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Chronic impact of sulfamethoxazole on acetate utilization kinetics and population dynamics of fast growing microbial culture

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HIGHLIGHTS

- Chronic exposure to SMX changes the composition of the microbial community.
- SMX inactivated vulnerable microbial fractions in favor of resistant species.
- *Paracoccus* and *Meganema* spp. proliferated owing to resistance and SMX co-metabolism.
- SMX enhances endogenous respiration due to higher maintenance energy demand.
- Part of acetate is utilized at slower rate due to binding/release effect of SMX.

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ABSTRACT

The study evaluated the chronic impact of sulfamethoxazole on metabolic activities of fast growing microbial culture. It focused on changes induced on utilization kinetics of acetate and composition of the microbial community. The experiments involved a fill and draw reactor, fed with acetate and continuous sulfamethoxazole dosing of 50 mg/L. The evaluation relied on model evaluation of the oxygen uptake rate profiles, with parallel assessment of microbial community structure by 454-pyrosequencing. Continuous sulfamethoxazole dosing inflicted a retardation effect on acetate utilization in a way commonly interpreted as competitive inhibition, blocked substrate storage and accelerated endogenous respiration. A fraction of acetate was utilized at a much lower rate with partial biodegradation of sulfamethoxazole. Results of pyrosequencing with a replacement mechanism within a richer more diversified microbial culture, through inactivation of vulnerable fractions in favor of species resistant to antibiotic, which made them capable of surviving and competing even with a slower metabolic response.

1. Introduction

Discovery of antibiotics should be considered as one of the most remarkable medical achievements of the past century. The term antibiotics literally means *against life*, mainly referring to microbial life. They are specifically designed to treat bacterial infections. While they act as major ingredients of health and life protection in modern society, they are also regarded as major pollutants, due to their wide use and persistence to biodegradation by virtue of their fatal impact on microbial metabolism (Kümmerer et al., 2004). They are extensively released through wastewater discharges and mostly accumulate in the environment, by-passing treatment systems like similar xenobiotics (Müller et al., 2013).

Sulfamethoxazole (SMX) is a synthetic antibiotic within the *sulfonamide* (*sulfa drugs*) family, a group widely used both in veterinary and human medicine because of its low cost of production, their efficiency and relatively low toxicity (Göbel et al., 2005). SMX prevents formation of dihydrofolic acid, a compound that bacteria must be able to produce in order to survive (Drillia et al., 2005). Since it cannot be completely eliminated by the typical conventional wastewater treatment technology, it is frequently detected in all environmental media, at concentrations of 70–150 ng/L in surface waters and 200–2000 ng/L in secondary wastewater effluents (Renew and Huang, 2004).

Occurrence of SMX in the environment has led many studies to investigate its fate and biodegradation in engineered microbial communities such as biological wastewater treatment systems. The biodegradation of SMX was investigated mostly using standard tests such as *Zahn Wellens Test* (OECD 302B) or CO_2 *Evolution Test*

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(OECD 301B), only capable of providing an index of biodegradability, which is often difficult to compare with continuously operated real systems. SMX proved to be persistent to biodegradation (Drillia et al., 2005; Kümmerer et al., 2004). Al-Ahmad et al. (1999) also reported poor biodegradability for SMX as the result of closed bottle tests during a period of 28 days seeded with biomass taken from an activated sludge plant. Potential degradation mechanisms of SMX were examined in different studies. Drillia et al. (2005), observed SMX co-metabolism with acetate in a lab-scale sequencing batch reactor, and stated that microorganisms might use SMX as a nitrogen source in the absence of readily biodegradable nitrogen source. Additionally, the ability of activated sludge to utilize SMX as both carbon and nitrogen source was pointed out by Müller et al. (2013). They also stated that biodegradation of SMX was enhanced in the presence of acetate.

SMX is also expected to have an inhibitory/toxic impact on microbial systems. The traditional approach for evaluating this impact relies on simple enzyme analogy applied to rate expressions associated with microbial growth (Pala-Ozkok et al., 2011; Nitisoravut and Klomjek, 2005). This approach is quite out-dated, because it is now well known that utilization of complex substrate mixtures such as wastewaters do not solely involve a single overall process – i.e. microbial growth – but a number of biochemical processes associated with substrate components with different biodegradation characteristics, all likely to be affected by the inhibitory impact (Pala-Ozkok et al., 2011). In this context, understanding the interactions between chemicals such as SMX and microbial cultures remain to be a major challenge. A number of developments may contribute to the interpretation of these interactions: Respirometric analysis generate oxygen uptake rate (OUR) profiles reflecting specific biodegradation fingerprints of the system under investigation (Orhon et al., 2009). New multi-component models incorporating all relevant processes and components may be used to interpret experimental data in terms of applicable process kinetics, i.e. numerical values of model coefficients through calibration; by comparing changes in model parameters it is also possible to determine the level of inhibition induced by the chemical/SMX antibiotic dosing (Pala-Ozkok et al., 2013a).

