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Enhanced Biodegradation of Iopromide and Trimethoprim in Nitrifying Activated Sludge†

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Iopromide (an X-ray contrast agent) and trimethoprim (an antibacterial drug) are frequently detected pharmaceuticals in effluents of wastewater treatment plants (WWTPs) and in surface waters due to their persistence and high usage. Laboratory-scale experiments showed that a significantly higher removal rate in nitrifying activated sludge as compared to conventional activated sludge was observed for both iopromide and trimethoprim. When the activity of the nitrifying bacteria was inhibited, the percent removal of iopromide decreased from 97 to 86% while trimethoprim removal decreased from 70 to 25%. The metabolite of iopromide identified when nitrification was not inhibited was a dehydroxylated iopromide at the two side chains. However, when the nitrifying bacteria were inhibited the metabolite identified was a carboxylate, formed during the oxidation of the primary alcohol on the side chain of iopromide. These results suggest that the nitrifying bacteria are important in the observed biodegradation of iopromide in the activated sludge with higher solid retention time (SRT). Results from the laboratory-scale study were corroborated by the observed removal efficiencies in a full-scale municipal WWTP, which showed that iopromide (ranging from 0.10 to 0.27 μg/L) and trimethoprim (ranging from 0.0.08 to 0.53 μg/L) were removed more effectively in the nitrifying activate sludge which has a higher SRT (49 days) than in the conventional activated sludge (SRT of 6 days). In nitrifying activated sludge, the percent removal of iopromide in the WWTP reached 61%, while in conventional activated sludge, average removal was negligible. For trimethoprim, removal was limited to about 1% in the conventional activated sludge, while in the nitrifying activated sludge, the removal was increased to 50%.

Introduction

The frequent detection of many pharmaceutical compounds in the environment has become an increasing concern because of their potential to cause undesirable ecological and human health effects. Pharmaceutical residues from human use are introduced via a number of pathways, but primarily from discharges of wastewater treatment plants (WWTPs) (*1*−*4*). Wastewater treatment generally consists of a primary, secondary, and sometimes an advanced treatment stage, with different biological, physical, and chemical processes available for each stage of treatment. Several reports have shown that the current wastewater treatment procedures do not completely eliminate many of these micro-contaminants (*4*−*9*).

Recently, there has been an increased effort to investigate the fate of pharmaceutical agents in WWTPs, particularly in biological treatment processes that rely on the activated sludge systems. While a few studies have implicated a correlation between the removal of selected pharmaceuticals and sludge age or solid retention time (SRT) (*10*−*12*), a significant variation in percent removal has been observed for the same compounds in other full-scale conventional activated sludge systems (*6*). High SRT allows the enrichment of slow growing bacteria, such as the nitrifying bacteria in the activated sludge. Currently, several studies have reported that nitrifying bacteria are capable of co-metabolizing a variety of organic micro-pollutants (*13*, *14*) that typically resist biodegradation. Therefore, we hypothesize that these nitrifying bacteria play a key role in the biodegradation of persistent pharmaceuticals in wastewater. The distinct capability of nitrifying bacteria to degrade recalcitrant compounds has been reported in several studies for a wide array of aromatic xenobiotics, such as anisol (*15*), aniline (*16*), and naphthalene (*17*), in which ammonia-oxidizing bacteria (AOB) were suggested to co-metabolize the organic substrates.

The objectives of the present work were to investigate the aerobic biodegradability of iopromide and trimethoprim in laboratory-scale bioreactors containing mixed liquor from nitrifying activated sludge and to corroborate the results with their fate in the full-scale WWTP. The chemical structures and selected physicochemical properties of these compounds are shown in Table 1. Iopromide is a drug that belongs to the family of iodated X-ray contrast media widely used in human medicine for imaging of organs or blood vessels during diagnostic tests. Iopromide is metabolically stable in the human body and is excreted almost completely within a day. As such, iopromide is one of the most frequently detected X-ray contrast media in WWTP effluents (*18*). Although it has been shown that iopromide can undergo some biodegradation in the laboratory scale tests (*19*, *20*), monitoring of iopromide in municipal WWTP showed no significant removal of this compound throughout the plant (*5*, *18*). Ternes and Hirsch detected iopromide at almost the same levels in the influents and effluents of municipal WWTPs, with levels reported between 0.75 and 11 μg/L (*18*). Trimethoprim is an antimicrobial drug commonly prescribed in combination with the sulfonamide sulfamethoxazole for the treatment of infectious diseases in humans. The removal of trimethoprim in WWTPs has been reported from negligible (*6*, *21*) to below 10% (*22*). Reported wastewater effluent concentrations in the United States have ranged from 0.011 to 0.53 μg/L (*3*, *4*). In laboratory settings, trimethoprim also exhibited strong resistance to microbial breakdown by activated sludge bacteria even after a prolonged adaptation phase of several weeks (*14*, *21*). The role of nitrifying bacteria in the biodegradation of pharmaceuticals in activated sludge has not been explored and is the subject of this paper.

