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Biodegradation of a mixture of benzophenone, benzophenone-3, caffeine and carbamazepine in a laboratory test filter

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Abstract: The biodegradation of a mixture of four pharmaceutical and personal care products (PPCPs) (benzophenone (BP), benzophenone-3 (BP-3), caffeine (CF) and carbamazepine (CBZ)) was studied in a laboratory test filter. The column was filled with inert material to exclude the adsorption processes and to enable the development of the biofilm, while river water was recirculated. High removal for BP, BP-3 and CF was observed from the beginning of the experiment at the initial concentration of 20 μg L^{-1} (90–99 %). In the case of CBZ analytical difficulties were experienced. The efficacy of biodegradation reflected as a change of the overall toxicity of initial mixture of selected PPCPs *vs*. toxicity of samples which were undergone different biodegradation phases was assessed with two standard laboratory tests with apical endpoint – acute toxicity test with *Daphnia magna* (immobilisation) and bioluminescence inhibition with *Vibrio fisheri*. Toxicity tests showed the substantial reduction of the overall mixture toxicity in a laboratory test filter. The residual toxicity to *D. magna* might be attributed to undetected transformation products.

Keywords: PPCPs mixture; toxicity; surface water.

INTRODUCTION

It is well known that the presence of pharmaceutical and personal care products (PPCPs) in surface water, groundwater and drinking water presents a potential risk to human and ecosystem health.[1] Among the substances frequently detected in river water worldwide particular attention is being paid to the so called anthropogenic markers, *e.g.*, carbamazepine (CBZ) – anticonvulsant and mood stabilizing medicine, applied in the treatment of epilepsy, bipolar disorder,

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and trigeminal neuralgia, known to be recalcitrant under standard biological wastewater treatment conditions,[2] and potent central nervous system stimulant caffeine (CF), one of the most widespread pharmaceuticals in the environment. Among the ingredients of personal care products, a group of benzophenones stands out, regarding the frequency of detection.[3] Their concentrations in river water vary for benzophenones between 0.3 and 550 ng L^{-1},[4] for carbamazepine Ternes *et al.*[2] reported value of 820 ng L^{-1} (as 90-percentile for 20 rivers), while Loos *et al.*[5] reported caffeine and carbamazepine at highest maximum concentrations of 40 and 12 μg L^{-1}, respectively in European rivers. Even in very large rivers, with extremely high mixing and dilution capacity, such as, *e.g.*, the river Danube, CF concentrations may reach 4 μg L^{-1} close to sewage discharge point and remain high (up to 0.16 μg L^{-1}) several km downstream, while CBZ concentration of 0.074 μg L^{-1} were reported in the river Danube sections not directly affected by municipal effluents and even 0.2 μg L^{-1} in a river section close to the sewage discharge point.[6]

In general, the acute toxicity of PPCPs, particularly pharmaceuticals, to aquatic organisms is rather low, as evidenced by the standard aquatic ecotoxicity tests,[7] but sub-chronic, chronic toxicity and very specific,[8] potentially highly ecologically relevant biological effects, such as (anti)estrogenicity, genotoxicity and mutagenicity, have been reported for some compounds (and their transformation products) as well as the simulated or the real waste water treatment plant (WWTP) effluent samples, assessed by the specific, mainly *in vitro* assay.[9,10] Since chemicals are metabolised in humans and domestic animals (pharmaceuticals, illicit drugs) and transformed during the (advanced) waste water processes and in the environment, some of the resulting transformation products (TPs) are known to be more abundant in the aquatic environment than their parent compounds, *e.g.*, CBZ metabolites 10,11-dihydro-10,11-dihydroxycarbamazepine and carbamazepine epoxide.[11,8] In many cases, TPs are by far more toxic than the parent compound, which is true in the case of CBZ and its photodegradation products acridine and acridone, as proved by a battery of laboratory tests including algae, bacteria and daphnids.[12] On the other hand, the most commonly reported metabolites of BP-3, seem to be less toxic to the freshwater invertebrates than the parent compound.[13] However, as biotransformation pathways are not often known,[8] the majority of TPs present in WWTP effluents and surface waters most likely have not even been identified yet,[10] and therefore their individual toxicity is completely unknown.[8] Takita *et al.*[14] tested the ability of sixteen fungal strains to degrade BP at the concentration level of 7.5 ppm in artificial wastewater. Results showed 99 % of BP degraded. Chang *et al.*[15] showed that microbial degradation of BP exists in real water samples (initial concentration 10 μg L^{-1}).

