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Relative contribution of ammonia oxidizing bacteria and other members of nitrifying activated sludge communities to micropollutant biotransformation

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ABSTRACT

Improved micropollutant (MP) biotransformation during biological wastewater treatment has been associated with high ammonia oxidation activities, suggesting co-metabolic biotransformation by ammonia oxidizing bacteria as an underlying mechanism. The goal of this study was to clarify the contribution of ammonia oxidizing bacteria to increased MP degradation in nitrifying activated sludge (NAS) communities using a series of inhibition experiments. To this end, we treated a NAS community with two different ammonia oxidation inhibitors, namely octyne (OCT), a mechanistic inhibitor that covalently binds to ammonia monooxygenases, and allylthiourea (ATU), a copper chelator that depletes copper ions from the active center of ammonia monooxygenases. We investigated the biotransformation of 79 structurally different MPs by the inhibitor-treated and untreated sludge communities. Fifty-five compounds exhibited over 20% removal in the untreated control after a 46 h-incubation. Of these, 31 compounds were significantly inhibited by either ATU and/or OCT. For 17 of the 31 MPs, the inhibition by ATU at 46 h was substantially higher than by OCT despite the full inhibition of ammonia oxidation by both inhibitors. This was particularly the case for almost all thioether and phenylurea compounds tested, suggesting that in nitrifying activated sludge communities, ATU does not exclusively act as an inhibitor of bacterial ammonia oxidation. Rather, ATU also inhibited enzymes contributing to MP biotransformation but not to bulk ammonia oxidation. Thus, inhibition studies with ATU tend to overestimate the contribution of ammonia-oxidizing bacteria to MP biotransformation in nitrifying activated sludge communities. Biolog tests revealed only minor effects of ATU on the heterotrophic respiration of common organic substrates by the sludge community, suggesting that ATU did not affect enzymes that were essential in energy conservation and central metabolism of heterotrophs. By comparing ATU- and OCT-treated samples, as well as before and after ammonia oxidation was recovered in OCT-treated samples, we were able to demonstrate that ammonia-oxidizing bacteria were highly involved in the biotransformation of four compounds: asulam, clomazone, monuron and trimethoprim.

1. Introduction

Micropollutants (MPs) such as pesticides, pharmaceuticals and personal care products (PPCPs) are increasingly detected in the environment and may adversely affect ecosystems and public health (Luo et al., 2014; Petrie et al., 2015). Extensive studies on MP biotransformation during the secondary biological treatment stage of WWTPs have revealed that many MPs were subject to partial removal, although biological treatment was not originally designed for MP removal (Pomies et al., 2013). Because only very few WWTPs are currently equipped with a tertiary treatment stage with the specific purpose of MP removal, such as advanced oxidation, the

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secondary treatment stage has a major effect on the mitigation of MPs before entering into the receiving environments. Therefore, a better understanding of the fate and transformation of MPs during biological wastewater treatment is of particular importance to evaluate the environmental impacts of both parent compound residues and transformation products (TPs).

A number of studies have pointed towards significant associations between MP biotransformation and nitrification activities of nitrifying activated sludge (NAS) in the biological treatment step. For example, biotransformation was enhanced for a variety of MPs, including pharmaceuticals, pesticides, and estrogens, as higher ammonia oxidation activities were reached in NAS (Fernandez-Fontaina et al., 2012; Helbling et al., 2012; Tran et al., 2009; Yi and Harper, 2007). Based on these observations, a causal relationship has been widely hypothesized between ammonia oxidizers in NAS communities and the biotransformation of certain MPs. However, correlation does not necessarily equate to causation, and explicit tests of this hypothesis are needed (Johnson et al., 2015b).

Two controlled laboratory experimental approaches have typically been used to corroborate the importance of ammonia oxidizers for MP biotransformation: i) studies that test the MP biotransformation capabilities of pure cultures of different ammonia-oxidizing microorganisms (AOMs); and, ii) studies that investigate changes of MP biotransformation in NAS communities treated with inhibitors of ammonia oxidation. The biotransformation capabilities of AOMs for several MPs, such as mianserin and ranitidine (Men et al., 2016), triclosan and bisphenol A (Roh et al., 2009), *p*-cresol (Kjeldal et al., 2014), and estrogens (*i.e.*, E1, E2, E3 and EE2) (Khunjar et al., 2011; Shi et al., 2004; Skotnicka-Pitak et al., 2009) have been demonstrated by pure culture studies, whereas no biotransformation activity has been observed in AOM pure cultures for compounds such as ibuprofen (Shi et al., 2004), trimethoprim (Khunjar et al., 2011), and eight other pesticides and pharmaceuticals (Men et al., 2016). These pure culture studies provide fundamental insights into biotransformation capability, reaction types and mechanisms, and the potential formation of TPs, but no concrete conclusion on MP biotransformation by AOMs in NAS communities can be drawn if negative results were obtained in the pure culture studies.

