

Report No. 396

**PROTECTING RECEIVING WATERS: REMOVAL OF BIOCHEMICALLY
ACTIVE COMPOUNDS FROM WASTEWATER BY SEQUENTIAL
PHOTOCHEMICAL AND BIOLOGICAL OXIDATION PROCESSES**

By

Detlef R.U. Knappe and Carolina Baeza

Department of Civil, Construction, and Environmental Engineering
North Carolina State University
208 Mann Hall, 2501 Stinson Drive
Raleigh, North Carolina 27695-7908

PROTECTING RECEIVING WATERS: REMOVAL OF BIOCHEMICALLY ACTIVE
COMPOUNDS FROM WASTEWATER BY SEQUENTIAL PHOTOCHEMICAL AND
BIOLOGICAL OXIDATION PROCESSES

Detlef R.U. Knappe and Carolina Baeza

Department of Civil, Construction, and Environmental Engineering
North Carolina State University
208 Mann Hall, 2501 Stinson Drive
Raleigh, North Carolina 27695-7908

The research on which this report is based was supported by funds provided by the North Carolina General Assembly through the North Carolina Department of Environment and Natural Resources.

Contents of this publication do not necessarily reflect the views and policies of the WRRI, nor does mention of trade names or commercial products constitute their endorsement by the WRRI or the State of North Carolina.

This report fulfills the requirements for a project completion report of the Water Resources Research Institute of The University of North Carolina. The authors are solely responsible for the content and completeness of the report.

WRRI Project No. 70234

July 1, 2010

ACKNOWLEDGMENTS

The authors of this report would like to thank the following people and organizations for their cooperation, participation, and support of this project:

- The Water Resources Research Institute (WRI) of the University of North Carolina for funding this project
- The WRI staff their excellent project management support
- David Black, Anjali Viswakumar, and Erik Rosenfeldt of the NC State Environmental Engineering Research Laboratory for their help with data collection
- Laurie Nielsen and JoAnn Burkholder of the NC State University Center for Applied Aquatic Ecology for supporting flow cytometric analyses

ABSTRACT

Biochemically active compounds (BACs) such as endocrine disrupting chemicals, antimicrobial compounds, and other pharmaceutically active compounds are ubiquitous in wastewater treatment plant effluents (WWTPEs). The principal objective of this study was to evaluate the effectiveness of combining photochemical and biological oxidation processes for the mineralization of ^{14}C -labeled sulfadiazine (SDZ), bisphenol A (BPA) and diclofenac (DCL) that were spiked into a North Carolina WWTPE. The three BACs were added at equivalent carbon concentrations of 36 $\mu\text{g-C/L}$. An advanced oxidation process based on the combined use of ultraviolet light and hydrogen peroxide (UV/ H_2O_2) was used to transform the ^{14}C -labeled BACs, and $^{14}\text{CO}_2$ production from the oxidation intermediates was evaluated in microcosms inoculated with lake water bacteria and lake sediments. $^{14}\text{CO}_2$ production rates from the oxidation intermediates were compared to those obtained with the respective parent BACs. At the tested conditions, the UV/ H_2O_2 advanced oxidation step transformed 94%, 88%, and >99% of SDZ, BPA, and DCL, respectively. Mineralization during UV/ H_2O_2 treatment was 27% for DCL and negligible for SDZ and BPA (<2%). Mineralization of ^{14}C -labeled oxidation intermediates by lake water bacteria was extremely slow (<1.1% for SDZ, <0.8% for BPA and <0.8% for DCL after 30 days), and mineralization rates of the non-oxidized parent compounds were even slower. The use of lake sediments enhanced the biodegradation rate of SDZ and its UV/ H_2O_2 oxidation intermediates, but mineralization rates were still slow (1.1% for SDZ and 5.2% for SDZ UV/ H_2O_2 oxidation intermediates after 30 days). Overall, the results of this research suggest that BAC oxidation intermediates are relatively persistent, and residence times that commonly exist in rivers between wastewater treatment plant discharges and either drinking water treatment plant intakes or ecologically sensitive areas are insufficient to yield substantial levels of biological mineralization.

Keywords: Biochemically active compounds, biodegradation, UV/ H_2O_2 advanced oxidation, wastewater treatment

TABLE OF CONTENTS	
CHAPTER 1 – INTRODUCTION	4
CHAPTER 2 – MATERIALS AND METHOS	6
2.1. Reagents	6
2.2. Experimental design	6
<i>Water matrix</i>	6
<i>Photochemical oxidation</i>	7
<i>Biological oxidation</i>	7
2.3. Bacteria sources	11
<i>Microbial consortium</i>	11
<i>Sediments</i>	11
<i>Positive controls</i>	11
2.4. Analytical methods	12
<i>BAC concentration</i>	12
<i>Liquid scintillation counting (LCS)</i>	12
<i>Flow Cytometry</i>	12
<i>Assimilable Organic Carbon (AOC)</i>	12
CHAPTER 3 – RESULTS AND DISCUSSION	14
3.1. ¹⁴ C-acetate biodegradation	14
3.2. Biodegradation of BACs and oxidized BAC intermediates by a natural bacteria consortium	17
<i>Sulfadiazine</i>	17
<i>Bisphenol A</i>	19
<i>Diclofenac</i>	20

3.3. Biodegradability of ^{14}C -SDZ oxidation intermediates by bacteria present in lake sediments	22
CHAPTER 4 – CONCLUSIONS AND ENVIRONMENTAL SIGNIFICANCE	24
REFERENCES	26
APPENDIX A – NaOH trap effectiveness for $^{14}\text{CO}_2$ recovery	30
APPENDIX B – Assimilable organic carbon (AOC) method	31

LIST OF TABLES

Table 1. Characteristics of ^{14}C -labeled BACs and conditions used to conduct UV/ H_2O_2 oxidation experiments	8
---	---

LIST OF FIGURES

Figure 1. Reactor design for aerobic biodegradation test	10
Figure 2. Mineralization of ^{14}C -labeled acetate by lake water bacteria in (a) buffered ultrapure water ($C_0 = 1 \mu\text{g L}^{-1}$), (b) buffered, UV/ H_2O_2 treated WWTPPE ($C_0 = 1 \mu\text{g L}^{-1}$), and (c) buffered, UV/ H_2O_2 treated WWTPPE ($C_0 = 36 \mu\text{g-C L}^{-1}$, of which $6 \mu\text{g L}^{-1}$ was ^{14}C -labeled). Panel (d) depicts mineralization of ^{14}C -acetate by bacteria associated with lake sediments in buffered, UV/ H_2O_2 treated WWTPPE ($C_0 = 1 \mu\text{g L}^{-1}$).	16
Figure 3. Mineralization of ^{14}C associated with (a) SDZ oxidation intermediates in buffered, ultrapure water, (b) SDZ in buffered UV/ H_2O_2 treated WWTPPE, and (c) SDZ oxidation intermediates in buffered UV/ H_2O_2 treated WWTPPE by lake water bacteria.	18
Figure 4. Mineralization of ^{14}C associated with (a) BPA and (b) BPA oxidation intermediates by lake water bacteria.	19
Figure 5. Mineralization of ^{14}C associated with (a) DCL and (b) DCL oxidation intermediates by lake water bacteria.	21
Figure 6. Mineralization of ^{14}C associated with (a) SDZ and (b) SDZ oxidation intermediates by bacteria in lake sediments.	23
Figure A.1. ^{14}C recovery in the NaOH trap for $^{14}\text{CO}_2$ and ^{14}C -acetate experiments as a function of time.	30
Figure B.1. Calibration relating cell counts to acetate concentration ($\mu\text{g-C L}^{-1}$) for consortia A and B.	34
Figure B.2. Flow cytometer output, with gating, for (a) pure culture of <i>E. coli</i> ATCC® 25922, (b) Jordan Lake natural consortium A and (c) Jordan Lake natural consortium B.	35

