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Frédéric Orias, Laurent Simon, Yves Perrodin. Respective contributions of diet and medium to the bioaccumulation of pharmaceutical compounds in the first levels of an aquatic trophic web. *Environmental Science and Pollution Research*, 2015, 22 (24), pp.20207-20214. <10.1007/s11356-015-5243-7>. <hal-01281625>

**HAL Id: hal-01281625**

**<https://sde.hal.science/hal-01281625v1>**

Submitted on 15 Sep 2023

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# Respective contributions of diet and medium to the bioaccumulation of pharmaceutical compounds in the first levels of an aquatic trophic web

Frédéric Orias<sup>1</sup>, Laurent Simon<sup>1</sup>, Yves Perrodin<sup>1</sup>

**Abstract** Nowadays, pharmaceuticals (PCs) are ubiquitous in aquatic ecosystems. It is known that these compounds have ecotoxic effects on aquatic organisms at low concentrations. Moreover, some of them can bioaccumulate inside organisms or trophic webs exposed at environmental concentrations and amplify ecotoxic impacts. PCs can bioaccumulate in two ways: exposure to a medium (e.g., respiration, diffusion, etc.) and/or through the dietary route. Here, we try to assess the respective contributions of these two forms of contamination of the first two levels of an aquatic trophic web. We exposed *Daphnia magna* for 5 days to 0, 5, and 50 µg/L <sup>15</sup>N-tamoxifen and then fed them with control and contaminated diets. We used an isotopic method to measure the tamoxifen content inside the daphnids after several minutes' exposure and every day before and after feeding. We found that tamoxifen is very bioaccumulative inside daphnids (BCF up to 12,000) and that the dietary route has a significant impact on contamination by tamoxifen (BAF up to 22,000), especially at low concentrations in medium.

**Keywords** Bioconcentration · Bioaccumulation · Pharmaceutical compound · Tamoxifen · Stable isotopes

## Introduction

Nowadays, pharmaceutical compounds (PCs) are ubiquitous in aquatic ecosystems. Indeed, PCs are found in surface waters (Blair et al. 2013; Fick et al. 2009; Kolpin et al. 2002), groundwater (Barnes et al. 2008; Loos et al. 2010), coastal zone (Claessens et al. 2013; Thomas and Hilton 2004), sediment (Schultz et al. 2010; Vazquez-Roig et al. 2012), and in biota (Zenker et al. 2014) all over the world. They are found at concentrations ranging from several picograms per liter to several milligrams per liter (Loos et al. 2009). Among these PCs, bioaccumulative PCs potentially threaten aquatic ecosystems due to their bioaccumulation inside organisms and along trophic webs (Lajeunesse et al. 2011). These compounds often have a high octanol/water partition coefficient  $K_{ow}$ .

Tamoxifen, an anticancer drug used to treat breast cancer, is one of these bioaccumulative PCs (Jean et al. 2012). Tamoxifen is a hydrophobic molecule, having high octanol/water partition coefficients with  $\log K_{ow}$  up to 7.1. QSAR models predicted bioconcentration factors between 1650 and 385,000 (Jean et al. 2012). This molecule has already been detected numerous times in the water (several hundred ng/L) and sediments of aquatic ecosystems (Roberts and Thomas 2006; Coetsier et al. 2009; Yang et al. 2011; López-Serna et al. 2012). In previous works, we demonstrated experimentally that tamoxifen was highly bioconcentrated in the first level of the aquatic trophic web, with substantial accumulation in unicellular algae when they are exposed to dissolved tamoxifen in water (Orias et al. 2015). The consumption of contaminated food sources (i.e., aquatic primary producers exposed to dissolved tamoxifen) might therefore constitute an important route of exposure for primary consumers.

The aim of this work is to determine experimentally the transfer of tamoxifen between a primary producer (photosynthetic algae) and a primary consumer (*Daphnia magna*), and to assess

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the respective contributions of exposure through feeding and through surrounding water to tamoxifen bioaccumulation in the first levels of a simplified freshwater food chain. Daphnids were exposed simultaneously to a medium and to a diet (i.e., microalgae), both contaminated by tamoxifen. We assess the respective contribution of each route to the bioaccumulation of tamoxifen by comparing these organisms to control organisms exposed only via diet or only via water or not exposed at all.

Because of analytic difficulties in measuring PCs within complex biological matrices, tamoxifen transfers between water, algae, and daphnids were quantified by stable isotopic labelling with  $^{15}\text{N}$  using [ $^{15}\text{N}$ ]-tamoxifen and by monitoring the evolution of  $^{15}\text{N}$  contents inside daphnids through time.

