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A MIXTURE OF ENVIRONMENTAL ORGANIC CONTAMINANTS IN LAKE SEDIMENTS AFFECTS HATCHING FROM *DAPHNIA* RESTING EGGS

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Abstract: Despite the relevance of resting eggs for ecology and evolution of many aquatic organisms and their exposure to contaminants accumulating in sediments, ecotoxicological studies using resting eggs are vastly underrepresented. The authors established a method to perform exposure assays with resting eggs produced by the *Daphnia longispina* species complex, key species in large lake ecosystems. A mixture of organic contaminants previously detected in sediments of Lake Greifensee was selected to test the potential effect of organic contaminants present in sediments on the hatching process. Resting eggs were exposed to a mix of 10 chemicals, which included corrosion inhibitors, biocides, pesticides, and personal care products, for a period of 15 d. Using an automated counting software, the authors found a significant increase in hatching success in the exposed resting eggs compared with controls. Such an effect has not yet been reported from ecotoxicological assays with resting eggs. Possible mechanistic explanations as well as the potential implications on the ecology and evolution of aquatic species that rely on a resting egg banks are discussed. Observed increased mortality and developmental abnormalities for hatchlings in the exposure treatments can be explained by toxic contaminant concentrations. The results of the present study highlight the need for additional studies assessing the effects of organic contaminants on resting egg banks and aquatic ecosystems. *Environ Toxicol Chem* 2015;34:338–345. © 2014 SETAC

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INTRODUCTION

Waterfleas of the genus *Daphnia* (Crustacea: Anomopoda) constitute a major food source for fish and invertebrates and are important planktonic grazers; they are, therefore, attributed as keystone species in lentic ecosystems. *Daphnia spp*. have been established as important model organisms in aquatic toxicology and have recently been adopted as 1 of 13 model organisms for biomedical research by the National Institutes of Health [1].

Most *Daphnia* species reproduce clonally (parthenogenetic cycle) during favorable conditions but switch to sexual reproduction (sexual cycle) when environmental conditions are not ideal, triggered by changes in food level, crowding, and photoperiod, for example [1,2]. During sexual reproduction, *Daphnia* produce dormant eggs, enclosed in a protective case called ephippium [2]. A portion of these ephippia may float on the lake surface, whereas another portion may sink to the bottom of the lake, where they contribute to the buildup of a so-called resting egg bank [3].

Resting egg banks are common among many aquatic organisms and play a crucial role in their ecology and evolution (for comprehensive reviews on egg banks see, for example, Brendonck and De Meester [3] and Gyllstrom and Hansson [4]). Briefly, parts of or even the entire active pelagial population are recruited from the egg bank during each growing season via transgenerational hatching, and the egg bank is in return restocked with dormant eggs produced by the active population. This interdependence between the active pelagial population and the resting egg bank has been termed benthic-pelagic coupling, a process that strongly determines population dynamics and genetics of many zooplankton species [4,5].

Genetic analyses of resting egg banks of the *Daphnia longispina* species complex from 5 European lakes revealed that human-induced changes in total phosphorus levels (i.e., eutrophication) resulted in profound taxonomic and genetic changes over time [6,7]. Today, many water bodies in Western countries have recovered from anthropogenically induced eutrophication. Nevertheless, pollutants, in particular so-called micropollutants, are still introduced into these systems, representing future threats to natural populations of aquatic organisms [8–10]. Depending on their physicochemical properties, organic contaminants can sorb to sediments and form an excellent archive of environmental contamination, as had been shown for highly lipophilic compounds such as polychlorinated biphenyls [11,12] and recently for medium polar contaminants such as pesticides, personal care products, biocides, and corrosion inhibitors [13,14]. Organic contaminants from the particulate and interstitial components of sediments, as well as from the water column, constitute a primary source of exposure for benthic organisms and their life cycle stages [15,16].

Despite the general importance of egg banks for many planktonic organisms and the known accumulation of contaminants in lake sediments, studies addressing a potential effect of pollutants on the function of egg banks are very limited and in some cases have inconclusive results, as summarized by

