Occurrence of emerging contaminants in a river system and characterization of the microbial community in the river sediments

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Abstract The presence of four non-steroidal anti-inflammatory drugs (Ibuprofen, Naproxen, Diclofenac, Ketoprofen) and five endocrine disrupters (Nonylphenol, Nonylphenol monoethoxylate, Nonylphenol diethoxylate, Bisphenol A, Triclosan) in a Greek river system (Spercheios river) and in one of its tributaries (German ditch) which receives treated municipal wastewater was investigated. Water samples were taken from six points along the river, five samples along the ditch, while samples were also collected from the outfall of the wastewater treatment plant located nearby. The tested compounds were frequently detected in the river water while the concentrations were higher in ditch water and wastewater. Among the compounds, phenolic substances were detected in all samples, with nonylphenol and its selected ethoxylates being the dominant pollutants in the water column while the calculation of the daily loads of these compounds showed substantial discharged quantities in the Maliakos Gulf. Although the sewage plant seemed to be an important source for the compounds to the river system, all the compounds were also detected upstream the sewage treatment plant outfall, reflecting additional sources in the upstream basin. Finally, although the microbial community varies between different redox conditions, the removal efficiency is similar; also high micropollutants' concentration seems to decrease slightly Archaea population.

Keywords: emerging contaminants; surface water; wastewater; Fluorescence In Situ Hybridization analysis

1 Introduction

Endocrine disrupting chemicals (EDCs) and non-steroidal anti-inflammatory drugs (NSAIDs) are categories of hazardous substances that have attracted increasing attention internationally and generated some concerns among the scientific community. In recent years, evidence has emerged showing that some chemicals at certain concentrations can cause direct effects on aquatic organisms and wildlife with hormone-like effects and disruption to their endocrine system (Naidoo *et al.*, 2010; Saravanan *et al.*, 2011; Bedoux *et al.*, 2012). During the last years, several studies have been published indicating the detection of several compounds included in these two aforementioned categories in worldwide surface water and in effluents of sewage treatment plants (Loos *et al.*, 2009; Loos *et al.*, 2013; Shanmugam *et al.*, 2014; Barber *et al.*, 2015; Inam *et al.*, 2015; Osorio *et al.*, 2016).

Based on the wide use, the frequent detection and the estrogenic activity, the compounds selected in this study as representatives of the EDCs are: Nonylphenol (NP), Nonylphenol monoethoxylate (NP1EO), Nonylphenol diethoxylate (NP2EO), Bisphenol A (BPA), Triclosan (TCS) and of the NSAIDs are: Naproxen (NPX), Ketoprofen (KTP), Diclofenac (DCF) and Ibuprofen (IBU). The objectives of this study were to investigate the presence of selected EDCs and NSAIDs along a Greek river (Spercheios River) and along one of its tributaries (German Ditch) as well as from the effluents of a sewage treatment plant discharging into this tributary whereas the daily amounts transferred through the river to the Maliakos Gulf was also estimated. Finally, additional sample was collected to identify the composition of microbial community presented on the river sediment while further batch experiments were conducted under different redox conditions with two micropollutants' concentrations, aimed to determine the impact of various conditions present along the length and depth of the river.

2 Material and Methods

2.1 Materials and reagents

Methanol, ethyl acetate and ethanol were of high performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany) and were used as received. BPA (>97%), TCS (>97%) and deuterated BPA (BPA-16) were purchased from Fluka (Heidelberg, Germany). Analytical standards of NP, NP1EO, NP2EO, IBU, NPX, KTP, DCF, and meclofenamic acid (MCF) were supplied by Dr. Ehrenstorfer (Germany). All compounds were used without further purification (minimum purity >99%). Stock solutions of individual compounds were prepared in methanol at 1000 mg L^{-1} and kept at -18 °C. HPLC grade water was prepared in the laboratory using a MilliQ/Milli-RO Millipore system (Millipore, Billerica, Massachusetts USA). Ultra-pure HCl (32%) was used for acidification of the samples (Merck, Germany). Phosphate buffered saline solution (PBS; 10 M) and paraformaldehyde powder were purchased from Sigma-Aldrich (US).

2.2 Study area and sampling sites

The Spercheios river is located in the Central-Eastern Greece (Fig. 1). The total area of river basin is 1.829 km2 and the distance from source to mouth is 82.5 km. In addition, Spercheios river has a mean water discharge of 62 m3 sec^{-1}, varying between 110 m^3 sec^{-1} (in wet period) and 22 m^3 sec^{-1} (in dry period) (Psomiadis *et al*., 2004). This makes the river seasonal with high flows during winter and low flows during summer. Moreover, Spercheios river receives water from 20 major tributaries and 63 small tributaries among which is the "German ditch", which constitutes one of the most important flood protection and irrigation project in the area. Consequently, the German ditch has very low water flow during the dry period of the year, while it receives the treated wastewater from the Wastewater Treatment Plant (WWTP) of Lamia city. The WWTP has an average sewage flow of 0.2 m^3 sec^{-1} and it is equipped with secondary treatment (activated sludge process). The discharge point of the WWTP is located 2.8 km upstream of the confluence with the river (Fig. 1).