## Materials and methods

### Algal cultures and rearing of daphnids

Algal culture (*Pseudokirchneriella subcapitata*) and daphnid (*Daphnia magna*) rearing conditions were described in previous works (Clément and Zaid 2004).

### Chemicals

Tamoxifen- $^{13}\text{C}_2$ ,  $^{15}\text{N}$  solution (CAS Number 10540-29-1) was purchased from Sigma-Aldrich Chemical (St. Quentin Fallavier, France). The stock solution of tamoxifen was prepared in methanol (1 mg/mL) and kept at  $-20\text{ }^\circ\text{C}$ .

### Exposure of algae to tamoxifen

An algal solution of 500 mL aged less than 10 days with a classical AFNOR medium (AFNOR 1980) and a density of  $10^6$  cells/mL (10 mg dry weight of algae) was exposed to  $50\text{ }\mu\text{g}$  of  $^{15}\text{N}$ -tamoxifen ( $100\text{ }\mu\text{g/L}$ ). After exposure for 24 h, the algal solution was centrifuged at 3000 rpm for 20 min in glass tubes. A “control” solution was also centrifuged at the same time. The supernatant was removed, and the bottoms of the “control” and “exposed” tubes were stored in glass tubes at  $5\text{ }^\circ\text{C}$  throughout the experiment.

### Exposure of daphnids to tamoxifen

#### Exposure via water

Daphnids were exposed to three concentrations of tamoxifen: 0, 5, and  $50\text{ }\mu\text{g/L}$ , for 5 days. For each concentration, four beakers containing 1 L of rearing medium with ten daphnids aged 10 days were prepared. These beakers were then placed in a climatic room at  $20\text{ }^\circ\text{C}$  with a photoperiod of 16 h (500 lx). For each treatment (0, 5, and  $50\text{ }\mu\text{g/L}$ ), two beakers

were reserved for the  $^{15}\text{N}$ -tamoxifen-contaminated diet. In order to limit the ingestion of dissolved contaminant by neonate daphnids, the latter were removed daily.

#### Exposure via feeding

Daphnids were fed for 2 h a day with contaminated or control algae by placing the daphnids in beakers containing 80 mL of medium with an algal density of 400,000 cells/mL. This discontinuous diet prevented the development of algae within the exposure medium and the dilution of tamoxifen in the algal biomass, thus feeding the daphnids with algae in isolation reduced the adsorption of tamoxifen on the algae.

### Determination of $^{15}\text{N}$ -tamoxifen content in algae and daphnids

#### Sample preparation

A sample of algae (0.5 mL of concentrated) was extracted daily before feeding the daphnids. Algae were placed in 2-mL Eppendorf tubes frozen for 5 min in liquid nitrogen and freeze-dried before EA-IRMS analyses in order to quantify the tamoxifen content in the algae before each feeding step.

The daphnids were extracted daily before (3, 12, 24, 48, 72, and 96 h) and after (5, 27, 48, 51, 75, 96, and 99 h) feeding. They were then frozen for 5 min in liquid nitrogen and freeze-dried.

#### $^{15}\text{N}$ measurements

After freeze-drying, the algae samples were weighed at 1 mg in tin capsules before analysis. Nitrogen concentration and  $^{15}\text{N}/^{14}\text{N}$  ratio were measured using an isotope ratio mass spectrometer (Isoprime 100, Isoprime Ltd, Manchester, UK) coupled in continuous flow with an elemental analyser (FlashEA 1112 Thermo Electron, Milan, Italy). Aspartic acid was used to calibrate the N concentration measurements. For the isotope ratio measurements, two-point normalization of measured values was performed using international reference material IAEA-N1 and IAEA-305 according to IUPAC guidelines.  $^{15}\text{N}/^{14}\text{N}$  ratio is expressed as  $\delta^{15}\text{N}$  in ‰ relative to atmospheric  $\text{N}_2$  (air):

$$\delta^{15}\text{N} = \left( \frac{{}^{15}\text{N}/{}^{14}\text{N}_{\text{sample}}}{{}^{15}\text{N}/{}^{14}\text{N}_{\text{Air}}} - 1 \right) \times 10^3$$

and converted to atomic fraction (i.e., atomic %, Coplen 2011):

$$x(^{15}\text{N}) = \left( \frac{{}^{15}\text{N}/{}^{14}\text{N}_{\text{sample}}}{1 + {}^{15}\text{N}/{}^{14}\text{N}_{\text{sample}}} \right)$$

In-house standards calibrated against IAEA-N1 and IAEA-N2 were interspersed within the samples, and the standard deviations for the replicate analyses were lower than 0.2‰.