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Navis et al. [17]. Moreover, most toxicological studies are conducted with the large-bodied *Daphnia magna* [18]. Whereas *D. magna* is a typical pond species that is usually absent from larger water bodies, the members of the *D. longispina* complex are representative of large lakes and play a crucial role in these ecosystems, including important drinking water reservoirs such as Lake Zurich, Lake Constance, and the Great Lakes [1,21]. Lakes and ponds are expected to vary considerably in their contamination profile as a result of substantial differences in their morphology as well as in the size and quality of their watersheds. In addition, *D. magna* and *D. longispina* complex members differ not only in their habitat but also in body size, physiology, ephippia size and structure, and other characteristics that are likely to affect their sensitivity toward pollutants [1,22]. Although there are no data on the chemicals we used in the experiment available for the *D. longispina* group, studies indicate that members of the *D. longispina* species complex may be more sensitive than *D. magna* toward heavy metals [23,24], nanoparticles [25], and organic contaminants [26]. These differences in habitat preference and sensitivity suggest that resting eggs of the *D. longispina* complex should be the preferred choice when studying effects of pollutants on the resting egg bank in large lakes. Working with ephippia from the *D. longispina* complex can be technically more challenging, however, because of difficulties with inducing the production of sufficient amounts of ephippia in the lab, the often high proportion of empty ephippia containing no resting eggs, high variability in hatching [27], and small size and strong adhesion of ephippia to glassware and plasticware. Therefore, novel approaches are needed to facilitate exposure studies with *D. longispina* complex resting eggs and to increase their use in ecotoxicological risk assessments.

In the present study, we collected ephippia of a natural population of the *D. longispina* species complex from the surface of Lake Greifensee (Switzerland) during peaks of sexual reproduction and exposed the ephippia to a mixture of organic contaminants that were detected in the sediments from this lake. In our previous work, we showed that organic contaminants can actually bioconcentrate in ephippia of these species. Ephippia can uptake and eliminate organic contaminants from the porewater in the sediments or from the water column by passive uptake and depuration mechanisms only, because ephippia represent a resting stage and metabolic activities are negligible, and ephippial internal concentrations in the past and present were predicted based on the temporal contamination of lake sediments, using the equilibrium partitioning model [28]. Overall, the main objectives of the present study were to establish a feasible experimental setup for ecotoxicological tests using *D. longispina* species complex ephippia and to assess whether a mixture of organic contaminants detected in the sediments of Lake Greifensee has, in general, the potential to impact hatching from *Daphnia* resting eggs, using a 1000-fold of the maximum concentrations measured in sediments from Lake Greifensee in a replicated toxicity assay.

MATERIAL AND METHODS

Standards and reagents

Reference standards used in the experiment had a purity of 97% or greater. Irgarol, triclocarban, benzotriazole, 5-methylbenzotriazole and octocrylene were purchased from Sigma-Aldrich. Propiconazole, terbutryn and prochloraz were purchased from Dr. Ehrenstorfer. Triclosan was provided by Ciba, and tonalide was purchased from LGC Standards.

Ephippia collection and preparation

To avoid using resting eggs that had already been pre-exposed to contaminants in the sediments and to ensure sufficiently high numbers of ephippia for several experiments, we regularly monitored the *Daphnia* population to determine the onset of sexual reproduction and obtained ephippia directly from the lake surface within a few days after production. Ephippia produced by the *D. longispina* species complex were collected with nets pulled after a boat from Lake Greifensee (47.3539500°N, 8.6758917°E) during annual peaks of sexual reproduction in spring 2011, spring 2012, and fall 2011, to avoid potential sampling biases, and stored in 10-L polyethylene bottles (Hünersdorff GmbH). Ephippia were thoroughly cleaned by repeated steps of sieving using nested sieves of different mesh sizes (180 μm, 250 μm, 1000 μm, 2000 μm, and 4000 μm) and resuspension in filtered lake water. Remaining particles were manually removed with forceps and the purified ephippia were rinsed with filtered and double autoclaved lake water and stored in the dark at 4 °C in 500 mL Schott bottles (Schott Duran) to break diapause (storage time, 168–580 d), as reported by Keller and Spaak [27]. Prior to the experiment, ephippia were dried on drying frames made with plankton net fabric in dark at 4 °C to remove the storage medium, reduce the risk of microbial contamination, and facilitate handling. Ephippia are resistant to desiccation, and we observed no effect of the drying process on hatchability.

The lake water used for storage and exposure experiments was collected from Lake Greifensee, filtered through a glass fiber filter (pore size, 0.45 μm; Sartorius Stedim AG) and double autoclaved at 120 °C for 30 min each time with a Vapoklav 500 (HP Medizintechnik GmbH). The lake water, collected simultaneously with the ephippia, was tested after filtration and contained none of the studied chemicals in background concentrations, which is consistent with the reported low-ng/L concentrations of organic contaminants found in Lake Greifensee [29].