CHAPTER 1 - INTRODUCTION

Biochemically active compounds (BACs) include endocrine disrupting chemicals (EDCs), antimicrobial compounds, and other pharmaceutically active compounds (PhACs). The presence of BACs in water sources at trace levels has been of concern because (a) bacteria may develop antimicrobial resistance in the presence of antibiotics at sub-therapeutic concentrations (Kümmerer, 2001), (b) EDCs may cause intersexuality in fish (Vajda *et al.*, 2008) and (c) little is known about the possible synergistic effects on aquatic life when BAC mixture are present in surface waters (Snyder *et al.*, 2005). The Washington Post (Farenthold, 2006) reported that a USGS study found that at least 80% of male smallmouth bass caught in Virginia and Maryland tributaries of the Potomac River grew eggs. In addition, 54% of male largemouth bass caught in the Potomac River near the Blue Plains wastewater treatment plant (WWTP) of Washington D.C. showed signs of feminization and 23% were intersex. While the specific cause of the feminization of male fish in the Potomac watershed has not yet been identified, other studies have linked incidents of intersexuality to the presence of EDCs that enter streams through WWTP discharges (Guillette *et al.* 1994, Hayes *et al.*, 2002, Fry and Toone, 1981). One can argue that similarities exist between the Potomac watershed and the Neuse River watershed in North Carolina. For example, rainfall, water usage, and population growth patterns are somewhat similar. Furthermore, the Potomac flows into the ecologically sensitive Chesapeake Bay, which serves as a breeding and rearing ground for many species of fish in the Atlantic Ocean. In North Carolina, the Neuse River flows into the similarly sensitive Pamlico Sound, and its watershed includes rapidly expanding urban areas (Durham, Cary, Raleigh). Conventionally treated wastewater from these municipalities is discharged into the Neuse or its tributaries. In addition, some water reuse projects have been implemented or are in the planning stage (irrigation, stream augmentation), which may also affect the water quality of the Neuse and its tributaries.

Depending on utilization and disposal patterns, BACs will enter the environment by different pathways. BACs used in human and veterinary medicine are subject to metabolic reactions and are released from the organism either unchanged or as metabolites via urine and feces. Most of the drugs used for veterinary purposes will be (1) directly discharged into water in aquaculture applications, (2) excreted directly on pastures or (3) spread onto agricultural land during land application of manure or sludge from lagoons containing animal waste. BACs that are present in land-applied manure/sludge or are disposed of in landfills can eventually contaminate groundwater (Jørgensen and Halling-Sørensen, 2000). BAC residues are also released into the environment through the effluents from municipal wastewater treatment plant. BACs are only partially removed during conventional wastewater treatment (Nasu *et al.*, 2001, Ternes, 1998, Paxeus, 2004), and WWTP discharges are therefore an important source, through which BACs are introduced into the environment. Recent studies have shown that BACs are now ubiquitous in United States surface waters and are detected at concentrations that range from ng L^{-1} to $\mu\text{g L}^{-1}$ levels (Lindsey *et al.*, 2001, Kolpin *et al.*, 2002). The most frequently detected antimicrobial

compounds were triclosan (~60% of samples), trimethoprim (~27% of samples), lincomycin (~19% of samples), and sulfamethoxazole (~19% of samples). Concentrations of antibiotics in US surface waters are typically below 1 µg/L (Kolpin *et al.* 2002). Similar surveys have been done in the European Union (EU). Hirsch *et al.* (1999) detected erythromycin (max. 1.7 µg/L), roxithromycin (max. 0.56 µg/L), and sulfamethoxazole (max. 0.48 µg/L) in German surface water samples. Zuccato *et al.* (2006) detected at least 14 pharmaceuticals in the Po river in Italy; lincomycin was the major pharmaceutical detected with a maximum concentration of 0.25 µg/L and a median concentration of 0.033 µg/L.

BAC classes and concentrations vary widely in conventional WWTP influents and effluents. For example, antibiotics have been found in wastewater treatment plant effluents at concentrations of up to 2000 ng L⁻¹ (e.g. Göbel *et al.*, 2005, Hartig *et al.*, 1999, Hirsch *et al.*, 1999, Miao *et al.*, 2004). Removal of antimicrobial compounds such as β-lactams, sulfonamides, macrolides and fluoroquinolones during conventional activated sludge treatment ranged from 30 - 90 % (Carballa *et al.*, 2005, Göbel *et al.*, 2005, Batt *et al.*, 2007). Also, the presence of EDCs in WWTP effluents has been extensively reported; e.g., 17α-ethinylestradiol (EE2) was found at concentrations of up to 3.4 ng L⁻¹ (Baronti *et al.*, 2000, Williams *et al.*, 2003) and BPA at concentrations ranging from <20 – 7625 ng L⁻¹ (Fuerhacker, 2003, Aguayo *et al.*, 2004, Höhne and Püttmann, 2008). In conventional activated sludge WWTPs, the removal of EE2 ranges from about 62 – 98% (Johnson and Williams, 2004) and that of BPA from about 73 – 93% (Fuerhacker, 2003, Höhne and Püttmann, 2008)

Advanced oxidation processes, such as the UV/H₂O₂ process, rely on the hydroxyl radical for the rapid oxidative transformation of many organic contaminants. However, complete mineralization of BACs is not achieved at typical UV doses and H₂O₂ concentrations. Hence, many unknown photolysis and oxidation intermediates are formed during the UV/H₂O₂ treatment of BACs. The hypothesis that was tested in this research is that BAC photooxidation products are readily biodegradable by microorganisms in NC surface waters. The principal objective of this study was to quantify the effectiveness of combining UV/H₂O₂ and biological oxidation processes for the mineralization of three ¹⁴C-labeled BACs. The BACs selected for this study were the antimicrobial compound sulfadiazine (SDZ), the EDC bisphenol-A (BPA) and the analgesic diclofenac (DCL), all of which commonly occur in conventionally treated WWTP effluents. The mineralization potential of ¹⁴C-labeled BAC parent compounds and oxidation products was quantified by measuring ¹⁴CO₂ production in samples inoculated with lake water bacteria and bacteria associated with lake sediments.

CHAPTER 2 – MATERIALS AND METHODS

2.1. Reagents

All non-labeled (^{12}C) compounds were purchased in pure form from Sigma Chemical Corporation (St. Louis, MO, USA). ^{12}C -SDZ (>99%), ^{12}C -BPA (>99%) and ^{12}C -DCL (>99%) were stored at room temperature and in the dark. All ^{14}C -labeled BACs were purchased in aqueous solution from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and stored at 4°C in the dark. The structures and stock solution characteristics of the selected BACs are shown in Table 1. The radiochemical purity and specific activity of the three ^{14}C -labeled BACs was verified by high-performance liquid chromatography (HPLC) and liquid scintillation counting. ^{14}C -labeled acetate was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and ^{14}C -sodium bicarbonate from Sigma Chemical Corporation (St. Louis, MO, USA). Acetonitrile used in HPLC analyses was HPLC grade (Fisher Scientific, Pittsburgh, PA, USA). Hionic Fluor Scintillation cocktail was purchased from Perkin Elmer (Groningen, Netherlands).

