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Pyrosequencing reveals the inhibitory impact of chronic exposure to erythromycin on activated sludge bacterial community structure

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ABSTRACT

The study investigated changes in the microbial population structure sustained at two different sludge ages of 10 d and 2 d under chronic impact of erythromycin. It intended to observe the experimental correlation between variable process kinetics and changes in the composition of the microbial community induced by erythromycin. Samples from two fill/draw reactors operated with continuous erythromycin dosing of 50 mg/L were collected for the analysis of microbial population structure using high-throughput sequencing of 16SrRNA gene. Significant changes were observed in the composition of microbial community during the exposure period. Richness analysis for slower growing culture indicated that most microbial fractions were inactivated and eliminated in favor of fewer more resistant species in different phyla. Sludge age appeared to control the impact of erythromycin on microbial composition. At a sludge age of 2 d, erythromycin appeared to generate richer community with faster growing and more compatible species. For slower growing culture, elimination of vulnerable species was supported by decrease in the number of shared level OTUs. For faster growing culture, shared species level OTUs also decreased significantly upon exposure to erythromycin, suggesting rapid washout and replacement by more resistant species. Resistance gene analysis yielded positive results for *mph*(A) gene indicating presence of erythromycin-resistant components in the microbial community.

1. Introduction

Activated sludge process represents one of the most intriguing microbial systems engineering for different specific purposes. Its operation may be adjusted to sustain microbial fractions that can utilize organic matter, oxidize and remove nitrogen, and perform preferential enhanced phosphorus removal. Particularly, biodegradation of organic substrate through this process has been an area of remarkable achievements: adopting chemical oxygen demand (COD) as the collective substrate parameter and then better defining it in terms of COD fractions with different biodegradation characteristics [1,2]. However, similar refinements could not be achieved in the assessment of the structure and composition of the microbial community sustained in the system. The available activated sludge models (ASMs) include only a single heterotrophic biomass component, which is not further explored under different operating conditions [3–5]. Extensive effort was only directed towards identification of microorganisms, mostly filamentous bacteria, capable of causing bulking and foaming, such as members of genera *Nostocoida*, *Nocardia* and *Microthrix* [6–8], but they were not extrapolated to cover the entire microbial community. However, this information would be of vital importance for better evaluating the effect of environmental conditions, especially the inhibitory impact of different chemicals on the microbial community and process performance.

Different methodologies, such as polymerase chain 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 have been used to determine the composition of microbial population in engineered and natural ecosystems [9–13]. Generally, they were short of characterizing the total community in activated sludge, mainly due to the wide diversity of the microbial community

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[14]. Recent methods such as 454-pyrosequencing and microarrays have been approved to be much more promising, as they provided significantly higher throughput than the conventional methods. High-throughput pyrosequencing technology has been used in different microbial ecology branches, such as microbial diversity and functional genes diversity [15], and for analysis of environmental samples including marine water and wastewater treatment plant influent [14,16,17]. This technology was also applied to activated sludge, mostly without any effort to relate the results to observed process kinetics [14,18–21]. Only in a recent study, observation of a variable kinetics as a function of culture history was related to induced changes in the microbial community, assessed by means of 454-pyrosequencing [22]. It should be noted that culture history generally refers to all conditions dictating the growth characteristics of the microbial community and in microbial systems sustained for wastewater treatment these conditions may be simplified into and expressed as the sludge age. Furthermore, sequencing results of the plasmid metagenome of a wastewater treatment plant revealed that the wastewater bacteria were important reservoirs for clinically important resistance determinants [23]. Another study by Schlüter et al. [24] investigated the genetic diversity of a plasmid metagenome of a wastewater treatment plant by 454-pyrosequencing, stating wastewater treatment plants play an important role as hotspots for circulation of antibiotic resistance determinants.

Earlier parts of the study were focused on adverse impact of antibiotics and particularly erythromycin on substrate utilization by mixed microbial culture. Erythromycin (ERY) is a macrolide antibiotic, which inhibits the aminoacyl translocation, thereby preventing the amino acid chain elongation [25]. This bacteriostatic antibiotic is used both in veterinary and human medicine with a broad activity against Gram-positive and few Gram-negative bacterial pathogens. Resistance is based on decreased cell permeability, alteration of 50S or 23S ribosomal unit or enzymatic inactivation. Many studies were conducted for assessing the fate and the impact of erythromycin on activated sludge systems; they remained mostly empirical in nature, without conclusive results on substrate utilization and certainly without any scientific clues on the response of the microbial community [26–29].