Hatching experiment

We performed 2 experiments with a total of 120 experimental units (2 experiments × 2 treatments × 30 replicates). For these exposure experiments, dried ephippia were moisturized with deionized water, and ephippia aliquots with a volume of 0.15 mL were transferred to 15-mL polypropylene centrifuge tubes (VWR). Assay tubes were randomized and filled with 14 mL of exposure medium or control medium, respectively. For each treatment (exposure and control), 30 tubes were prepared, resulting in a total of 60 assays per experiment. The amount of ephippia and number of replicates were chosen based on power analysis simulations with data from pretests on hatching. The exposure medium consisted of filtered and double autoclaved lake water containing a mixture of 10 organic contaminants with nominal concentrations between 2 μg/L and 800 μg/L. Concentrations were selected according to the predicted pore water maximum concentration based on sediment analyses from Lake Greifensee [13,28] and multiplied by a factor of 1000. The exposure mixture included pesticides, corrosion inhibitors, biocides, and personal care products. The chemicals were spiked as a mixture in ethanol with a final ethanol content of 0.2% (v/v). The complete list of chemicals used in the experiment, with measured concentrations and physicochemical characteristics, is reported in Table 1. The control medium contained filtered and double autoclaved lake water with a final ethanol content of 0.2% (v/v).

Table 1. Organic contaminants with measured concentrations (C_{med}) and physicochemical characteristics used in the exposure experiment

Name	CAS #	Compound class	log D_{OW} at pH 8.2[a]	$C_{ipwmax} \times 10^3$ (μg/L)	C_{med} (μg/L)	$C_{med\text{-}final\text{-}D}$ (μg/L)	$C_{med\text{-}final\text{-}L}$ (μg/L)	Degradation by light at 20 °C($t = 72$h)[b]	Sorption to PP tubes ($t = 72$h; %)[b]	EC50 (μg/L)[c]
Benzotriazole	95-14-7	Corrosion inhibitor	1.2	660	650 ± 60	550 ± 20	630 ± 40	Stable	0	35 000–280 000[e]
Irgarol	28159-98-0	Biocide	3.0	210	450 ± 40	360 ± 20	350 ± 30	Stable	7	8100[f]
Methyl-benzotriazole	49636-02-4	Corrosion inhibitor	1.4	510	740 ± 50	650 ± 20	700 ± 70	Stable	0	35 000–280 000[e]
Octocrylene	6197-30-4	PCP	6.8	2	<5	<5	<5	Stable	19	> 23[g]
Prochloraz[d]	67747-09-5	Pesticide	3.6	35	35 ± 3	23 ± 3	24 ± 2	Stable	6	4300[e,h,i]
Propiconazole[d]	60207-90-1	Biocide	4.3	24	19 ± 1	15 ± 1	16 ± 1	Stable	6	2600–13 000[h]
Terbutryn	886-50-0	Biocide	2.9	28	29 ± 2	23 ± 1	23 ± 2	Stable	7	2600–7100[e]
Tonalide	21145–77-7	PCP	5.0	510	500 ± 100	47 ± 4	13 ± 5	62%	86	239–249[j]
Triclocarban[d]	101-20-2	Biocide	4.9	820	260 ± 40	320 ± 2	300 ± 100	Stable	0	10[e]
Triclosan[d]	3380-34-5	Biocide	4.4	320	430 ± 330	120 ± 20	73 ± 13	73%	67	390–560[e]

[a]log D_{ow} (log octanol-water distribution coefficient (log K_{ow}) corrected for the dissociation at pH 8.2 of the medium) values were predicted using MarvinSketch 5.11 (ChemAxon http://www.chemaxon.com).
[b]Degradation by light using glass containers and sorption to polyethylene tubes under dark conditions were studied independently under similar experimental conditions. Concentrations of chemicals over time for the photodegradation experiment are reported in Supplemental Data, Figure S1.
[c]48-h median effect concentration (EC50) for *Daphnia magna*.
[d]Compounds known to have antimicrobial activity.
[e]ECOTOX AQUIRE database [57].
[f]Tóth et al. [58].
[g]European Chemicals Agency [61].
[h]University of Hertfordshire FOOTPRINT PPDB database [59].
[i]Ashauer et al. [60].
[j]EC50 in terms of reduction of growth rate - 21 d (ErC50-21 d) [49].
C_{ipwmax} = maximum predicted porewater concentration based on analysis of sediment from Lake Greifensee as previously reported [13,28]; C_{med} = average concentration at $t = 0$, before distribution and exchange of the medium in the assays; $C_{med\text{-}final\text{-}D}$ = average medium concentration during incubation at dark and at 4 °C after 72-h exposure; $C_{med\text{-}final\text{-}L}$ = average concentration of the medium under light exposure and at room temperature after 72 h exposure; PP = polypropylene.

