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Biodegradation potential of wastewater micropollutants by ammonia-oxidizing bacteria

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ABSTRACT

This study examined the biodegradation potential of three wastewater micropollutants (triclosan, bisphenol A, and ibuprofen) by *Nitrosomonas europaea* and mixed ammonia-oxidizing bacteria in nitrifying activated sludge. *N. europaea* could degrade triclosan and bisphenol A, but not ibuprofen. The degradation was observed only in the absence of allylthiourea (an inhibitor for ammonia monooxygenase (AMO)), suggesting that AMO might be responsible for triclosan and bisphenol A degradation. Competitive inhibition among ammonia, triclosan, and bisphenol A was observed. Inactivation of *N. europaea* was observed after degrading a mixture of triclosan and bisphenol A. The inactivation might be due to product toxicity and/or antimicrobial effect of triclosan; however, the causes of the inactivation were not determined.

Regardless of the presence of the AMO inhibitor, three micropollutants were degraded by two different nitrified activated sludge samples. The results suggested that both ammonia-oxidizing bacteria and heterotrophic microorganisms in the activated sludge can degrade triclosan and bisphenol A. On the other hand, ibuprofen was more likely degraded by heterotrophic microorganisms in the activated sludge.

1. Introduction

Approximately 80% of US streams (108 out of 139 US streams surveyed) were contaminated with a wide range of wastewater micropollutants, including bisphenol A (BPA, a plasticizer), triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol, an antimicrobial agent), and ibuprofen (a non-steroidal anti-inflammatory drug) (Kolpin et al., 2002).

Triclosan is a synthetic, broad-spectrum antimicrobial agent that has been widely used in the United States since the 1960s (Jungermann, 1968). As an antimicrobial agent and preservative, triclosan has been incorporated into a broad array of personal care products (e.g., hand disinfecting soaps, medical skin creams, dental products, deodorants, toothpastes), consumer products (e.g., fabrics, plastic kitchenware, sport footwear), and cleaners or disinfectants in hospitals or households (Singer et al., 2002). Between 1992 and 1999, over 700 consumer products contained antibacterial properties, and the vast majority of the products contained triclosan (Schweizer, 2001). The disposal of the triclosan-containing products caused triclosan contamination in wastewater, streams, seawater, sediments, fish, and human milk (Okumura and Nishikawa, 1996; Adolfsson-Erici et al., 2002; Kolpin et al., 2002; Singer et al., 2002). There are many concerns about the widespread contamination of triclosan in the environment, including an aid to the development of cross-resistance to antibiotics (Braoudaki and Hilton, 2004), the adverse effects on ecological health (Tatarazako et al., 2004), and the formation of more toxic pollutants under different conditions. For example, during photodegradation, triclosan forms chlorodioxins and other concerned metabolites (Latch et al., 2003). During drinking water treatment, triclosan could react with free chlorine to form chlorophenols, including *tetra*- and *penta*-chlorinated hydroxylated diphenyl ether, 2,4-dichlorophenol, and 2,4,6- triclorophenol (Canosa et al., 2005). Furthermore, a recent study suggested that triclosan is potentially a weak androgen (Foran et al., 2000).

BPA is a building compound for the manufacturing of plastics, epoxy resins, and polycarbonate resins. BPA is also used in a number of products like adhesives, building materials, powder paints, inner coating of cans, automotive lenses, compact discs, thermal paper, electrical and electronic parts (Staples et al., 1998). Production of BPA was approximately 2.5 Mt in 2001 and rapidly increased to 2.8 Mt in 2002 (Staples et al., 2002). Discharges from manufacturing facilities as well as leaching from various

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BPA-containing products, particularly poly vinyl chloride plastics (Cousins et al., 2002), contribute to the widespread contamination of BPA in the environment. BPA was detected at a concentration up to 0.4 μg L^{-1} in German surface waters (Bolz et al., 2001). The USGS survey of 139 US streams reported that the frequency of detection for BPA was 41%, with a maximum concentration of 12 μg L^{-1} and a medium concentration of 0.14 μg L^{-1} (Kolpin et al., 2002). BPA is also an endocrine disrupter (Krishnan et al., 1993; Fujimoto et al., 2006) and known to cause acute toxicity to aquatic organisms between concentrations of 1–10 μg L^{-1} (Alexander et al., 1988).