Experimental Section

Chemicals and Reagents. Iopromide (IOP) was purchased from USP (Rockville, MD), and trimethoprim (TRI) was purchased from ICN Biomedicals, Inc. (Aurora, OH). Individual standard solutions at a concentration of 1 mg/mL were prepared in methanol and stored at −40 °C for a maximum of 3 months. Working standard solutions were prepared daily by dilution with water. An isotopically labeled

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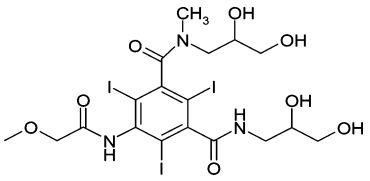
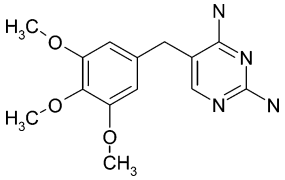
TABLE 1. Chemical Structures and Properties of the Investigated Pharmaceuticals

compound (abbreviation)	structure	log K_{OW} log K_D	precursor ion	product ion 1	product ion 2
Iopromide (IOP)		−2.33[a] 1.0[a,b]	792	559	774
Trimethoprim (TRI)		0.91[c] 0.28[d]	291	230	258

[a] From ref *23*. [b] As measured in secondary activated sludge. [c] From ref *24*. [d] Taken from SciFinder Scholar Substance Identifier as calculated using Advanced Chemistry Development (ACD/Labs) software version V8.14 for Solaris (1994−2005 ACD/Labs).

sulfonamide, $^{13}C_6$-sulfamethazine (Cambridge Isotope Laboratories, Andover, MA), was used as an internal standard. Water was prepared using a NANOpure Diamond Ultrapure water system (Barnstead International, Dubuque, Iowa).

LC/MS/MS Analysis. Samples were analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) using a LCQ Advantage ion trap mass spectrometer (IT−MS) equipped with an electrospray ionization source (ESI) operated in positive ion mode (Thermo Finnigan, San Jose, CA). The column used was a BetaBasic-18 C_{18} column (100 × 2.1 mm internal diameter with 3 μm particle size) equipped with a UNIPHASE guard cartridge (10 × 2.1 mm internal diameter with 3 μm particle size), both purchased from Thermo Hypersil-Keystone (Bellefonte, PA). The flow rate was 200 μL per minute, the column oven temperature was 30 °C, and the full loop injection volume was 20 μL. The separation was performed using a gradient mobile phase consisting of methanol (A) and water with 0.3% formic acid (B). The initial mobile phase conditions were 5% A and 95% B. After 1 min, A was increased to 30% over 1 min, and this condition was held for 14 min. Then, mobile phase A was further increased to 80% in 1 min and was held for 2 min. The initial mobile phase composition was then restored within 1 min and maintained for 15 min, resulting in a total analysis time of 35 min.