Thus, humans and aquatic ecosystems are exposed to a highly variable and unknown cocktail of chemicals. Although individual chemicals are typically present at low concentrations, TPs often (particularly in the case of pharmaceuticals and biocides which have been designed to exhibit biological activity) retain the biological activity and the same mode of toxic action as parent compounds and act concentration-additive in mixtures, meaning that the effects from TPs and parent compounds must be considered additive.[10] However, some studies have indicated the potential synergistic effects which could even enhance the overall toxicity of mixtures, such as in case of a mixture of CBZ and the lipid lowering agent, clofibric acid (two substances from very different therapeutic classes and expectant modes of action), which exhibited stronger effects than the single compounds at the same concentration.[16] Due to the lack of knowledge on PPCPs biodegradation pathways, potential TPs (and consequently analytical standards), their individual toxicity and the possible interactions with other constituents in the effluents and environment, the commonly applied approach in the assessment of water treatment efficacy to eliminate PPCPs is an effect driven approach.[10] A battery of standard aquatic toxicity tests of integrative nature and apical endpoints (*e.g.*, fish, daphnia, bacteria, algae short term tests) and/or specific *in vitro* assays (for detection of specific biological effects) of the mixture are used for screening of a reaction mixture. Effect-driven approach provides information on whether the toxicity changes during a transformation process and give an indication whether or not toxic TPs are likely to have been formed, or the change in toxicity can be fully explained by the decrease of parent compound concentration.[10,12] Primary processes which reduce and/or totally remove organic pollutants in natural waters are sorption and biodegradation. They usually occur together and can be simulated for the research purposes by the so called "test-filter", firstly introduced by Sontheimer, and later applied to monitor attenuation of organic pollutants in river water and sediment.[17] Some methodological drawbacks of the test filter approach are discusses by Bartelkamp *et al.*[18] since they can lead to overestimation of the biodegradation rate. However, it is a widely applied research tool. Börnick and Worch *et al.*[19,20] examined biodegradation and adsorption of aromatic amines to give a prognosis on the behaviour of organic compounds during riverbank filtration and to prioritize the substances with regard to drinking water quality. They also investigated if the biodegradation rate could be related to the physicochemical properties or functional groups of the aromatic amine. D'Alessio *et al.*[21] investigated impact of the seasonal variation, the oxygen level, and the level of organic matter on the removal of the selected pharmaceutical during simulated riverbank filtration. Trinh *et al.*[22] used the fixed-bad column recirculation system to investigate the sorption and biodegradation of two herbicides at different saturated materials (hidrofilt and river sediment). The separate investigation of the biodegradation process, apart from the adsorption

process, is simulated if inert column filling is used for the experimental set up.[20] It was shown that biodegradation can be very efficient for removal of certain organic substances and thus used in water treatment. Maeng *et al.*[23] confirmed high biodegradation rate for CF (>94 %), while studies at pilot plant at Louisville Water Company, Kentucky,[24] showed high removals of BP-3 (97 %) and CF (97 %) at three sequential biologically-active sand filter columns. Reported removal of CBZ was lower (17.5 %).

The aim of this study was to assess the biodegradation in a laboratory test filter for the mixture of four frequently used and detected PPCPs in river water. In addition to chemical analysis, toxicity after biodegradation process was assessed with two standard laboratory tests with apical endpoint — acute toxicity test with *Daphnia magna* (immobilisation) and bioluminescence inhibition with *Vibrio fisheri*.

EXPERIMENTAL

Standards and reagents

Four selected PPCPs (benzophenone, benzophenone-3, carbamazepine and caffeine) were purchased from Sigma–Aldrich with the purity of 99 % (properties of selected compounds are shown in Supplementary material to this paper, Table S-II). A stock solution of the mixture of benzophenone, benzephenone-3, carbamazepine and caffeine, was prepared in distilled water (5 mg L^{-1} each). The mixture was sonicated for 3 h, filtered through a 0.45 μm cellulose nitrate membrane filter and stored at 4 °C. This solution was used for spiking of the Danube river water for the experiment.