Samples were taken at the same period for two years, in June 2014 and June 2015, and made at twelve sampling points among which were 6 along Spercheios river (sampling stations R1-R6), 5 along German ditch (sampling stations G1-G5) as well as from the effluents of WWTP (sampling point WWTP) (Fig. 1).

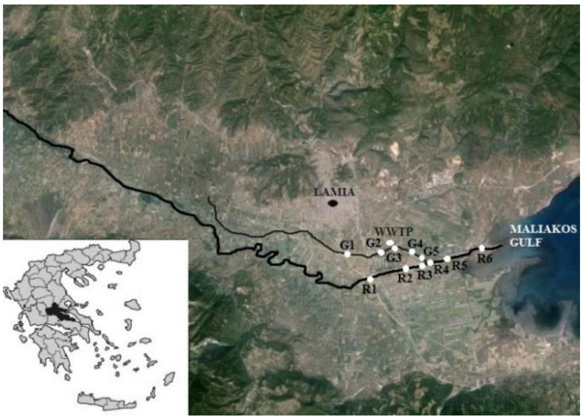

Figure 1. Map of the studying area indicating the sampling points and the outfall of WWTP serving the city of Lamia

2.3 Microcosms preparation

Sediment was collected from the outfall of the WWTP (G3) in order to identify and enumerate the microorganisms' population present by Fluorescence in situ hybridization (FISH). Moreover, batch experiments were carried out with two micropollutants' concentrations (40 and 500 μg L^{-1}) under aerobic (AER), methanogenic (MTH), anoxic (ANX) and sulfate reducing (SLF) conditions to reproduce the different states that can occur along Spercheios river. More specifically, experiments were performed in duplicate using 160 mL serum bottles containing 37.5 g of wet sediment and 112.5 mL of river water (1:3 volume ratio). Aerobic tests were performed by continuously aerating the water phase with pressurized air. Additionally, in the case of experiments in the absence of oxygen, the bottles were filled with river water and sediment, sealed with caps and butyl rubber stoppers and flushed with N_2 gas (99%), before incubated without shaking at 24 °C in darkness. Resazurin, a redox indicator dye, was added to confirm anaerobic conditions in the incubations throughout the experimental period. The anaerobic batch experiments were equilibrated for two weeks in order to consume electron acceptors in the incubations. Then, two incubations were periodically dosed with NO_3^- and two with SO_4^{2-} to reach a default concentration of 20 mg L^{-1} and 40 mg L^{-1} respectively. Moreover, two batch tests were left without any electron acceptor addition and therefore methane producing conditions was established. All experiments were carried out in pH 8.0 ± 0.3 during the course of the tests and all compounds were incubated as a mixture.

2.4 Fluorescence In Situ Hybridization (FISH) analysis

At the outfall of the WWTP (G3) in Spercheios river and at the end of the batch experiments described above, sediment samples were collected to perform FISH analysis. Cell fixation and hybridization were conducted as described by Llobet-Brossa *et al*. (1998) and Manz *et al*. (1999) respectively. The oligonucleotide probes used, along with the corresponding target group and optimal stringency in parenthesis, were: EUB338 (Bacteria; 35%), ARCH915 (Archaea; 35%), DEN67 (methanol-utilizing denitrifying cluster; 35%), ALF968 (α-proteobacteria; 20%), BET42a (β-proteobacteria; 35%), GAM42a (γ-proteobacteria; 35%), Delta495a (δ-proteobacteria; 35%), LGC354b (Firmicutes; 35%), SPH120 (*Sphingomonas* spp.; 30%), NSE1472 (*Nitrosomonas* spp.; 50%), PAE997 (*Pseudomonas* spp.; 0%), and LGC353b (*Bacillus* spp.; 20%). The probes were commercially synthesized with fluorescent dye Cy3 or Texas red (final concentration 5 μg ml-1; ThermoFisher Scientific, US). The amount of total cells was also determined by adding DAPI (4',6'-diamidino-2-phenylindole; Sigma-Aldrich, US) directly to the hybridization buffer (final concentration 20 μg ml-1). Slides were mounted with anti-fading AF-1 solution (Citifluor Ltd, UK) and viewed under oil immersion at 100x magnification with an epifluorescence microscope (Nikon E50i) fitted with filter sets for DAPI, CY3 and Texas red. Approximately forty images per sample were captured with Nikon Infinity-1 camera and quantified using the software Image Pro. Results are expressed as average percentage of the targeted microorganism out of the DAPI positive cells, along with standard deviations.

