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Evaluation of the influence of surfactants in the bioaccumulation kinetics of sulfamethoxazole and oxazepam in benthic invertebrates

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Keywords

Pharmaceuticals, antibiotic, sentinel species, river ecosystem, uptake, bioconcentration

Abstract

The potential ecotoxicological effects of mixtures of contaminants in the aquatic environment are generating a global concern. Benthic invertebrates, such as the crustacean *Gammarus fossarum,* are key in the functioning of aquatic ecosystems, and are frequently used as sentinel species of water quality status. The aim of this work was to study the effects of a mixture of the most frequently detected surfactants in the bioconcentration kinetics of two pharmaceuticals in *G. fossarum*, evaluating their potential enhancing or suppressing effects. Laboratory exposure experiments for both pharmaceuticals and surfactants (concentration ratio 1:25) were set up for two individual compounds, the anxiolytic oxazepam and the antibiotic sulfamethoxazole. Gammarid samples were processed using microQuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction. Pharmaceuticals concentration in the organisms was followed-up by means of nanoliquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Results indicated a similar mode of action of the surfactants in the bioconcentration kinetics of both drugs, decreasing the accumulation rate in the organism.. Oxazepam showed a higher accumulation potential than sulfamethoxazole in all cases. Depuration experiments for oxazepam also demonstrated the high depurative capacity of gammarids, eliminating >50% of the concentration of oxazepam in less than 6 h.

1. INTRODUCTION

The environmental presence of pharmaceuticals (PhACs) has been studied in detail in the last two decades, providing enough data to evidence their ubiquity in both terrestrial and aquatic ecosystems [1-4]. PhACs are very bioactive molecules, designed to remain active for long periods of time, even after being metabolized in the organism. In order to keep that activity, they are generally highly resilient to biodegradation and so their removal during conventional wastewater treatment is usually incomplete. These results in their regular entrance onto the aquatic environment via discharge of wastewater effluents, constituting an ecotoxicological threat to different non-target aquatic organisms that can be exposed directly or indirectly to these substances [5-8]: directly by the discharge of these pollutants in wastewater treatment plants (WWTPs) and indirectly by the accumulation and biomagnificaction of these PhACs in the foodweb [9]. However, most of the studies have focused so far in bioaccumulation and toxicity of single PhACs in superior aquatic taxa such as fish ([10-16]), neglecting the potential effects in inferior taxonomic groups such as benthic macro and microinvertebrates. In a recent work by Huerta et al., [17], the antiinflamatories diclofenac and ibuprofen were found in larvae of caddisflies (Hydrosipche sp.) at concentrations of 183 ng g-1 and 12.4 ng g-1 (dry weight), respectively In a different study

by Grabicova et al. [18], six different PhACs were found also in Hydrosipche sp. in rivers from the Czech Republic, reaching concentrations of 85 ng g-1 (wet weight, w.w.) in the case of the antibiotic azithromycin. Bioaccumulation in Erpobdella sp. (larvae of leeches) was also investigated in this study, and four different PhACs were detected, being diclofenac the compound found at the highest concentrations, ranging between 12-33 ng g^{-1} w.w. Recently, Lagesson et al. [19] reached the conclusion that bottom-living organisms were the main receivers of PhACs, as they showed the highest PhACs concentrations in their tissues. Despite the little data available on their bioaccumulation capacity, most of the species aforementioned are frequently used as sentinel species, as they play a key role in the food chain and any alteration or loss of some of them could alter or degrade critical ecosystem processes. For instance, leaf litter detritivores species are responsible for shredding coarse particulate organic matter (CPOM) and releasing high amounts of nutrients in solution for other aquatic species such as bacteria, fungi or algae. The freshwater crustacean Gammarus sp. is one of the most frequently used species in ecotoxicology. They are widespread in European freshwater ecosystems, their physiology is well known and they are easy to culture in the laboratory. Furthermore, their role in the food web is key, as they are very efficient as detritivores, being able to process up to the 73% of riparian dead leaf inputs in the water course[20]. Gammarids also constitute themselves a major food source for other aquatic and terrestrial vertebrates such as fishes, turtles or birds. Despite their high capacity to accumulate pollutants ([21-23]), they are very sensitive to them and any physiological changes in the gammarid population may have effects at higher levels of the food chain [24, 25].

In the last years, however, scientific concern has shifted from the conventional monitoring studies on the presence of these PhACs and other pollutants in different environmental compartments towards a broader vision of the problem, considering on one hand the presence not only of the parent drugs but also of metabolites and transformation products and their derived potential ecotoxicological effects [2, 26-33] and on the other the combined effects of the presence of mixtures of different PhACs (with or without other organic pollutants) and the potential synergetic, antagonistic or cumulative effects derived [13, 34-38]. Scientific efforts devoted to study mixture toxicity in aquatic environments have increased noticeably in the last years and had led to the creation and modification of new chemicals safety regulations, such as the existing European Union regulation REACH (Registration, Evaluation and Authorization of Chemicals) [39].

Taking all this into consideration it is very likely that PhACs coexist in the aquatic environment with other frequently detected pollutants such as perfluorinated compounds (PFOs), pesticides, flame-retardants or surfactants. The latter are one of the chemicals most produced and consumed worldwide [40, 41]. Contrary to PhACs, surfactants can be eliminated during wastewater treatment up to a 90-95% under optimized conditions, and they usually have low half-life times [42, 43]. However, their input in the receiving waters is regular, similarly to what happens to PhACs. Aquatic ecosystems are consequently facing a chronic exposure to both classes of contaminants, despite at low concentrations.

Surfactants show both hydrophilic and hydrophobic properties in their molecular structure, which gives them the ability to form micelles in solution (once they have reached a given concentration, the critical micelle concentration, (CMC)). These micellar structures confer them strong detergency and solubilisation properties that may have effects in other surrounding molecules. For instance, in soil ecosystems, hydrophobic organic compounds (HOCs) such as polycyclic aromatic hydrocarbons (PAHs) or perchloroethylene (PCE) can be solubilized by micelles (hydrocarbon core) and be transferred from soil to the aqueous pseudo-phase, leading to their desorption from soils. In fact, this remediation of contaminated soils and aquifer enhanced by surfactants application has become an effective and common treatment strategy [44]. Other works have also addressed their influence in the solubility of other hydrophobic compounds such as trichlorobenzene or dichlorodiphenyltrichloroethane (DDT) [45]. Surfactants, especially non-ionic species, are also widely used in pesticide formulations to increase their effectiveness in penetrating through cuticle structures of weeds, crops, and insects in crop fields [46]. In water ecosystems, it is well known that surfactants increase the water solubility of chemicals, depending equally on the physico-chemical properties and the nature of both substances. Recently, Chen et al. proved that the cationic surfactant cetyltrimethylammonium bromide (CTAB) significantly enhanced the photodegradation rate of triclosan in water, whereas anionic and neutral surfactants (sodium dodecyl benzene sulfonate, sodium dodecyl sulfate (SDS) and neutral polyoxyethylene (20) sorbitan monooleate, inhibited it [47]. The same CTAB, together with other surfactants (Triton X-100 and 4-dodecylbenzene sulfonic acid (SDBS)) seemed to reduce considerably the toxicity of different nanoparticles against the freshwater crustacean *Daphnia magna*, presumably due to the formation of aggregates of these nanoparticles [48]. On the contrary, other studies confirmed that, in presence of surfactants, the toxicity of these nanoparticles increased against certain plant species [49]. Ecotoxicity of surfactants themselves is usually low. Generally, cationic surfactants are more

toxic against aquatic species (fish, water flea and algae) than anionic surfactants [40]. EC_{50} values for cationic surfactants range from 0.1 to 10 ppm, whereas those for anionic formulations are 1 to 50 ppm [46].

All in all, it is obvious that aquatic ecosystems are submitted to a constant anthropogenic stress, as a wide variety of organic pollutants of very different nature enter surface waters on a daily basis, overcoming the equilibrium of these ecosystems and their capacity to neutralize their effects (dilution, in-stream attenuation, biodegradation etc). Reliable biomonitoring tools are therefore required to follow up the quality status of these ecosystems.

The aim of this study is to evaluate the influence of the background presence of a mixture of the most commonly detected surfactants on the bioaccumulation of selected PhACs in *Gammarus fossarum* in laboratory experiments. A novel method based on miniaturized QuEChERS, followed by nanoLC-MS/MS was applied to quantify the bioaccumulation of both PhACs individually in *G. fossarum*, allowing discerning bioconcentration kinetics under different conditions. The antibiotic sulfamethoxazole (SMX) and the benzodiazepine oxazepam (OXA) were chosen as target compounds, as they are frequently detected in environmental waters.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Ultra-pure water (18.2 MΩcm) was obtained from a milliQ water system from Millipore (Saint-Quentin-en-Yvelines, France). HPLC-grade solvents methanol, acetonitrile, hexane, formic acid and ammonium acetate were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Analytical standards for sulfamethoxazole (SMX) and oxazepam (OXA) (purity > 99%) were also purchased from Sigma-Aldrich. Stock standard solutions for each of the PhACs were prepared individually in MeOH at 1mg mL^{-1} and stored in the dark at −20 °C. Solutions at the corresponding working concentrations were prepared accordingly. The commercial surfactants benzyldodecyldimethylammonium chloride (BDDAC), benzyldimethyltetradecyl ammonium chloride (BDTAC), cetyl betaïn, lauryl pyridinium, sodium 2-ethylhexyl sulfate, Triton X-100 and sodium dodecyl sulfate (SDS) were supplied by Sigma-Aldrich; Stepanquat GA 90 was supplied by Stepan Company (Chassieu, France); Texapon N 701 S and benzotriazole by BASF (Lyon, France); Incromine SD by Crodarom SAS

(Chanac, France), Comperlan 100 by Cognis (Boussens, France) and the LAS C10-13 mixture by Marseille soap company (Marseille, France). Stock solutions of the surfactants (200 mg/l) were prepared in pure water/LC grade methanol (50/50; v/v) and stored at 4°C in the dark. A mixture containing all the surfactants was prepared at 5 mg L^{-1} using the appropriate dilution factor.