Ephippia were preincubated (day 0) in the dark for 4 d at 4 °C (to prevent early hatching) to allow them to equilibrate with the exposure medium and to reach a steady state for the bioconcentration of contaminants, as was determined in previous toxicokinetic experiments [28]. After preincubation, ephippia were transferred to an incubator to stimulate hatching at a temperature of 20 °C and a 16:8-h light:dark cycle (Memmert GmbH & Co. KG). This two-step exposure protocol ensures that the embryos, which are protected by the ephippial case and egg membranes, are actually already exposed to a constant concentration of the contaminants when they receive the hatching stimulus and proceed with their development. In the incubator, the assay tubes were randomized and placed at a 10° angle. The use of tubes in such an arrangement allows optimum contact between the ephippia and the medium and eliminates the issue of ephippia adhering to the side wall of a petri dish or floating on the medium, which may affect exposure concentration as well as hatching. Assay tubes were shaken gently and randomized daily and examined on days 4, 7, 10, 13, and 15 under a stereo microscope. Alive and dead hatchlings (i.e., immobilized fully developed embryos that had at minimum hatched from the chorion) were removed from the assay tubes and counted separately, and hatchling mortality was calculated by dividing the number of dead hatchlings by the total number of hatchlings. Simultaneously, exposure and control media were renewed to avoid sorption and degradation of chemicals in the exposure medium. The old medium was almost entirely removed (residual volume < 100 μL; < 0.8%) using a syringe and replaced by fresh medium containing chemicals at the same initial concentrations, to keep the experimental conditions constant during the entire experiment. After 15 d, the experiment was stopped based on data from pretests that consistently showed that hatching ceased between day 13 and day 15. The complete experiment was performed twice during different seasons of the year—December 2012 (experiment A) and March 2013 (experiment B).

Quality control samples were taken during the experiment and included freshly prepared control and exposure media, as well as control and exposure media taken from random assay tubes while exchanging the media. The quality control samples were collected on days 0, 4, 7, 10, 13, and 15 in duplicates and analyzed for possible cross contamination and stability of the exposure medium. No contamination was found in the quality control samples. The measured concentrations are reported in Table 1 and Supplemental Data, Table S1. Control and exposure media were directly analyzed by transferring 940 μL of medium to 1-mL high-performance liquid chromatography vials (BGB Analytics AG) followed by addition of 60 μL of internal standard mix solution and analyzed by liquid chromatography–high-resolution mass spectrometry (LC-HRMS) as described elsewhere [28]. In addition, pH was monitored (713 pH meter; Metrohm AG) during the experiment (pH 8.2 ± 0.2). Furthermore, dissolved oxygen was measured for a subset of samples to check for potential differences between control and exposure medium; however, no such differences were detected.

Chemical stability

The stability of the chemicals under experimental conditions was explored in independent studies. First, photodegradation alone was assessed by spiking all chemicals as a mixture (Table 1) into filtered and autoclaved lake water with a nominal final concentration of 200 μg/L. The spiked solution was distributed into glass jars and exposed to light at room temperature. Aliquots were taken at the beginning of the experiment (0 h) and every 24 h for 6 d in triplicates.

To test sorption to polypropylene tubes, chemicals were spiked as a mixture in filtered and autoclaved lake water at a nominal final concentration of 200 μg/L. Samples were taken at

the beginning of the experiment (0 h) and every 24 h for 6 d under light and dark conditions in triplicates at 20 °C and 4 °C, respectively. All samples were analyzed by LC-HRMS as described elsewhere [28].

Ephippia and egg counting

Ephippia were counted at the end of the experiment (after 15 d) to account for differences in the absolute number of ephippia per assay tube. To render counting of the large number of ephippia possible within a reasonable time, they were arranged on glass slides (8 cm × 11 cm) with a white background, photographed with a digital camera (Panasonic DMC-FZ50 camera with a Leica DC-Vario-Elmarit 1:2.8–3.7/7.4–88.8 ASPH zoom lens) and automatically counted using CellC 1.2. software in batch processing mode [30]. The implemented cluster division algorithm based on cell shape for light microscopy images was used with cluster division set to a value of 1, with a background correction applied prior to processing. Moreover, 2 different intensity thresholds (0.4 and 0.25) were employed, representing the upper and lower limits of values for which reliable results in previous tests had been obtained. The difference between the 2 ephippia count datasets was minor, with an average difference of 2.1% (standard deviation, 1.1%) and a maximum difference of 6.1%, and no bias for controls nor exposed ephippia was found. Therefore, only results obtained for intensity threshold 0.25, which yielded a slightly better ephippia count, are reported. The counted number of ephippia in each treatment was nearly identical (127 471 ephippia in the control and 127 479 ephippia in the exposure treatments).