Water matrix

The samples of the river Danube water, taken upstream the city of Novi Sad (Serbia) sewage discharge points were used in all experiments. The main characteristics of the water used for experiments were as follows: pH 8.03±0.15 (n = 8), conductivity 360±24.2 μS cm^{-1} (n = 8), temperature 21±2.9 °C and $KMnO_4$ consumption 12±4.09 mg $KMnO_4$ L^{-1} (n = 8). The selected PPCPs in the river water were not detected by the method applied.

Test filter experimental set-up

The quartz sand was used as an inert column filler. In this way sorption process is excluded from the investigation and it was considered that all the removal that was observed was due to biodegradation.[20] Particle size of quartz sand was less 30 times than the diameter of the column ($d \leq 0.67$ mm).[29] The test filter set-up is presented in Supplementary material (Fig. S-1). The steel column with inner diameter of 2 cm and length of 20 cm was filled with quartz sand. A 2 L glass bottle was used as a reservoir and filled with 1.7 L river water spiked with stock solution of PPCPs mixture. Experiments were performed at initial nominal concentrations of 20 and 60 μg L^{-1}, for each substance that is a compromise between the environmental concentrations and the requirements of the analytical method that was used. The system was kept in the dark at room temperature. The water was pumped bottom-up in recirculation with a flow rate of 0.5 mL min^{-1}. Samples (100 mL) were collected once a day for liquid-liquid extraction. Non-filtered river water was used in the phase of biofilm development, while in subsequent phases of experiment the water was filtered through a 0.45 μm cel-

lulose nitrate membrane filter before the experiment, in accordance with the method described by Börnick.[19]

Assessment of PPCPs biodegradation

Experiment of the biodegradation of selected PPCPs in test filter was performed in four consecutive phases after the initial phase of biofilm development (BFD). Overview of the experiment is given in Table S-I.

Biofilm development phase was performed for four weeks. Dissolved oxygen, pH, conductivity, temperature and the $KMnO_4$ consumption were measured to assess are the main water characteristics suitable for the biofilm development (with enough oxygen and carbon). Börnick in his study proved that 11 days of recirculation of river Elbe water (*DOC* = 6.64 mg L^{-1}, t = 20°C) was sufficient under similar conditions.[19] Adaptation phase (A1) followed the biofilm development phase (BFD): microorganisms were adapted to the concentration of 20 μg L^{-1} of the tested substances for 8 days. Subsequently, the biodegradation phase 1 (B1) was performed for 8 days with the same initial concentration of each test substance. After the biodegradation phase 1, the initial concentrations of substances were raised to 60 μg L^{-1} and fresh river water spiked with higher concentrations was recirculated through the test filter for 7 days without determination of PPCPs with goal to adapt microorganisms (adaptation phase 2, A2). Finally, biodegradation phase 2 (B2) was conducted under the same conditions with measurements of tested compounds. From the results collected during the experiment it was possible to determine the biodegradation rate constant (k / h^{-1}) from the pseudo-first order rate law.[18–20]

Additionally, the half-life ($t_{1/2}$) of the studied PPCPs was calculated. The effective test filter contact time is calculated because of the decrease in the volume of water due to sampling and it is given in the following equation:

$$t_{\mathrm{fi,i}} = t_{\mathrm{fi,i-1}} + \frac{V_{\mathrm{fi}}\left(t_{\mathrm{r}} - t_{\mathrm{r-1}}\right)}{V_{\mathrm{w}} - V_{\mathrm{ww}} - \left(n-1\right)V_{\mathrm{s}}} \quad (1)$$

$t_{\mathrm{fi,i}}$ – effective test filter contact time, h; n – number of previous taken sample; V_{fi} – effective volume of filter, mL; t_{r} – total retention time, h; V_{w} – total volume of water, L; V_{ww} – volume of water sample which is not recycled during rinsing at the beginning of an experiment, mL; V_{s} – sample volume taken for analysis, mL.[19]

In parallel to the test filter experiment, the sub-samples of the filtered Danube river water (0.45 μm) were spiked with mixture of test substances at both concentration levels and kept in glass beakers, under night-day circle and dark conditions, to check out the weathering effects (due to possible photodegradation and biodegradation in natural river water). All samples from the beakers were analysed for ecotoxicity. Samples from the beakers during phases B1 and B2 were analysed for PPCPs.

