson, Hall	Scientific reports
21	27771567	0	0	3	3	2017	Chronic toxicity effects of ZnSO₄ and ZnO nanoparticles in Daphnia magna.	0	Bacchetta, Santo, Marelli, Nosengo, Tremolad	Environmental research
22	27037768	0	0	3	3	2016	Evaluation of the detoxication efficiencies for acrylonitrile wastewater treated by a combined anaerobic oxic-aer	0	Na, Zhang, Deng, Quan, Chen, Zhang	Chemosphere
23	26376098	0	1	2	3	2016	Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on Daphnia magna.	0	Nasser, Lynch	Journal of proteomics
24	25855616	0	0	3	3	2015	Phosphogypsum as a soil fertilizer: Ecotoxicity of amended soil and elutriates to bacteria, invertebrates, algae an	0	Hentati, Abrantes, Caetano, Bouguerra, Gonça	Journal of hazardous materials
25	25826796	0	3	0	3	2015	Assessing toxicity of copper nanoparticles across five cladoceran species.	0	Song, Vijver, de Snoo, Peijnenburg	Environmental toxicology and chemistry
26	25450943	0	0	3	3	2015	Effects of silver nanoparticle properties, media pH and dissolved organic matter on toxicity to Daphnia magna.	0	Seitz, Rosenfeldt, Storm, Metreveli, Schauman	Ecotoxicology and environmental safety
27	25240117	0	3	0	3	2014	Beating the blues: is there any music in fighting cyanobacteria with ultrasound?	0	Lürling, Tolman	Water research
28	24736707	0	0	3	3	2014	Effects of juvenile host density and food availability on adult immune response, parasite resistance and virulence	0	Schoebel, Auld, Spaak, Little	PloS one
29	24495458	0	0	3	3	2014	Impact of agglomeration on the bioaccumulation of sub-100 nm sized TiO_2.	0	Kwon, Jeon, Yoon	Colloids and surfaces. B, Biointerfaces
30	24028854	0	0	3	3	2013	The effect of dissolved organic matter (DOM) on sodium transport and nitrogenous waste excretion of the fresh	0	Al-Reasi, Yusuf, Smith, Wood	Comparative biochemistry and physiology. Toxicology & pharmacolo
31	23095291	0	3	0	3	2013	Filtering capacity of Daphnia magna on sludge particles in treated wastewater.	0	Pau, Serra, Colomer, Casamitjana, Sala, Kamp	Water research
32	19759452	0	0	3	3	2009	Effectiveness of ozonation and chlorination on municipal wastewater treatment evaluated by a battery of bioassa	0	Kontana, Papadimitriou, Samaras, Zdragas, Yi	Water science and technology : a journal of the International Associat
33	18504841	0	0	3	3	2008	Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): effects on survival,	0	Haeba, Hilscherová, Mazurová, Bláha	Environmental science and pollution research international
34	12112633	0	0	3	3	2002	Quality of water types in Ukraine evaluated by WaterTox bioassays.	0	Arkhipchuk, Malinovskaya	Environmental toxicology
35	9504971	0	3	0	3	1998	Quantitative structure-activity relationships and volume fraction analysis for nonpolar narcotic chemicals to the a	0	Rose, St J Warne M, Lim	Archives of environmental contamination and toxicology
36	32744726	2	0	0	2	2020	Application of Dispersive Liquid-Liquid Microextraction Followed by HPLC/MS-MS Analysis to Determine Tetrabrom	0	Macêdo, Bernegossi, Sabatini, Corbi, Zaiat	Environmental toxicology and chemistry
37	32763603	0	0	2	2	2020	Ecotoxicological effects of micropollutant-loaded powdered activated carbon emitted from wastewater treatmen	0	Woermann, Sures	The Science of the total environment
38	32521362	0	0	2	2	2020	Perfluorinated alkyl substances impede growth, reproduction, lipid metabolism and lifespan in Daphnia magna.	0	Seyoum, Pradhan, Jass, Olsson	The Science of the total environment
39	32247803	0	0	2	2	2020	The volatile oils from the oleo-gum-resins of Ferula assa-foetida and Ferula gummosa: A comprehensive investig	0	Pavela, Morshedloo, Lupidi, Carolla, Barboni, C	Food and chemical toxicology : an international journal published for
40	31959775	0	0	2	2	2020	Characterization of key bacterial species in the Daphnia magna microbiota using shotgun metagenomics.	0	Cooper, Cressler	Scientific reports
41	31952101	0	0	2	2	2020	Translocation, trophic transfer, accumulation and depuration of polystyrene microplastics in Daphnia magna and	0	Elizalde-Velázquez, Carcano, Crago, Green, Sh	Environmental pollution (Barking, Essex : 1987)
42	31428869	0	0	2	2	2019	Does differential iron supply to algae affect Daphnia life history? An ionome-wide study.	0	Jeyasingh, Pulkkinen	Oecologia

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