The capillary temperature was 235 °C and the spray voltage was 5.0 kV. Nitrogen was used as a sheath gas at a flow rate of 20 arbitrary units and helium gas was used to induce dissociation for the acquisition of MS/MS data. The MS acquisition was divided into two time segments, with the first time segment (0 min to 10 min) including a full scan and full scan MS/MS scan events for IOP and its respective metabolites. For the second time segment (11−23 min), a full scan and full scan MS/MS scan event for trimethoprim and its respective metabolites were included. The collision energy for TRI and its metabolites was 50% and the isolation width was 1.0, while the collision energy for IOP and its metabolites was 40% and the isolation width was 1.5. Precursor ions and product ions used for the monitored compounds are listed in Table 1. At least two product ions collected in the full scan MS/MS events were used for identification purposes, while the product ion of highest intensity (product ion 1, Table 1) was used for quantification. During the first minute and last 10 min of the chromatographic run, the flow was diverted to waste. The instrument limit of detection (LOD) and limit of quantification (LOQ) were determined using an approach described previously (*25*). Briefly, the LOD and LOQ were determined using a replicate injection ($n = 10$) of a 0.005 μg/L standard of IOP and TRI. The LOD was defined as the concentration corresponding to three times the standard deviation of analyte/internal standard area ratio and the LOQ was defined as the concentration corresponding to 10 times the standard deviation of the analyte/internal standard area ratio. The LOQ was set as the lower limit of the linear range (LR).

Solid-Phase Extraction. Samples were prepared by solid-phase extraction (SPE) according to a modification of a previously reported method (*26*). Five-hundred mg Oasis HLB cartridges were conditioned with 5 mL acetonitrile followed by 5 mL water. Samples were loaded at a rate of 3−5 mL per minute, and the cartridges were eluted twice with 4 mL acetonitrile into a glass tube containing 50 μL of the internal standard (corresponding to 50 μg of $^{13}C_6$-sulfamethazine). The volume of the acetonitrile in the extracts was reduced to 0.2 mL under a stream of air, water was added to a final volume of 1.0 mL, and the samples were immediately analyzed by LC/MS/MS. The extraction recoveries, which are similar to those previously reported using HLB cartridges for these compounds (*26*, *27*), along with the method limit of detection (LOD), limit of quantification (LOQ), and linear range (LR) obtained in various matrices are listed in Table 2.

Biodegradation by Nitrifying Activated Sludge. The influence of nitrifying bacteria on the biodegradation of IOP and TRI was investigated under laboratory conditions using biomass obtained from the stage-2 activated sludge of Amherst WWTP. Prior to treatment with the pharmaceuticals, the collected biomass was aerated for 1 day to reduce dissolved organic matter content. Four batch reactors, consisting of glass flasks wrapped in aluminum foil, were setup to contain 5-L of biomass in each reactor, with the initial mixed liquor suspended solids (MLSS) in the reactors being 3300 mg/L. All batch reactors were spiked with aqueous stock solutions of ammonia (final concentration 50 mg/L of NH_4-N). Two of the reactors were treated with IOP and the other two were treated with TRI (final concentration of 250 μg/L of each compound). The IOP-treated and TRI-treated batch reactors were further subdivided into Batch-1 (no allylthiourea) and Batch-2, with allylthiourea (5 mg/L final concentration) added to inhibit nitrification by the ammonia oxidizing bacteria (AOB). These reactors were mixed using magnetic stirrers at 300 rpm. A 500-mL aliquot was withdrawn from each reactor at 0.1, 5, 24, 60, and 96 h after treatment. Samples were centrifuged at 3500 rpm for 10 min. The final concentrations of each IOP and TRI in the dissolved phase from each batch reactor were determined by directly injecting 20 μL aliquots into the LC/MS/MS. The reported results are

TABLE 2. Extraction Recoveries for Iopromide and Trimethoprim in Water and Effluents, Along with the Limit of Detection (LOD), Limit of Quantification (LOQ), and Linear Range (LR) Observed in Distilled Water and Wastewater Matrices

	Water				
compound	recovery %[a] 0.25 μg/L	recovery %[a] 2.5 μg/L	LOD (μg/L)	LOQ (μg/L)	LR (μg/L)
IOP	97 ± 10	104 ± 11	0.04	0.04	0.08−10
TRI	97 ± 9	98 ± 13	0.007	0.01	0.01−10
	Primary Effluent				
compound	recovery %[b] 1.0 μg/L		LOD (μg/L)	LOQ (μg/L)	LR (μg/L)
IOP	93 ± 10		0.04	0.13	0.13−10
TRI	81 ± 10		0.06	0.16	0.16−10
	Secondary Effluent				
compound	recovery %[b] 1.0 μg/L		LOD (μg/L)	LOQ (μg/L)	LR (μg/L)
IOP	107 ± 4		0.04	0.07	0.07−10
TRI	71 ± 2		0.05	0.09	0.09−10

