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Environmental concentrations of pharmaceuticals directly affect phytoplankton and effects propagate through trophic interactions

Malgorzata Grzesiuk[a,b,c,*], Elly Spijkerman[b], Sabrina C. Lachmann[b], Alexander Wacker[c]

[a] Department of Hydrobiology, Faculty of Biology, University of Warsaw at Biological and Chemical Research Centre, Żwirki i Wigury 101, 02-089 Warsaw, Poland
[b] Department of Ecology and Ecosystem Modelling, Institute for Biochemistry and Biology University of Potsdam, Am Neuen Palais 10, 14469 Potsdam, Germany
[c] Department of Theoretical Aquatic Ecology and Ecophysiology, Institute for Biochemistry and Biology, University of Potsdam, Am Neuen Palais 10, 14469 Potsdam, Germany

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ABSTRACT

Pharmaceuticals are found in freshwater ecosystems where even low concentrations in the range of ng L^{-1} may affect aquatic organisms. In the current study, we investigated the effects of chronic exposure to three pharmaceuticals on two microalgae, a potential modulation of the effects by additional inorganic phosphorus (P_i) limitation, and a potential propagation of the pharmaceuticals' effect across a trophic interaction. The latter considers that pharmaceuticals are bioaccumulated by algae, potentially metabolized into more (or less) toxic derivates and consequently consumed by zooplankton. We cultured *Acutodesmus obliquus* and *Nannochloropsis limnetica* in P_i-replete and P_i-limited medium contaminated with one of three commonly human used pharmaceuticals: fluoxetine, ibuprofen, and propranolol. Secondly, we tested to what extent first level consumers *(Daphnia magna)* were affected when fed with pharmaceutical-grown algae. Chronic exposure, covering 30 generations, led to (i) decreased cell numbers of *A. obliquus* in the presence of fluoxetine (under P_i-replete conditions) (ii) increased carotenoid to chlorophyll ratios in *N. limnetica* (under P_i-limited conditions), and (iii) increased photosynthetic yields in *A. obliquus* (in both P_i-conditions). In addition, ibuprofen affected both algae and their consumer: Feeding ibuprofen-contaminated algae to P_i-stressed *D. magna* improved their survival. We demonstrate, that even very low concentrations of pharmaceuticals present in freshwater ecosystems can significantly affect aquatic organisms when chronically exposed. Our study indicates that pharmaceutical effects can cross trophic levels and travel up the food chain.

1. Introduction

The production, prescription and use of pharmaceuticals has steeply increased during the last decades (Kookana et al., 2014; Bernhardt et al., 2017). Removal efficiency of these pharmaceuticals from sewage ranges from 0% to 97% depending on the specific technology used (Wennmalm and Gunnarsson, 2009). Therefore, pharmaceuticals are commonly entering and contaminating freshwater ecosystems all over the world (Hughes et al., 2013; Osorio et al., 2016). Although, the pharmaceuticals and their metabolites are diluted in large freshwater bodies, the compounds are constantly discharged from wastewater plants (Zuccato et al., 2006). This results in the chronic exposure of aquatic organisms to these pharmaceuticals. Being biologically active by design, these chemicals are of concern for their potential interactions with non-targeted organisms in the environment such as phytoplankton and zooplankton. Up to now, the majority of conducted experiments used concentrations far higher than those detected in aquatic systems (e.g see Heckmann et al., 2007; González-Péreza et al., 2016 for ibuprofen). Therefore, for many pharmaceuticals, a more ecological relevant investigation of the effects of chronic low concentration exposure on aquatic organisms is needed (e.g. Fent et al., 2006). One such study has recently been done on the bacterial community in stream biofilms (Rosi et al., 2018).

Complicating the picture further is that environmental factors may modulate the effects of pharmaceuticals. For example previous research has shown that P_i-limited and P_i-replete algae have a different sensitivity to acute drug exposures (Grzesiuk et al., 2016). Algae are an important component of freshwater ecosystems, since they are often the key primary producers and constitute the basis of food webs. Observations of parameters associated with photosynthesis (e.g. quantum yield, pigment concentration) and biomass composition in algae cultured with pharmaceuticals can be useful in assessing their effects on

* Corresponding author at: Department of Hydrobiology, Faculty of Biology, University of Warsaw at Biological and Chemical Research Centre, Żwirki i Wigury 101, 02-089 Warsaw, Poland.
E-mail address: m.grzesiuk@uw.edu.pl (M. Grzesiuk).