Custom QuEChERS salts (500 mg) were obtained from Agilent Technologies (Massy, France), and contained $MgSO_4$ (61.5 %), NaCl (15.4%), sodium citrate (15.4%) and disodium citrate sesquihydrate (7.7%). The pH of the salts was 5-5.5 and its purity was > 99.3%.

2.2. Wastewater sampling

Effluent wastewater was analyzed to fathom out the amounts of surfactants discharged into the aquatic environment. Composite samples were taken during 4 consecutive days in November 2015 in the wastewater treatment plant (WWTP) of La Feyssine (Lyon), which receives an average inflow of 66000 m^3 day^{-1} of sewage from the Lyon peripheral area (capacity of 300,000 equivalent inhabitants). The collected water is first introduced into one of three Sedipac® 3D (lamellar settling tanks) to remove grease and grit, then the pre-treated water is forwarded to three biological treatment lines to remove carbonated pollution via oxygenation and nitrogen pollution via nitrification/denitrification. Samples were immediately filtered through a 0.7 µm pore size and directly pretreated for analysis.

2.3. Test organisms

Gammarids were collected at the beginning of November 2015 in a small affluent of the Bourbre river located in a pristine site located near the municipality of Bourgoin-Jallieu (coordinates 45°36'13.9"N, 5°15'31.6" E). Mature individuals were collected and brought to the laboratory, where they were kept in 30 L aerated tanks filled with groundwater that was constantly renewed during an acclimatization and stabilization period of 3 weeks. At this stage, only males were selected and kept in order to avoid potential sensitivity differences between sexes [50]. Maturity and size homogeneity amongst individuals was also considered. The light cycle was set to 10 h of light and 14 h of darkness, and the temperature in the tanks was kept at 12 °C ± 1°C. Gammarids were fed *ad libitum* with alder

leaves (*Alnus glutinosa*) that were collected in an unpolluted site and preconditioned (rehydrated in groundwater during a week approximately).

2.4. Experimental set up

Laboratory experiments were carried out in December 2015. Organisms were exposed to SMX and OXA separatedly (triplicated experiments for each target compound), with and without background concentration of surfactants; in both cases, three replicates of 20 male gammarids were exposed for 14 days to a concentration of 1 µg L^{-1} of the corresponding drug; beakers were filled up with 500 mL of doped groundwater , and the gammarids were added at the beginning of the experiment. The same procedure was repeated in paralell, this time adding groundwater doped with a mix of surfactants at a final concentration of 25 µg L^{-1}. In both cases, the exposed gammarids were fed *ad libitum*. The experiments were performed in semi-static conditions to avoid aeration in the beakers and prevent potential degradation of the contaminants. Therefore, and in order to maintain the same dissolved oxygen (DO) level in the experiments, solutions were made new and replaced every day with groundwater previously aerated during 24 h. This procedure also prevented potential bacterial growth in the beakers and adsorption of the pollutants to the leaves, contributing to keep the same exposure concentration throughout the experiment. Doping volumes of SMX and OXA were calculated to have a proportion of MeOH ≤ 0.1 ‰ (v/v) in the solutions. Two sets of blank controls (without PhACs) were also set up in triplicates: three with only a background concentration of surfactants and the other three without it. In the latter case, the same amount of MeOH as in the experiments doped with PhACs was added. All the beakers were placed in a water bath at 12 °C ± 1°C, and submitted to the same light cycle as used during the acclimatization period.

2.5. Gammarids and water follow up

During the experimental period, gammarids were collected right before the start of the experiments (t_0), then after 1d, 2d, 7d and 14d (t_1, t_2, t_3 and t_4 respectively). Five individuals were collected at each sampling time for each set of triplicates, immediately frozen at -80°C and finally lyophilized. In the case of the gammarids sampled from the surfactants experiments, they were previously rinsed in deionized water during two minutes before freezing. Individuals from both blank controls were also sampled at t_3 and t_4 as quality controls.

In order to check and confirm the PhACs concentration in the solutions, water was sampled directly from the beakers and analyzed at t_2 and t_4. Temperature, DO and pH were monitored every day during the experiment to check for constant conditions.At the end of the exposure, the remaining gammarids were transferred to new beakers with groundwater to evaluate their depuration capacity. They were sampled individually after 6h, 24h and 48h.

2.6. Analytical methodology

2.6.1. HPLC-MS/MS analysis

Both effluent wastewater samples and water samples from the different experimental tests were analyzed by means of HPLC-MS/MS, working with an Agilent Series 1200 RRLC system (Agilent Technologies, Massy, France) coupled to a 3200 QTrap (ABSciex®, Les Ulis, France). For surfactants analysis in effluent wastewaters, samples were pretreated following the methodology of Bergé et al. [51]. Briefly, SPE extraction was carried out using cartridges Cunax2 (3 cc, 200 mg). A volume of 200 mL of wastewater was loaded onto the cartridges, and taken to a final reconstitution volume of 2 mL. Further information on the methodology is given in the Supplementary Information (SI) (Tables S1 and S2).

Regarding the water samples from the tests to verify the PhAC concentration during the experimental period, they did not need any pretreatment (SPE) due to the high working concentration of the PhACs used and the neglectable amount of suspended solids in the solutions. An Agilent ZORBAX Eclipse Plus C_{18} column was used (50 x 2.1 mm, 1.8 µm) with a mobile phase composition of (a) H_2O + 0.1% acetic acid and (B) MeOH. The gradient was a follows: from 10 to 100% (B) in 2 minutes then 100% (B) during 2 minutes and back to initial conditions in 3 min. The optimization of the MS/MS experimental conditions for SMX and OXA was performed by direct syringe infusion of standard solutions of the individual compounds at 10 ng mL^{-1}. Identification of the precursor ions was performed in the full scan mode by recording mass spectra from *m*/*z* 50 to 500. Analyses were performed in the selected reaction monitoring (SRM) mode, recording two SRM transitions per compound, one for quantitation and the other for positive confirmation. The MS/MS settings are given in Table S3 (SI).

2.6.2. Gammarids analyses

Gammarids were pre-treated and analyzed individually, following and adapting the method developed by Berlioz-Barbier et al. [23]. Briefly, the sample (1 organism) was placed

in a 2 mL centrifuge tube and ground with two stainless steel grinding balls by means of a 2010 Geno/Grinder (SPEX Sample Prep, Stanmore, UK) for 2 min at 1000 spm. Once the sample was ready, 500 µL of milliQ water, 500 µL of ACN and 200 µL of hexane were added to the centrifuge tube, followed by the QuEChERS citrate buffer. Tubes were shaken vigorously to avoid agglomeration of the salts, then vortexed during 30 sec and then centrifuged during 2 min (10000 rpm). Afterward, hexane was removed and 400 µL of ACN were transferred from the tube to an LC vial, evaporated to dryness under a low N_2 stream and finally reconstituted to 120 µL of H_2O:ACN:MeOH (70:15:15) for nanoLC-MS/MS analysis.

2.6.3. NanoLC-MS/MS analysis

Analyses were performed on an Ultimate 3000 nanoLC system (Thermofisher®, Villebon sur Yvette, France) coupled to a 5500 Qtrap (ABSciex®, Les Ulis, France) equipped with a Nanospray II interface (nano-ESI) (ABSciex®). Chromatographic separation of SMX and OXA was achieved by means of an Acclaim C_{18} PepMap 100 nanocolumn (5cm x 75 µm, 3µm particle size, 100 Å pore size) preceded by a C_{18} PepMap 100 µm-pre-column cartridge (5 mm × 300 µm, 5 µm, 100 Å pore size), both from (Thermofisher®). The auto sampler temperature was fixed at 5 °C and the column oven was set to 40 °C. An injection volume of 1 µL was used in full loop. More information on the methodology can be found elsewhere [23]. Briefly, the analysis can be divided into two steps. First, a loading step in which the target compounds are loaded and trapped in the pre-column by means of a loading pump. The loading solvent used was H_2O:ACN:MeOH (96:2:2) at a flow rate of 20 µL min^{-1}. Then, a separation step when both pre-column and separation column are connected by the switch of a 10 port-valve (at 1.5 min of the gradient), and the analytes are back-flushed from the pre-column to the nanoLC column by means of a micropump. The nanoLC flow was 300 nL min^{-1}. Mobile phases and the gradient used are described in Table S4 in SI. The valve switches again at the end of the gradient (19 min) and the pre-column is put offline, washed and equilibrated for the next sample. The micro-pump keeps connected to the analytical column till the end of the gradient.

The optimization of the MS/MS experimental conditions was performed as indicated in section 2.6.1. The nano-ESI source parameters were optimized by on-column injection of the standards into the chromatographic system.

2.6.4. Quantification

289 Matrix-matched calibration was used for quantification for both water and biota. For
290 gammarids analysis, ten calibration points were prepared for each experimental set. A pool
291 of blank gammarids was used, working with individual samples of 5.5 mg (average weight of
292 an individual gammarid). Each sample was doped with the corresponding concentration of
293 the PhAC, ranging between the LOQ and 50LOQ, and extracted following the same
294 procedure as the experimental samples.