Toxicity tests

The efficacy of the biodegradation process, in terms of overall toxicity reduction, was estimated with two standard toxicity tests: *Daphnia magna* acute toxicity test and *Vibrio fischeri* luminescence inhibition test. The toxicity of freshly prepared mixture test solutions – filtered river Danube water spiked with low initial concentration (*FDLIC*) and filtered river Danube water spiked with high initial concentration (*FDHIC*) of test substances was compared to the toxicity of the following samples: A1 – sample taken after biofilm adaptation to low concentration mixture, B1 – sample taken after biodegradation phase 1, A2 – sample taken after biofilm adaptation to high concentration mixture, B2 – sample taken after biodeg-

radation phase 2, FDLICWL – sample taken after weathering of the corresponding FDLIC under light, FDLICWD – sample taken after the weathering of the corresponding FDLIC in dark, FDHICWL – sample taken after weathering of the corresponding FDHIC under light and FDHICWD – sample taken after weathering of the corresponding FDHIC under dark.

D. magna acute toxicity tests were performed according to the standard ISO 6341 method.[30] The detailed procedure is given in the Supplementary material. *V. fischeri* luminescence inhibition tests were performed according to the standard ISO 11348-3:2007 method.[31] A standard test endpoint, bacterial luminescence, was measured using the BioFix LUMI 10 luminometer by the manufacturer Macherey-Nagel GmbH&Co. KG, Duren, Germany. The details related to test procedures are given in the Supplementary material.

The chosen biological responses (percentage of immobilisation in *D. magna* tests / percentage of luminescence inhibition in *V. fischeri* tests) in different control sample solutions as well as between corresponding control samples and the test solutions were compared using the t-test (independent sample). For the comparison of biological responses in test solutions before and upon biodegradation, as well as between the weathering under different light regime (light *vs.* dark) the *t*-test based on dependent samples was used. The significance was assigned uniformly at $p \leq 0.05$. Statistical analyses were done using Statistica, ver. 8.

Assessment of the biofilm development

Separate smaller column (diameter 1cm and length 20 cm, sand particles $d \leq 0.33$ mm, flow rate of 0.13 mL min^{-1}) was filled and run under the same conditions as the test filter to be able to analyse biofilm after the four weeks of operation. Two types of samples taken from the column were used for microscopic analysis — native and stained heat fixed samples. Gram staining technique was used to prepare stained slides. Native sample slides were examined with light microscope Olympus BX 51 with total magnification of 100 or 400 times. Stained samples were analysed using immersion light microscope Olympus BX 51 with total magnification of 1000 times. Photographs were taken with Colour View Olympus camera U--CMAD3, Japan, and processed with B Cell Imaging Software (Olympus, Japan).

PPCPs analysis

The water samples (100 mL) were extracted by 2×10 mL of dichloromethane. Extracts were dried with anhydrous sodium sulphate, evaporated to dryness under a gentle nitrogen stream and dissolved in 0.3 mL of 1:1 dichlormethane/hexane mixture. Description of GC/MS analysis is given in the Supplementary material. Target ions (Table S-III of the Supplementary material) for each compound were used for the assessment of the biodegradation rate by tracking the ratio of the surface of the target ion peaks in samples taken during experiment and target ion peaks from the initial solution (A/A_0) for each experimental phase (spiked river water). All the samples within the same experimental phase (A1, B1 and B2) were analyzed within one series of measurements with the same instrumental conditions related to a liner and chromatographic column to eliminate possible matrix influence on the results. The quality of the measurements was demonstrated by measuring the target peak surface area in triplicates for BP, BP-3, CBZ and CF in spiked river water. Relative standard deviations of 4, 3, 8 and 12 % at nominal concentration level of 5 μg L^{-1} and relative standard deviations of 15, 10, 9 and 17 % at 20 μg L^{-1} were obtained, respectively. The linearity of response was confirmed for the range of 5–60 μg L^{-1} in river water (0.95–0.99). *S*/*N* ratio of 3 was observed at the level of 5 μg L^{-1} due to a very small sample volume. Regarding the assessment of CBZ removal, the analytical difficulties were experienced. It is known that iminostilbene (IMS) is formed in the liner of GC inlet during analysis,[32] and that it was found as an obstacle for analytical measure-

ments in real water matrix. Only a few results that showed the same ratio of IMS/CBZ peak areas in chromatogram, within the same batch of the samples, were taken into account for the rough assessment of the biodegradation of CBZ.