2.2. Experimental design

Tests were conducted to determine the biological mineralization potential of ^{14}C -labeled BAC photooxidation intermediates. ^{14}C -labeled BACs were individually spiked at trace levels into wastewater treatment plant effluent (WWTPE), and the biological process was initiated following UV/ H_2O_2 oxidation treatment. BAC (SDZ, BPA and DCL) oxidation was performed at a UV dose and H_2O_2 concentration (Table 1) that would yield a high degree of parent compound transformation (>85%). Batch microcosms were constructed to evaluate the biological mineralization of ^{14}C -labeled BAC oxidation intermediates with bacteria isolated from lake water and with bacteria associated with lake sediments. Additional bioreactors were set up with the non-treated ^{14}C -labeled parent compound, to establish a biodegradability comparison between the parent compound and its oxidation intermediates.

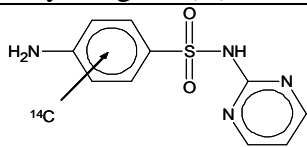
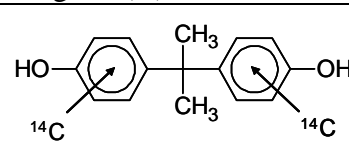
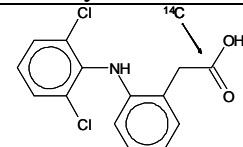
Water Matrix. Experiments were conducted in ultrapure water and in wastewater treatment plant effluent (WWTPE). The pH of the ultrapure water and WWTPE was adjusted to 7 with 1 mM phosphate buffer. WWTPE was obtained from the North Cary Water Reclamation facility (Cary, NC, USA), stored at 4 °C and filtered through a 0.22- μm membrane filter prior to use. The dissolved organic carbon (DOC) concentration of the WWTPE was quantified with a Total Organic Carbon Analyzer (Model TOC-5000A, Shimadzu, Columbia, MD) and was 7.3 mg-C L⁻¹.

Photochemical oxidation. UV/H₂O₂ oxidation experiments were carried out in a quasi-collimated beam (QCB) apparatus. The QCB apparatus was equipped with four low pressure UV lamps ($\lambda=254$ nm), and the delivered UV fluence to the sample was calculated as described by Bolton and Linden (2003). UPW and WWTPE were spiked with a mixture of ¹²C/¹⁴C-labeled BACs as described in Table 1. At predetermined oxidation levels (Table 1-Applied UV dose), the reaction was stopped by turning off the UV lamp and quenching the remaining hydrogen peroxide with sodium thiosulfate (Liu *et al.*, 2003). Additional UV/H₂O₂ tests were conducted with the WWTPE without the spiked ¹²C/¹⁴C compound blend to measure the assimilable organic carbon (AOC) present in the oxidized WWTPE. AOC was measured to determine the overall amount of readily biodegradable carbon that could be used by the bacteria as primary substrate.

Biological oxidation. Upon photochemical oxidation, 50 mL of quenched sample was transferred to batch bioreactors. The batch bioreactors for these tests were described previously by Chen *et al.* (2004) (Figure 1). To obtain reliable results, reactors were set up at least in duplicate. To each bioreactor, 50 μ L of mineral buffer solution (section 2.3) was added to assure that nutrients and minerals (e.g.: nitrogen, phosphorous, copper, iron, manganese, etc.) were not limiting substrates in the reactor. Each bioreactor was inoculated with a consortium of bacteria that were isolated from lake water as described in section 2.3. Inoculation was done such that the initial concentration of bacteria in the bioreactor was $\sim 1.6 \times 10^4$ cells mL⁻¹. Two inocula were prepared during the course of this study (consortia A and B). For ¹⁴C-SDZ, additional tests were completed, in which sediments from a local lake served as the source of bacteria. Sediments were added to the batch bioreactors before the aqueous sample containing ¹⁴C-SDZ oxidation intermediates was transferred to yield a sediment to water ratio (weight/weight) of approximately 0.5. The recorded wet weight of sediment inside the bioreactors was 25.54 ± 0.14 g.

To assess the improvement in biodegradability due to UV/H₂O₂ oxidation, bioreactors were also set up with the non-treated ¹⁴C-labeled parent compound. In these tests, the WWTPE was oxidized before spiking the BAC parent compound. UV/H₂O₂ conditions during the oxidation step were the same as those shown in Table 1 to assure that initial AOC concentrations were similar to those in biodegradation tests involving BAC oxidation intermediates.

Table 1: Characteristics of ¹⁴C- labeled BACs and conditions used to conduct UV/H₂O₂ oxidation experiments.

Characteristics of ¹⁴C stock solution				
Compound	¹⁴ C-Sulfadiazine		¹⁴ C-Bisphenol A	
Molecular weight	250.25		228.29	
	pK _{a,1} = 2.02 ; pK _{a,2} = 6.43		pK _{a,1} = 9.78 ; pK _{a,2} = 10.52	
Radiolabel site	Phenyl-ring- ¹⁴ C(U)		Ring- ¹⁴ C(U)	
Structure				
				
Solvent	Sterile water		Sterile water	
Specific activity	67 mCi/mmol		200 mCi/mmol	
	50 mCi/mmol		50 mCi/mmol	
UV/H₂O₂ experimental design				
Water matrix	UPW	WWTPE	WWTPE	WWTPE
Initial parent compound concentration (μg L ⁻¹)*	75	75	46	68
¹² C/ ¹⁴ C initial mass ratio	0.8 / 0.2	0.8 / 0.2	0.8 / 0.2	0.8 / 0.2
Applied UV dose (mJ cm ⁻²)	340	2000	2000	575
H ₂ O ₂ initial concentration (mg L ⁻¹)	6	6	6	6
Expected parent compound degradation (%)**	90	90	85	99
Measured parent compound degradation (%)	91	94	88	>99 (BDL***)

* All initial BAC concentrations were equal on a carbon basis (36 μg-C L⁻¹)

** Expected compound degradation was calculated with fluence-based pseudo first order rate constants (Baeza 2008)

*** Final concentration after UV/H₂O₂ treatment was below detection limit

Bioreactors were equipped with a NaOH trap that captured $^{14}\text{CO}_2$ produced from the mineralization of ^{14}C -labeled photooxidation intermediates. Aerobic conditions were maintained in all reactors. In initial experiments, a vial containing 1 mL of 30% (v/v) H_2O_2 and 0.5 mL of 0.5% (wt/wt) FeCl_2 served as a source of oxygen to maintain aerobic conditions. During the course of this study, it was realized that the reactor headspace and the aqueous oxygen concentration was sufficient to maintain an aerobic environment inside the bioreactors and therefore the H_2O_2 vial was no longer filled in later experiments. The bioreactors were incubated at $34 \pm 1^\circ\text{C}$ in an incubator shaker operated at 120 rpm (New Brunswick Scientific C24 Classic series, Edison, NJ). The content of the NaOH trap was analyzed periodically to determine the rate of $^{14}\text{CO}_2$ production. At each sampling time, the content of the NaOH trap was removed for analysis by scintillation counting and replaced with fresh 2N NaOH solution. All bioreactors were compared to an abiotic control reactor (negative control), to determine $^{14}\text{CO}_2$ that was only formed by chemical oxidation and not by microbial activity. Abiotic controls were not inoculated and were spiked with sodium azide (10-15 mg per reactor), which inhibits aerobic biological activity.

Additional abiotic tests were completed by spiking ^{14}C -acetate and ^{14}C -sodium bicarbonate into ultrapure water buffer at pH 7. The purpose of these tests was to determine the effectiveness of the NaOH trap for trapping $^{14}\text{CO}_2$ relative to ^{14}C -labeled volatile fatty acids that can form during the UV/ H_2O_2 oxidation process. These reactors showed that the NaOH trap recovered 96% of the spiked bicarbonate after 100 hours, while only 1% of the spiked ^{14}C acetate was recovered after 100 hours (For more details see Appendix A).