In the past, ERY has been the subject to a number of studies, mainly in conjunction with its potential for deteriorating the efficiency of biological treatment. In most of these studies, reported information was only limited to simple assessment of influent and effluent ERY concentrations in wastewater treatment plants and resulting levels in the water environment erythromycin on all related biochemical processes of microbial metabolism [26,29,30]. They usually report very low levels of antibiotics, largely diluted in the wastewater, which bypass treatment as they are mostly resistant to biodegradation. This way they accumulate in the environment. A better approach would be to detect and remove these chemicals at different primary sources, such as hospitals, pharmaceutical plants, etc., where they are first generated, and implement effective treatment on segregated and concentrated wastewater streams. Removal at source requires assessment of adverse effects at high concentrations. In this context, ERY levels selected in this study are compatible with effluent discharges from hospitals and pharmaceutical plants. Reported results in several studies indicate a range of 100–500 mg/L for antibiotic concentrations in hospital and pharmaceutical plant effluents [31,32]. For example, tylosin concentration in the mixed antibiotic wastewater of Eli Lilly & Company in Liverpool, UK, was measured between 20 and 200 mg/L [33]. Furthermore, the selection of tested ERY dose of 50 mg/L was specifically confirmed on the basis of respirometric studies conducted with three different antibiotics as significant concentrations, which inhibited and reduced the oxygen uptake rate of the same organic substrate—the *peptone mixture*—by more than 50%, i.e. lower than the LC_{50} level, in the standard ISO8192 inhibition test [34]. This approach also justifies the relatively high ERY concentration tested in this study for its chronic inhibitory impact.

The scientific insights provided by the observed results summarized above would be greatly improved by clarifying the following major question: Are variable process kinetics and changes in the metabolic activities directly associated with the impact of erythromycin on the metabolic machinery of the same microbial community? Or, is it the result of a different microbial community changed under chronic exposure to ERY? In this context, the main objective of the study was to assess changes in the composition of the microbial community, i.e. microbial population dynamics, sustained at different sludge ages, during the chronic impact of erythromycin. It is intended to clarify the relationship between observed metabolic responses and the composition of the microbial community using the results of pyrosequencing.

2. Materials and methods

2.1. Reactor setup and operation

Experimental setup first involved two 14 L laboratory-scale fill/draw reactors acclimated to a synthetic organic substrate (peptone mixture) with a hydraulic retention time of 1 day. Peptone mixture was selected mainly because it was prescribed as standard substrate for inhibition studies based on respirometry [34–37] and its COD composition includes a number of fractions with different characteristics similar to real wastewaters [34].

The seed sludge used for the reactor setup was taken from the aeration tank of a domestic wastewater treatment plant in Istanbul (Turkey). One liter of the peptone–meat extract mixture [34] consisted of 16 g of peptone (Acumedia–Pancreatic Digest of Gelatin (Pepton G) 7182A), 11 g of beef extract (Acumedia–Beef Extraxt Powder 7228A), 3 g of urea (Acumedia–Urea Agar Base 7226), 0.7 g of NaCl, 0.4 g of $CaCl_2{\cdot}2H_2O$, 0.2 g of $MgSO_4{\cdot}7H_2O$ and 2.8 g of K_2HPO_4. Additionally, a macronutrient solution consisting of K_2HPO_4 (320 g/L) and KH_2PO_4 (160 g/L) and a micronutrient solution consisting of $MgSO_4{\cdot}7H_2O$ (15 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) and $CaCl_2{\cdot}2H_2O$ (2.65 g/L) were added to the reactors. For each 1000 mg of COD feeding, 20 mL of both macro- and micronutrient solutions were added to the reactors together with the carbon source.

Reactors were operated until they reached steady state, at sludge ages–sludge retention times–(SRT) of 10 and 2 d, respectively. Both reactors were aerated continuously at a rate to ensure a DO concentration in excess of 3.0 mg/L for the purpose of eliminating any possible interference/adverse effect on substrate utilization due to dissolved oxygen limitations. At steady-state conditions, biomass concentrations in the control reactors were 2000 mg VSS/L and 570 mg VSS/L, yielding F/M ratios of 0.30 mg COD/mg VSS and 1.26 mg COD/mg VSS, for sludge ages of 10 d and 2 d, respectively.

Biomass taken from the control reactors at steady state were used as seed for two fully aerated fill/draw reactors sustained at the same sludge ages, for chronic impact studies. The concentration of organic substrate was adjusted to 720 mg COD/L for both chronic exposure reactors operated at sludge ages of 10 d and 2 d. Macro- and micronutrient solutions were also added to the reactors as defined in the ISO 8192 procedure [35,38]. In addition to peptone mixture, feed for chronic reactors also involved a continuous dosing of 50 mg ERY/L (Aldrich856193, CAS No.: 114-07-8).

Samples were collected to determine the chronic effect of ERY on the microbial community structure for the faster growing community by means of 454-pyrosequencing. At the sludge age of 2 d, the first sample was taken one on day 0 (C-2), prior to ERY dosing; the second on day 3 (E-2-3) and the third one on day 10 (E-2-10).