RESULTS AND DISCUSSION

The biofilm development

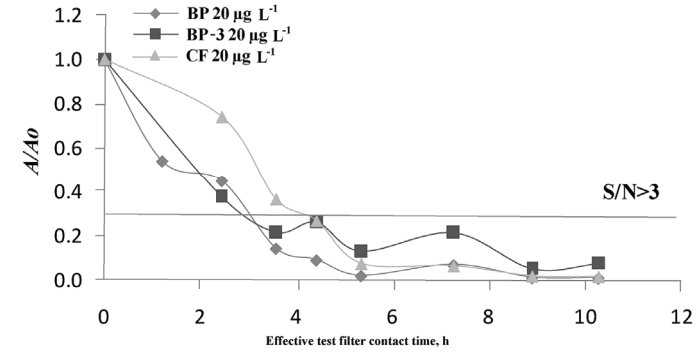
Water quality parameters during a phase of the biofilm development are presented in Supplementary material (Table S-IV). Based on the values shown in Table S-IV it can be concluded that the change of the water every 7^{th}–8^{th} day was frequent enough to prevent the lack of organic matter in the system, which is in accordance with literature findings.[19] The decrease of dissolved oxygen during the cycles was in the range 8–37 %. The characterisation of the biofilm after the four weeks circulation showed that it was formed (Figs. S2 and S3). Images of the stained sample are shown in Fig. S-2. Beside the presence of diatoms, the heat fixed stained samples enabled the identification of coccal and rod forms of bacteria in the biofilm. Among the detected bacteria the Gram-positive were prevalent. The analysis of the native sample slide showed the dominance of siliceous algae (diatoms) belonging to several genera *Sinedra* (Fig. S-3a), *Ciclotella* (Fig. S-3b), *Melosira* (Fig. S-3c), *Asterionella* (Fig. S-3d) and *Nitzschia* (Fig. S-3e).

Biodegradation of PPCPs

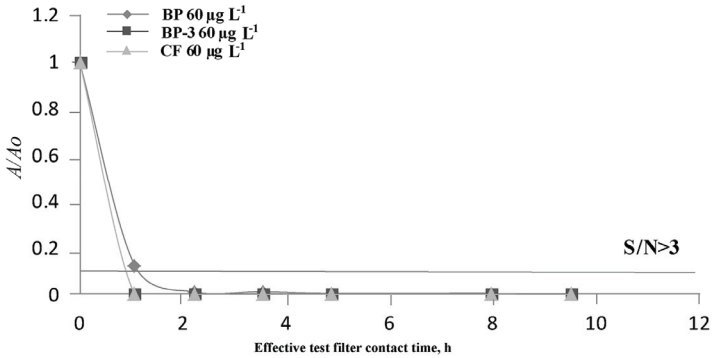
The biodegradation of BP, BP-3, CF and CBZ was monitored during the phases A1, B1 and B2. During the adaptation phase 1 after 8^{th} day the removal was already estimated as 90 % for BP, 95 % for BP-3, 98 % for CF but no removal was observed for CBZ. This is in accordance with results of Maeng *et al.*[23] for CF and Snyder *et al.*[24] for CF and BF-3. Possible reason for such an effective removal during the adaptation phase might be the already adapted microflora present in the Danube river water due to the possible long term exposure to small concentrations of the substances.[11] Fig. 1a and b show the biodegradation of BP, BP-3 and CF during phases B1 (initial concentration of 20 μg L^{-1}) and during B2 (initial concentration of 60 μg L^{-1}).

It can be concluded that the substances were effectively removed in both phases of biodegradation. During the B1 (Fig. 1a) the removal higher than 70 % for BP-3 and CF was observed after 4.4 h, while for BP, the removal was 86 % after 3.6 h the effective contact time (total run time 90 h). In B2 phase (Fig. 1b), the degradation was almost complete within only 1.07 h effective contact time (total run time 24 h) with estimated removal of 90 % for BP, and 99 % for BP-3 and CF. When it comes to CBZ it was concluded that there was no biodegradation in adaptation period based on two samples that have the same IMS/CBZ ratio (and thus they are comparable). The initial A/A_0 ratio and the ratio after the total test filter run time of 192 h were the same. During the B1 phase the

analytical difficulties occurred and no results for CBZ biodegradation were possible to discuss. During B2 phase an indication of CBZ biodegradation was observed based on three samples with similar IMS/CBZ ratio of 0.99, 0.91 and 0.87 for solutions after 0, 48 and 74 h total test filter run time, respectively. The last two showed removal of 87 and 65 %. It can be concluded that the obtained results are in accordance with already mentioned results of Maeng *et al.*[23] and with the results from a riverbank filtration pilot plant at Louisville Water Company, Louisville, KY.[24] The biodegradation rate constant and half-life of selected PPCPs in phase B1 are presented in Table I. It should be noted that those values are rough assessment and that they were calculated based on the removals presented in Fig. 1a including the first sample below *S*/*N* 3 threshold. B2 phase was not considered, since all the measurements of areas of target peaks were below *S*/*N* 3 (Fig. 1b).