Finally, changes induced in the composition of the microbial community may be assessed by molecular techniques. Different methodologies were used in order to determine the microbial community structure of various biological systems. Among these methods t-RFLP, FISH, PCR-DGGE coupled with cloning and sequencing can be given. However, activated sludge systems being very diverse and complex communities, was shown to exceed the capacity of detection of previously listed methods. For a better insight of microbial communities researchers developed high-throughput sequencing technologies. 454-pyrosequencing, producing large amounts of DNA reads, is accepted as one of the ideal tools to analyze complex microbial communities (Szczepanowski et al., 2008; Ye et al., 2011). Although in the literature increasing number of studies can be found, which applied high-throughput sequencing in determination of microbial community structure, only a limited number of studies are present that investigated activated sludge community structure and the effect of antibiotics.

Recently, the research group at ITU introduced a novel approach to the evaluation of acute impact of different antibiotics using all essential experimental ingredients, covering generation of respirometric data, process modeling and molecular analysis of microbial community: In studies conducted with peptone mixture, a complex substrate with similar biodegradation characteristics with domestic sewage, organic substrate although completely removed could not be fully utilized in microbial metabolism at first exposure to antibiotics, an observation compatible with *uncompetitive inhibition* (Pala-Ozkok et al., 2011). Acute impact of antibiotics was observed to affect all related processes together with microbial growth, decreasing the hydrolysis rate, suppressing substrate storage and enhancing endogenous respiration due to higher maintenance energy requirements (Pala-Ozkok et al., 2013b). The acute impact of SMX was different depending upon the nature of organic substrate: It was mainly substrate binding for the peptone mixture; whereas for acetate, it exerted slight inhibition on microbial growth kinetics, however with full substrate utilization (Pala-Ozkok et al., 2013a). The outcome of these studies was quite significant mainly because the adverse kinetic impact of the antibiotics could be numerically expressed not only on the growth process as in the traditional inhibition studies, but on all significant processes such as hydrolysis, substrate storage, endogenous respiration taking part in current biodegradation models. It left behind however an equally important question to be further clarified: Were the observed kinetic changes resulted from the impairment of the metabolic machinery of the same microbial community or else, did they in reality reflect changes inflicted on the composition of the microbial community due to adverse condition. This study attempted to clarify this basic issue by exploring changes in the microbial community structure under continuous exposure to SMX and also, evaluating the relationship between observed process kinetics and microbial population structure using both pyrosequencing and respirometric modeling.

While acute tests are useful for assessing the response of non-acclimated biomass at first exposure, occurring as pulse discharges in real systems, it is clear that there is also need for exploring the chronic impact trough experiments with continuous dosing of the inhibitor, which would basically evaluate the effect of acclimation, cumulative impact and possible changes in the composition of the microbial community. In this context, the objective the study was to provide a new understanding and interpretation to the chronic impact of SMX on the microbial community sustained in the system. Evaluation mainly relied on model evaluation of the OUR profiles generated at critical phases of the experimental period, with parallel assessment of the microbial community structure continuously exposed to SMX dosing. The study was designed to address the following major issues, in order to comply with its objectives: (i) How the impact would be translated into process kinetics and stoichiometry? (ii) Would the impact be permanent or reversible with time through acclimation of the microbial community? (iii) Would continuous exposure to SMX affect and change the composition of the microbial community? (iv) Would the acclimated community be capable of using SMX as an organic carbon source, leading to SMX biodegradation?

2. Methods

2.1. Experimental approach

Experiments were started with a laboratory-scale fill and draw control reactor with a net aeration volume of 4.0 L. The reactor was initially inoculated with a biomass seed taken from an existing biological system sustained with acetate feeding. It was operated at steady state at a sludge age of 2.0 d. A fast growing microbial culture was selected with the adopted sludge age, mainly to better visualize different metabolic responses induced by continuous SMX dosing. Acetate was selected as the sole organic carbon source as a readily biodegradable substrate, because it can be easily measured and monitored; it also favors significant intracellular storage as *polyhydroxybutyrate* (PHB) under intermittent feeding conditions and this way, it offers a parallel substrate utilization mechanism likely to be affected by SMX. The daily acetate feeding was adjusted to 1.6 g COD/d (400 mg COD/L). At steady-state, the biomass concentration in the reactor was stabilized around 480 ± 25 mg VSS/L, corresponding to an average food to microorganism (F/M) ratio of 0.85 mg COD/mg VSS d.