2.5 Analytical Determinations

For the determination of the target compounds a chromatographic method developed by Samaras *et al*. (2011) was used. For the qualitative and quantitative analyses, an Agilent Gas Chromatograph 7890A connected to an Agilent 5975C Mass Selective Detector (MSD) was used.

3 Results and discussion

3.1 NSAIDs and EDCs occurrence

All EDCs were identified in Spercheios river, whereas NSAIDs were detected in very low concentrations. More specifically, nonylphenol and nonylphenol ethoxylates (NP1EO, NP2EO) were dominant compounds in the river water and their concentrations in ng L^{-1} ranged from 1050 to 3112 for NP, 187 to 715 for NP1EO and 51 to 98 for NP2EO. On the other hand, IBF and NPX were detected in negligible concentrations in all samples taken along the river while DCF and KTP were below detection limits in all river water samples. Furthermore, based on the results for the samples taken from the German ditch, all target compounds were detected in higher concentrations compared with these from Spercheios river. The concentration of the compounds increased downstream the discharge point of the sewage treatment plant and this indicates that the effluent affects the quality of the water in sampling points G3, G4 and G5. Moreover, similarly to the river water samples, phenolic compounds (NP, NP1EO, NP2EO and BPA) exhibited higher concentrations than all other compounds. However, it should be noted that, the compounds' concentrations in the river and the ditch could be affected strongly by the flow rate and hence the dilution of the substances. More specifically, the flow rate fluctuates widely depending on the season, the weather conditions and in the case of German ditch depends on the contribution of wastewater effluents as well. Average daily loads of the target compounds which discharged to the Maliakos Gulf were calculated using mass balances. More specifically, the mean daily load of each compound was calculated for the Spercheios river as well as the contribution of WWTP in total mass of the target compounds. The mass balances were applied to the sampling point R6, which was near the mouth of the river. According to the results, EDCs seem to have the highest daily load and specifically, NP presents the highest load among all compounds, around 1600 g per day (Fig. 2), while the nonylphenol ethoxylates show amounts ranging from 122 g d^{-1} (NP2EO) to 201 g d^{-1} (NP1EO). In addition, as it can be shown in Fig. 2, the contribution of WWTP to the total mass of target compounds in the river was rather small and ranged from 2% (TCS) to 8% (NPX). The minor contribution of the WWTP indicates the existence of other sources of these compounds along Spercheios river. This is possibly due to the existence of the industrial complex area of Lamia, scattered industries and small settlements in the wider area. On the other hand, DCF and KTP are estimated to be discharged in lower masses, of about 1 g per day (Fig. 2). Furthermore, DCF and KTP seem to enter the river almost exclusively through the sewage treatment plant, indicating that WWTP is the main source of these compounds into the river and hence into Maliakos Gulf. Finally, it should be noticed that, although the concentrations of most of the compounds in the samples taken from Spercheios river are relatively lower than the concentrations in samples taken from the German ditch and WWTP, however, Spercheios river is expected to contribute significantly to the total loads of the substances discharged into Maliakos Gulf because of its high flow rate.

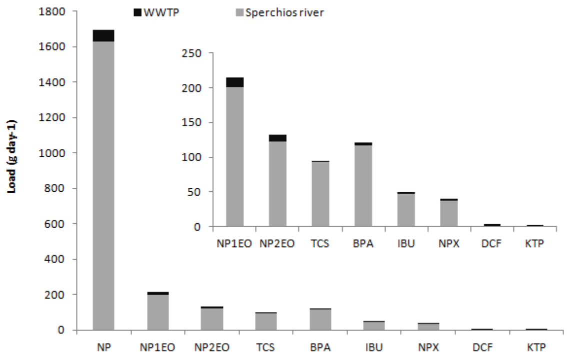

Figure 2. Contribution of WWTP and Sperchios river in daily loads of target compounds discharged to the Maliakos Gulf (mass balance in mouth of Sperchios river, R6 sampling point)

3.2 Microbial community composition of microcosms

Batch experiments were performed in order to identify the various microbial populations which grow in the river sediment under different redox conditions (aerobic, methane producing, anoxic and sulfate reducing conditions) and in the presence of two different target compounds' concentrations (40 and 500 μg L^{-1}). The replicates operated under anoxic and sulfate reducing conditions showed efficient consumption of NO_3^- and SO_4^{2-} corresponding to average rates of approximately 3.5 mg NO_3^- L^{-1} d^{-1} and 7 mg SO_4^{2-} L^{-1} d^{-1}. In addition, batch tests showed a satisfactory removal of the parent compounds under all operational conditions. More specifically, most of the substances showed high removals (>85%) after 30 days of incubation, with the exception of DCF which showed 10% less removal than the other substances. An important fact is that all compounds showed similar removal efficiency independently of the tested concentrations or redox conditions since the experiments showed an insignificant statistical difference.