To calculate the number of eggs for the analysis of hatching success, an average egg content per ephippium was determined by opening a subsample of 500 ephippia and counting the eggs under a stereo microscope. The number of exposed eggs in each assay tube was calculated by multiplying the ephippia count datasets with the average egg content (0.17 eggs/ephippium). In the present study, therefore, hatching success is defined as number of observed hatchlings per calculated total number of resting eggs.

Statistical analysis

The binomial response variables hatching success and mortality were evaluated with a generalized linear model (GLM). To account for overdispersion, present in both cases, a quasi-binomial model was employed.

For hatching success, the full model was tested first, with treatment and experiment as fixed factors and their interaction as explanatory variables. The interaction term was not significant in a partial F-test comparing the full model against a model without interaction. Therefore, the interaction term was removed and a simpler model containing the fixed factors treatment and experiment was used (Table 2). The analysis was performed with datasets based on both ephippia counts (intensity thresholds 0.4 and 0.25), and both analyses revealed a similar outcome with no relevant differences.

For mortality, the full model was tested using treatment and experiment as the fixed factors and their interaction as explanatory variables. The interaction term was highly significant, and the full model was retained (Table 3). One extreme outlier was removed from the mortality dataset to improve the model, with no relevant effect on the significance of terms.

Residual analyses conducted for all models revealed no violation of model assumptions.

All models were evaluated using the function glm and partial F-tests for the significance of single terms in the models and were performed using the function drop1 in R Ver 3.0.1 [31].

RESULTS AND DISCUSSION

The results of the present study show that reproducible toxicological assays with resting eggs of the *D. longispina* species complex are feasible when elaborate protocols are established and that organic contaminants found in lake sediments may potentially affect hatching from the resting egg bank, a finding that demands further attention.

Chemical stability

The stability of the chemicals under experimental conditions is reported in Table 1. The photodegradation study, excluding sorption to polypropylene tubes, shows degradation of 73% and 62% for triclosan and tonalide, respectively, after 72 h under light exposure. The results are consistent with the reported photolysis of triclosan and tonalide in surface water and wastewater [32,33]. No photolysis was observed for the remainder of the compounds, as illustrated in Table 1 and Supplemental Data, Figure S1. Additionally, the results show that most of the compounds do not significantly sorb to polypropylene within 72 h. However, 67% and 86% of triclosan and tonalide, respectively, are lost and therefore not bioavailable over the whole time range of the experiment. The 5-fold exchange of the medium during the experiment accounted for photolysis and sorption losses.

Hatching success

The main finding of our study was a highly significant treatment effect on hatching success ($p < 0.001$; Table 2 and Figures 1 and 2). In total we observed 3434 hatchlings from 254 950 ephippia containing 43 342 resting eggs. Ephippial egg content and overall hatching success were well in accordance with a previous study on reproduction in the same species complex (see Figure 2 in Keller and Spaak [27]). Intriguingly, hatching success increased in both experiments when ephippia were exposed to the mixture of organic contaminants. In absolute numbers, we observed a total of 1498 hatchlings in the control and 1936 hatchlings in the exposure treatments. Although a few studies have reported a negative impact of organic and inorganic contaminants on hatching success [17,34–39], we provide novel

Table 2. Estimates and test statistics for a quasibinomial generalized linear model examining hatching success using fixed factors treatment and experiment as explanatory variables[a]

	Estimate	Standard error	t	$p(>\|t\|)$[b]	F	$p(>\|F\|)$[b]
Intercept	−2.689	0.069	–38.806	< 0.001		
Treatment	0.276	0.076	3.661	< 0.001	13.322	<0.001
Experiment	0.172	0.075	2.285	0.024	5.166	0.025

[a]Null deviance: 610.27, $df = 119$; residual deviance: 526.29, $df = 117$, dispersion parameter: 4.438.
[b]p values are given for individual hypothesis tests and partial F tests.
t = Student's t-value ; F = Fisher-Sendecor's F-value.