295

296 **3. RESULTS AND DISCUSSION**

297

298 **3.1. Experimental set up**

299 *3.1.1. Selection of working concentrations for SMX and OXA*

300 Physicochemical properties of both studied compounds are summarized in Table 1.
301 Given the high number of publications dealing with PhACs presence in wastewaters and
302 surface waters, a bibliographic research was carried out in order to know the usual
303 concentrations detected for SMX and OXA and choose a representative dope level. A
304 summary is given in Table S5 (SI).

305 SMX is one of the most ubiquitous antibiotics, being frequently detected in all water
306 ecosystems probably due to its solubility and high excretion rates [52-54]. Its environmental
307 occurrence and fate has been studied intensively due to its potential, even at low
308 concentrations, to promote antibiotic resistance [55, 56]. Concentrations in urban WWTP
309 effluents can range from 10 ng L^{-1} to 650 ng L^{-1} [52] and up to 8714 ng L^{-1} in hospital
310 effluents [57]. In surface waters, average concentrations of 89.2 ng L^{-1} were detected in the
311 Ebro river basin in Spain [52], and up to 653 ng L^{-1} in the Llobregat river mouth [58]. There is
312 still a huge uncertainty regarding the biodegradability of SMX during wastewater treatment,
313 considering that published removal rates range from negative values to 100% elimination
314 [59]. once released in surface waters, its elimination depends on the presence of
315 appropriate degrading microorganisms. Photodegradation may have a role in its elimination,
316 but only to a small extent and in the most superficial water layers.

317 On the other hand, OXA is a psychiatric drug belonging to the group of
318 benzodiazepines (anxiolytics). It is also the final degradation product of diazepam, one of the
319 most consumed antidepressant worlwide [60]. Loos et al. [61] found out that oxazepam was
320 present in the 90% of the 90 effluent samples analyzed in 18 different European countries,
321 at an average concentration of 162 ng L^{-1} Similar results were detected by Baker et al. [62]

in the UK . Furthermore, it has been demonstrated that OXA is resilient not only to aerobic and anaerobic biodegradation, but also to photodegradation [60, 63] and so it is likely that this compound can persist and bioaccumulate in aquatic ecosystems. Klaminder et al. [64] concluded that OXA could persist in water systems for decades.

The working concentration of both compounds was finally set at 1 μg L^{-1} to allow the evaluation of bioaccumulation and also to make possible future metabolite elucidation analyses.

3.1.2. Selection of working concentrations for surfactants

Levels of surfactants detected in the effluent wastewater sampled are shown in Table 2. As observed, the concentrations ranged from 0.13 μg L^{-1} to 34.2 μg L^{-1}. The highest concentrations corresponded to the anionic surfactants LAS $C_{10\text{-}13}$, specifically to LAS C_{12} (34.2 μg L^{-1}). Similar and usually higher concentrations in effluent wastewaters are found in the literature [43, 51]. A working concentration of 25 μg L^{-1} was finally selected as representative of a real scenario.

3.2. Exposure experiments

Only the free dissolved concentration in water of a given compound is bioavailable and can pass through biological membranes [65]. The use of groundwater in the experiments, containing negligible amounts of suspended solids, ensured that none of the spiked drugs were sorbed or bound to these particles, and so all the pollutant was available for potential bioconcentration in the test organisms. Working at semi-static conditions aided to renew the media and the available concentration on a daily basis, avoiding any potential degradation during the 14 d exposure. Furthermore, the information provided in section 3.1.1 also confirms the resilience of both PhACs in water environment in short-term experiments.

The concentration of both compounds in the water from the different beakers was measured twice (once per week) during the experiment (Table 3). Variation between both weeks was negligible for OXA (0.7%-4.4%) and low for SMX (9.9%-13%). Other control parameters such as pH, DO and T were also measured (see S.I., Table S6).

Regarding nano-LC-MS/MS analyses, the methodology used allowed the analysis at individual scale for five gammarids individuals per day of sampling. Inter-individual variability was estimated as the relative standard deviation (RSD) and is shown in Table S7

(SI). No background concentration was detected in any of the individuals from the blank controls.

3.2.1. Mortality

After one week of exposure, the mortality of gammarids stayed below 30% in both controls (with and without surfactants). The overall mortality at the end of the experiment was of 35% of the individuals in the control without surfactants and a 20% in the control with the surfactants mixture (see Figure 1). Micelles formed by the surfactants may have a protective function against external conditions for the gammarids; for instance, against the doped MeOH that was added in both controls to equal the amount of PhACs added in the rest of the experiments (despite it remained ≤ 0.1 ‰ (v/v) in all the solutions). Unfortunately, there are currently no standard exposure tests for gammarids. Different publications put forward a cut-off level of 10% mortality in the blank controls, as quality measure [66, 67]. However, it should be taken into account that the exposure conditions in these papers were different to those in our work; for instance, the exposure was shorter (10 d), the gammarids were not fed, and the species used (*Gammarid pulex*) and population origin were completely different. Nevertheless, considering the proposed threshold, mortality was highly acceptable at least in the control with surfactants after 10 d (11.6%). On the other hand, for other sensitive crustaceans such as daphnia, test validity is fixed to 20% of mortality in the controls (for exposures of 7 days, ISO (2008) International Standard 20665). In our experiment, this 20% mortality is reached after 14 days of exposure in the control with surfactants, but it was reached after only 4 days in the control without surfactants. This could also be due to a lack of homogeneity of the individuals: differences in age and degree of maturity (despite the selection was done carefully), tolerance to the organic matter in the beakers (although the containers were cleaned and refilled daily), or some errors during the manipulation of the beakers.

In the experiments without surfactants, SMX showed a rapid increase in the mortality rate, reaching 23% after the first week, and 28% at the end of the experiment, with an average of 14 survivors out of 20 in each triplicate experiment (no considering the individuals sampled) (see Figure 1a). The mortality registered in the experiments with OXA was slightly lower, with a 15% of mortality after a week and a final mortality of 22% at the end of the experiment (average of 16 survivors in each triplicate). The potential adverse effects of SMX in the gammarids acted more rapidly than those of OXA during the 14 days of exposure. To the author´s knowledge, there are no data regarding the ecotoxicity of SMX against benthic invertebrates. The most restrictive ecotoxicity values found in the literature

388 are half maximal effective concentrations (EC_{50}) of 27 µg L^{-1} against blue green algae and up
389 to 25.2 mg L^{-1} against daphnids [68]. In both cases, these EC_{50} values are much higher than
390 the concentration used in the experiments in this study (1 µg L^{-1}). Regading OXA, its
391 ecotoxicity is poorly described in the literature. There is only one published study from 1994
392 by Calleja et al. [69], in which the hazard quotient for this compound was estimated
393 considering the environmental measured concentrations (MECs), resulting in "unlikely risk".
394 More recently, Brody et al. [70] observed behavioural alterations in wild European perch
395 exposed to1.8 µg $L^{-1}$of this drug.

396 In contrast, the mortality rate was higher in both exposure experiments of OXA and
397 SMX with background concentration of surfactants, and equal to 33.3% in both cases at the
398 end of the experiments (with an average of 13 survivors out of 20 in each triplicate). This
399 could be explained in terms of the additive effect of the background concentration of the
400 surfactants. As observed in Figure 1b, the mortality was higher in the PhACs doped
401 experiments than in the corresponding control, more markedly during the second week. It
402 seems that the extended exposure to surfactants had an effect on the vulnerability of these
403 organisms to other external factors, in this case SMX or OXA simultaneously present in the
404 same media. However, this is not reflected in an increased bioconcentration of SMX or OXA
405 in the organisms (see section 3.3).

406 *3.2.2. Feeding test*

407 Due to the low number of survivors by the end of the experiments, feed tests could
408 only be carried out in both control experiments (with and without detergents), in order to
409 check if the background presence of surfactants, independently from the effects of the
410 studied PhACs, affected the efficiency of gammarids as CPOM shredders. The feeding rate is
411 frequently used as a sensitive and robust endpoint in bioassays, indicating relevant changes
412 in the equilibrium of fresh water ecosystems [71].

413 At the end of the experiments, the remaining food was removed from the beakers and 10
414 leaf discs were added in each replicate of the controls. An average of 10 gammarids
415 remained in the control without surfactants, and 13 in the controls with the surfactant
416 concentration. Differences were not very significant, as feeding was only slightly higher in
417 those controls with a background concentration of surfactants (5.9% higher), which could be
418 attributed to stress in the organisms (see Figure S1 in SI).

419

420 **3.3. Exposure over time and bioconcentration**

In all the experimental sets, 5 organisms were taken at each sampling time and analyzed. Detected values were above LOQ in all cases. With the results obtained, kinetic curves were built by means of the SciDAVIs free software, using the Levenberg-Marquardt algorithm [72].

3.3.1. Oxazepam

Figure 2 shows the bioconcentration kinetic curves of OXA in the gammarids. In the experiment without surfactants (exposure 1µg L^{-1}), an increasing trend was observed from the beginning, which was strong during the first day, going from 0 to an average value of 50.2 ng g^{-1} after only 24 h. After that, the concentration in the organisms kept accumulating but at a more moderate pace. At the end of the experiment, the steady state hadn´t been achieved, and an average concentration of 106 ng g^{-1} was detected in the organisms. Sordet et al. [21]carried out a similar experiment with a lower concentration exposure to the drug (200 ng L^{-1}). This resulted in a much slower bioconcentration process, reaching an average value of 2.73 ng g^{-1} after 1 day. It decreased at day 2 (2.03 ng g^{-1}), but kept increasing till day 7 (3.98 ng g^{-1}). Steady state was achieved during the second week, and the bioconcentration started decreasing from day 14 on. Comparison with this previous study shows that the capacity of gammarids to accumulate OXA could be proportional to the exposure concentration. If we look at the kinetic curve estimated in the present study, it seems that the bioconcentration plateau was reached between day 4 and 6 of exposure. The mortality associated to that first week of exposure went up to 15%, higher than the mortality registered from that point till the end of the experiment (from 15% to 22%).

