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Toxicity assessment of pharmaceutical compounds on mixed culture from activated sludge using respirometric technique: The role of microbial community structure

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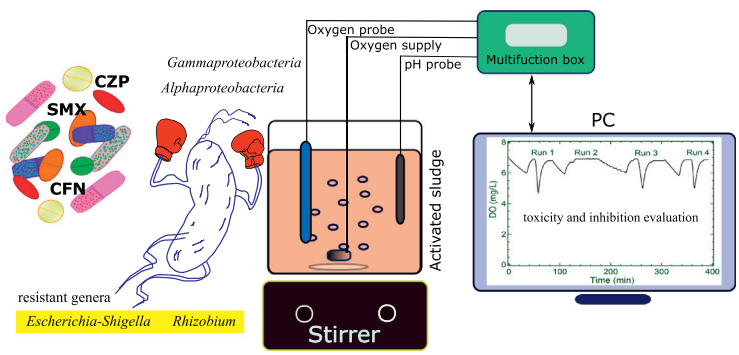
HIGHLIGHTS

- Evaluation of toxicity and inhibition of pharmaceuticals using respirometry
- Pharmaceuticals exhibited high toxic/inhibition effects on a non-acclimated culture.
- Pharmaceuticals altered bacterial community structure after acclimation process.
- Acclimation enhanced the resistance to pharmaceuticals' toxicity and inhibition.
- Dominant resistant genera of *Proteobacteria* (*Escherichia-Shigella, Rhizobium*) were detected.

GRAPHICAL ABSTRACT



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ABSTRACT

Micropollutants of emerging concern such as pharmaceuticals can significantly affect the performance of secondary biological processes in wastewater treatment plants. The present study is aimed to evaluate the toxicity and inhibition of three pharmaceutical compounds (caffeine, sulfamethoxazole and carbamazepine) on two cultures of microbial consortia enriched from wastewater aerobic activated sludge. One of them was acclimated to pharmaceuticals and the other was non-acclimated as control bioassay. The toxic and inhibitory effects on these cultures were assessed by respirometric tests through the oxygen uptake rate as an indicator of their capacity to degrade a readily available carbon source. Higher values of toxicity and inhibition of pharmaceutical compounds were observed for the control culture as compared to the acclimated one. Sulfamethoxazole and carbamazepine exhibited higher toxicity and inhibition effects than caffeine in both acclimated and control cultures. The microbial diversity of the two cultures was also studied. The composition of microbial community of acclimated and control cultures, was determined by targeting the 16S ribosomal RNA gene. It was observed that *Proteobacteria* was the most abundant phylum, with *Gammaproteobacteria* dominating both cultures. Control culture was dominated by *Gammaproteobacteria* and mostly by the genera *Pseudomonas* and *Sodalis*, which belong to common families present in wastewater. Results suggested that the acclimated culture to the three pharmaceuticals was mostly comprised of the extremely multiresistant genera *Escherichia-Shigella* (38%) of *Gammaproteobacteria*, resulting to higher resistance as compared to the control culture (*Escherichia-Shigella*, 7%). Finally, the microbial structure of the microorganisms present in a real bioreactor, which was initially seeded with the acclimated culture and fed in a continuous mode with the selected pharmaceuticals, was also analyzed. The continuous loading

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of pharmaceuticals in the bioreactor affected its microbial diversity, leading to the dominance of *Betaproteobacteria* and to the resistant genus *Rhizobium* of *Alphaproteobacteria*.

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1. Introduction

Pharmaceutical compounds are active substances that most of the times enter the water environment mainly through the effluent of wastewater treatment plants (WWTPs). The presence of pharmaceutical compounds in the natural environment has received great attention, since it is correlated with possible ecological risk due to toxicity, the spread of antibacterial resistance and the threat to drinking water quality (Hernando et al., 2006; Barcelo, 2007; Parrott and Bennie, 2009; Ortiz de García et al., 2014).

To date, the ecotoxic effect of various pharmaceuticals has been documented using Microtox bioassays of bioluminescence tests with *Vibrio fischeri* (Santos et al., 2010; Reungoat et al., 2011; Vasiliadou et al., 2014; Aydin, 2016) as well as using *Daphnia magna* and algal tests (Cleuvers, 2004). Moreover, Onesios and Bouwer (2012) based on biofilm protein measurements in column experiments suggested toxicity of pharmaceuticals to the biofilm communities. Recently, Ortiz de García et al. (2014) studied the ecotoxicity of pharmaceuticals in aquatic environments and WWTPs using both bioluminescence and respirometry assays, respectively.

Microtox and respirometry are common techniques for toxicity assessment of xenobiotic compounds present in wastewaters. Microtox is considered the standard method to evaluate acute toxicity. However, this bioluminescence test has inherent problems since the single microbial species (*Vibrio fischeri*) do not represent the microbial complexity of the communities present in activated sludge of WWTPs (Ricco et al., 2004; Brandt et al., 2015).