Ibuprofen is a popular chiral, non-steroidal anti-inflammatory drug with a propionic acid group. Ibuprofen is also an analgesic and antipyretic drug that can be obtained without a prescription. Ibuprofen was detected in most lakes (2–8 ng L^{-1}), and some rivers (up to 139 ng L^{-1}) in Germany (Buser et al., 1999). In the United States, ibuprofen was detected at a 9.5% detection frequency, a maximum concentration of 1 μg L^{-1}, and a medium concentration of 0.2 μg L^{-1} (Kolpin et al., 2002). Ibuprofen is known to effect the growth of certain species of aquatic plants, *Synechocystis* sp. and *Lemna minor* (Pomati et al., 2004).

Biodegradation of these three micropollutants had been reported by mixed cultures in river water (Kang et al., 2006) and in wastewater treatment plants (WWTPs) (Staples et al., 1998; Buser et al., 1999; Federle et al., 2002; Singer et al., 2002; Kanda et al., 2003; Thompson et al., 2005). Singers et al. reported that approximately 79% of triclosan in wastewater was biodegraded; 15% was sorbed into biosolids, and 6% was released into the receiving water bodies (Singer et al., 2002). Several studies have reported removal of triclosan by different biological treatment processes (Kanda et al., 2003), including activated sludge (Federle et al., 2002; Thompson et al., 2005), rotating biological contactors and trickling filters (Thompson et al., 2005). Unlike triclosan, greater than 90% of removal has been reported for BPA (Staples et al., 1998) and ibuprofen and its metabolites (Buser et al., 1999).

Activated sludge is a complex biological wastewater treatment system that is currently designed for removing carbon and/or nitrogen constituents in the wastewater. The nitrogen constituents in wastewater are converted from ammonia to nitrate through a process called nitrification. The first step in the nitrification processes (i.e. from ammonia to nitrite) is carried out by ammonia-oxidizing bacteria, a group of ubiquitous lithoautotrophic microorganisms. The ammonia-oxidizing bacteria can express ammonia monooxygenase (AMO) enzyme to oxidize ammonia to nitrite. In addition to the ammonia oxidation, AMO enzymes are known to oxidize a wide range of aliphatic and aromatic hydrocarbons (Hyman et al., 1988; Rasche et al., 1991; Keener and Arp, 1994; Chang et al., 2003). More recently, Shi et al. (2004) reported that *Nitrosomonas europaea*, a well studied ammonia-oxidizing bacterium, was capable of degrading steroidal estrogens in wastewater.

While removal of triclosan, BPA and ibuprofen by biological treatment processes has been observed, little is known about the microorganisms that are responsible for their degradation in wastewater. Since AMO has a wide, non-specific range for substrates, we hypothesized that ammonia-oxidizing bacteria might be involved in triclosan, BPA and ibuprofen biodegradation during wastewater treatment. This study reported the biodegradation potential of triclosan, BPA and ibuprofen by pure and mixed ammonia-oxidizing bacteria. The role of AMO in triclosan, BPA and ibuprofen biodegradation was also examined. The results of this study could enhance the understanding of the fate of triclosan, BPA and ibuprofen in wastewater and the environment.

2. Methodology

2.1. Chemicals

Triclosan (>97% pure) and allylthiourea were obtained from Sigma–Aldrich Inc. (Milwaukee, WI). BPA (99% purity) was obtained from TCI America, Inc. (Portland, OR). Ibuprofen (USP grade) was obtained from MP Biomedicals (Solon, OH). Stock solutions of triclosan, BPA, and ibuprofen were prepared in acetone. Dimethylformanide (DMF) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ) and N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Biotechnology Inc. (Rockford, IL). All other reagents used were commercial products of highest grade available.