a)



b)

Fig. 1. Biodegradation of BP, BP-3 and CF during: a) phase B1 (initial concentration of 20 µg L^{-1}) and b) phase B2 (initial concentration of 60 µg L^{-1}).

In accordance with classification of the biodegradability of compounds based on filter tests results from Börnick *et al.*[33] and based on results presented in Table I,

it can be concluded that BP, BP-3, CF are good biodegradable substances (effective $t_{½}$, 1–3 h). Bertelkamp *et al.*[18] investigated biodegradation behaviour of 14 organic micropollutants at concentrations of 200 ng L^{-1}, in lab-scale soil columns under the oxic conditions and reported half-life for CF ($t_{½}$ = 2 h) with biodegradation rate of 0.4 h^{-1} and no biodegradation rate for CBZ. The results of weathering tests performed in glass beakers, compared to the tests in the laboratory test filter, for those two phases, are shown in Supplementary material (Table S-V). Results show that the degradation of substances happens in the filtered river water during 7 days as well, except for CBZ. BP degradation was enhanced under the night-day circle conditions in comparison to dark conditions. It is known that the direct phototransformation may come from the direct absorption of sunlight by the compound, which either enables it to react with the constituents of the water or induce self-decomposition.[3] However, this was not the case with BP-3 that degraded better under dark conditions, while in case of CF there was no difference. Kotnik *et al.*[3] showed that the photosensitizers present in natural waters significantly affect the photolytic behaviour of benzophenones. Comparing the results of the weathering tests in river water (Table S-V) with the results from the laboratory test filter that was running under dark conditions, it can be concluded that the biofilm development at sand material significantly contributed to the removal of those three PPCPs.

TABLE I. Biodegradation parameters of selected PPCPs during B1 phase

Analyte	Rate constant, h^{-1}	Half-life, h
BP	0.50	1.4
BP-3	0.33	2.1
CF	0.29	2.4

Toxicity tests

No *Dophina magna* neonates immobilisation was observed in either of the control samples – filtered river Danube sample (FD) and standard (M_4) medium after 24 and 48 h, therefore the immobilisation in test solutions was compared to the FD control sample only. Likewise, in tests with *Vibrio fischeri*, the I_{30}/I_0 ratio did not significantly differ between the two different control samples – standard 2 % NaCl solution and the filtered sample of the river Danube water adjusted for salinity with NaCl (FD+2 % NaCl), so the effects observed in tests solutions were compared to FD+2 % NaCl control sample only. Due to the absence of any adverse effects in FD (daphnids) and FD+2 % NaCl (bacteria), all the effects observed in test solutions can be attributed to the mixture of effects of test substances and their degradation products in test solutions.

Toxicity of low (20 μg L^{-1}) and high (60 μg L^{-1}) concentration mixtures (FDLIC and FDHIC, respectively) was tested in freshly prepared solutions, in

solutions after the biofilm adaptation phase (A1 and A2 samples) and in solutions after the column biodegradation phase (B1 and B2 samples, Table II). Exposure to

TABLE II. The results of the toxicity tests with *Daphnia magna* and *Vibrio fisheri*;* – significantly different from the corresponding control sample; # – significantly different from the corresponding freshly prepared solution in Danube river water (FDLIC / FDHIC)