### Calculation of excess $^{15}\text{N}$ and tamoxifen content

Excess  $^{15}\text{N}$  concentration,  $[\text{}^{15}\text{N}]$ , was calculated from the difference between the  $^{15}\text{N}$  fraction ( $x(^{15}\text{N})$ ) of algae exposed to  $^{15}\text{N}$ -tamoxifen and the  $^{15}\text{N}$  fraction ( $x(^{15}\text{N})_{\text{control}}$ ) measured in the control treatment without exposure as follows:

$$[\text{}^{15}\text{N}] = \left( x(^{15}\text{N}) - x(^{15}\text{N})_{\text{control}} \right) \cdot [\text{N}],$$

where  $[\text{N}]$  is the N concentration in the sample.

As  $^{15}\text{N}$ -tamoxifen was the only  $^{15}\text{N}$  tracer source in the experiment, and assuming no dissociation of tamoxifen by chemical reactions or metabolic pathways, the tamoxifen concentration was calculated from the  $^{15}\text{N}$  concentration:

$$[\text{Tamoxifen}] \left( \mu\text{g/g} \right) = [\text{}^{15}\text{N}] / 15 \cdot M_{\text{Tamoxifen}} \cdot 10^6.$$

with  $M_{\text{tamoxifen}}$  (374.49 g/mol) being the molar mass of [ $^{13}\text{C}$ ,  $^{15}\text{N}$ ]-tamoxifen.

### Determination of dissolved tamoxifen concentration in water

At the end of the experiment, 150 mL of medium was collected in each beaker in which the daphnids were exposed. Because of the amount of water required for tamoxifen quantification, samples of each three replicates were pooled, resulting in a single tamoxifen concentration per treatment. Analytical measurements were performed by the Analytical Sciences Institute (CNRS, Lyon) using HPLC-MS/MS (Waters: UHPLC H-Class/Xevo TQ-S). Analytes were separated on a C18 column (Phenomenex Kinetex C18 Phényl Hexyl 100×2.1 mm, 2.6  $\mu\text{m}$ ) using as mobile phase a binary gradient formed by (A) Milli-Q quality water+0.01 % acetic acid, and (B) acetonitrile (ACN). The gradient started at 40 % B, rose to 100 % B in 2 min, leveled for 2 min at 100 % ACN, and was followed by column repackaging at 40 % ACN for 2 min. The flow was 0.5 mL/min, the furnace temperature was 40 °C, and the volume of injection was 2  $\mu\text{L}$ . Eluted analyses were monitored by MS/MS equipped with an electrospray ion source operating in positive-ion mode, and the acquisition was performed in multi-reaction monitoring (MRM) mode. Detection and quantification limits were 0.15  $\mu\text{g/L}$  (LOD) and 0.5  $\mu\text{g/L}$  (LOQ), respectively.

### Statistical analysis

The differences of  $\delta^{15}\text{N}$  values of daphnids between the different treatments (water contamination; food contamination) were tested using three-way repeated measure analysis of variance (RM-ANOVA), with “time” being the repeated factor, and “water contamination level” and “algae contamination” the independent factors. Mauchly’s test was used to test for

sphericity and univariate test degrees of freedom were adjusted using the Huynh-Feldt epsilon correction factor when necessary. Post hoc tests (contrast method) were performed to determine whether significant pairwise differences occurred between treatments. Significance for statistical tests was accepted for  $\alpha < 0.05$ . All the statistical analyses were performed using JMP 9 (SAS Institute Inc., Cary, NC) software.

## Results

### Evolution of $\delta^{15}\text{N}$ in daphnids

$\delta^{15}\text{N}$  values of daphnids fed with noncontaminated algae ( $\delta^{15}\text{N} = -1.6 \pm 0.5\text{‰}$ ) remained constant during the course of the experiment, with a mean value of  $+3 \pm 1\text{‰}$ . When exposed to algae artificially contaminated with  $^{15}\text{N}$ -tamoxifen ( $\delta^{15}\text{N} = 53 \pm 1\text{‰}$ ), the  $\delta^{15}\text{N}$  value of daphnids increased from  $+3$  to  $+6\text{‰}$  (Fig. 1). This significant increase of  $^{15}\text{N}$  in the organisms when exposed to contaminated algae compared to noncontaminated algae demonstrated a transfer of  $^{15}\text{N}$ -tamoxifen via the trophic route.