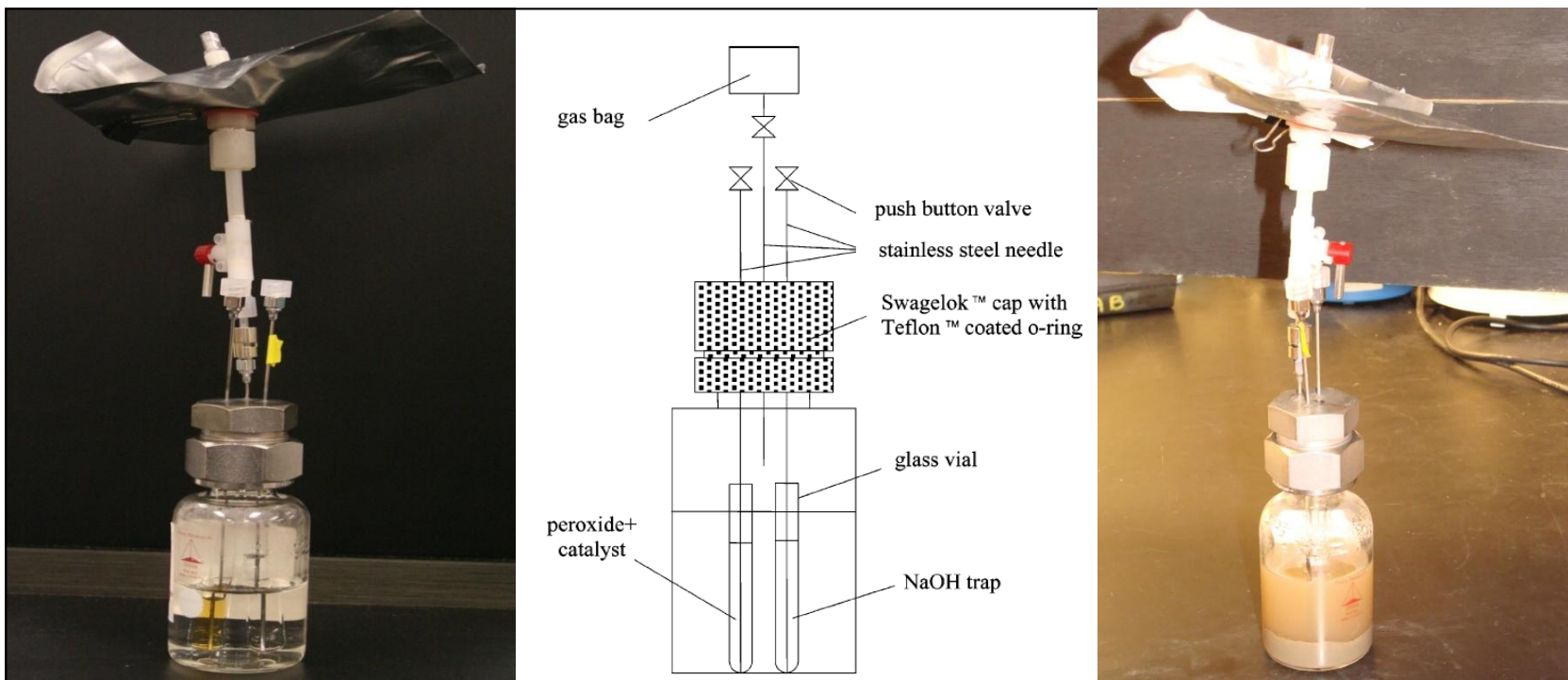


Figure 1. Reactor design for aerobic biodegradation test. (Chen *et al.*, 2004). Photo on the left depicts reactor inoculated with lake water bacteria. Photo on the right depicts reactor inoculated with lake sediments.

2.3. Bacteria sources

Microbial consortium. The microbial consortium was obtained from Jordan Lake, North Carolina, USA. To obtain the inoculum, 40 mL of lake water was filtered through PVDF filter membranes (0.22- μm pore size, Millex-GV). Prior to use, filters were made AOC-free by rinsing the filters with 2L of ultrapure water at a rate of $\sim 4 \text{ ml min}^{-1}$ (Berger *et al.*, 2005). The filtrate was inoculated with $\sim 100 \mu\text{L}$ of unfiltered water and incubated at 34°C for 14 days. The cells were harvested by centrifugation (3000 rpm, 30 minutes), and subsequently re-suspended in HPLC grade water amended with mineral buffer (for 1L: 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 3.0 g KH_2PO_4 , 7.0 g K_2HPO_4 , 0.2 g KCl , 0.1 g NaCl , 50 mg MgSO_4 , 4.1 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 5.4 mg $\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$, 5.0 mg $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.1 mg ZnCl_2 , 1.3 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; Berger *et al.*, 2005). The re-suspended cells were incubated for 7 additional days to assure that the AOC concentration of the inoculum was negligible. To obtain a cell count of the prepared inoculum, a sub-sample was analyzed by flow cytometry. The inoculum was stored for at least six months at 4°C and; remained viable and maintained similar growth characteristics throughout this period as confirmed by tests that were periodically conducted with ^{14}C -acetate. This procedure was adapted from Hammes and Egli (2005).

Sediments. Sediments were collected from Lake Wheeler, North Carolina, USA. One day prior to setting up experiments, 1-L of sediments was collected from the top layer of a shallow part of the lake to assure that sediments were aerobic. Sediments were stored overnight at 4°C . The collected sediments had a moisture content of $30.4 \pm 0.4\%$ at the start of the experiment. Additional tests were completed with abiotic sediments to determine the partitioning of ^{14}C -SDZ and ^{14}C -SDZ oxidation byproducts between the water and sediments. Abiotic partitioning tests were prepared in baked, amber 20 mL vials at the same sediment-to-water mass ratio as the bioreactors. Sediments were sterilized by the following succeeding steps: (1) dried over 2 days at 105°C , (2) autoclaved for 20 minutes at 121°C , and (3) mixed with a sodium azide solution (240 mg L^{-1}) such that sediments regained their initial moisture content of 30.4 %. Vials were shaken at $34 \pm 1^\circ\text{C}$ in an incubator shaker operated at 120 rpm. After 40 days, the supernatant of the samples was removed and filtered through a 0.22 μm membrane (Millex-GV, PVDF). Sample aliquots of 1 mL were analyzed by liquid scintillation counting. Blank samples (without sediments) were also analyzed to determine the amount of initially spiked ^{14}C -SDZ, and the sorbed ^{14}C -SDZ concentration was calculated using a mass balance approach:

$$q = \frac{(C_0 - C)V}{m}$$

where q is the solid-phase concentration (mg/g), C is the aqueous-phase concentration (mg/L), V is the volume of water (L), and m is the dry sediment mass (g).

Positive controls. To determine the viability of both, lake water and sediment bacteria, additional bioreactor experiments were completed with ^{14}C -acetate (positive controls). ^{14}C -

acetate was added to ultrapure water buffered at pH 7 and to WWTPE that was preoxidized by UV/H₂O₂ at the same experimental conditions as those used for BAC biodegradation tests (Table 1).