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the algae. Our previous results have indicated that taxonomically different phytoplankton species have species-specific tolerances to pharmaceuticals (Grzesiuk et al., 2016). Therefore, the first aim of our current study was to chronically expose microalgae to pharmaceutical concentrations detected in the environment.

The effects of these contaminants on primary producers may also result in adverse effects on the physiology of organisms at higher trophic levels due to feeding and bioaccumulation (Hylland and Vethaak, 2011). Many pharmaceuticals are dispersed within aquatic habitats and in some cases bio-accumulated in the food chain (Meredith-Williams et al., 2012), e.g. in the filter feeder *Daphnia*. Proper functioning of food webs is complex and fragile to environmental change, where even a small disturbance can influence the whole ecosystem. This was recently illustrated by van Donk et al. (2015), who indicated that pharmaceuticals can affect the stability of ecosystems via unexpected pathways (e.g. infochemicals changing physiology, morphology or behavior of competitors or enemies). Following Scheffer et al. (2001) the recovery of such a disturbed (bi-stable) system can be extremely difficult.

Next to the need for research of the direct responses to the presence of low concentrations of pharmaceuticals, alternative secondary effects via the contamination of food items with drugs might well be present and important. We hypothesize that a propagation of the pharmaceuticals' effect can emerge if these compounds are bioaccumulated by algae, potentially metabolized into more (or less) toxic derivates and consequently consumed. Also, pharmaceuticals affecting the algal physiology and changing its nutritive value for a consumer is such a propagation. Therefore, the second aim of our study was to describe the effect of pharmaceutical-exposed microalgae on *Daphnia*. In the current study, we (i) quantified the direct effect of pharmaceuticals on photosynthetic performance, biochemical composition and growth after chronic exposure in ecologically relevant concentrations in two species of phytoplankton, (ii) tested for the propagation of any effects on consumers by feeding *Daphnia* phytoplankton grown in the presence of pharmaceuticals, and (iii) studied a potential modulation of the above mentioned effects by applying P_i limitation on phytoplankton and zooplankton. To obtain our goals we cultured the two algae species *Acutodesmus obliquus* and *Nannochloropsis limnetica* in P_i-replete and P_i-limited medium with one of three pharmaceuticals: fluoxetine, ibuprofen, and propranolol, which are abundantly and commonly used by humans (OECD Health Statistics, 2014).

2. Materials and methods

2.1. Pharmaceuticals

Fluoxetine, ibuprofen, and propranolol were used as model compounds to test the effect of low level chronic exposure of pharmaceuticals on algae. The algal cultures grown in the presence of these compounds were then fed to *D. magna*. All three pharmaceuticals were obtained from SIGMA, and were minimum 98% pure. The concentrations of the drugs in the culture medium were chosen following literature reports of the concentrations of pharmaceuticals detected in several freshwater ecosystems (Hughes et al., 2013; Huggett et al., 2003): (i) the antidepressant fluoxetine 360 ng L^{-1} (max. concentration found 596 ng L^{-1}), (ii) the anti-inflammatory and analgesic drug ibuprofen 2 μg L^{-1} (max. concentration found 31.3 μg L^{-1}), (iii) the beta-blocker propranolol 1 μg L^{-1} (max. concentration found 1.9 μg L^{-1}).