Daphnids exposed to  $^{15}\text{N}$ -tamoxifen via surrounding water exhibited a significant increase of  $\delta^{15}\text{N}$  values, up to  $+9\text{‰}$  at 5  $\mu\text{g/L}$  and up to  $+60\text{‰}$  at 50  $\mu\text{g/L}$  (Fig. 2). The  $^{15}\text{N}$  enrichment showed that tamoxifen transfer from environmental water is a major contamination route.

When exposed to  $^{15}\text{N}$ -tamoxifen via the trophic route and the surrounding medium, the  $\delta^{15}\text{N}$  values of daphnids were close to the  $\delta^{15}\text{N}$  values measured during exposure via surrounding water only (Fig. 3).

### $^{15}\text{N}$ tamoxifen quantification in algae and daphnids

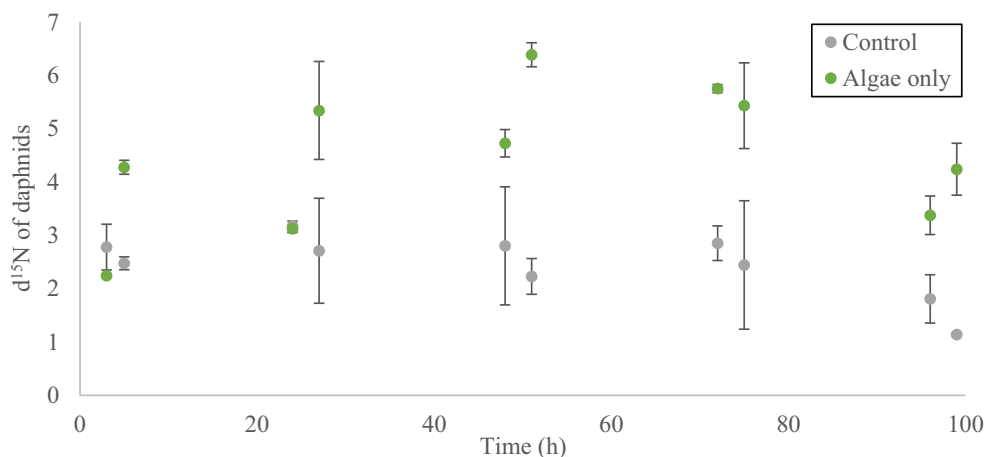
#### Tamoxifen content in algae

The average tamoxifen content in algae measured before each feeding step was 390  $\mu\text{g/g}$  ( $\pm\text{SD}$  3.32  $\mu\text{g/g}$ ).

#### Daphnids exposed via the dietary route only (Fig. 4)

During the experiment, the tamoxifen concentration in daphnids increased from 15 to 20  $\mu\text{g/g}$  after each feeding step, except at the fourth step (Fig. 4). This increase was due to the tamoxifen inside the algae grazed by daphnids during the 2-h feeding periods. This increase of content was systematically followed by a depuration phase, complete after the first feeding step (no more tamoxifen inside the daphnids), and then partial for the other feeding steps. This first step showed that 20 h was enough to purge the gut. However, the next steps showed that tamoxifen accumulated inside the daphnia even after the gut was purged. Consequently, considering the whole

**Fig. 1** Evolution of ratios  $^{15}\text{N}/^{14}\text{N}$  ( $\delta^{15}\text{N}$ ) in daphnids without toxicant (control: *gray circles*) and in daphnids exposed to  $^{15}\text{N}$ -tamoxifen via the dietary route (algae only: *green circles*). Error bars show standard deviation



curve, we observed progressive bioaccumulation of tamoxifen in daphnids as the experiment progressed. This clearly showed that the dietary route was a significant route of contamination under our experimental conditions.