[a] Number of samples = 5. [b] Number of samples = 3.

an average of duplicate samples collected from each reactor. Ammonium-nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), and pH were monitored to track the nitrification activity in each batch reactor. Concentrations of NH_4-N and NO_3-N were determined using the colorimetric tests following the protocol of HACH ammonia nitrogen test kit (model NI-SA, cat. no. 24287−00) and HACH nitrate test kit (model NI-11, cat. no.1468−03), respectively. The pH of the reactors was measured daily, and if the pH dropped below 6.5, the pH was adjusted to around 7.5 with sodium hydroxide. For the two iopromide batch reactors, after the 96 h monitoring period, a 150 mL aliquot was centrifuged and concentrated by solid-phase extraction to detect novel metabolites formed. Because there are no reference standards for the IOP metabolites detected, their concentrations were estimated using the response curve of the parent IOP, with the assumption that the ionization efficiencies in the LC/MS/MS were very similar. The effect of sorption on the removal of both IOP and TRI in this specific activated sludge has previously been determined to be negligible by the use of a control batch reactor either with the addition of 5% formaldehyde (*28*) or autoclaved sterilized sewage (*29*). Therefore, any removal can be attributed solely to biodegradation.

Description of WWTP and Sample Collection. The WWTP selected for this study is located in Amherst, NY, and its schematic diagram and operating conditions are shown in Table 3. For the secondary treatment processes, the Amherst WWTP includes a two-stage secondary biological degradation, with both stages being slurry systems. Stage-1 is a conventional activated sludge with relatively short SRT (6 days) for substrate removal. Stage-2 is a separate activated sludge process with longer SRT (49 days) and is optimized for effective nitrification. Samples were collected during the month of March 2006. Triplicate 1-L, flow proportional 24-h composite samples were collected once a week for three consecutive weeks using an automated sampler in clean, baked amber glass bottles from the primary effluent and the secondary effluent of both the stage-1 and stage-2 secondary treatments. All samples were stored at 4 °C for no longer than 12 h prior to extraction.

WWTP Sample Analysis. Sample LODs and LOQs were determined in primary and secondary effluents as described in ref *25* and are listed in Table 2. Since no isotopically labeled standards are available for either IOP or TRI, matrix effects on ionization were accounted for by using standard addition to quantify the amounts present in wastewater. The internal standard was added to the samples after extraction to account for any variations in extract or injection volume only. The applicability of the extraction method to primary and secondary effluent samples was also determined prior to sample collection. Triplicate 1-L volumes of both primary and secondary effluent was spiked with 1.0 ppb of both TRI and IOP, with the recoveries and method LODs, LOQs, and LR in the specific matrices being listed in Table 2.

Results and Discussion

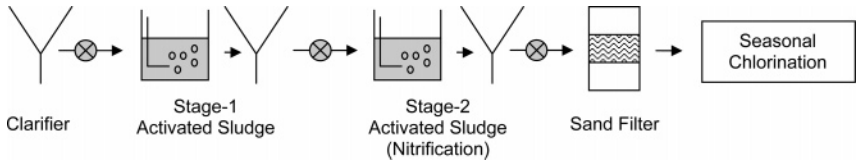
Biodegradation by Nitrifying Activated Sludge. A laboratory study was conducted to investigate the importance of nitrifying bacteria in activated sludge in the biodegradation of IOP and TRI. Biomass from the stage-2 activated sludge of the Amherst WWTP was used because this bioreactor is operated at relatively long SRT (49 days) to ensure stable nitrification. The nitrification process is catalyzed by two guilds of aerobic, chemolithoautotrophic bacteria, the ammonia oxidizing bacteria (AOB), and the nitrite-oxidizing bacteria (NOB). These bacteria, collectively known as nitrifying bacteria, are extremely slow-growing microorganisms, hence their growth in activated sludge is favored at relatively longer SRT.