The activated sludge respirometric test is considered a direct method for evaluating the toxic effect of chemicals (e.g. pharmaceuticals) on sludge activity and thus the potential effect on its microbial population (Gutierrez et al., 2002; Ren, 2004; Ortiz de García et al., 2014; Tobajas et al., 2016; Amariei et al., 2017). The respirometry is a sensitive and cost effective method, which is based on the consumption of oxygen by activated sludge during biological activities (Ortiz de García et al., 2014). By this reason, the respirometry has been widely used to estimate the potential toxicity of many different chemical compounds on the mixed microbial community of WWTP sludge (Ricco et al., 2004; Oviedo et al., 2009; Rizzo, 2011; Ortiz de García et al., 2014).

It is worth noting that the presence of toxicants in wastewater streams can affect microbial metabolic and growing activities of bacteria in activated sludge, due to their acute and chronic inhibitory effects (Foladori et al., 2014; Aydin, 2016). Consequently, it has been recently proposed that toxicological tests should not only evaluate the activity of a microbial community, but also its microbial diversity (Brandt et al., 2015). The exposition of bacteria in activated sludge to toxic compounds can affect the quality of the treated effluent in WWTPs. Therefore, the observed differences in pharmaceuticals' removal from WWTPs (Carballa et al., 2004; Kosma et al., 2010; Sui et al., 2011) may be attributed to environmental and operating conditions as well as to differences in the microbial community among WWTPs (Chonova et al., 2016). Moreover, it has been previously reported that the time of biomass adaptation on pharmaceuticals can enhance the removal efficiency of the culture (Kraigher et al., 2008; Vasiliadou et al., 2013). This is because the exposure of a microbial culture to pharmaceuticals can gradually accumulate microbial species with pharmaceutical-resistance (Xia et al., 2015; Aydin et al., 2015).

Pharmaceuticals are stable pollutants, however, depending on the compound they have been characterized as hardly biodegraded (e.g. carbamazepine, CZP), moderately biodegraded (e.g. sulfametzoxazole, SMX) and in some cases well biodegraded (e.g. caffeine, CFN) (Yu et al., 2006; Zorita et al., 2009; Petrovic et al., 2009; Kosma et al., 2010). CZP, SMX and CFN belong to three different therapeutic classes; CZP is an anti-epileptic drug, SMX is an antibiotic and CFN is used as stimulant. Despite their different use, these three compounds belong to the group of the most studied pharmaceuticals in the literature, in terms of biodegradability, sorption, etc., using different methodologies, conditions and biomass systems (Carballa et al., 2004; Kosma et al., 2010; Sui et al., 2011; Vasiliadou et al., 2014; Vasiliadou et al., 2016; Cruz del Álamo et al., 2018). Finally, CZP has been reported to be one of the most persistent compounds in conventional biological wastewater treatments due to its low or no-biodegradable behaviour (Sui et al., 2011). On the other hand, CFN it is known to be easily biodegradable (>98%) in WWTPs by activated sludge (Grandclement et al., 2017). Considering SMX, the removals that have been documented from WWTPs are in the wide range of 20 to 80% (Rosal et al., 2010; Verlicchi et al., 2013).

The aim of the present study was to evaluate the toxic and inhibition effect of three pharmaceutical compounds (CZP, SMX, CFN) on a mixed culture of activated sludge, which was previously acclimated to artificial wastewater with the three pharmaceuticals, by tracking oxygen uptake rate using a respirometric technique. These results were compared to a control culture, which was not acclimated to pharmaceuticals. Considering that pharmaceuticals have been reported to be degraded mostly under aerobic conditions (Shi et al., 2013; Stadler et al., 2015), both cultures were seeded with aerobic activated sludge from the same WWTP. The composition and diversity of bacterial communities in both acclimated and control cultures were determined by targeting the 16S ribosomal RNA gene. Finally, the microbial diversity of a bioreactor which was inoculated with the acclimated culture and operated for pharmaceuticals' removal in our previous study, was analyzed (Vasiliadou et al., 2014).

To the best of our knowledge this work will produce relevant results in four major points not well established in this field, which are: 1) the toxic and inhibitory effects of pharmaceuticals on adapted or not microbial communities of activated sludge, 2) the influence of pharmaceuticals on the microbial community structure, 3) the effect of microbial composition on the functional abilities of the community (e.g. resistance to pharmaceuticals) and 4) the effect of operating conditions (e.g. continuous presence of pharmaceuticals) on the microbial diversity.

2. Materials and methods

2.1. Pharmaceutical compounds

Three pharmaceutical compounds were selected as target chemicals: the stimulant caffeine (CFN, $C_8H_{10}N_4O_2$), the antibiotic sulfamethoxazole (SMX, $C_{10}H_{11}N_3O_3S$) and the antiepileptic carbamazepine (CZP, $C_{15}H_{12}N_2O$). These pharmaceuticals were selected in this study due to their widespread occurrence in WWTPs and the suspected toxicity as well as their varying removal efficiency in WWTPs (Sui et al., 2011; Vasiliadou et al., 2014; Aymerich et al., 2016). All the chemicals used were purchased from Sigma-Aldrich. Stock solutions (1 g/L) were prepared in methanol. The standard solutions of each compound (100 mg/L) were prepared by dilution of individual stock solutions in ultra-pure water (Vasiliadou et al., 2014).

2.2. Mixed culture preparation

Two cultures of various microbial consortia were used in this study. Both of them were enriched from aerobic activated sludge taken from

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