2.2. Algal culture conditions

Two freshwater phytoplankton species were semi-continuously cultured by daily diluting 20% of the culture volume. Species were cultured under both P_i-replete and P_i-limited conditions. The two species used were the Chlorophyceae *Acutodesmus obliquus* Turpin, Hegewald et Hanagata (formerly called *Scenedesmus obliquus*, SAG276-3a), and the other was the Eustigmatophyceae *Nannochloropsis limnetica* Krienitz, Hepperle, Stich et Weiler (SAG18.99). Both species were cultivated in 600 mL WC-medium (as in Grzesiuk et al., 2016) adjusted to pH 7.0 in 1 L Erlenmeyer flasks. Fluorescent tubes placed above the flasks (Osram LUMILUX L30W/830, warm white) provided a light intensity of 290 ± 34 μmol photons m^{-2} s^{-1} (measured with a 4π quantum sensor inside the flask, US-SQS, Walz, Germany) for 18 h per day in a climate cabinet set to 20 °C (Vötsch GmbH, Balingen-Frommern, Germany, VB 1514). P_i-limited conditions were obtained by decreasing the P_i-concentration in the medium from 100 μM P_i (P_i-replete) to 2 μM P_i. Nitrate concentrations were replete (2 mM). Species were cultured in triplicate in both P_i conditions. Daily measurements of optical density (OD 800 nm; 5 cm cuvette; UV-2401 PC; Shimadzu, Berlin, Germany) were performed to monitor cell density in all cultures.

The P_i-replete and P_i-limited phytoplankton species were exposed to a pharmaceutical for 50 days, reflecting 30 algal generations in order allow for acclimation and evolutionary adaptation. Stock solutions of fluoxetine, ibuprofen and propranolol were dissolved in sterilized water (Millipore) and stored in dark bottles in a refrigerator. Directly after daily dilution, 110 μL water (control i.e., 0 mg drug L^{-1}) or one of the three pharmaceutical stock solutions was added to restore the concentration of the pharmaceutical. Photodegradation of fluoxetine which has a half time of 55.6 h ± 2.3 h (Heimke and Hartter, 2000) was compensated by a higher concentration of the stock solution (i.e. 0.592 mg of fluoxetine L^{-1}).

After 50 days of acclimation the chlorophyll *a* concentration, absorption cross section, photosynthetic light response curve, and fatty acid composition was measured for each algal culture. In addition, cell densities, pH (which did not exceed 7.30 during the experiment), particulate P and C concentrations were measured (see below).

2.3. Daily food preparation for Daphnia

Algae exposed to pharmaceuticals for 50 days were centrifuged (1500 × g, 5 min, 20 °C), after which medium was discarded in order to prevent direct contact of *D. magna* with the pharmaceuticals. Phytoplankton cells were resuspended in Artificial *Daphnia* Medium (ADaM), an artificial freshwater for the cultivation of zooplankton (Klüttgen et al., 1994). From the measured OD of the resuspended algae, the concentration of organic carbon (C_{org}) was estimated using previously determined conversion factors for each algal species and P_i-condition. (e.g. Marzetz et al., 2017). Using these estimations, we calculated the volume of both algae suspensions necessary to prepare food mixtures for *D. magna* containing 1.35 mg C_{org} L^{-1} of *A. obliquus* and 0.15 mg C_{org} L^{-1} of *N. limnetica* resulting in a total food concentration of 1.5 mg C_{org} L^{-1}.

2.4. Daphnia magna clone cultivation

Two *D. magna* clones A and B who originated from Warsaw city park pond (52.23 N, 21.02E; Poland), isolated in 2010, were used in the experiment. Before the start of the experiment, *D. magna* were cultured at 20 °C and darkness. *D. magna* mothers were fed daily with 2 mg C_{org} L^{-1} of P_i-replete *A. obliquus* until they had eggs from the second clutch in their brood chamber, from this point P_i-limited *A. obliquus* in the same amount (2 mg C_{org} L^{-1}) was fed.

The second clutch of *D. magna* neonates from clones A and B, were used for the experiment and divided randomly into experimental groups. All neonates were less than 18 h old.

Experimental animals were cultured at 20 °C in ADaM medium (pH of cultures: 7.3 – 7.5), and summer photoperiod (16: 8, light: dark, with 27 μmol photons m^{-2} s^{-1} during the day). Two mg C_{org} L^{-1} food consisted of *A. obliqus* and *N. limnetica* (for details see above). Each treatment consisted of three replicates each starting with ten juvenile *D. magna* per beaker. To establish the effect of pharmaceutical-exposed algae on their consumers (*D. magna*) we measured the following