The biodegradation of each IOP and TRI were conducted in two bioreactors, Batch-1 (no inhibition of nitrification) and Batch-2 (nitrification was inhibited). As expected, the batch reactors where the activity of the AOB in the activated sludge was not inhibited with allylthiourea showed a decrease in ammonia concentration over time and a corresponding increase in nitrate (Figures 1A and 2A). On the other hand, the changes in ammonia and nitrate concentrations were negligible in the Batch-2 reactors, indicating that the AOB activity in the activated sludge was inhibited (Figures 1B and 2B). Moreover, the pH of Batch-1 reactors significantly decreased (from 8.2 to 6.1) on a daily basis even with pH adjustment, while the pH of the Batch-2 reactors was only slightly decreased (from 8.2 to 7.7) after 96 h of incubation, further establishing that nitrification occurred only in the Batch-1 reactors.

When the activity of the nitrifying bacteria in the sludge were not inhibited (Batch-1) the removal of IOP was about 97%, while when nitrification was inhibited (Batch-2) the removal decreased to about 86%, as shown in Figure 1. The calculated degradation half-life of IOP using pseudo first-order rate kinetics in Batch-1 reactor was 20 h (degradation rate of 0.035/day), while in Batch-2 reactor the half-life was 34 h (rate of 0.020/day). Similarly, a relatively high removal of TRI (70%) in the Batch-1 reactor was observed, while removal was markedly decreased in the Batch-2 reactor (only up to about 25%), as shown in Figure 2. The calculated degradation half-life of TRI in Batch-1 reactor was 67 h (degradation rate of 0.010/hour), while in Batch-2 reactor the half-life was 315 h (rate of 0.0020/hour). The removal rates of IOP and TRI in the nitrifying activated sludge is higher than those previously reported in WWTPs (*6*, *18*, *22*), which ranged from no significant removal to 10%, indicating that the nitrifying bacteria play a key role in the biodegradation of IOP and TRI in activated sludge.

The presence of IOP metabolites was determined in the laboratory scale bioreactors after the 96 h incubation period had been completed, with the formation of IOP metabolites in nitrifying activated sludge over time being previously reported (*28*). In the Batch-1 reactor, the only metabolite observed above the LOD (calibrated using IOP) was the dehydroxylated IOP (m/z 759), which was formed at 0.39% of the initial IOP concentration. The chemical structure and full scan MS/MS spectrum of this metabolite is shown in Figure 3A indicating the characteristic fragment ions. The complete mass spectral characterization of this novel me-

TABLE 3. Representation of the WWTP Design (with the Sampling Points Indicated by a Cross) and a Summary of the Average Characteristics and Operating Conditions[a]



					BOD (mg/L)		TSS (mg/L)	
flow rate (m^3/d)	secondary treatment	MLSS (mg/L)	SRT (days)	HRT (hrs)	influent	effluent	influent	effluent
113562	stage-1 activated sludge	3581	6	1	68	17[b]	65	31[b]
	stage-2 nitrification	3312	49	2	17[b]	6	31[b]	11

[a] MLSS is the mixed liquor suspended solids; SRT is the solids retention time; HRT is the hydraulic retention time; BOD is the biological oxygen demand; TSS is the total suspended solids [b] Stage 1 effluent is stage 2 influent.