Lagesson et al. [19]found out bioaccumulation concentrations around 10 ng g^{-1} in different benthic invertebrates such as *Ephemeroptera sp*. or *Zygoptera sp*. in a natural pond spiked at an initial concentration of 400 ng L^{-1}. Despite the potential decrease in the water concentration with time, it barely affected the bioaccumulation levels during the length of the experiment (65 days).

In the experiment with the background concentration of surfactants, the trend was similar during the first week, reaching body burdens around 74-76 ng g^{-1}. Bioconcentration of OXA reached 33.7 ng g^{-1} after one day (compared to the 50 ng g^{-1} in the experiment without surfactants). Between days 2 and 7 of the experiment, average concentrations went from 47.5 ng g^{-1} to 74.03 ng g^{-1} (an increase of 55.7%). It correlates with a quick increase in the mortality during the first week (25% on day 7). Between days 7 and 14, the bioconcentration decreased a 12%, reaching an average value of 65 ng g^{-1}, suggesting that

454 the steady state would have been achieved during the last days of the experiment and after
455 that, a depuration process would have started. In contrast, mortality was higher during the
456 first week (25%) and increased during that last week, but a much lower pace, reaching a final
457 value of 33% of dead individuals. As observed, the body burden of the gammarids in this
458 case could have been diminished by the presence of surfactants, which seem to decrease
459 the capacity of gammarids to accumulate this drug. Contrary to what was expected, the
460 presence of surfactants may have some effect on the physiology of gammarids, as mortality
461 was higher at the end of this experiment but the bioconcentration of OXA was lower (see
462 Figure 1b and 2b).

463 *3.3.2. Sulfamethoxazole*

464 Regarding SMX, bioconcentration kinetics followed a different trend (see Figure 3).
465 In the experiment without surfactants, bioaccumulation was observed from the beginning of
466 the experiment but at a much slower rate than that of OXA (18 ng g^{-1} on day 1); it did not
467 vary on day 2 and then increased during the rest of the experiment, reaching 44.4 ng g^{-1}
468 after two weeks (see Figure 3a). In this case, the mortality increased rapidly during the first
469 week, reaching a 23% at day 7, and then increased to a final value of 28% during the second
470 week (Figure 1a).

471 In the experiment with surfactants, it can be observed that the average
472 concentration detected in the organisms was nearly the same from the first day till the end
473 of the experiment, indicating that the steady state was reached between day 1 and 2 at a
474 concentration around 9 ng g^{-1} (see Figure 3b). Mortality reached a 10% on day 3 and
475 remained stable till day 6 and increasing much more markedly during the second week (see
476 Figure 1b). As observed with OXA, it seemed that the background presence of the
477 surfactants mix decreased the bioconcentration rate of the antibiotic but enhanced the
478 mortality rate throughout the experiment, especially during the second week. These results
479 diverge with those observed in the experiment with OXA, in which mortality was more
480 severe during the first week of exposure. Nevertheless, differences in the bioconcentration
481 amount between OXA and SMX could be attributed to a higher solubility of the antibiotic,
482 making it less prone to accumulate in the gammarid tissues. This could also explain why the
483 background presence of surfactants hampered the bioaccumulation of SMX in comparison
484 with the exposure experiments without that mix (four times fold).

485 *3.3.3. Bioconcentration factor*

The bioconcentration factor (BCF) reflects that capacity of the organism to accumulate a given compound from the environment, but only through its respiratory and dermal surfaces (exposure in the diet is excluded) [65]. It can be calculated following equation 1:

[1]

$$\frac{dC_{organism}}{dt} = k_{in} \times C_w(t) - k_{out} \times C_{organism}(t)$$

where t is time, $C_{organism}$ is the concentration in the organism, k_{in} is the uptake rate constant from the water (mL g^{-1} d^{-1}), C_w the concentration in the water and k_{out} is the depuration rate constant (d^{-1}), which gathers different elimination pathways in the organism such as respiratory surface, fecal egestion, metabolic biotransformation and/or growth dilution [73]. K_{in} and k_{out} are given in Table 4 and were estimated by the SciDAVIs software, considering first order kinetics described with a one-compartment toxicokinetic model [74]. Only when $C_{organism}$ and C_w do not change with time, the system has reached the steady state condition and BCF can be calculated as follows (equation [2]):

[2]

$$\frac{k_{in}}{k_{out}} = \frac{C_{org}}{C_w} = BCF$$

Steady state was reached only in two of the four experiments.

Following the indications from REACH legislation, those compounds with a BCF > 2000 are considered bioaccumulative, and very bioaccumulative if the corresponding BCF > 5000. Despite BCFs are higher for OXA than for SMX, both are far below these threshold limits established by the UE.

3.4. Depuration experiments

At the end of the exposure experiments, survivors were placed in beakers with groundwater during 72 hours to evaluate their depuration capacity. The limiting factor in this case was the number of survivors at the end of each experiment, and the evaluation could only be carried out after the exposure experiments for OXA without surfactants, with a total of 6 gammarids in the three bakers. As shown in Figure 4, depuration of OXA

happened quickly, as levels decreased from 105.4 ng g^{-1} to 50 ng g^{-1} in barely 6 h, and it was fully eliminated before 72 h. Data seemed to follow and first order exponential decay.

Only 4 gammarids survived in both SMX experiments, and after only 6 h, no trace of sulfamethoxazole could be detected. There were no survivors in the experiment with OXA and surfactants.

4. CONCLUSIONS

The structural and functional equilibrium of freshwater ecosystems is currently compromised by the environmental presence of a large number of organic pollutants which are being discharged into natural waters on a regular basis. Data on this regard is still insufficient for a full risk assessment, particularly on the direct or indirect effects of mixtures of these contaminants in the first trophic levels. Bibliographic research demonstrates that uptake data available for aquatic organisms is mostly devoted to fish and aquatic plants. Recent works have shown the bioaccumulation of selected pharmaceuticals in gammarids, but have not fully addressed their bioconcentration kinetics. This work demonstrates the highest capacity of gammarids to accumulate OXA than SMX at the same exposure conditions, even under environmental stress situations such as the background presence of other contaminants, and also their capacity to quickly eliminate one of these compounds, OXA, from their tissues. Mortality registered was higher in the exposure experiments with surfactants (33% vs 28% after 14 d). Bioconcentration of OXA was more relevant than that of SMX in both experiments with and without the presence of surfactants. Further studies will focus on the gammarids metabolism of the bioconcentrated PhACs, in order to evaluate to which extent metabolism is key in the elimination of this compounds and which TPs are generated.

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804 **FIGURE CAPTIONS**

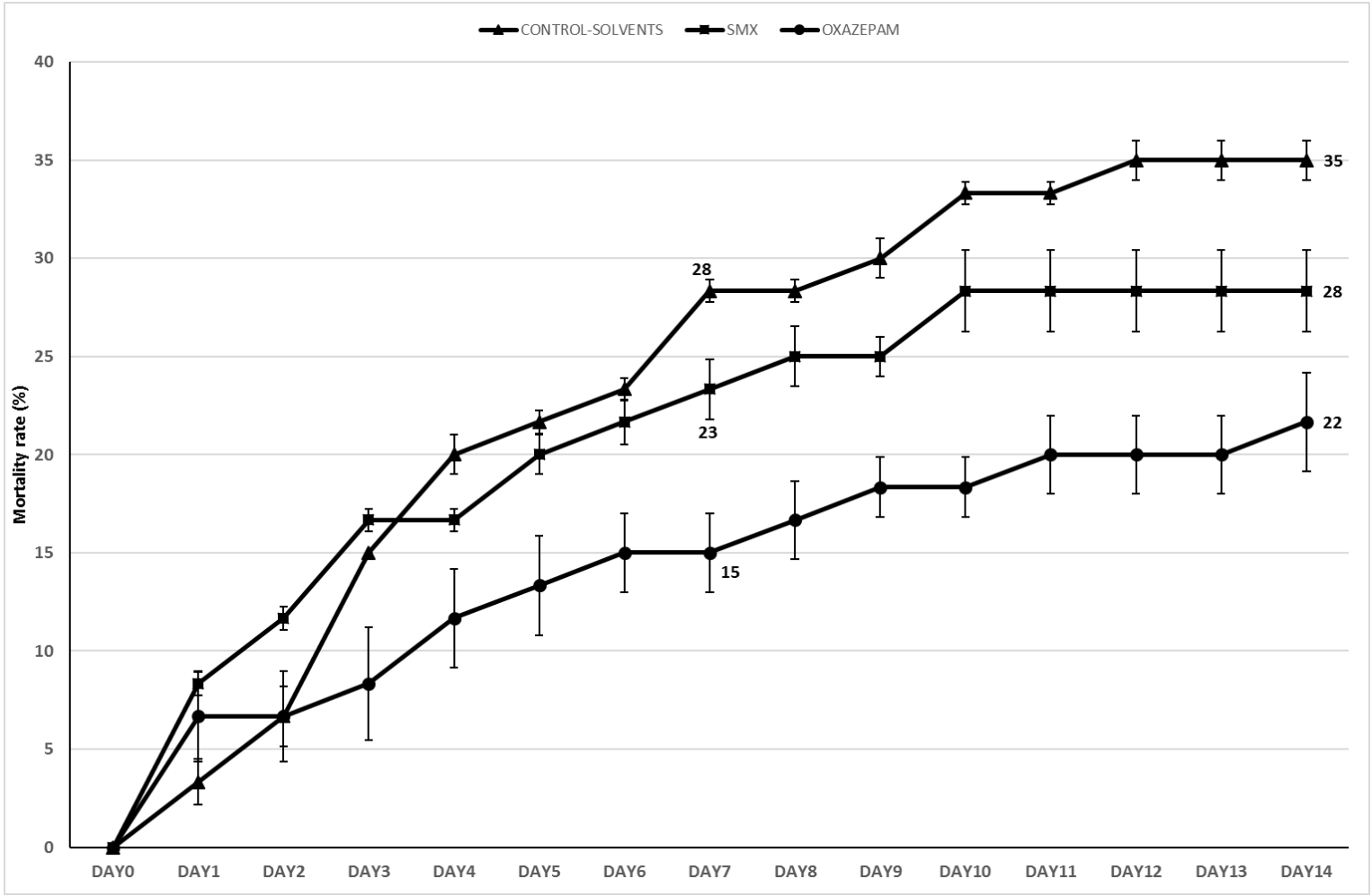
805 **Figure 1.** Mortality, expressed as a percentage of dead individuals, during the exposure
806 experiments. A. Exposure experiments without surfactants. B. Exposure experiments with
807 surfactants.