2.2. Pure culture and growth conditions

N. europaea was generously provided by Dr. Michael Hyman, Department of Microbiology, North Carolina State University. The cells were grown in 2 L glass flasks containing 1 L of mineral salt medium (Hyman et al., 1988) and 25 mM of $(NH_4)_2SO_4$ in the dark at 150 rpm, at 30 °C for 3 d. The initial pH of the growth medium was adjusted to 8.0. The change of pH during the cell growth was minimized due to the strong buffer in the growth medium [5.4 M KH_2PO_4 and 0.6 M KH_2PO_4]. The cells were pelleted by centrifugation (at 10,000*g* and 4 °C for 30 min), washed once with phosphate buffer solution (50 mM NaH_2PO_4 [pH 7.8], and 2 mM $MgSO_4{\cdot}7H_2O$), and then resuspended in the mineral salt medium for experimental use. The resting cell concentrations were measured as protein content using a BCA Protein Assay Reagent Kit (Pierce Biotechnology Inc., Rockford, IL), or as optical density at 600 nm (OD_{600}) using a Hewlett Packard G1130A UV–visible spectrophotometer.

2.3. Nitrifying activated sludge

Nitrifying activated sludge samples were collected from two different wastewater treatment plants (WWTP #1 and WWTP #2). The WWTP #1 is located in Houston, Texas, and treats 265,000 $m^3\ d^{-1}$ of wastewater by using two-sludge nitrification with pure oxygen. The WWTP #2 is located in College Station, Texas and employs a single-sludge nitrification process to treat an average flow of 9500 $m^3\ d^{-1}$ of wastewater. The solids retention times (SRTs) for WWTP #1 and WWTP #2 are 20–30 d and 7–8 d, respectively. The activated sludge samples were collected from the nitrification tank of WWTP #1 and from the aeration tank of WWTP #2. The samples were pelleted by centrifugation (at 12,000*g* and 4 °C for 20 min), washed twice with the phosphate buffer solution, and resuspended in the growth medium with reduced ammonia concentrations (5 or 10 mM of $(NH_4)_2SO_4$). The reduced ammonium concentration was used because $(NH_4)_2SO_4$-sensitive ammonia-oxidizing bacteria in activated sludge have been reported (Suwa et al., 1997).

2.4. Biodegradation tests

Biodegradation experiments were conducted in a series of 1 L-flasks containing resting cell suspension of *N. europaea* and a known amount of triclosan (0.5–2 mg L^{-1}), BPA (0.12–1.6 mg L^{-1}), or ibuprofen (0.4 mg L^{-1}) in an acetone-free medium. The acetone-free medium was prepared similarly as described by Yu et al. (2007). Autoclave-killed cells were used as controls. Due to analytical detection limits and potential analytical error in low concentrations, higher than ambient concentrations of micropollutants

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were used in the biodegradation tests. The use of higher concentrations was also justified as the objective of this study was to determine whether AOB can degrade these wastewater micropollutants. Samples were collected over time and used for nitrate, nitrite, and micropollutants analyses. Concentrated H_2SO_4 was added to samples to stop microbial activity. Another set of experiments with allylthiourea (10 mg L^{-1}) was conducted similarly to determine whether AMO is responsible for the biodegradation of selected micropollutants. Allylthiourea is an AMO inhibitor (Rasche et al., 1991). It is expected that no degradation of micropollutants will be observed in the presence of allylthiourea if AMO is responsible for the biodegradation. Nitrifying activated sludge was also used for biodegradation tests. The concentration of mixed liquor volatile suspended solids (MLVSS) of 1000 mg L^{-1} was used.