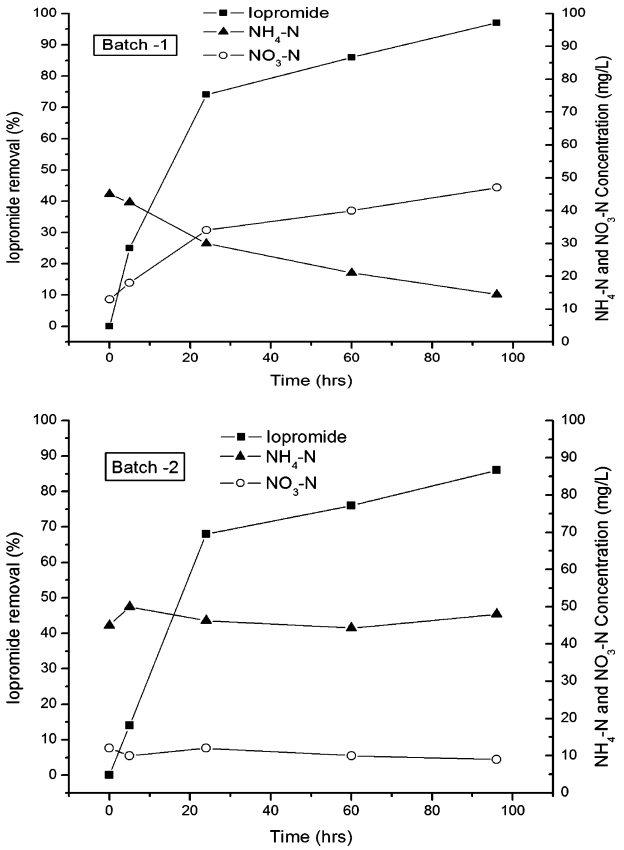


FIGURE 1. Iopromide removal in nitrifying activated sludge: (A) without AOB inhibition (Batch-1), and (B) with AOB inhibition (Batch-2).

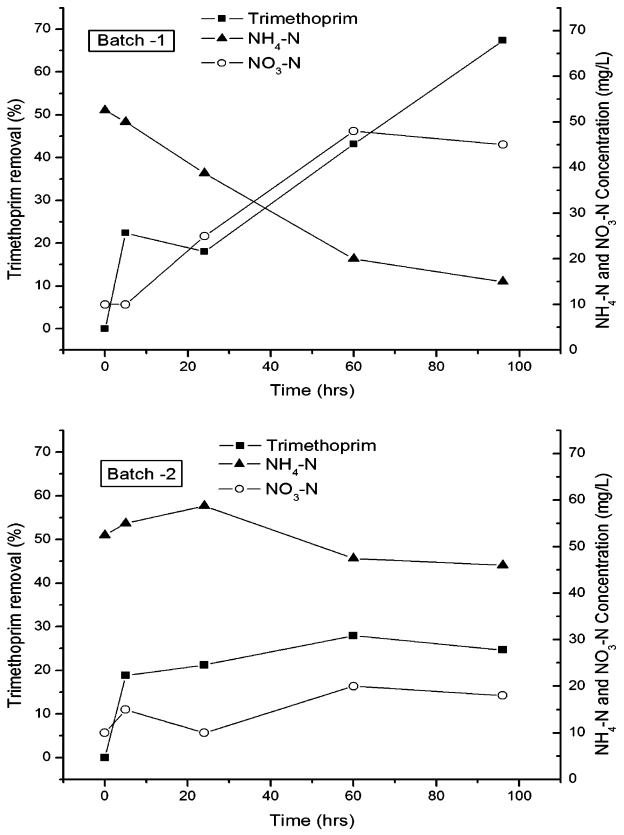


FIGURE 2. Trimethoprim removal in nitrifying activated sludge: (A) without AOB inhibition (Batch-1), and (B) with AOB inhibition (Batch-2).

tabolite establishing its identity can be found in our previous publication (*28*). In the Batch-2 reactor, a different metabolite (m/z 805) was formed at 0.14% of the initial IOP. Figure 3B shows the chemical structure and full scan MS/MS of this metabolite, indicating the characteristic loss of the side chains (m/z 104) and HI. The loss of HCOOH (m/z 46) indicates presence of a carboxylic acid group in the side chain, as previously confirmed by a derivatization reaction (*28*). It should be noted that since the presence of the metabolites was determined several days after spiking, the formed metabolites may also have partially degraded. The metabolites of IOP have been shown to reach up to 30% of the initial IOP concentration in nitrifying and conventional activated sludges (*28*).

Removal in Full-Scale WWTP. The results from the batch reactor experiments were corroborated in a full-scale WWTP by determining the removal rates of IOP and TRI at the two stages of the activated sludge process of Amherst WWTP. The concentrations found in the triplicate composite samples from the three separate sampling occasions ranged from below the LOD to 0.27 μg/L for IOP. The concentrations of TRI were found to range from 0.08 to 0.53 μg/L. The complete results are reported in Table 4. While trimethoprim was detected in all sampling locations on all three occasions, IOP was only detected during the first sampling campaign. Therefore, while the average percent removal of TRI can be determined in both stages of the WWTP from three sampling times, the percent removal of IOP can only be estimated