2.4. Analytical methods

BAC concentration. ¹²C-BAC concentrations were measured with a HPLC system (Breeze, Waters, Milford, MA) equipped with a C18-AQ HPLC column (5 μm, 4.6 x 250 mm, Alltima HP, Grace) and a dual-wavelength UV detector. The mobile phase flow rate was 1.0 mL/min. For SDZ, the mobile phase was 20% v/v acetonitrile and 80% v/v 25 mM ammonium acetate buffer (pH 5). For BPA and DCL, the mobile phase was 42% v/v acetonitrile and 58% v/v 25 mM ammonium acetate buffer (pH 5). The detector wavelength was set at 266 nm for SDZ, 225 nm for BPA, and 220 nm for DCL.

Liquid scintillation counting (LCS). To measure ¹⁴CO₂ production the entire content of the NaOH trap was removed at each sampling point and divided into 1 mL aliquots that were each mixed with 12 mL of Hionic Fluor Scintillation cocktail (Perkin Elmer, Groningen, Netherlands) in 20 mL scintillation vials. The Hionic Fluor Scintillation cocktail reduces the chemiluminescence that occurs when analyzing basic samples with traditional scintillation cocktails. LCS samples were counted with a TRI-CARB 2800TR scintillation counter (Packard Instrument Company, Downers Grove, IL, USA).

Flow cytometry. Bacteria in the lake water consortium were enumerated with a Beckman-Coulter EPICS Altra flow cytometer (Fullerton, CA) at The Center for Applied Aquatic Ecology at NC State University. Samples were prepared as described in Hammes and Egli (2005). Samples were stained with 10 μL mL⁻¹ of SYBR green I (Invitrogen, Molecular Probes, 10,000X concentrate in DMSO) previously diluted in DMSO to a concentration of 1:100. The samples were left in the dark for at least 20 minutes prior to analysis. SYBR green stains total nucleic acids and emits a bright fluorescent signal at 530nm +/- 30nm (Green Fluorescence) upon excitation with a laser at 488nm. Another signal is also detected above 590nm (Red Fluorescence). Data were analyzed with the shareware WinMDI Version 2.8 (Windows Multiple Document Interface for Flow Cytometry) developed by Joe Trotter, Scripps Institute.

Assimilable Organic Carbon (AOC). AOC concentrations were measured according to the method of Hammes and Egli (2005). Details of the AOC method are presented in Appendix B. For this method, all glassware and materials were made AOC-free as described in Standard Method 9217 (APHA et al., 1998) and Charnock and Kjonno (2000) (the same method was used for the oxidation experiments and bioreactors glassware). The microbial consortium utilized in the AOC protocol was the same as that for the bioreactors. The growth of the microbial consortium was converted to AOC concentration by a yield factor,

which represents the number of cells produced per μg organic carbon used. To obtain the yield factor for the inoculum, a calibration curve was developed with acetate as the carbon source. The same mineral buffer described in section 2.3.1 was added to all samples to assure that minerals were not limiting growth. The mineral buffer was added at a ratio of 1 μL of mineral buffer per mL of sample. Two inocula were prepared during the course of this study. Using acetate concentrations ranging from 0 – 300 $\mu\text{g-C L}^{-1}$, a yield factor of 6.35×10^6 cells $(\mu\text{g-C})^{-1}$ was obtained for consortium A and 4.36×10^6 cells $(\mu\text{g-C})^{-1}$ for consortium B.

CHAPTER 3 – RESULTS AND DISCUSSION

3.1. ^{14}C -acetate biodegradation.

To assess the aptitude of the microbial consortia to mineralize an easily biodegradable carbon source at trace levels, bioreactor experiments with ^{14}C -acetate were performed. These experiments served as positive controls. The recovery of ^{14}C in the NaOH trap as a function of time after inoculation is shown in Figure 2. In Figure 2, results obtained for inoculated reactors are compared to those obtained for matching abiotic reactors (negative controls with 10-15 mg of sodium azide per reactor). Figure 2(a) depicts the rate of ^{14}C -acetate mineralization in ultrapure water buffered at pH 7. The AOC of the ultrapure water prior to spiking ^{14}C -acetate was negligible ($0.018 \pm 0.092 \mu\text{g L}^{-1}$); therefore, the sole carbon source that was available to the bacteria was the ^{14}C -acetate spiked at a concentration of $1 \mu\text{g L}^{-1}$ ($0.33 \mu\text{g-C L}^{-1}$). As shown in Figure 2(a) the consortium was clearly able to mineralize acetate at trace levels. However, the rate of $^{14}\text{CO}_2$ evolution was slow, and a comparison between the inoculated sample and the negative control suggests that only 3.2% of ^{14}C -acetate was mineralized after 53 days. It was expected that no exponential growth and/or substrate uptake would take place in these reactors knowing that (1) van der Kooij (1992) suggested that to prevent microbial growth and create a stable water, AOC should not exceed a concentration of $10 \mu\text{g acetate-C L}^{-1}$ and (2) Rittmann and McCarty (2001) stated that S_{\min} for easily biodegradable organic matter is of $37 \mu\text{g BOD}_L \text{ L}^{-1}$.

Figure 2(b) depicts the mineralization rate of ^{14}C -acetate in UV/ H_2O_2 oxidized WWTPPE that was buffered at pH 7 and spiked with $1 \mu\text{g L}^{-1}$ ^{14}C -acetate concentration. Prior to spiking ^{14}C -acetate, the WWTPPE was treated with a UV dose of 2000 mJ cm^{-2} and 6 mg L^{-1} H_2O_2 . After UV/ H_2O_2 treatment the AOC of the oxidized WWTPPE was $137 \pm 4 \mu\text{g L}^{-1}$. For comparison, the non-treated WWTPPE had an AOC of $86 \pm 1 \mu\text{g L}^{-1}$. The AOC increase of 60% during UV/ H_2O_2 treatment can be explained by the oxidation of organic matter to more biodegradable compounds (e.g. lower molecular weight organic compounds with higher oxygen content).

For the bioreactor, for which results are shown in Figure 2(b), a lag phase was observed for the first 3-5 days, and 48.5% of ^{14}C -acetate was mineralized after 25 days. Although the ^{14}C -acetate concentration in the UPW reactor (Figure 2a) and the pre-oxidized WWTPPE reactor (Figure 2b) was the same, the rate of ^{14}C -acetate mineralization was higher in the WWTPPE reactor. This result can be explained by the higher AOC of the pre-oxidized WWTPPE ($136.6 \pm 4.3 \mu\text{g L}^{-1}$) that provided an additional carbon source for the bacteria.

Results shown in Figure 2(c) were obtained with bioreactors that contained pre-oxidized WWTPPE and $36 \mu\text{g-C L}^{-1}$ of a $^{12}\text{C}/^{14}\text{C}$ -acetate mixture ($6 \mu\text{g L}^{-1}$ of ^{14}C -acetate). The purpose of these experiments was to (1) assess the mineralization rate of a readily

biodegradable organic molecule at the same initial carbon concentration at which tests were performed with ^{14}C -labeled BACs and (2) test the variability between consortium A and B. WWTPPE for bioreactors inoculated with consortium A was oxidized with a UV dose of 2000 mJ cm^{-2} and an initial H_2O_2 concentration of 6 mg L^{-1} . WWTPPE for bioreactors inoculated with consortium B was oxidized with a UV dose of 575 mJ cm^{-2} and an initial H_2O_2 concentration of 6 mg L^{-1} . For the treatment with the lower UV dose, the AOC of the oxidized WWTPPE was $88 \pm 1 \mu\text{g L}^{-1}$; compared to the non-treated WWTPPE ($86 \pm 1 \mu\text{g L}^{-1}$) no significant change in AOC content was observed. Figure 2(c) results showed that for the bioreactors inoculated with consortia A and B the ^{14}C -acetate mineralization after 20 days ranged from 45% to 64%, and a lag phase of 3-5 days was apparent for both consortia. The lag-phase may represent the time that the consortium needed to transition from a maintenance mode to an actively growing population before a more rapid ^{14}C -acetate mineralization could proceed.

