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Is the chronic impact of sulfamethoxazole different for slow growing culture? The effect of culture history

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HIGHLIGHTS

- Acute impact of SMX boosted endogenous respiration due to higher cell maintenance.
- Chronic exposure to SMX resulted in retardation and reduction of substrate storage.
- Part of acetate is utilized at slower rate due to binding/release effect of SMX.
- Microbial population structure was assessed using sequencing of 16S rRNA gene.
- *Amaricoccus* and *Bacteroidetes* spp. proliferated owing to co-metabolism of SMX.

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ABSTRACT

The study evaluated impact of *sulfamethoxazole* on acetate utilization kinetics and microbial community structure using respirometric analysis and pyrosequencing. A fill and draw reactor fed with acetate was sustained at a sludge age of 10 days. Acute impact was assessed by modeling of respirometric data in batch reactors started with sulfamethoxazole doses in the range of 25–200 mg/L. Fill and draw operation resumed with continuous sulfamethoxazole dosing of 50 mg/L and the chronic impact was evaluated with acclimated biomass after 20 days. Acute impact revealed higher maintenance energy requirements, activity reduction and slight substrate binding. Chronic impact resulted in retardation of substrate storage. A fraction of acetate was utilized at a much lower rate with partial biodegradation of sulfamethoxazole by the acclimated biomass. Pyrosequencing indicated that *Amaricoccus* sp. and an *unclassified Bacteroidetes* sp., possibly with the ability to co-metabolize sulfamethoxazole, dominated the community.

1. Introduction

Antibiotics are intensively used for human and veterinary medicine, livestock farming and aqua culture purposes; consequently, they are continuously discharged to wastewater treatment plants (WWTPs) either as parent compounds and/or metabolites, through excretion, runoff/leaching of manure or imprecise disposal of unused/expired drugs (Kümmerer, 2009; Michael et al., 2013). As they resist biodegradation, they by-pass conventional treatment and accumulate in the environment. Adverse effects of gradually increasing levels of antibiotics in different environments have been extensively studied. Similarly, their fate and impact on the performance of treatment systems and especially, on the characteristics of microbial community sustained in biological treatment systems have also been a major concern. This issue is much more important for concentrated waste streams generated by hospitals, pharmaceutical plants and animal feeding operations, as point sources with much higher antibiotic concentrations (Cetecioglu et al., 2012).

Sulfamethoxazole (SMX) is one of the most popularly prescribed and consumed polar, low-adsorptive synthetic sulfonamides (Müller et al., 2013). Much like other pharmaceuticals, its persistence due to bacterial resistance remains to be the major issue both in the environment and in treatment systems. Results of extensive research efforts on its fate and biodegradation are still quite inconclusive: While some studies indicated efficient removal of SMX by different biological processes (Kümmerer, 2009; Michael et al., 2013), others reported very low or practically no removal potential for the same systems (Al-Ahmad et al., 1999; Joss et al., 2006). The

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observed discrepancy between different results is largely due to the fact that most related studies relied on empirical black-box approaches conducted with no emphasis on kinetic analysis, modeling, and dynamics of the microbial community (Larcher and Yargeau, 2012; Müller et al., 2013).

Recently, evaluation of the impact of antibiotics based on variations in process kinetics was reported using multi-component modeling of oxygen uptake rate (OUR) profiles under aerobic conditions. Ozkok et al. (2011) assessed the acute impact of SMX, together with tetracycline (TET) and erythromycin (ERY) on the biodegradation of peptone mixture selected as a complex organic substrate at two antibiotics concentrations (50 and 200 mg/L), where modeling of respirometric data revealed inhibitory impact on all processes involved. Similar study also compared chronic impact of ERY on the utilization of peptone mixture (Pala-Ozkok and Orhon, 2013). Acute impact of SMX was also investigated both on complex substrate (peptone mixture) and simple substrate (acetate) substrate using the same respirometric modeling approach by Pala-Ozkok et al. (2014a). Furthermore, acute impacts of different antimicrobials under anaerobic conditions were explored by Cetecioglu et al. (2012). A similar study on the inhibitory impact of SMX would obviously be quite useful both for setting a basis for benchmarking with other antibiotics and also, for comparing the effect of chronic exposure to SMX with its acute impact.

While kinetic analysis of respirometric data gives significant clues on how the adverse impact may be visualized in terms of different biochemical processes involved, it remains insufficient in determining why these changes occur, because it does not properly cover the role of population dynamics on process kinetics and the spread/persistence of both antibiotic-resistant bacteria and antibiotic resistance determinants in the WWTPs. Many different studies characterized microbial community structures of laboratory and full-scale engineered activated sludge systems and natural systems using a variety of methods, which included reaction denaturant gradient gel electrophoresis (PCR-DGGE), terminal restriction fragment length polymorphism (t-RFLP), fluorescent *in situ* hybridization (FISH), ribosomal intergenic spacer analysis (RISA) and analysis of 16S rRNA clone libraries (Hu et al., 2012). However these methods fail to characterize the total community of activated sludge biomass due to the exceptionally rich microbial diversity (Hu et al., 2012; Ye et al., 2011; Pala-Ozkok et al., 2012). For a better sense of microbial composition, researchers improved next-generation DNA sequencing to produce thousands or millions of sequences concurrently. 454-pyrosequencing used widely to analyse the microbial community in various environmental samples including soil, marine water and WWTP influent (Ye et al., 2011). Nevertheless, applications of this technology on activated sludge still remain limited to a small number of references in literature (Pala-Ozkok et al., 2012, 2014b; Hu et al., 2012; Ye et al., 2011).