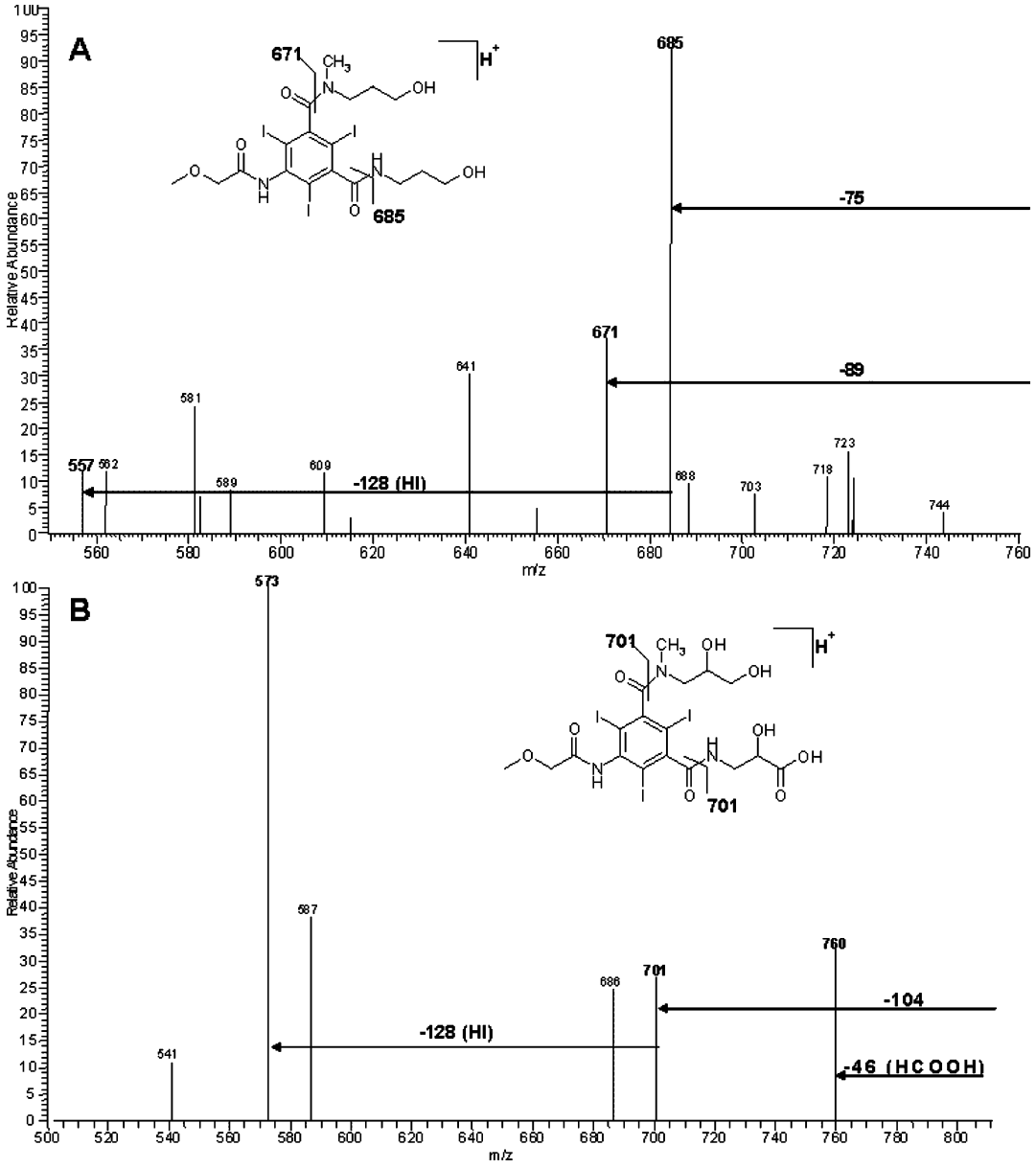


FIGURE 3. (A) Fullscan MS/MS spectra for iopromide metabolite M759 detected in non-inhibited nitrifying sludge and (B) Fullscan MS/MS spectra for iopromide metabolite M805B detected in inhibited nitrifying sludge.