Figure 2(d) shows the results obtained with ^{14}C -acetate bioreactors that were inoculated with lake sediments. The ^{14}C -acetate concentration spiked into these reactors was $1 \mu\text{g L}^{-1}$ and the WWTPPE was previously oxidized with a UV dose of 2000 mJ cm^{-2} an initial H_2O_2 concentration of 6 mg L^{-1} . For these reactors, there was no measurable lag phase and ^{14}C -acetate mineralization reached 70.3% after 15 days (Figure 2d).

Mass balances for the ^{14}C -acetate reactors were determined once the $^{14}\text{CO}_2$ recovery began to reach a plateau. The mass balance for the abiotic samples was complete ($100 \pm 3\%$) and verified that losses to reactor components and/or leaks were negligible. For the biotic reactors, the mass balance ranged from 80 – 87 %. When ^{14}C is incorporated into biomass, reflection from intracellular walls can interfere with the measurement of radioactivity by LSC. Similar ^{14}C recovery percentages were found by Ingerselv and Nyholm (2000), who used aniline as a readily biodegradable substrate. No mass balances were performed for the bioreactors inoculated with sediments.

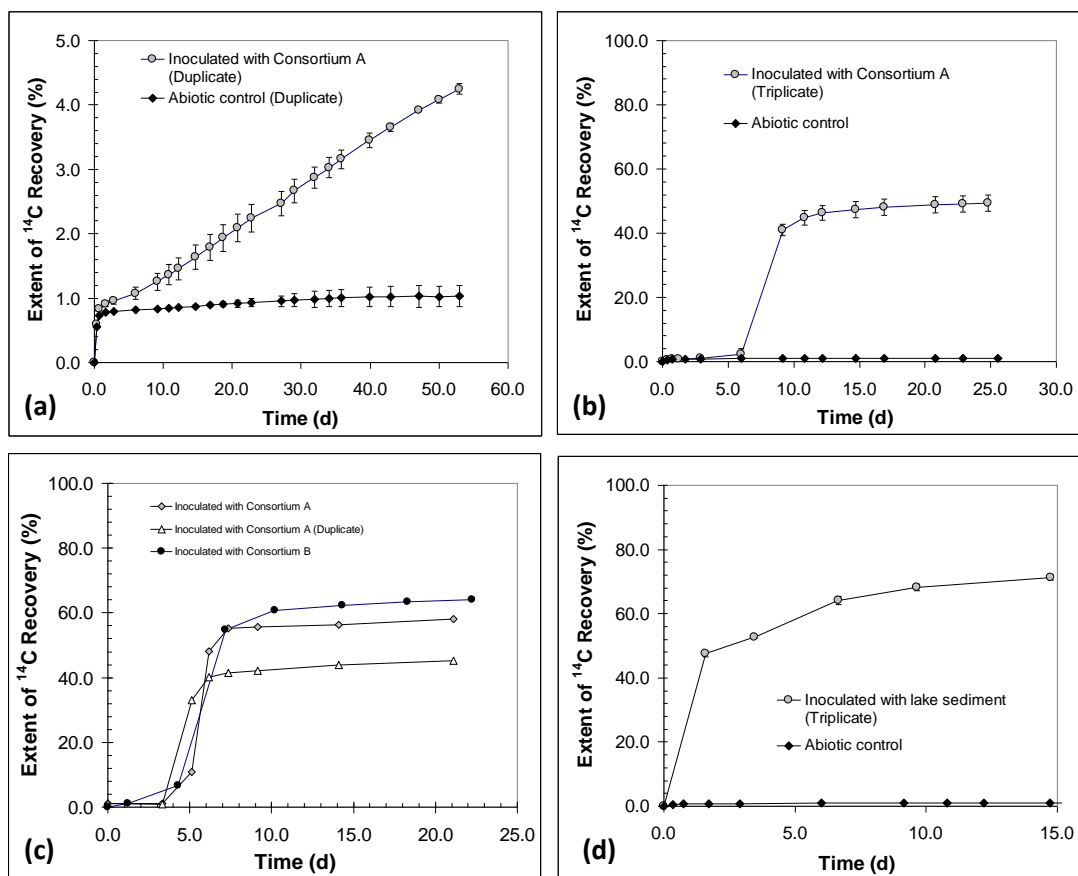


Figure 2. Mineralization of ^{14}C -labeled acetate by lake water bacteria in (a) buffered ultrapure water ($C_0 = 1 \mu\text{g L}^{-1}$), (b) buffered, UV/ H_2O_2 treated WWTPe ($C_0 = 1 \mu\text{g L}^{-1}$), and (c) buffered, UV/ H_2O_2 treated WWTPe ($C_0 = 36 \mu\text{g-C L}^{-1}$, of which $6 \mu\text{g L}^{-1}$ was ^{14}C -labeled). Panel (d) depicts mineralization of ^{14}C -acetate by bacteria associated with lake sediments in buffered, UV/ H_2O_2 treated WWTPe ($C_0 = 1 \mu\text{g L}^{-1}$).

3.2. Biodegradation of BACs and oxidized BAC intermediates by a natural microbial consortium

Sulfadiazine. Figure 3 shows results from $^{12}\text{C}/^{14}\text{C}$ -Sulfadiazine biodegradation tests that were inoculated with consortium A. Figure 3(a) represent results from a biodegradation test conducted in buffered ultrapure water after UV/ H_2O_2 treatment with a UV dose of 340 mJ cm^{-2} and an initial H_2O_2 concentration of 6 mg L^{-1} . For this experiment, 91% of the parent compound was transformed and the resulting photooxidation intermediates were the sole carbon source in solution due to the negligible AOC of the UPW. The biological mineralization of ^{14}C -labeled SDZ intermediates was negligible with a $^{14}\text{CO}_2$ recovery of 0.3% above the abiotic control after an incubation time of one month. Figure 3(b) depicts results from a biodegradation test conducted in buffered pre-oxidized WWTPPE into which the SDZ parent compound was spiked. No $^{14}\text{CO}_2$ beyond that from the abiotic controls was recovered in these bioreactors over a period of 65 days. Studies evaluating the biodegradability of sulfonamides in activated sludge systems showed that degradation of structurally similar sulfonamides occurred at similar rates on previously acclimated sludge (Ingerslev and Halling-Sørensen, 2000, Perez *et al.*, 2005), suggesting that the enzyme required for sulfonamide degradation attacks the common 4-aminobenzene moiety (phenyl ring) of sulfonamide compounds (Perez *et al.*, 2005). However, no mineralization of the ^{14}C -labeled phenyl ring of the parent compound was observed in bioreactors that were inoculated with non-acclimated surface water bacteria (Figure 2b).

Figure 3(c) depicts results from a biodegradation test conducted with SDZ-spiked ($36 \mu\text{g-C L}^{-1}$) buffered WWTPPE after UV/ H_2O_2 oxidation treatment with a UV dose of 2000 mJ cm^{-2} and a H_2O_2 dose of 6 mg L^{-1} . In this experiment, 94 % of the parent compound was transformed during UV/ H_2O_2 treatment. The biodegradation test showed that only 1.8% of ^{14}C -labeled oxidation intermediates was mineralized after 65 days. Also, a 10-day lag phase was observed before the bacteria began to mineralize the ^{14}C -labeled oxidation intermediates. A longer lag period compared to that observed in ^{14}C -acetate biodegradation tests was expected because the cells in the consortium were not adapted to the ^{14}C -labeled oxidation intermediates. The SDZ phenyl ring, which was uniformly ^{14}C labeled, is a preferable site to for an $\bullet\text{OH}$ radical attack via electrophilic addition to the aromatic ring; in addition, ring cleavage can be expected upon addition of two or more OH groups to the aromatic ring (Ho, 1986). Therefore, ^{14}C -labeled oxidation products should be more biodegradable than the SDZ parent compound. A comparison of results presented in Fig 3(b) and (c) shows that the oxidation intermediate were indeed more biodegradable, but the difference was small.