808 **Figure 2**. Bioconcentration curves of oxazepam in *Gammarus fossarum* sp. (a) Exposure
809 media with oxazepam at 1 µg L^{-1}. (b). Exposure media with surfactants at 25 µg L^{-1} and
810 oxazepam at 1 µg L^{-1}. Curves obtained with SciDavies software.

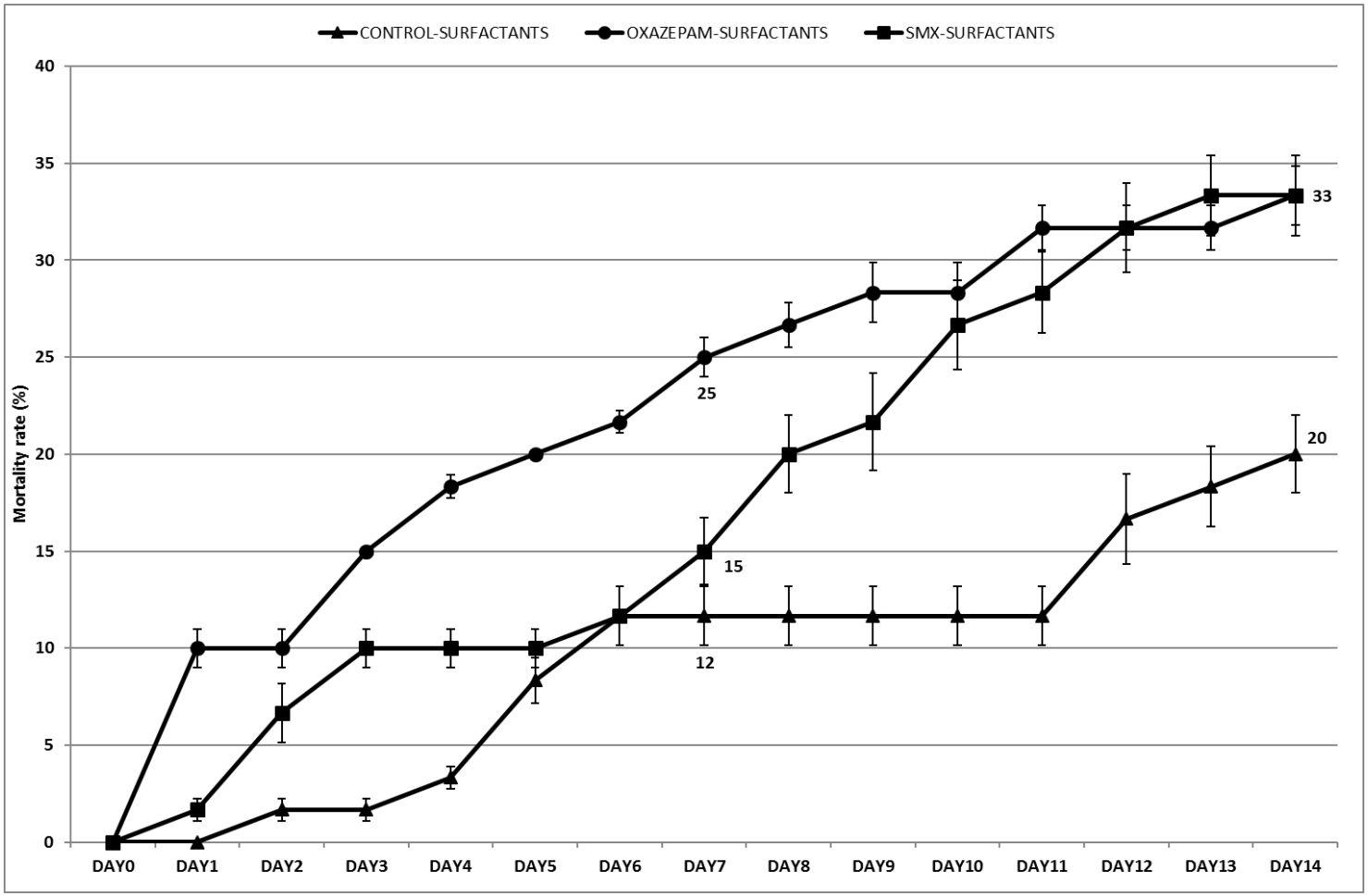
811 **Figure 3**. Bioconcentration curves of sulfamethoxazole in *Gammarus fossarum* sp. (a)
812 Exposure media with sulfamethoxazole at 1 µg L^{-1}. (b) Exposure media with surfactants at µg
813 L^{-1} and sulfamethoxazole at 1 µg L^{-1}. For comparative purposes, both graphs have been
814 depicted with the same scale on Y axis. Curves obtained with SciDavies software.

815 **Figure 4**. Depuration of oxazepam accumulated in *Gammarus fossarum* (without
816 surfactants) over < 72 h.

a.


CONTROL-SOLVENTS
SMX
OXAZEPAM
Mortality rate (%)
40
35
30
25
20
15
10
5
0
DAY0
DAY1
DAY2
DAY3
DAY4
DAY5
DAY6
DAY7
DAY8
DAY9
DAY10
DAY11
DAY12
DAY13
DAY14
35
28
28
23
22
15

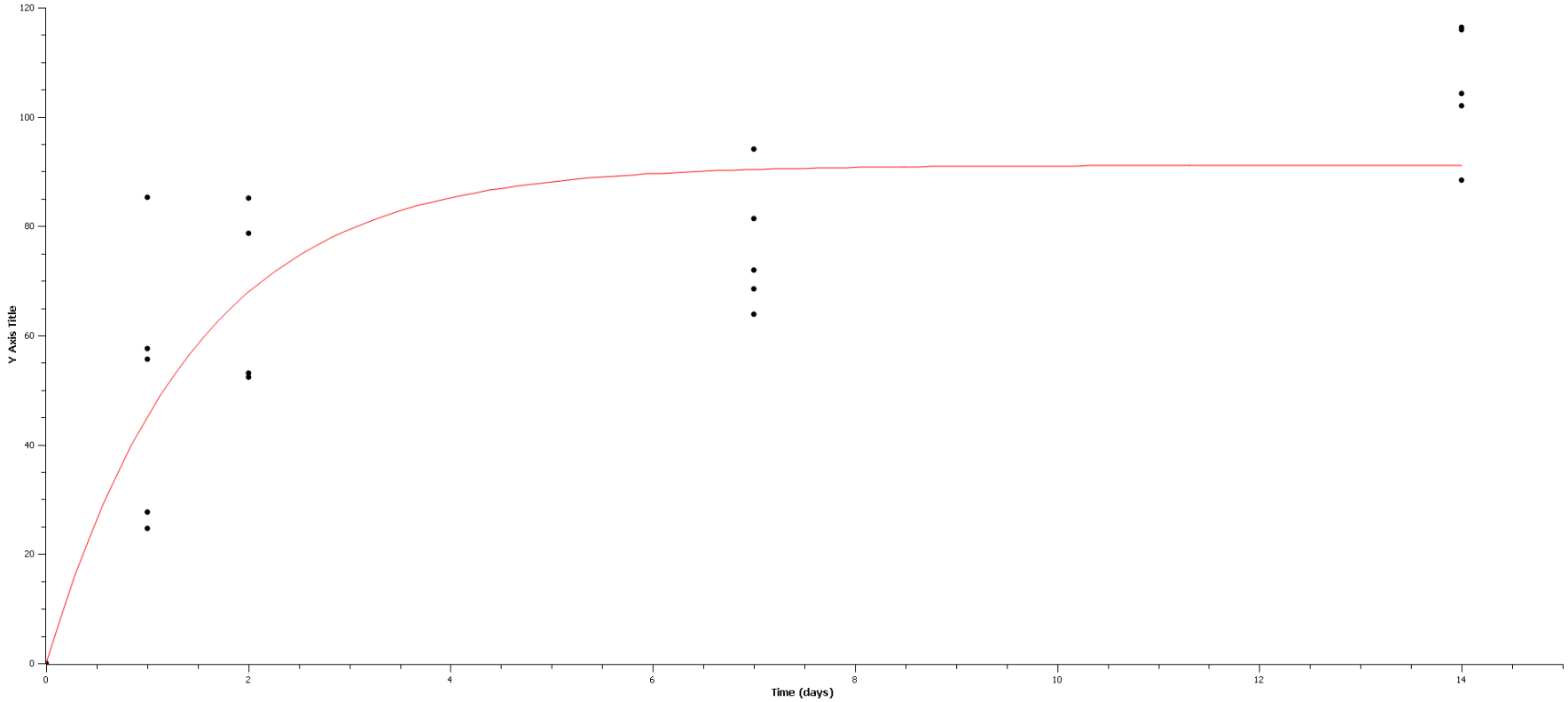
821 b.