TABLE 4. Summary of the Average Concentrations and Standard Deviations in μg/L Detected for Triplicate Composite Samples for Iopromide and Trimethoprim[a]

compound	sampling week	primary effluent	stage-1 secondary effluent	stage-2 secondary effluent
IOP	1	0.20 ± 0.03	0.27 ± 0.06	0.10 ± 0.01
	2	nd	nd	nd
	3	nd	nd	nd
TRI	1	0.53 ± 0.06	0.52 ± 0.06	0.25 ± 0.1
	2	0.15 ± 0.04	0.16 ± 0.03	0.08 ± 0.03
	3	0.38 ± 0.07	0.39 ± 0.05	0.21 ± 0.03

[a] Observed on the three different sampling occasions ($n = 3$). [b] ND = not detected.

using the triplicate samples collected during the first week of sampling. Comparison of the average percent removals for both pharmaceuticals in the full scale WWTP are presented in Figure 4.

No significant removal was observed for iopromide in the stage-1 activated sludge of the Amherst WWTP. Although degradation of iopromide has been shown to occur in the lab scale batch reactors utilizing biomass from this stage-1 activated sludge over an 8 day period (*28*), the hydraulic retention time (HRT) of this process in the full scale WWTP is only 1 h (Table 2), resulting in negligible removal. However, in the stage 2 nitrifying activated sludge, approximately 61% of iopromide was removed. An increased removal in the nitrifying activated sludge was also observed for TRI. While the average removal for trimethoprim was limited to less than 1% in stage-1 activated sludge, trimethoprim concentration was reduced to about 50% in stage-2 nitrifying activated sludge. No metabolites of either IOP or TRI were detected in the WWTP effluents. It is highly likely that the metabolites formed in the WWTP are below the LODs, considering that the concentrations of IOP and TRI detected during the sampling period (Table 4) were relatively low, and the expected amounts of metabolites are below 30% and 2% for IOP (*28*) and TRI (*29*), respectively.

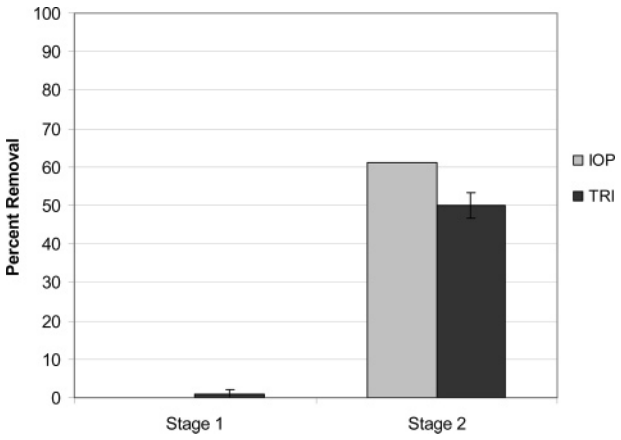


FIGURE 4. Percent removals achieved for iopromide and trimethoprim in stage-1 conventional activated sludge and stage-2 nitrifying activated sludge of the Amherst WWTP.

Implications to Other Emerging Contaminants. The significantly higher biodegradation of both IOP and TRI in the laboratory scale batch reactors, where activity of nitrifying bacteria was not inhibited relative to that where nitrification was inhibited, provides a clear evidence that these nitrifying bacteria play a key role in the biodegradation of pharmaceuticals in the stage-2 activated sludge (high SRT) at the full-scale WWTP. As mentioned earlier, because of the slow growth rate of nitrifying bacteria, nitrification is enhanced by increasing the SRT of bioreactors, such as the case for stage-2 activated sludge of Amherst WWTP. It appears that the prolonging of SRT to achieve stable nitrification in the activated sludge has an added benefit of increasing the removal efficiencies of microcontaminants, such as pharmaceutical compounds. A similar observation relating SRT and percent removal was reported recently for other pharmaceuticals and personal care products in full scale WWTPs with varying SRTs (*11*, *30*, *31*). The enhanced biodegradation in nitrifying activated sludge and the different metabolites formed compared to conventional activated sludge may be a consequence of the physiological changes in the AOB community (*32*) in the activated sludge when longer SRT is employed. The results of our study imply that it is possible to optimize the configuration of biological wastewater treatment plants to improve removal of some of the more persistent pharmaceuticals and other emerging contaminants in wastewater before they are discharged into the environment.

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