Finally, the mineralization due to chemical oxidation only was determined by comparing the cumulative ^{14}C -recovery of the abiotic controls. For the non-treated SDZ parent compound, 0.3 % of the ^{14}C -labeled carbon was recovered in the NaOH traps; it is likely that this result was attributable to ^{14}C impurities in the ^{14}C -SDZ, and this value was used to

correct the mineralization percentages obtained during UV/H₂O₂ treatment. For the experiment conducted in ultrapure water at a UV dose of 340 mJ cm⁻², the ¹⁴C recovery of the abiotic control was equal to that obtained with non-treated SDZ; consequently no mineralization of the ¹⁴C-phenyl ring was achieved during the chemical oxidation step. For the experiment conducted in WWTPE at a UV dose of 2000 mJ cm⁻², the ¹⁴C recovery of the abiotic control was of 2.2 %, suggesting therefore, that 1.9% of the ¹⁴C-phenyl ring in SDZ was mineralized in the chemical oxidation step.

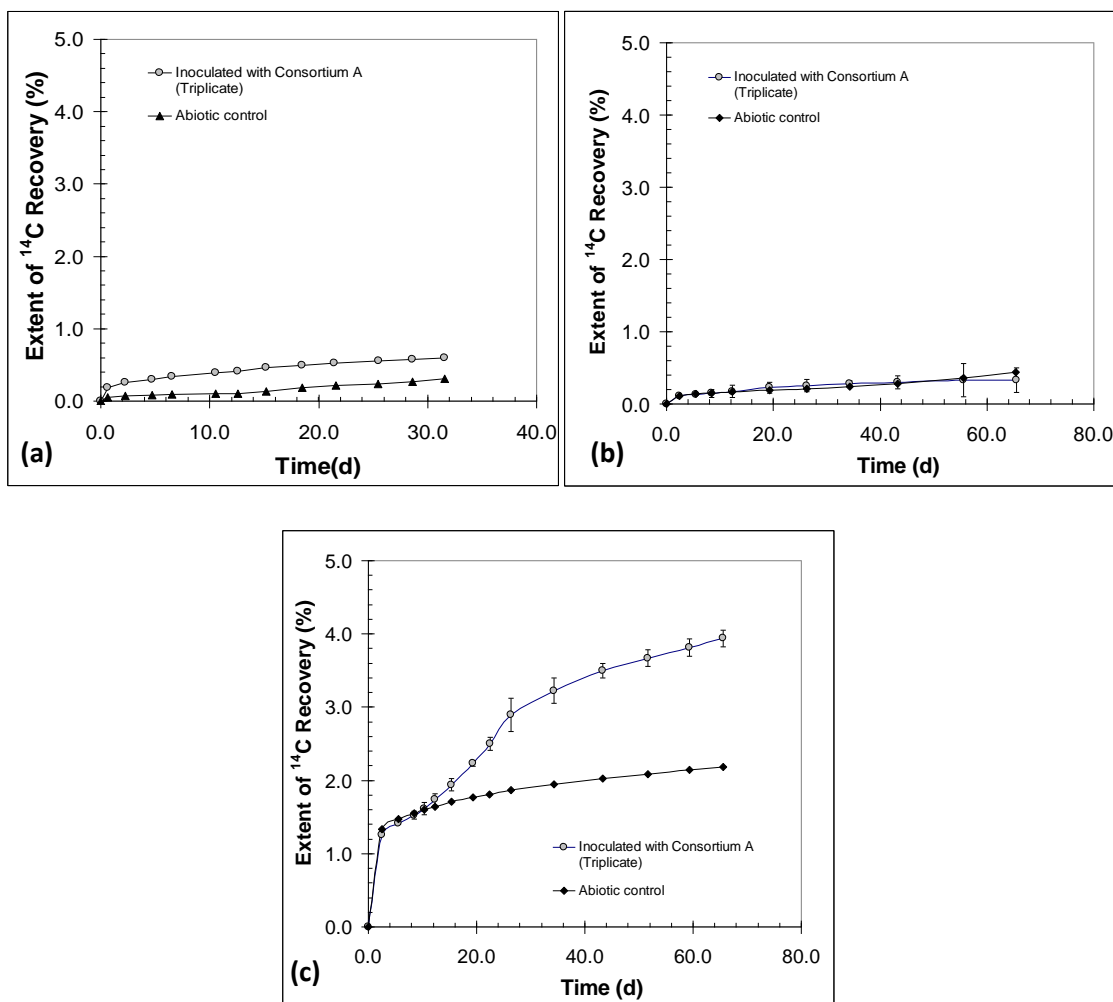


Figure 3. Mineralization of ¹⁴C associated with (a) SDZ oxidation intermediates in buffered, ultrapure water, (b) SDZ in buffered UV/H₂O₂ treated WWTPE, and (c) SDZ oxidation intermediates in buffered UV/H₂O₂ treated WWTPE by lake water bacteria. UV/H₂O₂ oxidation conditions are shown in Table 1.

Bisphenol A. Results of the bisphenol A biodegradation tests are summarized in Figure 4. Figure 4(a) depicts results of the biodegradation test conducted with buffered oxidized WWTPe into which the bisphenol A parent compound was spiked. Comparing ^{14}C recoveries between the inoculated and the abiotic bioreactors, only a 0.2% difference in ^{14}C recovery was achieved after 36 days. This result shows that bisphenol A biodegradability is poor in the oxidized WWTPe matrix

Figure 4(b) shows results from the biodegradation test conducted with BPA oxidation intermediates in buffered WWTPe after UV/ H_2O_2 oxidation treatment with a UV dose of 2000 mJ cm^{-2} and an initial H_2O_2 dose of 6 mg L^{-1} . BPA parent compound transformation during UV/ H_2O_2 treatment was 88%, and 2.0% of the ^{14}C was mineralized during the photochemical oxidation step. Ring-labeled ^{14}C -BPA was used in this study; therefore, a small percentage of the phenolic rings was mineralized via hydroxyl radical oxidation. In terms of biodegradability, ^{14}C recovery in the inoculated reactors after 36 days was only 0.8% above that measured in the abiotic reactor. Therefore, the UV/ H_2O_2 oxidation step offered little advantage in terms of improving biodegradability. Fukahori *et al.* (2003) studied BPA oxidation by TiO_2 photocatalysis and suggested an oxidation pathway in which the ring structure was largely preserved during $\cdot\text{OH}$ radical attack, and this may be the reason why UV/ H_2O_2 treatment had little effect on biodegradability.