#### *Daphnids exposed via medium only (Fig. 5a)*

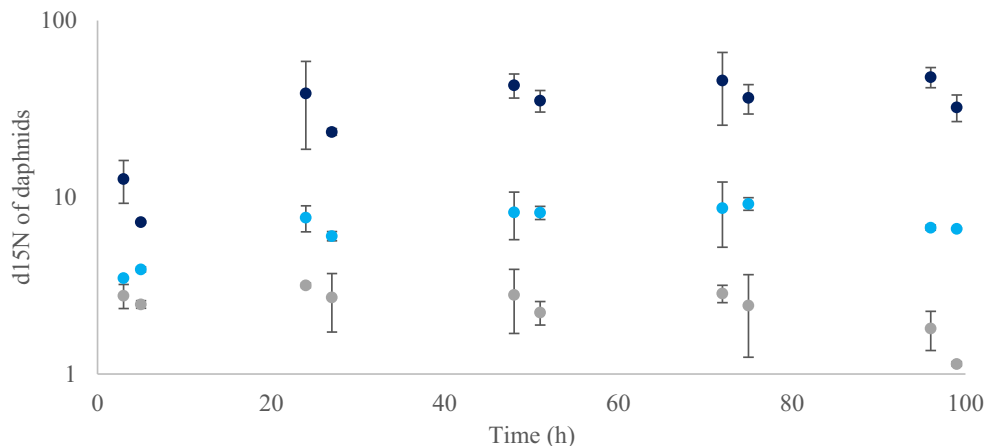
Figure 5a shows the tamoxifen content in daphnids exposed to tamoxifen via only the medium. We observed that daphnids had a very high tamoxifen content within the first minutes of exposure, about  $10 \mu\text{g/g}$  and about a hundred micrograms per gram for daphnids exposed to  $5 \mu\text{g/L}$  and  $50 \mu\text{g/L}$  tamoxifen, respectively. We could also see that the content in daphnids decreased slightly during the feeding step. This can be explained by the fact that there was no tamoxifen in the feeding medium. The algae was not contaminated either so the tamoxifen content in the daphnids decreased via a depuration mechanism. Steady state was rapidly reached with a stable tamoxifen content in the daphnids. After the third feeding step, the tamoxifen content in the daphnids was stable in both

treatments. At the end of this third feeding step, we reached a content in daphnids of up to  $26 \mu\text{g/g}$  ( $\text{SD}\pm 6$ ) and  $234 \mu\text{g/g}$  ( $\text{SD}\pm 64$ ) for  $5 \mu\text{g/L}$  and  $50 \mu\text{g/L}$ , respectively, in water. It is noteworthy that the same order of magnitude was obtained between the water of two treatments and the daphnids of two treatments (ten times more in each case). Thus, the bioconcentration factor of tamoxifen in daphnids was not linked to the initial concentration in water for the concentration range tested.

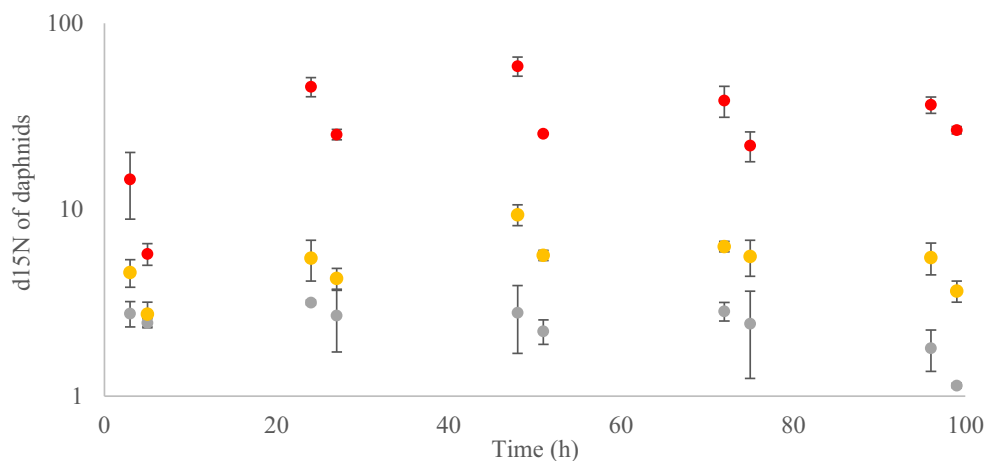
#### *Daphnids exposed via medium and dietary routes (Fig. 5b)*

Figure 5b shows the tamoxifen content in daphnids exposed via the medium and dietary routes. The dynamic bioaccumulation in these daphnids is close to that of daphnids exposed only via water: rapid bioaccumulation during the first few hours and at steady state reached around 48 h. Tamoxifen contents in daphnids were  $46 \mu\text{g/g}$  ( $\text{SD}\pm 13$ ) and  $299 \mu\text{g/g}$  ( $\text{SD}\pm 95$ ) for daphnids exposed to  $5 \mu\text{g/L}$  and  $50 \mu\text{g/L}$  in water and to contaminated algae, respectively.