Table 3. Estimates and test statistics for a quasi-binomial generalized linear model examining the mortality using fixed factors treatment, experiment, and their interaction as explanatory variables[a]

	Estimate	Standard error	*t*	$p(>\|t\|)$[b]	*F*	$p(>\|F\|)$[b]
Intercept	1.111	0.138	8.050	< 0.001		
Treatment	0.464	0.192	2.414	0.017		
Experiment	–1.417	0.174	8.138	< 0.001		
Treatment × experiment	–2.977	0.327	9.091	< 0.001	89.95	< 0.001

[a]Null deviance: 1044.79, $df = 118$; residual deviance: 294.41, $df = 115$, dispersion parameter: 2.321.
[b]p values are given for individual hypothesis tests and partial F tests.
t = Student's t-value ; F = Fisher-Sendecor's F-value.

evidence that exposure to organic contaminants can also have an opposite effect. The present study shows the potential of organic contaminants found in lake sediments to increase hatching success; at this stage, however, we can only speculate about possible underlying mechanisms.

One obvious explanation for the observed result could be an interaction of a single or several compounds used in the experiment with physiological pathways that control dormancy, such as regulatory pathways that maintain cell cycle arrest or control transmission of hatching cues. To date, there is little or no information about the physiology of dormancy control and the involved regulatory pathways. Navis et al. [17] found evidence that hatching can be affected by an endocrine disruptor in *D. magna*, albeit negatively. Among our selected compounds, several are known (the antibacterial agents triclocarban [40] and triclosan [41]) or suspected (the musk fragrance tonalide; the UV filter octocrylene; and the pesticides terbutryn, prochloraz, and propiconazole [42]) to act as endocrine disruptors. Triclocarban has been shown to stimulate embryo production in the freshwater mudsnail *Potamopyrgus antipodarum* at relevant environmental concentrations [43]. Clearly, much more work is needed to elucidate a potential role of 1 or several of these endocrine disrupters in the hatching process, and triclocarban might be a promising candidate for initial studies.

Another non-exclusive explanation for the observed increase in hatching is environmentally cued hatching [44]. Hatching of *Daphnia* resting eggs is known to be triggered by signals indicating favorable conditions [45], but there are also examples in the animal kingdom that show that early hatching may be initiated when conditions become unfavorable for an egg (i.e., escape hatching) [44]. Resting eggs, in theory, also may be capable of sensing a decrease of their chance of survival in the sediment and thus may try to escape by terminating diapause and hatching. To our knowledge, this possibility has not yet been considered or tested for *Daphnia* resting eggs. Such an escape hatching response may have been triggered by, for example, cellular damage caused by the chemicals in our experiment.

Finally, the increase of hatching observed could also be explained by an indirect effect, such as the reduction of microbial growth. Damaged resting eggs infested with fungi or bacteria are regularly encountered in ephippia collected from lake sediments (M. Möst, personal observation), and microbial growth is therefore likely to interfere with the development of hatchlings and hatching success. Four of the compounds used in the experiment (propiconazole, prochloraz, triclocarban, and triclosan) are known to have antimicrobial activity (Table 1). Although we used double autoclaved lake water and sterile tubes in all experiments, dried ephippia prior to the experiments to eliminate microbes, and observed no obvious microbial growth during hatching, we cannot exclude the possibility that resistant microbial spores adhered to the ephippia and microbial growth had been observed in pretests after several days of incubation. Therefore, inhibition of microbial growth by biocides remains as a possible explanation for the increase in hatching success.

In addition to the effect of organic contaminants, a weak significant effect for the experiment (p value = 0.025, Table 2) explained by a higher hatching success in the second experiment was found (Figures 1 and 2). One possible explanation for this observation may be seasonality. The first experiment was conducted in December 2012 (experiment A), and the second experiment was conducted in March 2013 (experiment B), which is the start of the growing season and onset of hatching in nature [3,46,47]. To date, however, our knowledge on the physiology of dormancy is scarce and further research is required to answer the question whether seasonal rhythms are maintained in resting eggs.

Hatchling mortality

We also recorded and analyzed the number of dead and alive hatchlings. However, because our experiment was designed primarily to test effects on hatching success, our sampling scheme was chosen to ensure minimum disturbance of the hatching process. As a consequence, hatchling mortality is defined in the present study as the proportion of dead hatchlings encountered after 3 d (day 4–7, day 7–10, day 10–13) and 2 d (day 13–15), respectively, and individuals may have hatched at any time during this period. Hatchlings that had hatched shortly after medium exchange thus remained in the assay tubes for up to 3 d, increasing their risk of mortality. Therefore, differences in hatchling mortality in the present study may, in part, also reflect slight temporal shifts in hatching and should be interpreted carefully and treated as additional information rather than a defined endpoint of the present study.