2.5. Analytical methods

Triclosan, BPA, and ibuprofen were extracted with ethyl ether overnight and derivitized with 50 μL BSTFA in 450 μL DMF (Zhao, 2006; Subramanya, 2007). The derivitized samples (1 μL) were injected into an Agilent 6890 Series II Gas Chromatograph (GC)/Agilent 5973 Mass Spectrometer system. The GC was equipped with a HP-5MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness). Helium (purity 99.999%) was used as the carrier gas at a constant flow of 1.2 mL min^{-1}. The injector temperature was set at 250 °C. The oven temperature was set at 80 °C, with an increasing rate of 30 °C min^{-1} to 280 °C, held at 280 °C for 3 min, and then increased to 300 °C for 3 min. The analysis was performed at selective ion monitoring mode. The primary ions used for quantification and identification were *m/z* 200 and 360 for triclosan, *m/z* 357 and 372 for BPA, and *m/z* 263 and 278 for ibuprofen. The standard curves, ranging from 0.005 to 5 mg L^{-1}, were used. Liquid samples were filtered with Millex-GP filter unit (0.22 μm pore size) before use for ammonia and nitrite analyses. Concentrations of ammonia were determined by an ammonia-selective electrode probe (Accumet benchtop meter, Fisher Scientific, Pittsburgh, PA). Concentrations of nitrite and nitrate were measured by injecting 10 μL of liquid sample into a DX-80 Ionic Chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with an IonPac AS14A – 5 μm Analytical Column (3 × 150 mm). An eluent solution, containing 0.16 M Na_2CO_3 and 0.02 M $NaHCO_3$, was used, at a flow rate of 0.5 mL min^{-1}.

3. Results and discussion

3.1. Degradation of three micropollutants by N. europaea

While *N. europaea* is known to express AMO to degrade a wide range of substrates (Hyman et al., 1988; Rasche et al., 1991; Keener and Arp, 1994), no study has explored its degradation ability toward emerging wastewater micro pollutants, except estrogens (Shi et al., 2004). In this study, experiments were designed to investigate whether a well-studied ammonia-oxidizing bacterium, *N. europaea*, can degrade triclosan, BPA, and ibuprofen. Production of nitrite in corresponding to decrease of ammonia was observed only in the absence of allylthiourea (data not shown), suggesting that *N. europaea* was active and allylthiourea is an effective inhibitor for AMO enzyme. None of the micropollutants was degraded by *N. europaea*, when allylthiourea was present. In the absence of allylthiourea, *N. europaea* quickly oxidized ammonium into nitrite within the first 10 h of incubation, but was unable to degrade ibuprofen (0.4 mg L^{-1}) (data not shown). Biodegradation of triclosan (0.5–2 mg L^{-1}) and BPA (0.12–1.6 mg L^{-1}) were observed in the absence of the AMO inhibitor (Fig. 1). These observations suggested that AMO might be responsible for the degradation of triclosan and BPA. Our degradation results also suggested that triclosan and BPA was cometabolically degraded by AMO.

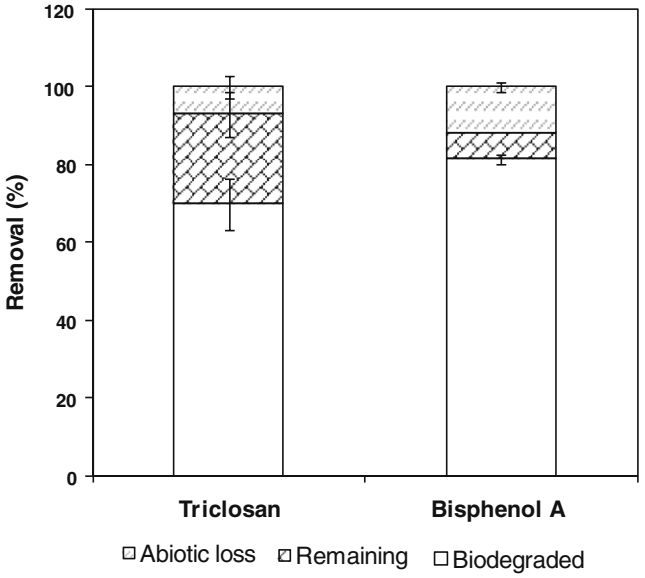

Fig. 1. Biodegradation of triclosan and BPA by *N. europaea*. Duplicate experiments were repeated using a range of initial concentrations (i.e., 0.5–2 mg L^{-1} for triclosan and 0.7–1.6 mg L^{-1} for BPA).