822

823 **Figure 1.** Mortality, expressed as percentage of dead individuals, during the exposure experiments. A. Exposure experiments without surfactants. B. Exposure
824 experiments with surfactants.

a)



b)

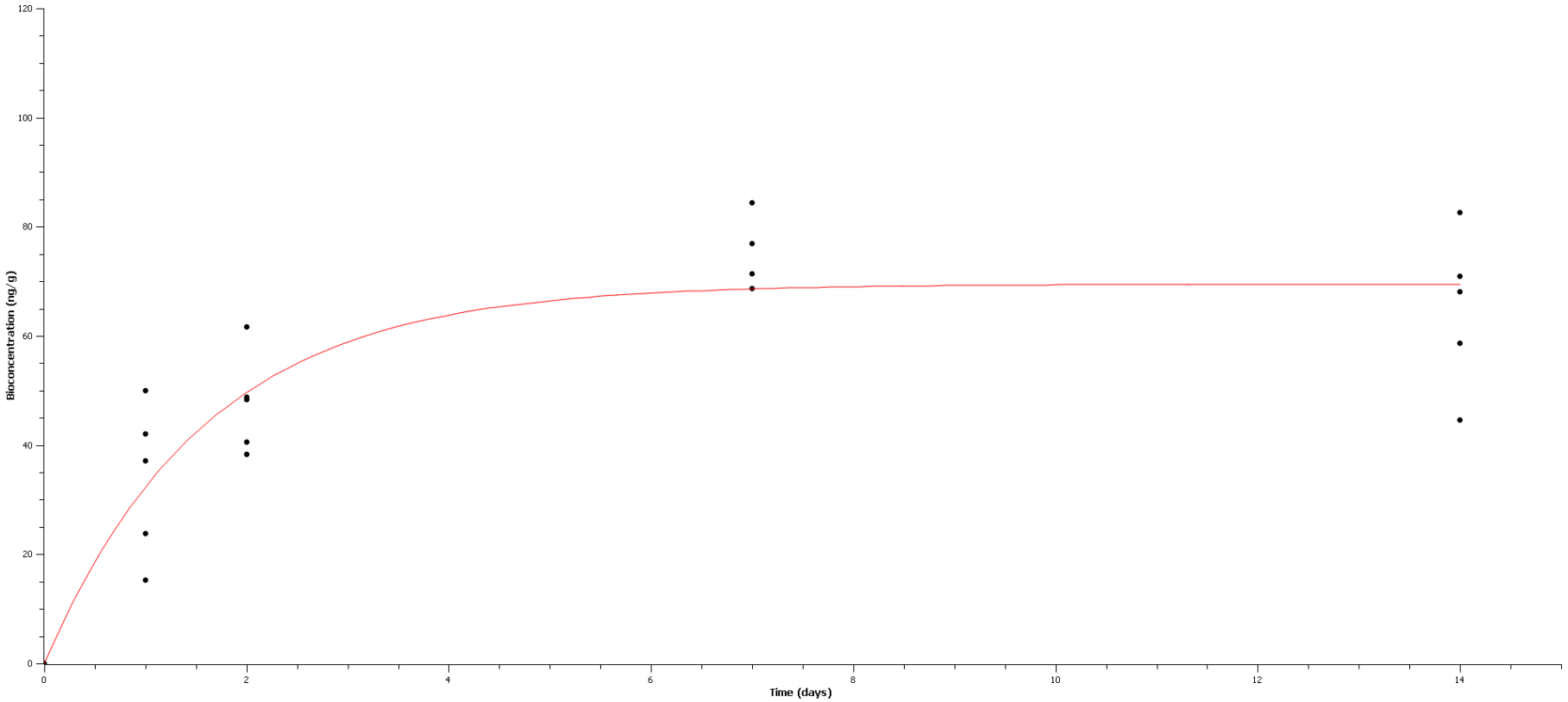
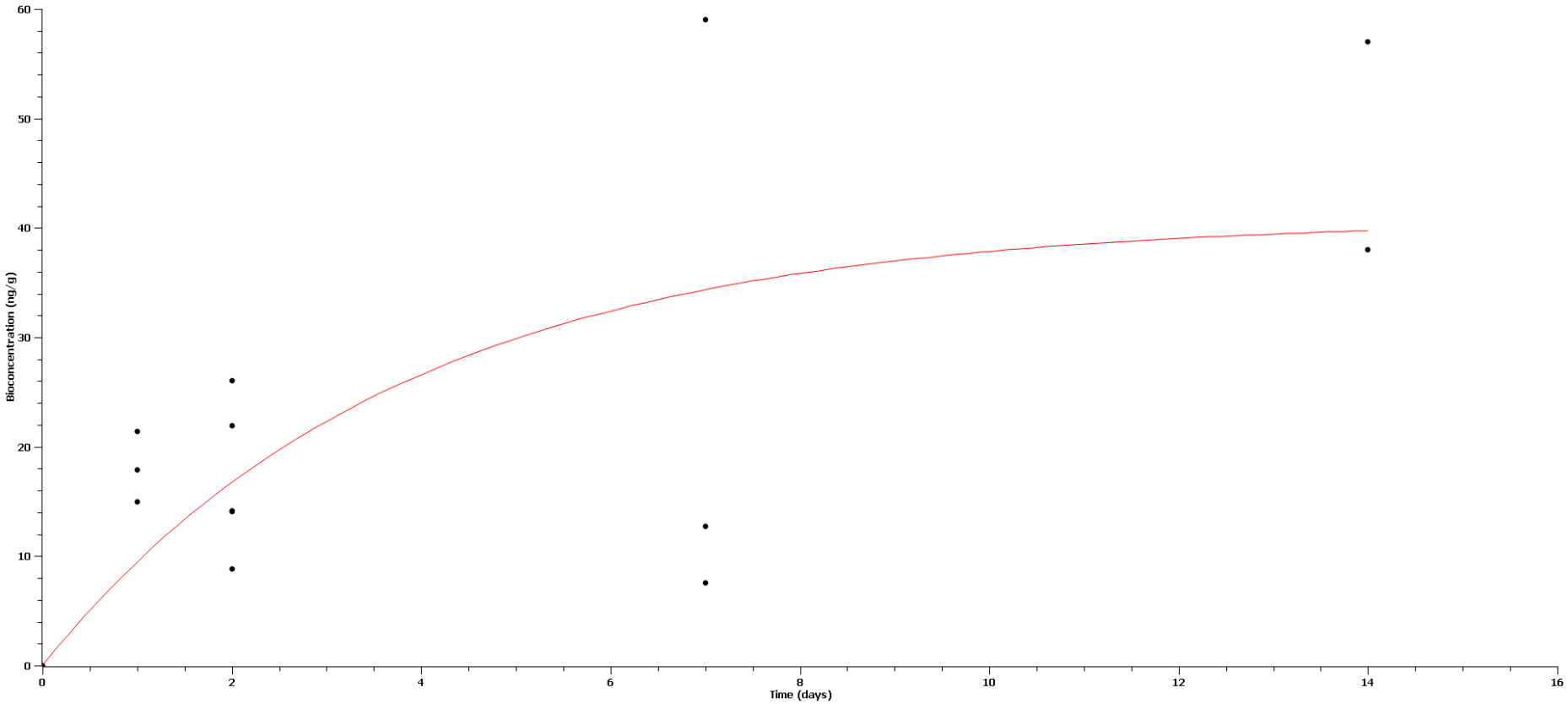


Figure 2. Bioconcentration curves of oxazepam in Gammarus fossarum sp. (a) Exposure media with oxazepam at 1 µg L-1. (b). Exposure media with surfactants at 25 µg L-1 and oxazepam at 1 µg L-1. Curves obtained with SciDavies software.

841 a.