In this context, the objective of this study was to evaluate the acute and chronic impacts of SMX on the readily biodegradable substrate (acetate) under aerobic conditions, emphasizing (i) inhibitory and toxic effects based on observed changes on process kinetics; (ii) differences revealed in the structure of the microbial population under chronic exposure to SMX dosing and this way, (iii) meaningful relationships between observed process kinetics and microbial community structure using both 454-pyrosequencing and respirometric modeling.

2. Methods

2.1. Experimental approach

The experimental approach essentially consisted of a laboratory-scale fill and draw reactor with a net volume of 10 L and operated at steady-state at sludge age of 10 days (d). This sludge age (SRT) was selected to approximate the operating conditions conventionally adopted in biological treatment systems (Orhon et al., 2010). The biomass was taken from a wastewater treatment facility operated in Istanbul (Turkey); it was cultured and acclimated to pulse feeding under aerobic conditions using laboratory-scale bioreactors operated at steady-state and fed with the selected organic substrate. After the start-up period, the steady-state operation was sustained for 30 d, corresponding to a period of three SRTs. After this period, the acclimated biomass was subjected to a series of batch experiments for the respirometric analysis of acute inhibitory impact of SMX. After the acute tests, the operation of the fill and draw reactor was continued for another 20 d with continuous dosing of SMX together with acetate. The same biomass was used for chronic SMX experiments. The last batch test was conducted at the end of the 20 d period for the assessment of the chronic impact of SMX on process kinetics and the composition of the microbial community.

Acetate was selected the sole organic carbon source for evaluating the impact of SMX on substrate utilization and storage. Acetate is a well studied, simple and readily biodegradable substrate, which can also be converted into polyhydroxybutyrate (PHB), a biopolymer stored in microbial cells under intermittent feeding conditions (Ciggin and Orhon, 2014). Acetate feeding solution was prepared from sodium acetate anhydrous (CH_3COONa) (MERCK ve 127-09-3) and daily acetate feeding was adjusted to 1.6 g COD/d (400 mg COD/L). Besides the carbon source, macro nutrients (NH_4Cl: 120 g/L, KH_2PO_4: 160 g/L, K_2HPO_4: 320 g/L) and micro nutrients ($MgSO_4{\cdot}7H_2O$: 15 g/L, $CaCl_2{\cdot}2H_2O$: 2.65 g/L, $FeSO_4{\cdot}7H_2O$: 0.5 g/L, $ZnSO_4{\cdot}7H_2O$: 0.5 g/L, $MnSO_4{\cdot}H_2O$: 0.41 g/L) were added to the reactor. Nutrients were supplied on a daily basis for the nutritional requirements of the cells and to provide enough buffer capacity to the reactor. The reactor was monitored for chemical oxygen demand (COD) and volatile suspended solids (VSS) concentrations for steady-state conditions. Biomass concentration was stabilized at approximately 1800 ± 50 mg VSS/L, associated with an average food to microorganism ratio (S_{AC0}/X_{T0}) of 0.22 mg COD/mg VSS d at steady state operation. pH was kept between 6.0 and 8.0, suitable for biological activity and the temperature was maintained at 20 ± 1 °C within the reactor. Aeration was continuously provided and the oxygen concentration was kept above 2 mg/L in order to maintain aerobic conditions. After the steady-state operation with biomass acclimated to acetate feeding, the reactor was fed with a combination of acetate (400 mg COD/L) and continuous dosing of SMX (50 mg SMX/L) to examine the chronic impact of the antibiotic.

2.2. Respirometric analysis in batch experiments

Respirometric analysis was performed in a series of batch experiments, essentially (i) to visualize and evaluate the acute impact of SMX on acetate biodegradation, (ii) to observe the variation of this impact as a function of antibiotic concentrations. Therefore, the first test (Run 1) was started only with around 200 mg COD/L of acetate and served as the *control reactor*; prior to SMX dosing; the other four runs (Runs 2–5) were conducted with initial SMX (Fluka S7507, CAS Number 723-46-6) doses of 25, 50, 100 and 200 mg/L, respectively, together with the same level of acetate in the control test. The last batch experiment (Run 6) was conducted after 20 d of continuous SMX feeding. To avoid possible photo-degradation of SMX, the reactor was covered with aluminum foil during the chronic experiments. All batch tests were run in duplicate and also served for the assessment of COD and PHB profiles parallel to respirometric assessment.

SMX can best be treated at source because it remains mainly in the aqueous phase due to its low sorption coefficient and also it