To our knowledge, this is the first study reporting that *N. europaea* can degrade triclosan and BPA, but not ibuprofen. The reason why *N. europaea* could not degrade ibuprofen was not clear. As *N. europaea* can degrade phenolic compounds (Keener and Arp, 1994), this might explain the observation of triclosan and BPA degradation since both compounds contain a phenolic functional group.

3.2. Inhibition and toxicity effects

Alternative substrates for AMO could often exert an inhibitory effect on ammonia oxidation (Keener and Arp, 1994), due to their competition for binding with AMO. This study also conducted experiments to determine whether triclosan would affect ammonia oxidation by *N. europaea*. In the absence of triclosan, nitrite concentrations increased from 0 to 20 mM in the first 24 h and then remained unchanged (Fig. 2). When triclosan was present (2 mg L^{-1}), the nitrite concentration only reached 6 mM (Fig. 2),

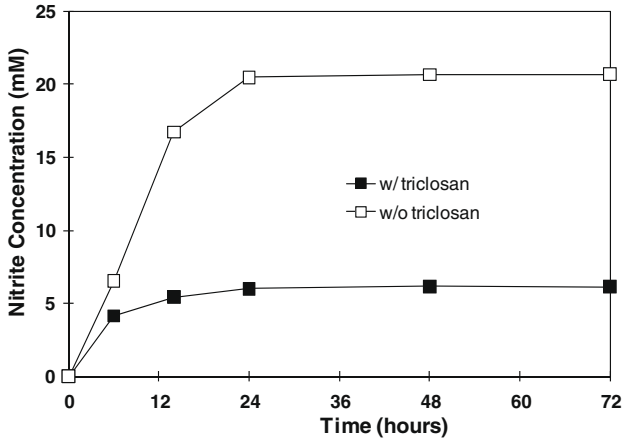

Fig. 2. Production of nitrite by *N. europaea* in the presence and absence of triclosan (2 mg L^{-1}). Solid squares represent samples with triclosan. Empty squares represent samples without triclosan.

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indicating that nitrite production was reduced as much as 70% due to the presence of triclosan. The reduction is likely due to competitive inhibition between ammonia and triclosan for AMO enzymes and/or toxic effects of triclosan on the cells.

Additional experiments were conducted to examine whether the resting cells of *N. europaea* can degrade a mixture of triclosan and BPA. As shown in Fig. 3, *N. europaea* could degrade the mixture of BPA and triclosan. Compared to triclosan, BPA was degraded to a lower concentration (0.2 mg L^{-1}) at a faster rate. The results suggested AMO of *N. europaea* might have a smaller affinity coefficient for BPA than for triclosan. The ability to oxidize ammonia after degrading the mixture of triclosan and BPA was examined. Ammonia (50 mM) was added into the vials at the end of the degradation test (i.e. after 25 h) and the vials were incubated with shaking for 5 d. No ammonia oxidation was observed after 5 d of incubation, suggesting that *N. europaea* were completely inactivated after degrading the mixture. The causes of the inactivation were not determined. Two possible causes are product toxicity generated from the degradation and the antimicrobial effects of triclosan.