842

843

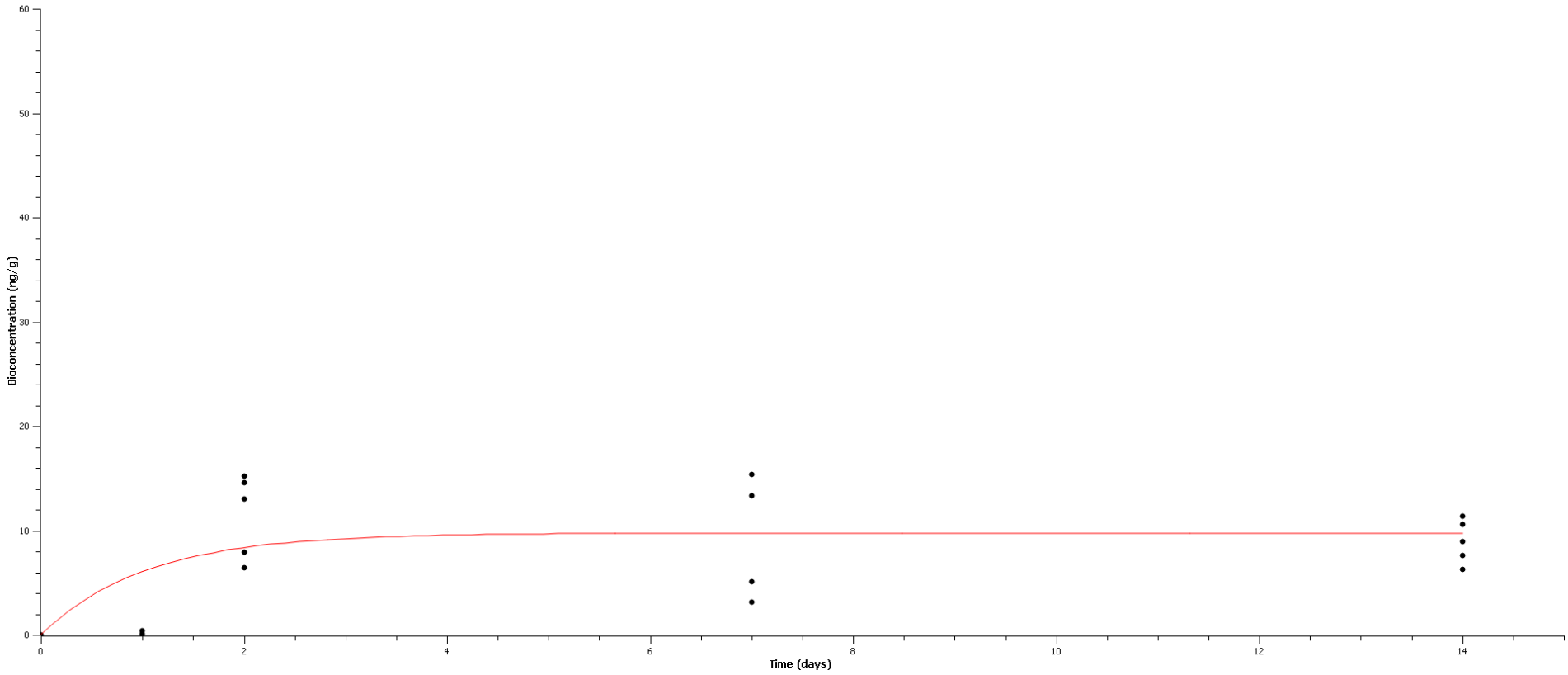
844

845

846

847 b.

848

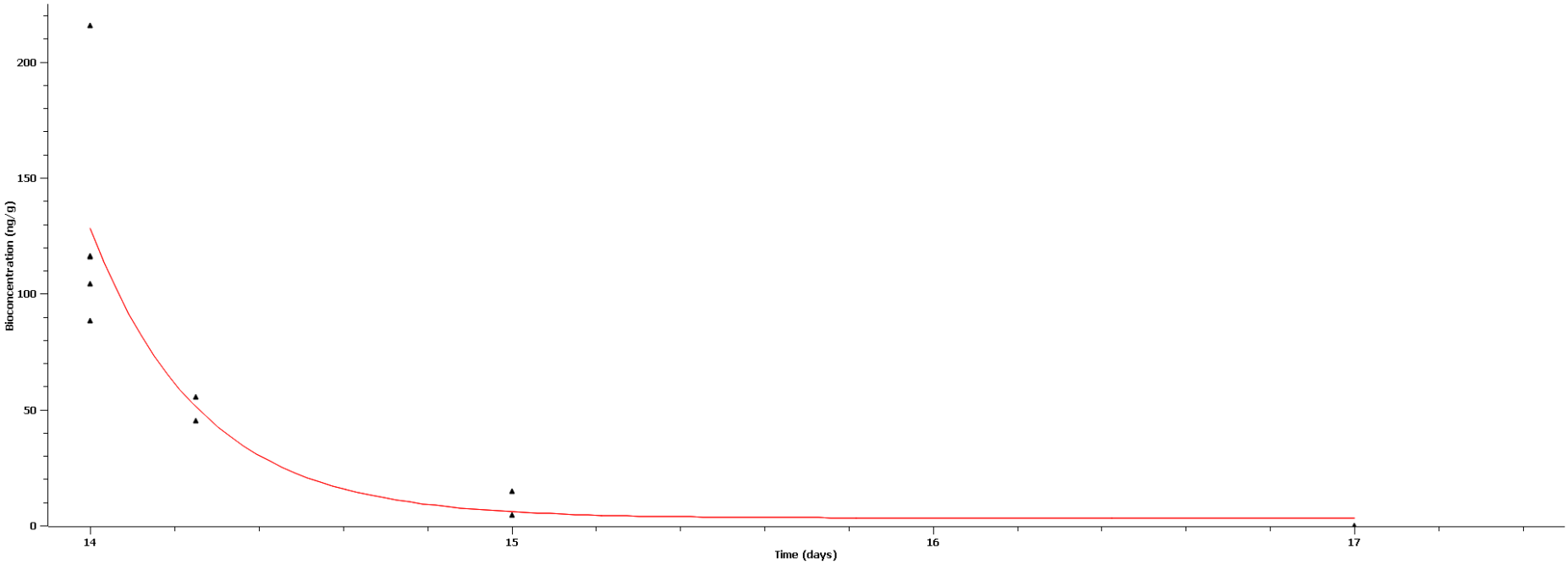


849

850

851 **Figure 3**. Bioconcentration curves of sulfamethoxazole in *Gammarus fossarum* sp. (a) Exposure media with sulfamethoxazole at 1 µg L^{-1}. (b)
852 Exposure media with surfactants at µg L^{-1} and sulfamethoxazole at 1 µg L^{-1}. For comparative purposes, both graphs have been depicted with the
853 same scale on Y axis. Curves obtained with SciDavies software.

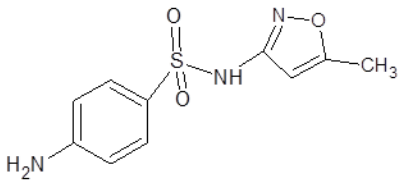
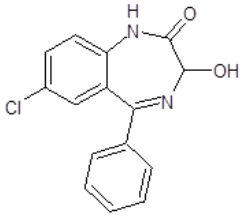
854



855

856 **Figure 4**. Elimination of oxazepam accumulated in *Gammarus fossarum* (without surfactants) over < 72 h.

857 **Table 1.** Experimental (*) and predicted physico-chemical properties of the studied pharmaceuticals.

	MOLECULAR STRUCTURE	ELEMENTAL COMPOSITION	CAS NUMBER	MW (average)	SOLUBILITY (mg/L)	Log P
SULFAMETHOXAZOLE		$C_{10}H_{11}N_3O_3S$	723-46-6	253.278	610* 459	0.89* 0.79
OXAZEPAM		$C_{15}H_{11}ClN_2O_2$	604-75-1	286.713	179* 88.1	2.24* 2.01-2.92

858 Source: www.drugbank.ca (07/2016)

859 **Table 2.** Surfactant levels determined in wastewater effluent from La Feyssine wastewater treatment plant. LOQs are given in Bergé et al. [39].

SURFACTANT	Day 1	Day 2	Day 3	Day 4
Benzotriazole	4.80	4.29	3.70	3.03
Comperlan 100	< loq	< loq	< loq	< loq
Cetyl Betain	0.41	< loq	< loq	< loq
Triton X100	3.03	19.62	2.27	3.21
Stepanquat GA 90	< loq	< loq	< loq	< loq
BDDAC	5.24	0.53	0.96	0.37
BDTAC	**7.20**	0.13	0.55	< loq
Lauryl pyridinium	1.52	< loq	< loq	< loq
Incromine SD	< loq	< loq	< loq	< loq
Sodium 2-ethylhexyl sulfate	< loq	< loq	< loq	< loq
SDS	< loq	< loq	< loq	< loq
LAS C10	3.69	23.15	21.25	28.20
LAS C11	< loq	**33.75**	2.51	19.55
LAS C12	< loq	**34.20**	< loq	7.87
LAS C13	< loq	24.55	< loq	< loq
Texapon N 701 S	< loq	< loq	< loq	< loq

860

861 **Table 3.** Measured concentration of the studied pharmaceuticals in the experimental tests (n=3)

		Day 3			Day 13		
		C (ng L^{-1})	SDV	RSD(%)	C (ng L^{-1})	SDV	RSD(%)
SULFAMETHOXAZOLE	***No-surfactants***	752.5	107.6	14.3	851.5	44.5	5.2
	Surfactants	755.5	91.1	12.1	830.3	39.6	4.8
OXAZEPAM	***No-surfactants***	1016.7	29.4	2.9	1009.7	22.1	2.2
	Surfactants	955.2	41.9	4.4	997.7	23.5	2.3

Table 4. Uptake and depuration constants (K_{in} and K_{out}, respectively) and bioconcentration factor (BCF) estimated by SciDAVIs Software

	K_{in}	K_{out}	BCF (L/Kg)
Oxazepam no surfactants	61.5 ± 12.3	0.68 ± 0.15	**90.4**
Oxazepam surfactants	44.5 ± 6.9	0.62 ± 0.11	**71.8**
Sulfamethoxazole no surfactants	13.6 ± 5.7	0.26 ± 0.15	**52.3**
Sulfamethoxazole surfactants	11.8 ± 7.4	0.97 ± 0.67	**12.2**

SUPPLEMENTARY INFORMATION

1. HPLC-MS/MS analysis of surfactants in for effluent wastewater (based on Bergé et al. J Chrom A 1450 (2016), 64-75

SPE EXTRACTION

Briefly, SPE extraction was carried out using cartridges Cunax2 (3 cc, 200 mg). They were conditioned with 8 mL of MeOH, 4 mL of miliQ water water and 4 mL of phosphate buffer (0.1 M at pH = 6). A volume of 200 mL was loaded onto the cartridges, and after that they were washed with 5 mL of phosphate buffer and dried under vacuum for 10 min. Elution was performed separately, first with 2 x 5 mL of ACN and then with 2 x 5 mL of ACN with 5% of NH_4OH. Both fractions were collected separately, evaporated to dryness under a gentle N_2 stream (45ºC) and reconstituted to 1 mL each, with ACN/H_2O (65/35; v/v). Finally, both extracts were put together in one vial.