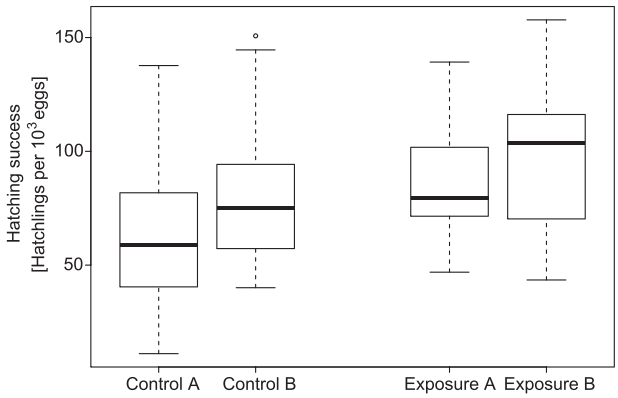

Figure 1. Boxplots of hatching success for control and exposure treatments in experiments A and B. Each boxplot is based on 30 observations at 4 time points. Whiskers indicate 1.5× interquartile range of the upper and the lower quartile, respectively. Outliers are shown as open circles.

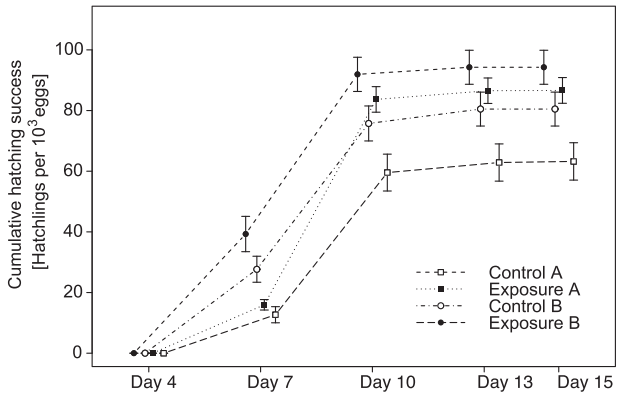

Figure 2. Mean cumulative hatching success over time for control and exposure treatments in experiments A and B. Error bars indicate standard errors of means (SEM).

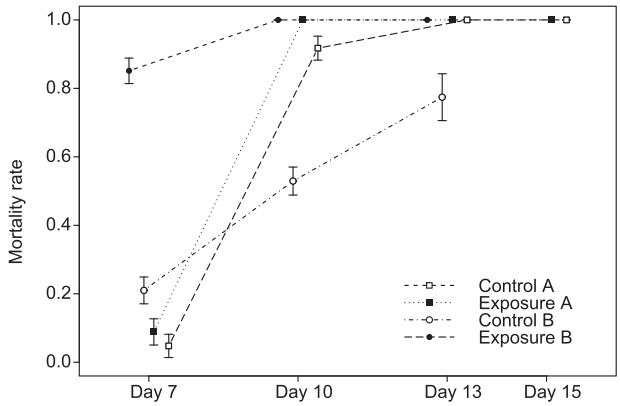

Figure 4. Mean mortality over time for control and exposure treatments in experiments A and B. Error bars indicate standard errors of means (SEM).

In total, we found 648 dead and 850 alive hatchlings in the control versus 1712 dead and only 67 alive in the exposure treatment. Although mortality was consistently higher in the exposure medium in both experiments ($p < 0.001$; Table 3 and Figures 3 and 4), the effect size differed between the 2 experiments. This difference is attributable to an increased mortality in the control treatment of the first experiment, which may be attributed to an effect of season or subtle differences in the timing of peak hatching between the 2 experiments. Mortality also showed an increase over time in the control treatments (Figure 4). This effect is most likely explained by the accumulation of embryos suffering from developmental retardations and defects with time . These embryos are expected to hatch later (i.e., toward the end of the experiment) and have a higher risk of mortality. This is supported by observations from several other hatching experiments in which fitter and larger individuals tended to hatch earlier (M. Möst, personal observation).

The most evident explanation for the observed increase in mortality in the exposure treatments is the concentrations of some of the selected compounds. Median effect concentration (EC50) values for *D. magna* (no data available for the *D. longispina* group) of most of the studied compounds are between 20 times and 200 times higher—with the exception of tonalide, triclosan and triclocarban—when compared with the measured exposure concentration (Table 1). *Daphnia magna* EC50 values for