Initial mixture	Sample	*Daphnia magna* immobilisation, % *vs.* control sample	Toxicity reduction, % *vs.* FDLIC/ FDHIC	*Vibrio fischeri* luminescence inhibition, % *vs.* control sample	Toxicity reduction, % *vs.* FDLIC/ FDHIC
Low concentration mixture (concentration of each substance 20 μg L^{-1})	FDLIC – filtered Danube with low initial concentration of PPCPs mixture (prior to degradation process)	42.5*	–	8.01	–
	A1 – sample taken after the biofilm adaptation phase (column)	45*	–	1.46	82
	B1 – sample taken after the biodegradation phase 1 (column)	25#	41	0.11	99
	FDLICWL – sample taken after the weathering under light (batch)	20#	53	8.78	–
	FDLICWD (dark) – sample taken after the weathering in the dark (batch)	37.5*	12	10.11	–
High concentration mixture (concentration of each substance 60 μg L^{-1})	FDHIC – filtered Danube with high initial concentration of PPCPs mixture freshly (prior to degradation process)	90*	–	58.01*	–
	A2 – sample taken after the biofilm adaptation phase (column)	45*	50	9.84#	83
	B2 – sample taken after the biodegradation phase 2 (column)	35*#	61	14.44#	75
	FDHICWL – sample taken after the weathering in light (batch)	50*	44	21.10#	64
	FDHIC WD – sample taken after the weathering in the dark (batch)	62.5*	30	26.07#	**55**

FDLIC and A1 samples resulted with over 40 % neonates immobilisation, while percentage of neonate immobilisation after 48 h exposure to FDHIC sample was as high as 90 %. The concentration-dependent effects were recorded in *V. fischeri* tests. The low concentration mixture (FDLIC) was not toxic, while the exposure to high concentration mixture (FDHIC sample) caused significant – 58 % inhibition of luminiscence *vs.* control samples (Table II).

High toxicity of freshly prepared samples is somehow surprising, given the low toxicity of individual substances to the selected test species. For benzophenone (BP) *D. magna* 24 h LC_{50} 7.63 mg L^{-1} and 48 h EC_{50} 9.51 mg L^{-1} have been reported by Sun *et al.*[34] and Liu *et al.*[35], respectively. According to the literature data, BP-3 is slightly more toxic to *D. magna* with 48 h EC_{50} ranging between 1 and 2 mg L^{-1}.[35–37] Benzophenons seem to be only slightly harmful to the bacteria *V. fischeri* as evidenced by 30 min IC_{50} over 10 mg L^{-1} for BP and BP-3 reported by Liu *et al.*[35] According to the results of laboratory tests with selected pharmaceuticals, CBZ and CF are only harmful to *D. magna* as EC_{50} are reported to be over 10 mg L^{-1},[38,39] while non-toxic to *V. fischeri*.[40] Therefore, the results of this study underline the risk of more than additive, potentially synergistic effects of PPCPs mixtures and modulating effects of NOM in the river water.

Sample taken after biodegradation phase 1 (B1 sample) was not toxic to daphnids – percentage of immobilisation of only 25 % after 48 h exposure was not significantly different from the control sample. Biodegradation phase 2 substantially reduced the overall toxicity of the high concentration mixture: percentage of the immobilisation was only 35 % in B2 sample, which was still high in comparison to the control sample, but significantly lower than FDHIC (90 %). In case of *V. fischeri* the mixture toxicity of the high concentration solution diminished after the column biodegradation, as the inhibition of luminescence in B2 sample was only 14.4 % and did not significantly differ from the control sample. In parallel to the column biodegradation tests, the sub-samples of the river Danube water spiked with low and high concentration mixture of test solutions were kept for a week in glass beakers, at a room temperature, under night- day circle and dark conditions, to check out the weathering effect (both photodegradeation and biodegradation potential of natural river water). The efficacy of the weathering under different light regimes was estimated with each of the selected toxicity tests and the results are also presented in Table II. The mixture toxicity, expressed as percentage of immobilisation of *D. magna* neonates, decreased after weathering process, from 42.5 % in FDLIC to 37.5 % (decrease is not statistically significant) and to 20 % (statistically significant decrease) after the weathering in dark (FDLICWD) and under the light (FDLICWL), respectively. Decrease of the toxicity after the weathering, though not statistically significant, was observed also in case of high concentration test solution. The initial immobilisation (after 48 h exposure) decreased from 90 % in FDHIC to 62.5 % and to