3.3. Biodegradation of three micropollutants by nitrifying activated sludge

Several bacteria are capable of degrading triclosan (Hay et al., 2001; Meade et al., 2001; Zhao, 2006), BPA (Lobos et al., 1992; Ike et al., 2000; Kang et al., 2004; Sasaki et al., 2005), or ibuprofen (Chen and Rosazza, 1994; Murdoch and Hay, 2005). For example, two wastewater isolates, *Sphingomonas sp.* RD1 (Hay et al., 2001) and *Sphingopyxis* strain KCY1 (Zhao, 2006), were able to degrade triclosan via cometabolism. Two soil bacteria, *Pseudomonas putida* and *Alcaligenes xylosoxidans* strains, have a high triclosan resistance and can use triclosan as a sole carbon source (Meade et al., 2001). Many wastewater bacterial isolates are known to degrade or use BPA as a growth substrate (Lobos et al., 1992; Ike et al., 2000; Kang et al., 2004; Sasaki et al., 2005). Two ibuprofen-utilizing cultures, a species of *Nocardia* (Chen and Rosazza, 1994) and a *Sphingomonas* sp., have been isolated from wastewater (Murdoch and Hay, 2005). Nevertheless, their roles and the abundance of these isolates in WWTPs remain unclear.

In this study, the nitrifying activated sludge collected from WWTP #1 was able to degrade 2 mg L^{-1} of triclosan within 5 d, regardless the presence of AMO inhibitor (Fig. 4a). The results suggested that both ammonia-oxidizing bacteria and non-ammonia-oxidizing bacteria play a role in triclosan biodegradation in the nitrifying activated sludge. Nitrate, but not nitrite, was detected throughout the course of experiments (5 d). In the absence of triclosan and AMO inhibitor (i.e. live controls), approximately 55% of the NH_4^+-N added was converted to NO_3^--N in 5 d (Fig. 4b). In the presence of triclosan, nitrate production reached the plateau around 2 mM, which was about one-fifth of that produced by live controls. Similar biodegradation trends were observed for BPA (1 mg L^{-1}) and ibuprofen (0.5 mg L^{-1}) in the WWTP #1 sludge samples, regardless of the presence of AMO inhibitor. BPA was completely removed by the WWTP #1 sludge within 2 d. In the presence of AMO inhibitor, complete removal of BPA was also observed within 3 d (from 60% on day 2 to 100% removal on day 3, data not shown). Ibuprofen was completely removed within 2 d.

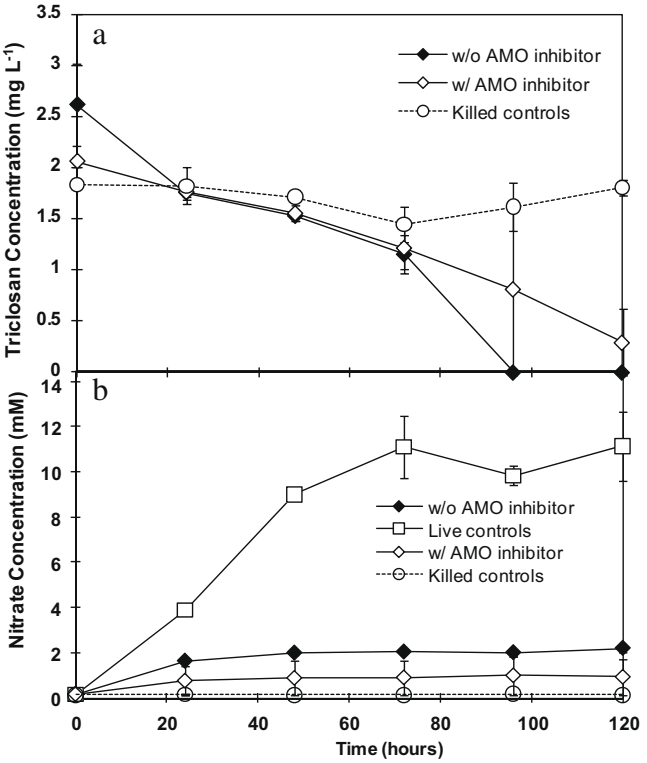

Fig. 4. Biodegradation of triclosan (A) and production of nitrate (B) by nitrifying activated sludge from WWTP #1. Solid diamonds (without AMO inhibitor), empty diamonds (with AMO inhibitor), empty circles (killed control), and empty squares (live controls, no triclosan).