**Fig. 2** Evolution of ratios  $^{15}\text{N}/^{14}\text{N}$  ( $\delta^{15}\text{N}$ ) in daphnids without toxicant (control: *gray circles*) and in daphnids exposed to  $^{15}\text{N}$ -tamoxifen via medium only ( $5 \mu\text{g/L}$ , *light blue circles*, and  $50 \mu\text{g/L}$ , *dark blue circles*). Error bars show standard deviations



**Fig. 3** Evolution of ratios  $^{15}\text{N}/^{14}\text{N}$  ( $\delta^{15}\text{N}$ ) in daphnids without toxicant (control: gray circles) and in daphnids exposed to  $^{15}\text{N}$  tamoxifen via medium (5  $\mu\text{g/L}$ , yellow circles, and 50  $\mu\text{g/L}$ , red circles) and the dietary route. Error bars show standard deviations



## Discussion

### Bioconcentration and bioaccumulation factors

For better comprehension of the respective contributions of medium and dietary routes to tamoxifen contamination, we compared tamoxifen concentrations dissolved in water and its content in daphnids at the end of the experiment (at steady state) (Table 1). Indeed, we could calculate BCF and BAF only when the concentrations were at steady state between medium and the organism.

We observed that the concentrations measured in water at the 99th hour were nearly half the initial concentration, most likely because of the adsorption of the tamoxifen onto the walls of the beakers (data not shown).

First, the results of these chemical analyses allowed calculating the bioconcentration factor (BCF) (for daphnids exposed only via water) and bioaccumulation factors (BAF) (for daphnids exposed via medium and the dietary route). These values (Table 1) show the high bioaccumulability of tamoxifen in daphnids.

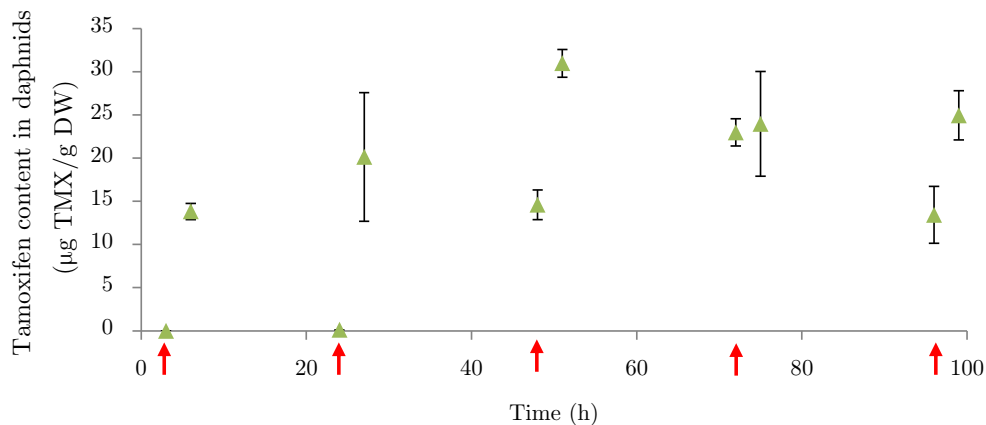
Then, by comparing the BCFs and BAFs calculated for all the treatments, we saw that the dietary route contributed more to bioaccumulation in daphnids exposed to 5  $\mu\text{g/L}$  in water than in daphnids exposed to 50  $\mu\text{g/L}$ .

Finally, we observed that the tamoxifen content in daphnids at the end of the experiment was always lower than that in algae used to feed them (390  $\mu\text{g/g}$ ). Thus, under these experimental conditions, we did not observe biomagnification of tamoxifen. This can be explained by the fact that, in our experiment, the tamoxifen concentration to which the algae were exposed (100  $\mu\text{g/L}$ ) was always higher than that to which the daphnids were exposed (5 and 50  $\mu\text{g/L}$ ).