50 % after the weathering under dark (sample FDHICWD) and light (FDHICWL) conditions, respectively. Both values differed significantly from the control sample (Table II). The freshly prepared low concentration solution (FDLIC) sample was not toxic to *V. fischeri* and the weathering process did not affect the toxicity of the solution – the luminescence values in samples FDLIC WL and FDLICWD did not significantly differ from the control sample. The freshly prepared high concentration solution (FDHIC) was toxic to *V. fischeri* – 58 % luminescence inhibition *vs.* control sample. After the weathering, the toxicity decreased significantly – the luminescence inhibition was only 21 and 26 % under the light (FDHICWL) and dark (FDHICWD) conditions, respectively. Neither of the values differed significantly from the control sample, meaning that the residual toxicity after the weathering is negligible. Taken together, the presented results indicate that a natural degradation of the selected PPCPs can occur in the river water, meaning that the inherent potential of microbial communities to degrade different organic substances includes also some PPCPs, although CBZ is considered to be highly resistant to biodegradation.[12] The mixture toxicity reduction recorded in the current study was more efficient in the light than in the dark, indicating that the potential role of photolytic degradation cannot be disregarded, especially for BP. Literature findings related to the laboratory simulation tests indicate that the photodegradation cannot be seen as a general degradation process for all PPCPs.[41] According to the comprehensive review of the degradation products of UV filters in aqueous solutions.[42] BP-3 is considered stabile under natural, UV and artificial sunlight, while photodegradation products have never been detected. The degradation of the compounds selected for the current study was apparently more efficient in the column tests, as the overall toxicity reduction was higher than in the glass beakers. Given the fact that the column is made of solid, not transparent material, the photodegradation is not likely to occur, so the overall reduction in toxicity can be attributed to the biodegradation (biofilm activity). Since the chemical analysis showed the decrease of the parent compounds after the various biodegradation phases, the residual toxicity might be attributed to the presence of unknown transformation products.

CONCLUSIONS

The biodegradation of the mixture of selected PPCPs rapidly occurred for BP, BP-3 and CF (higher than 90 %) even in the adaptation phase of experiment. The reason for such a high activity of microorganisms may be the already adapted microflora in the Danube river water where the chronic exposure to low level concentration is possible. When it comes to CBZ biodegradation the samples from the biodegradation phase 1 were not possible to analyze, while in the adaptation phase and under weathering conditions CBZ degradation was not observed. The enhanced biodegradation process in biodegradation phase 2 when

the initial concentration was increased from 20 to 60 μg L^{-1} was observed for BP, BP-3 and CF, while for CBZ only the rough indication that it was happening was obtained. The experiments without test filter recirculation confirmed the high degradation potential of substances in the Danube river water, both in dark and light conditions. BP degradation in river water was significantly enhanced by light (from 25–30 % in dark to more than 80 % in light), while for BP-3 it was found that the degradation is less efficient under light conditions. About 50 % of BP-3 and CF were degraded in river water under dark conditions without application of test filter. The overall toxicity reduction was higher in a laboratory test filter than in the glass beakers, based on *D. magna* and *V. fischeri tests*. Taken together, the presented results indicate that a natural degradation of selected PPCPs can occur in the river water, meaning that the inherent potential of microbial communities to degrade different organic substances includes also some PPCPs. The residual toxicity to *D. magna* might be attributed to the presence of unknown transformation products.

SUPPLEMENTARY MATERIAL

Experimental details and additional data are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

ИСПИТИВАЊЕ БИОДЕГРАДАЦИЈЕ СМЕШЕ БЕНЗОФЕНОНА, БЕНЗОФЕНОНА-3, КОФЕИНА И КАРБАМАЗЕПИНА ТОКОМ ЛАБОРАТОРИЈСКОГ ТЕСТА

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У раду је испитивана биодеградација бензофенона, бензофенона-3, кофеина и карбамазепина у води Дунава током лабораторијског теста. Утицај адсорпције је био искључен употребом инертне колонске испуне (челична колона пуњена кварцним песком) која је истовремено послужила као носач за развој биофилма при рециркулацији речне воде у филтру. Током експеримената са почетном концентрацијом супстанци од 20 μg L^{-1} уочено је ефикасно уклањање бензофенона, бензофенона-3 и кофеина (90-99 %). Паралелно са испитивањем биодеградације, испитивана је и токсичност смеша пре и након лабораторијског теста помоћу два стандардна теста: акутни тест токсичности помоћу *Daphnia magna* (имобилизација) и помоћу *Vibrio fisheri* (инхибиција биолуминисценције). Тестови токсичности показали су значајно смањење укупне токсичности смеша током лабораторијског теста. Резидуална токсичност у односу на *D. magna* може бити последица недетектованих трансформационих продуката.

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