Regarding results from FISH analysis (Fig. 3), Bacteria (72% ± 7%) outcompete over Archaea domain (9% ± 0%) in river sediment. Furthermore, as micropollutants' concentration increases the population of Archaea slightly decreases, since, in anaerobic microcosms with nominal concentrations of 40 μg L^{-1} and 500 μg L^{-1}, the population was approximately 30% and 18%, respectively. Thus, the decreased Archaea population did not affect the removal of the target compounds which remained in the same levels. However, the small decrease may be attributed to toxicity caused by micropollutants, considering the inhibition on methane yield under the high amount of emerging contaminants observed in literature (Symsaris *et al*., 2015; Stasinakis A., 2012). Besides Archaea inhibition, no other significant effect by the increased concentration was noted. The aerobic batch tests and the sediment sample taken from point G3 were very similar in terms of microbial community composition, suggesting that the two samples had approximately the same geochemical conditions. Moreover, dominant groups of microorganisms vary between different redox conditions. Thus, α-proteobacteria prevailed over other groups in aerobic (36%±8%) and anoxic (43%±2%) conditions and δ-proteobacteria in sulfate-reducing conditions (28%±7%), mainly due to

SRBs (Sulfate Reducing Bacteria). However, under methane-producing conditions, no predominant group was observed. The population of γ-proteobacteria was high in all batch experiments (17-28%), while under anoxic and anaerobic conditions was mostly represented by *Pseudomonas* spp. (16-21% of total cells). This trend is consistent with the findings of Yang *et al*. (2014), where γ-proteobacteria and α-proteobacteria were the largest two groups in BPA-degrading sediment microcosms under aerobic conditions. Although, *Sphingomonas* spp. are frequently present in micropollutants-degrading experiments (Zhang *et al*. 2013; Hay *et al*. 2001), only a small percentage (10-15%) was detected in the aerobic tests. Moreover, all the population of the phylum Firmicutes (2-21%) seems that belong to *Bacillus* spp. (5-20% of total cells) in all experiments. Regarding methanol-utilizing denitrifying bacteria, the percentage ranged between 10%-26%, while the highest presence was detected under anoxic conditions as expected.

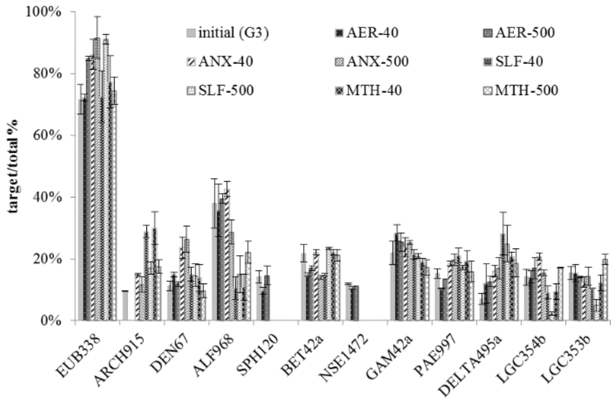

Figure 3. Average percentage of targeted groups/DAPI in microcosms studies

4 Conclusions

The occurrence of selected endocrine disrupters and non-steroidal anti-inflammatory drugs was investigated in Spercheios river and in one of its tributaries (German Ditch) which receives effluents from a wastewater treatment plant located nearby. The data showed that phenolic compounds were detected in all samples, with nonylphenol and its selected ethoxylates being the dominant pollutants in the water. According to the results, both river and ditch water discharged daily into the Maliakos Gulf contain significant quantities of the EDCs. Furthermore, wastewater treatment plant seems to be an important source for these compounds to the German ditch. However, all the compounds were detected in the German ditch upstream from the sewage treatment plant outfall, reflecting additional sources in the upstream basin.

Batch experiments in aerobic, anoxic and anaerobic conditions showed high removals (>85%) in most of the substances after 30 days of incubation, with the exception of DCF. The composition of microbial community seems to be affected primarily by redox conditions and not by the elevated concentration of the target compounds. However, lower percentage of Archaea was observed at higher micropollutants' concentration. Only γ-proteobacteria was similar in all batch experiments with different geochemical conditions.

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