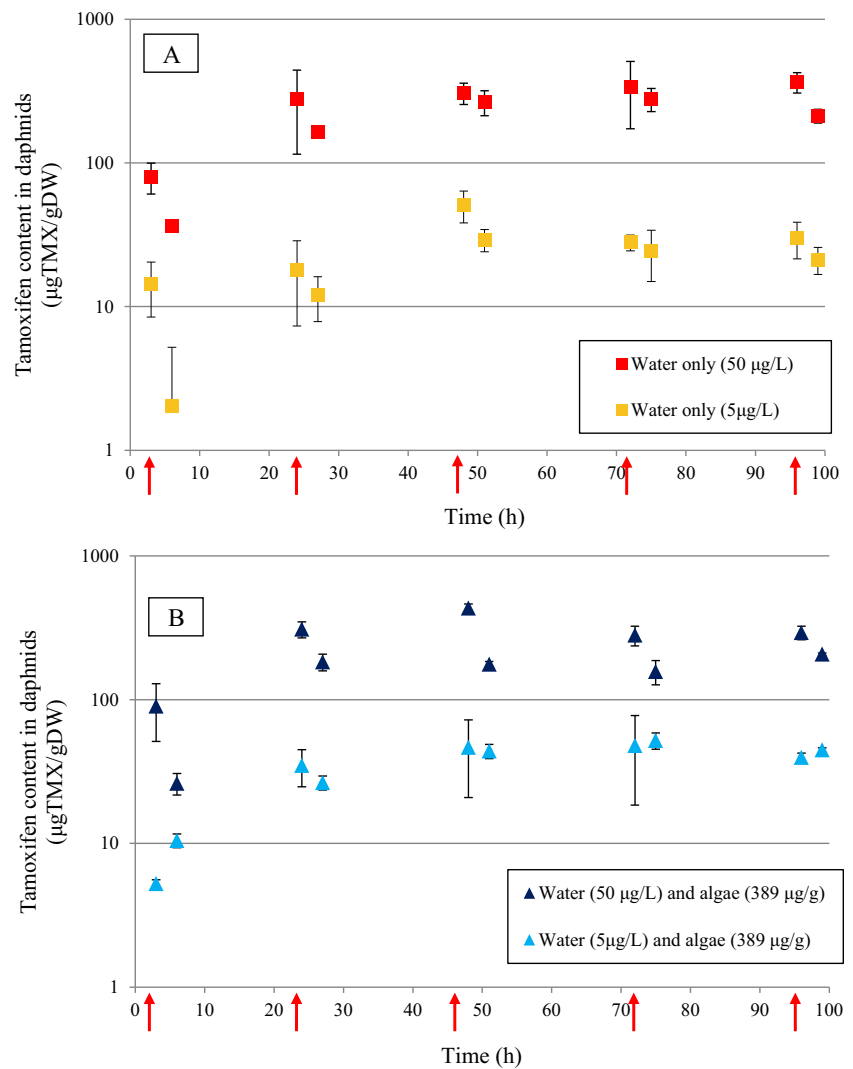
To our knowledge, tamoxifen is the most bioaccumulative PC ever measured experimentally in an invertebrate. However, other organic compounds have already been measured as more bioaccumulative, notably inside *Daphnia magna* (Geyer et al. 1991).

However, it is noteworthy that our method is an indirect analytic method. In our work, we consider that a nitrogen atom corresponds to a molecule of tamoxifen. However, it is possible that this nitrogen atom is not an unchanged

**Fig. 4** Tamoxifen content in daphnids ( $\mu\text{g}$  tamoxifen/g dry weight) exposed to tamoxifen via only the dietary route for 5 days. Error bars shows standard deviations. Red arrows show feeding steps



**Fig. 5 a, b** Evolution of tamoxifen content in daphnids ( $\mu\text{g}$  tamoxifen/g dry weight) exposed to tamoxifen via only water ( $5 \mu\text{g/L}$ , yellow square, and  $50 \mu\text{g/L}$ , red square) and via water and food ( $5 \mu\text{g/L}$ , dark blue triangle, and  $50 \mu\text{g/L}$ , light blue triangle). Error bars show standard deviations. Red arrows show feeding steps



molecule of tamoxifen but a metabolite of it. Indeed, it has already been demonstrated that human pharmaceuticals can be metabolized by freshwater crustaceans (Jeon et al. 2013). We note that the pharmaceuticals studied in the latter work were not bioaccumulative compounds.