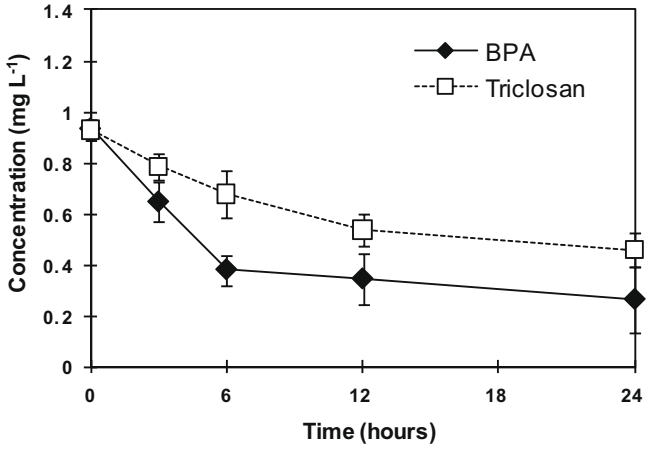

Fig. 3. Biodegradation of a mixture of BPA (1 mg L^{-1}) and triclosan (1 mg L^{-1}) by *N. europaea*. Triclosan concentrations showed as empty squares. BPA concentrations showed as solid squares.

The results of experiments using WWTP #2 activated sludge samples were similar to those using WWTP #1 activated sludge. Triclosan was degraded within 5 d by the activated sludge of WWTP #2 (Fig. 5a). In live controls of WWTP #2 sludge, the production of nitrite increased from 0 to 2.4 mM in 5 d (Fig. 5b), which was about one-fifth of that produced by the WWTP #1 sludge (Fig. 4b). Interestingly, approximately 80% of BPA was degraded on day 3 and remained unchanged on day 5. As expected, complete removal of ibuprofen was once again observed within 2 d. The degradation pattern or efficiency was not affected by the presence of AMO inhibitor.

As mentioned earlier, many wastewater bacterial isolates are known to degrade triclosan, BPA, and ibuprofen, it was not surprising to observe biodegradation of these three micropollutants by the nitrifying activated sludge of WWTP #1 and WWTP #2. High triclosan removal by activated sludge (ranging from 90–100% as shown in Figs. 4a and 5a) was observed regardless the presence of AMO inhibitor. While the experimental data was insufficient to determine the fraction of triclosan degraded by AOB, these

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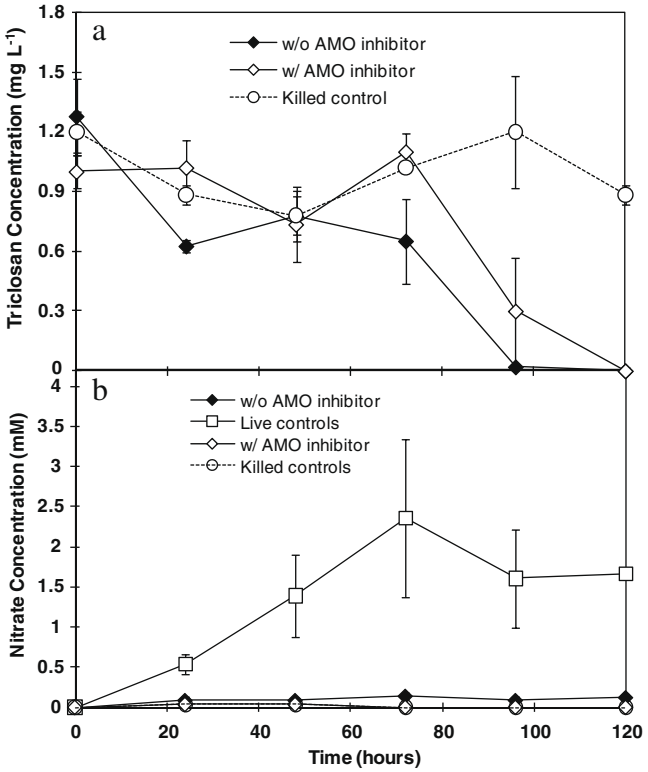