Inhibition studies are more used for understanding the contribution of AOMs to MP biotransformation in complex communities such as NAS. Given that most environmental microorganisms are uncultivable and tend to interact with other microbes in communities, pure culture studies may not always properly reflect the behavior of the microorganism in complex environmental microbial communities. In contrast, inhibition studies address the question of the actual role played by AOMs in a more environmentally relevant manner.

Known AOM inhibitors include 1-alkynes (C_2–C_{10}), which irreversibly inhibit ammonia monooxygenases (AMOs) (Hyman et al., 1988). Acetylene can inhibit both bacterial and archaeal AMOs, whereas octyne (OCT) was found to be more specific to ammonia-oxidizing bacteria (AOB) than to ammonia-oxidizing archaea (AOA). OCT has therefore previously been used to differentiate the roles played by AOB and AOA together with another inhibitor, PTIO, which is more specific to AOA (Martens-Habbena et al., 2015; Taylor et al., 2013). Besides acetylene and OCT, allylthiourea (ATU) is also considered a specific inhibitor of ammonia monooxygenases (AMOs), and has so far most commonly been used to investigate the linkage between MP biotransformation and nitrification (Batt et al., 2006; Khunjar et al., 2011; Li et al., 2015; Maeng et al., 2013; Rasche et al., 1991; Rattier et al., 2014; Roh et al., 2009; Sathyamoorthy et al., 2013; Tran et al., 2009; Zhou and Oleszkiewicz, 2010). The biotransformation of a variety of MPs was affected by the addition of ATU to different extents, suggesting the involvement of AOMs. However, similar to OCT, ATU is not an effective inhibitor for AOA. ATU exhibited similar inhibitory effects as OCT on the nitrification in soil communities containing both ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Taylor et al., 2013). In addition, the inhibitory effects of ATU on nitrification was incomplete (80%) in archaea-containing seawater samples (Jantti et al., 2013). Therefore, ATU and OCT are more suitable for inhibition studies on NAS communities with AOMs dominated by AOB. For municipal WWTPs, the AOM in NAS communities are generally dominated by AOBs (Gao et al., 2013; Wells et al., 2009), although AOA have been found to be more abundant in some municipal and industrial WWTPs (Limpiyakorn et al., 2013; Sauder et al., 2012).

However, there are two limitations for the inhibition studies conducted so far to address the contribution of AOMs to MP biotransformation. First, there is a lack of understanding how AOM-inhibitors interact with non-AOM members of complex microbial communities. This is because AOM-inhibitors have been primarily investigated in a biogeochemical context (*i.e.*, focusing on the roles of different nitrifying microbial groups in the global N cycle) or in a biochemical context (*i.e.*, focusing on roles of AMOs in pure or highly enriched cultures). Second, previous inhibition studies (Batt et al., 2006; Maeng et al., 2013; Margot et al., 2016; Rattier et al., 2014; Roh et al., 2009) only targeted a limited number of exposure-relevant, yet structurally diverse MPs, providing little information on the compound specificity of biotransformation performed by AOMs.

The goal of this study was to clarify the relative contribution of AOMs and other members of NAS microbial communities to MP biotransformation by specifically addressing the above-mentioned shortcomings of previous inhibition studies. To address questions of compound specificity, we used a total of 79 MPs for the inhibition study, which were selected to cover different chemical classes and/or groups of anticipated biotransformation reactions with multiple compounds in each of the selected categories. To address questions of inhibitor specificity, we compared the biotransformation of selected MPs with or without the addition of two inhibitors (*i.e.*, ATU and OCT) in a previously studied NAS community in which AOB were supposed to be the dominant group of AOMs (Men et al., 2016). The effects of ATU on heterotrophic respiration were investigated to further examine the specificity of ATU. Overall, we sought to provide a comprehensive view of the biotransformation of the tested MPs in NAS, and more specifically, the extent to which AOB are involved in their biotransformation.

2. Materials and methods

2.1. Compound selection

A total of 79 MPs (Table S1) were selected based on compound availability and to have multiple compounds cover the same chemical classes and/or anticipated biotransformation reactions. The reference compounds for the 79 MPs were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), Dr. Ehrenstorfer GmbH (Augsburg, Germany), HPC Standards GmbH (Cunnersdorf, Germany), Honeywell Specialty Chemicals (Seelze, Germany), and Toronto Research Chemicals (Toronto, Canada). Stock solutions were prepared in ethanol, with a concentration of 40–50 mg/L for each compound. Allylthiourea (ATU) and 1-octyne (OCT) were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland).

2.2. Inhibition experiments

The inhibition experiments were performed in batch reactors. Nitrifying activated sludge (NAS) was taken from the aeration tank