### Bioconcentration and bioaccumulation dynamics

When observing our tamoxifen bioconcentration dynamics inside daphnids, we saw that it was consistent with other works on the bioconcentration of pharmaceuticals inside

**Table 1** Tamoxifen  $^{15}\text{N}$  concentration in water and tamoxifen  $^{15}\text{N}$  content in daphnids exposed to tamoxifen via only the dietary route for 5 days

	Treatments			
	Water $5 \mu\text{g/L}$	Water $5 \mu\text{g/L}$ and algae $390 \mu\text{g/g}$	Water $50 \mu\text{g/L}$	Water $50 \mu\text{g/L}$ and algae $390 \mu\text{g/g}$
Measured TMX concentration in water at the end of the experiment ( $\mu\text{g/L}$ )	2	1.9	29.5	25.9
Mean ( $n=4$ ) TMX content ( $\pm$ SD) in daphnids at the end of the experiment ( $\mu\text{g/g}$ )	25.69 ( $\pm 7.6$ )	42.13 ( $\pm 3.5$ )	250.3 ( $\pm 53$ )	289.2 ( $\pm 95$ )
Bioconcentration factor ( $\pm$ SD)	12844 ( $\pm 3791$ )		8483 ( $\pm 1794$ )	
Bioaccumulation factor ( $\pm$ SD)		22172 ( $\pm 1844$ )		11167 ( $\pm 3689$ )

invertebrates (Meredith-Williams et al. 2012; Anskjær et al. 2013). In these studies, the authors observed a very high bioconcentration at the beginning of exposure and then a stabilization of the content inside organisms after 24 to 48 h. Nevertheless, the PC contents in these studies were lower than those observed in our works concerning tamoxifen.

There is an increasing number of works on PC bioconcentration in invertebrates in the literature, but studies on the bioaccumulation of these contaminants are still rare. These studies are more common for other organic contaminants (e.g., perfluoroalkyl, polychlorobiphenyls, and orhexachlorobenzene). However, all these studies demonstrated that for pelagic organisms, aqueous medium is the predominant route of contamination compared to the dietary route (Muñoz et al. 1996; Dai et al. 2013; Kim et al. 2014). However, for benthic organisms, the relative contribution to the bioaccumulation of the dietary route and water can be modified (Dussault et al. 2009; Lopes et al. 2012; Bertin et al. 2014).

### Ecosystemic consequences

We can describe at least two of the consequences of these bioaccumulations at ecosystem scale: biomagnification and decreasing fitness. Indeed, many works have already shown that organic contaminant transfer, notably of PCs, can occur along the trophic web and have harmful effects on the organisms exposed (Correa-Reyes et al. 2007; Lajeunesse et al. 2011; Dai et al. 2013). Moreover, it has been demonstrated that PCs can be transferred after several generations (Kim et al. 2014). This “multigenerational effect” is consistent with the work of Chiaia-Hernandez et al. (2013), who showed that several PCs could be significantly accumulated in daphnia eggs. Lastly, Montiglio and Royauté (2014) highlighted the need to take into account anthropogenic contaminants in the behavioral disturbances of several organisms (e.g., fish, crustaceans, insects, birds, etc.).

### Conclusion and perspectives

This work, as well as previous experiments on bioconcentration in microalgae (Orias et al. 2015), showed that tamoxifen has strong bioaccumulative potential in the first levels of the trophic web considered. The experiment described in this paper focused on the transfer of tamoxifen in daphnids, the second level of this experimental trophic web, on the one hand due to immersion in the contaminated medium and, on the other hand, from the dietary route via the consumption of contaminated algae. Under these experimental conditions, we observed that this consumption plays a key role, especially when the concentration of medium is lower. In other words, we can say that the lower the concentration in

medium, the greater the role played by the dietary route in contamination by tamoxifen. In the future, it would be interesting to test lower levels of food and medium contamination in order to get closer to environmental conditions. Moreover, assays integrating a secondary consumer (i.e., a fish species) could enrich knowledge on the bioaccumulation of pharmaceuticals such as tamoxifen along a longer trophic web.

In addition, in this study, we used stable isotopes for the first time in order to monitor an organic contaminant in an invertebrate. Our results highlighted the advantages of this method for studying the bioaccumulation of contaminants in aquatic invertebrates (especially the possibility of analyzing the material directly without prior extraction, and the small quantity of living material required for the analysis). However, stable isotope analysis does not allow identifying the potential metabolites of the molecule studied. Therefore, it is now necessary to validate our method using a direct analysis method such as chromatography.

**Acknowledgments** The authors thank the SIPIBEL field observatory on hospital effluents and urban wastewater treatment plants, the European Union, the Rhone-Mediterranean Corsica Water Agency, the Rhône-Alpes Region, the Ministries of Health and the Environment, the Regional Office of Health, and the General Council of Haute-Savoie for their support.

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