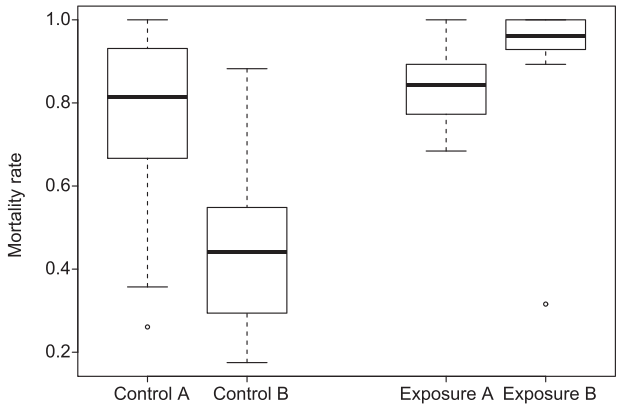

Figure 3. Boxplots of mortality for control and exposure treatments in experiments A and B. Each boxplot is based on 30 observations at 4 time points. Whiskers indicate 1.5× interquartile range of the upper and the lower quartile, respectively. Outliers are shown as open circles.

tonalide, triclosan, and triclocarban are similar or higher than the exposure concentrations used [48,49]. For example, the 48-h *D. magna* EC50 values for survival and reproduction have been reported to be 390 μg/L for triclosan [50,51], which is close to the exposure concentration in the experiment. Our experimental design does not allow us to disentangle an effect of the toxicants on the developing embryo in the resting egg from the effect on the hatched individuals. However, hatchlings that had died shortly after or even during the hatching process were frequently encountered in the exposure treatment, some showing obvious deformations and developmental abnormalities. This qualitative observation suggests that the mixture of chemicals already had an effect on the developing embryo in the egg, but a quantitative follow up study is required to confirm this finding.

Implications

In the present study, we provide evidence that organic contaminants that deposit in the sediments of large lakes have the potential to affect the function of egg banks and thereby affect benthic-pelagic coupling. The observed effects, if occurring in nature, will have severe consequences for the ecology and evolution of species that depend on egg banks. Changes in the contribution of ex-ephippial hatchlings, which differ from parthenogenetically produced individuals in life history traits and physiology [52,53], to the pelagic population may affect abundance and average life history traits of the whole population. Such an impact on the seasonal population dynamics of relevant zooplankton species is expected to have an effect not only on the ecology and survival of the respective species but also on the whole-lake ecosystem through food web interactions [17]. Moreover, the egg bank also represents a storage of genetic variation produced by sexual recombination over several generations [3,54,55], and altered hatching dynamics from the sediments may have severe implications on the evolutionary potential of species [3]. In contrast to the small number of ecotoxicological studies on egg banks conducted to date, which generally found detrimental effects on hatching success and hatchling fitness [17], the present study reveals a novel result: a significant increase in hatching success of resting eggs when exposed to organic contaminants. *Daphnia* resting eggs do not all hatch at the same occasion [56], which allows for transgenerational overlap and reduces the risk of extinction [4,54]. Therefore, the observed increase in hatching may interfere with bet-hedging strategies, lead to a depletion of the egg bank, and thus increase the extinction risk of local populations [3,4,54]. Furthermore, it may affect competition

between species in cases in which the reaction of hatching to organic contaminants differs between species.

CONCLUSION AND OUTLOOK

In the present study, we exposed resting eggs of a natural population of the *D. longispina* complex, occupying a key role in large lake ecosystems, for 15 d to a 1000-fold increased concentration of a complex mix of micropollutants previously detected in the sediments of the studied lake.

We established a method, using a novel exposure protocol and automated counting software, that allows us to overcome most technical limitations of ecotoxicological assays with ephippia produced by the *D. longispina* complex. This method easily can be adapted to test further substances and endpoints or resting eggs of other small-bodied zooplankton organisms.

Applying our new method, we found clear evidence that organic contaminants in lake sediments have the potential to affect hatching success in a previously unknown way. Clearly, follow-up studies are now required to identify the active component(s) in our mixture, establish dose-response curves, investigate the mode of action, and clarify the role of seasonality. Whether the observed effects actually occur in nature will depend on environmental concentrations, the mix of organic contaminants, and the exposure time. Resting eggs can stay in the active resting egg bank for decades, and chronic low-dose effects thus are likely to be relevant. Availability and uptake of organic contaminants may be altered by the presence of sediment and the microbial flora therein. Therefore, future research also needs to focus on more environmentally realistic scenarios, including, for example, long-term mesocosm experiments.

In conclusion, the present study reveals the potential for micropollutants found in lake sediments to affect ecology and evolution of key species in large-lake ecosystems via a thus far unnoticed mechanism and highlights the urgent need for further research in this direction to assess the risks that emanate from the ongoing input of organic pollutants into aquatic systems.

SUPPLEMENTAL DATA

Table S1 and Figure S1 (182 KB DOC).

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