Fig. 5. Biodegradation of triclosan (A) and production of nitrate (B) by nitrifying activated sludge from WWTP #2. Solid diamonds (without AMO inhibitor), empty diamonds (with AMO inhibitor), empty circles (killed control), and empty squares (live controls, no triclosan).

results suggested that both AOB and heterotrophic microorganisms in activated sludge can degrade triclosan. Nitrate production of the two WWTPs (Figs. 4b and 5b) was consistent with the common observation more nitrifying bacteria (both ammonia-oxidizing and nitrite-oxidizing bacteria) in WWTP #1 (two-stage activated sludge system) than in WWTP #2 (single-sludge activated sludge system). More than five times of nitrate was produced by the live controls of two WWTPs than those produced by the samples with triclosan addition. The reduction of nitrate can be explained by the combination of competitive inhibition between ammonia and triclosan for AMO enzymes (Fig. 2) and the inhibition effects of triclosan on nitrite-oxidizing bacteria (Dokianakis et al., 2004). It was unclear why BPA was completely degraded by activated sludge samples from WWTP #1, but not from WWTP #2. Nevertheless, our results suggested that the conditions favorable for nitrifying bacteria might be also important for microorganisms capable of degrading BPA in wastewater.

Conversely, degradation of ibuprofen was only observed in activated sludge samples. Combining the observation that *N. europaea* was unable to degrade ibuprofen, it strongly suggested that non-ammonia-oxidizing bacteria are important for ibuprofen removal in the wastewater. The two reported ibuprofen-utilizing isolates (a species of *Nocardia* (Chen and Rosazza, 1994) and a *Sphingomonas* sp. (Murdoch and Hay, 2005)) might be present in the activated sludge from two WWTPs. However, the microorganisms responsible for ibuprofen degradation in the activated sludge remain unclear.

Numerous emerging micropollutants, including triclosan, BPA and ibuprofen, are present in wastewater. However, these compounds are unlikely to support microbial growth in the activated sludge, in part, due to their low concentrations. The ability of ammonia-oxidizing bacteria to cometabolize triclosan and BPA offers another potential removal mechanism for these wastewater micropollutants. More study is needed to decipher the percentage of wastewater micropollutants degraded by AOB in nitrifying activated sludge. However, this might not apply to readily biodegradable wastewater micropollutant, like ibuprofen. As observed in this study, non-ammonia-oxidizing microorgansims were likely to be responsible for ibuprofen biodegradation. Our results explain in part why different removals of these micropollutants were observed in various biological treatment processes (Thompson et al., 2005). The pure culture studies have demonstrated that ammonia-oxidizing bacteria can also remove triclosan and BPA. However, the significance of their roles in degrading triclosan and BPA might vary significantly, depending on different microbial ecology in WWTPs. Before one can design an effective biological system for removing a myriad of concerned micropollutants in the wastewater, more studies are needed to identify and understand the active – micropollutant-degraders, other than ammonia oxidizers, in various activated sludge system.

4. Conclusions

Several conclusions can be drawn from this study. They are:

N. europaea can degrade triclosan and BPA but not ibuprofen. The degradation was likely catalyzed by AMO via cometabolic reactions. Competitive inhibition of ammonia oxidation, as shown by the decreased nitrite production, was observed when triclosan or BPA was present. Since *N. europaea* can also degrade estrogen comentabolically, future studies to examine degradation kinetic for BPA, triclosan, and estrogens, are warranted.

Inactivation of *N. europaea* was observed after degrading triclosan and a mixture of triclosan and BPA. The causes of inactivation were unclear, but likely due to product toxicity and/or antimicrobial effect of triclosan.

Both ammonia-oxidizing bacteria and non ammonia-oxidizing bacteria were responsible for biodegradation of triclosan, BPA, and ibuprofen in activated sludge samples. A better understanding of wastewater microorganisms responsible for the biodegradation is the key to design an effective treatment processes for the wastewater micropollutants.

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