HPLC-MS/MS ANALYSIS

HPLC-MS/MS analyses were performed with an Agilent 1200 LC system (Massy, France), equipped with a quaternary pump, followed by an API-3200 QTRAP from ABSciex (Les Ulis, France). ABSciex software Analyst 1.6.1 version was used for data collection and instrument control. Analyses in both positive and negative ionization modes were carried out using an Acclaim Surfactant Plus column (3 μm, 150×3 mm, 3.0 μm) preceeded by an Acclaim surfactant Plus guard-column, (5 μm, 3×10 mm), both from Thermo Fischer (Illkirch, France). The mobile phases were 0.1 M ammonium acetate in Milli-Q water (pH=5.0)(A), Milli-Q water (B) and ACN (C). The gradient is shown in Table S1.

Table S1. Elution gradient used for the surfactants analysis.

t (min)	%A	%B
0	70%	30%
1	70%	30%
8	15%	85%
20	15%	85%

The analyses were performed in multiple reaction monitoring (MRM) mode, using the two most abundant transitions, for quantification and confirmation respectively. MRM transitions for the surfactants are given in Table S2.

Table S2. MS/MS conditions for the surfactants studied (**Bergé et al. J Chrom A 1450 (2016), 64-75)**

SURFACTANT	Ionization mode	Precursor ion [M+H]+	MRM Transitions	RT (m)	DP (V)	EP (V)	CE (V)	CXP (V)
Benzotriazole	ESI +	120.1	65	1.7	46	10	23	4
			92		46	10	23	4
BDDAC	ESI +	304.2	91.1	2.6	51	12	45	4
			212.3		51	12	27	4
BDTAC	ESI +	332.4	91	3.7	46	11	49	4
			240.3		46	11	29	4
Stepanquat GA 90	ESI +	402.4	283.3	4.3	56	10.5	31	4
			57.1		56	10.5	65	4
Lauryl pyridinium	ESI +	248.3	80.1	1.7	66	10.5	33	4
			57.1		66	10.5	37	4
Incromine SD	ESI +	369.4	324.4	5.0	46	12	31	4
			57.1		46	12	57	4
Sodium 2-ethylhexyl sulfate	ESI -	209.2	96.8	7.4	-55	-3	-32	0
			79.8		-55	-3	-60	0
SDS	ESI -	265.1	96.9	9.2	-65	-3	-36	-2
			79.8		-65	-3	-82	0
LAS C10	ESI -	297.1	119	9.5	-75	-10	-62	0
			184		-75	-10	-38	0
LAS C11	ESI -	311.1	118.9	9.9	-80	-12	-68	-2
			182.8		-80	-12	-48	0
LAS C12	ESI -	325.1	118.8	9.9	-85	-12	-70	0
			182.8		-85	-12	-48	0
LAS C13	ESI -	339.2	118.9	10.3	-95	-12	-72	0
			182.8		-95	-12	-50	0
Texapon N 701 S	ESI -	293.1	79.8	10.7	-55	-2.5	-100	0
			96.2		-55	-2.5	-40	0
Comperlan 100	ESI +	244.2	62.1	5.6	31	10	33	4
			57.1		31	10	35	4
Triton X100	ESI +	532.4	89.1	5.4	46	10	41	4
			133.1		46	10	33	4
		576.4	89	5.4	51	10	47	4

			133.1		51	10	33	4
		620.5	89	5.3	61	9.5	51	4
			133.2		61	9.5	37	4
		664.5	89.2	5.2	61	12	51	4
			133.1		61	12	37	4
		708.5	89.1	5.1	66	10	51	4
			133.1		66	10	41	4
Cetyl Betain	ESI +	328.3	104.1	6.6	66	12	31	4
			85.1		66	12	35	4

Table S3. MS/MS optimization setting for the two pharmaceuticals used. SRM: selected reaction monitoring; DP: declustering postential; CE: collision energy; CXP: collision cell exit potential.

	Precursor ion $[M+H]^+$	**SRM transitions**	**DP(V)**	**CE(V)**	**CXP**
SULFAMETHOXAZOLE	254	156	81	21	12
		92	81	35	12
OXAZEPAM	287	241.1	106	29	12
		269	106	23	16

Nano-LC-MS/MS conditions

Both oxazepam and sulfamethoxazole were separated using (A) water with 0.1% of acetic acid and (B) MeOH:ACN:H2O(45:45:10) with 0.1% of acetic acid.

Table S4. Elution gradient used for oxazepam and sulfamethoxazole analysis in biota.

t (min)	**%A**	**%B**
0	90%	10%
1.5	90%	10%
7	0%	100%
15	0%	100%
15.5	90%	10%
20	90%	10%

Table S5. Environmental concentrations found in the literature for sulfamethoxazole and oxazepam.

	WWTP effluent	Surface water	References
Sulfamethoxazole	222	10-79	Gros et al, J Chrom A, 2012
	50-650	0,2-35,6	Garcia-Galan et al. Environ Int 2011
	10,8-284	-	Garcia-Galan et al. Sci Total Environ 2012
	55,1-532	-	Garcia-Galan et al. Anal Bioanal Chem 2012
	36,5-80	-	Papageorgiou et al. Sci Total Environ 2016
	10	-	Collado et al. Environ Pol, 2014
	41-8714*	-	Santos et al. Sci Total Environ 2013
	19-198	-	Gros et al. JCA, 2013
	142-280**	-	Loos et al. Water Res 2013
Oxazepam	1190-1700	28-53	Berlioz-Barbier et al, (submitted)
	162**	-	Loos et al. Water Res 2013
	80-160**	30-40**	Hass et al. Water Res 2012
	28-133	11-31	Kosjek et al. Water Res 2012

*: hospital effluents; **: average values

Table S6. Measured parameters during the experiment.

	Control solvents	Control surfactants	Oxazepam	Oxazepam +surfactants	Sulfamethoxazole	Sulfamethoxazole +surfactants
pH	7.73(0.34)	7.7(0.26)	7.9	7.9(0.2)	7.9(0.1)	7.8
Temperature (ºC)	14.82(0.54)	14.7(1.7)	14.6(0.4)	14.6(1.8)	14.7(0.8)	15.3
Conductivity (µS/cm)	409.6(0.4)	408.9(0.2)	408.5(0.2)	408.9(0.1)	302.1(0.2)	434
Dissolved O_2 (mg/L)	5.1(7.6)	4.4(3.7)	6.6(2.5)	7(1.5)	6.9(1.3)	5.7

*. Measurements were performed in the morning, right before changing the solutions.

Table S7. Inter individual variability (n=5) calculated for SMX and OXA analyses each day of sampling

Time of exposure (days)	OXAZEPAM	OXAZEPAM (with surfactants)	SMX	SMX (with surfactants)
1	36.40	36.64	19.00	42.40
2	17.76	12.52	22.60	20.60
3	10.30	13.80	36.40	31.80
4	15.10	15.05	15.00	36.60

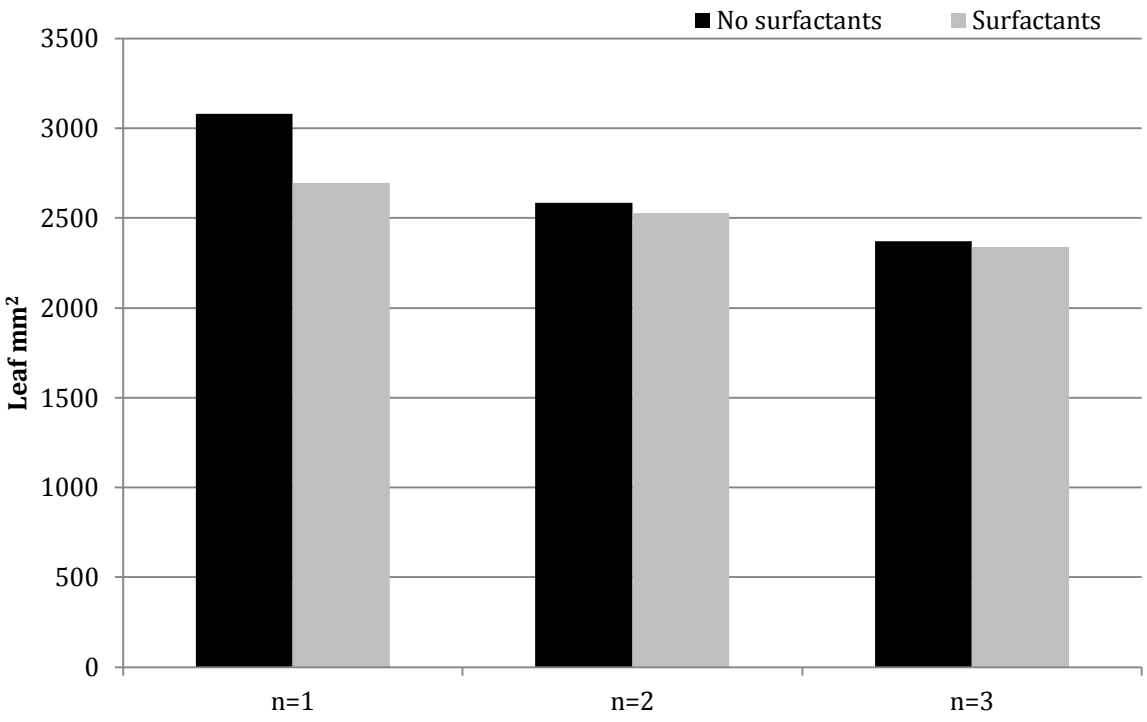


Figure S1. Feeding tests in the controls, expressed in leaf surface area