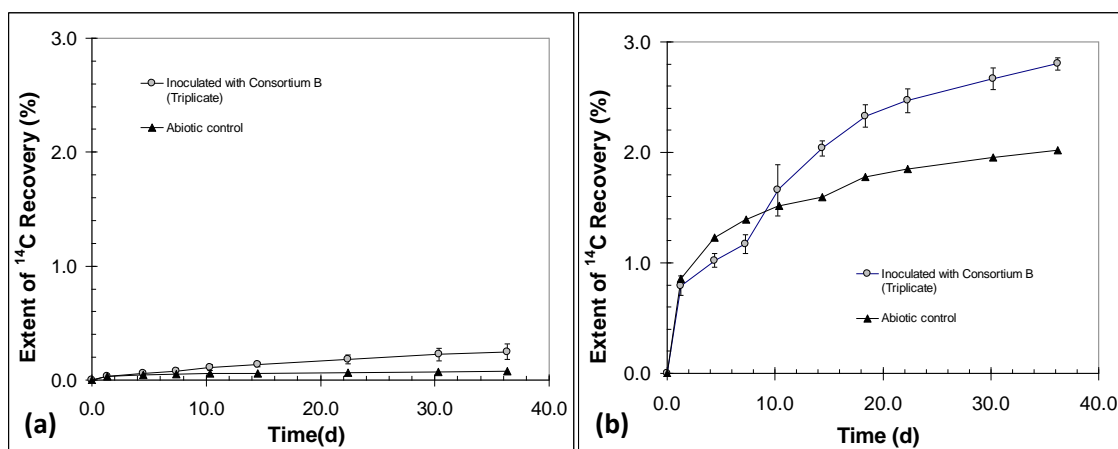


Figure 4. Mineralization of ^{14}C associated with (a) BPA and (b) BPA oxidation intermediates by lake water bacteria. Experiments were conducted in buffered, UV/ H_2O_2 treated WWTPe. UV/ H_2O_2 oxidation conditions are shown in Table 1.

Diclofenac. Results of diclofenac biodegradation tests are shown in Figure 5. When diclofenac was spiked into buffered oxidized WWTPPE, similar ^{14}C recoveries were obtained with the inoculated and the abiotic bioreactor over the study period of 36 days (Figure 5a). The ^{14}C recovery of 1.5% for non-treated diclofenac in the abiotic reactor was likely attributable to volatile ^{14}C -labeled impurities in the purchased diclofenac stock solution. A lack of biological diclofenac mineralization was expected based on the results of prior studies that showed very limited diclofenac removal in wastewater treatment plants (Heberer *et al.*, 2002 and Quintana *et al.*, 2005). Groning *et al.* (2007) identified p-benzoquinone imine and 5-hydroxyquinone as microbial transformation intermediates of diclofenac, but no evidence of complete mineralization was reported. In addition, Groning *et al.* (2007) pointed out that the biotransformation of diclofenac in different river sediments may be restricted only to a specific group of microorganisms and is not controlled by the total microbial activity. The results obtained here suggest that this group of microorganisms was not present in the tested microbial consortium.

Results from the biodegradation test conducted with diclofenac oxidation intermediates in buffered WWTPPE after UV/H₂O₂ oxidation treatment with a UV dose of 575 mJ cm⁻² and a H₂O₂ dose of 6 mg L⁻¹ are shown in Figure 5(b). Removal of the parent compound by UV/H₂O₂ treatment exceeded 99%, and 27 % of the ^{14}C was mineralized by the photochemical oxidation step. Diclofenac was labeled at the carboxyl carbon; therefore, the relatively high degree of ^{14}C mineralization suggests that the hydroxyl radical attacked the carboxylic acid moiety of diclofenac. Perez-Estrada *et al.* (2005) studied diclofenac transformation by photo-Fenton reactions and suggested a partial degradation pathway by which the hydroxyl radical reacts with diclofenac. Decarboxylation was one of the steps involved in the proposed pathway, and the results obtained here support the decarboxylation pathway. The ^{14}C recovery in the inoculated and abiotic bioreactors (Figure 5b) was similar over the 36-day study period and only 1.3% of ^{14}C was recovered above the abiotic control on day 36. This result suggests that mineralization of ^{14}C -labeled diclofenac oxidation intermediates by the microbial consortium was negligible. The relatively high ^{14}C recovery in the abiotic blank indicates the level of mineralization that was achieved at the carboxyl moiety during the UV/H₂O₂ photooxidation step.

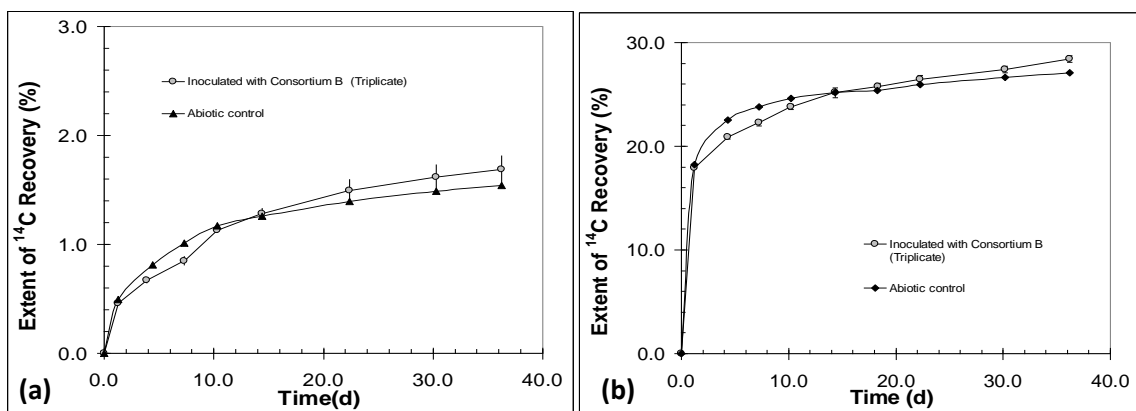


Figure 5. Mineralization of ^{14}C associated with (a) DCL and (b) DCL oxidation intermediates by lake water bacteria. Experiments were conducted in buffered, UV/ H_2O_2 treated WWTP. UV/ H_2O_2 oxidation conditions are shown in Table 1.

3.3. Biodegradability of ^{14}C -SDZ oxidation intermediates by bacteria present in lake sediments

Results for the biological mineralization of ^{14}C -labeled sulfadiazine and sulfadiazine oxidation intermediates after inoculation with lake sediments are shown in Figure 6. To test the biological mineralization of ^{14}C -labeled oxidation intermediates, SDZ was spiked into buffered WWTPPE prior to UV/H₂O₂ treatment (Table 1). A comparison of ^{14}C recoveries for the inoculated and abiotic reactors shows that both ^{14}C -SDZ and ^{14}C -labeled SDZ oxidation intermediates were mineralized by sediment-associated microbial activity (Figure 6). Mineralization of SDZ parent compound reached 1.6% after 52 days (Figure 6a), suggesting that the microbial consortium could degrade the ^{14}C -labeled 4-aminobenzene moiety of SDZ albeit at a very slow rate. For the ^{14}C -labeled UV/H₂O₂ oxidation intermediates, 6.7 % mineralization was measured after 52 days (Figure 6b). As seen in Figure 6, no lag period was observed and degradation rates were faster than those obtained with the lake water-derived microbial consortium (Figure 3). The sediments collected were not acclimated to sulfonamides in the laboratory prior to use in the bioreactors. It is possible that trace levels of sulfonamides were present in the lake water from which sediments were collected, suggesting a possible natural adaptation of the sediment bacteria to SDZ. However, the faster mineralization rate observed with sediments (Figure 6) compared to that observed with lake water bacteria (Figure 3) was more likely a result of the increased biomass and/or background AOC concentration present in the sediments. These reasons can also explain the absence of a lag phase in Figure 6.

Sorption of SDZ and SDZ oxidation intermediates is a factor that affects the availability of this carbon source for the bacteria. At the tested solid/liquid ratio (500 g L⁻¹) uptake of ^{14}C -SDZ and ^{14}C -SDZ oxidation intermediates was 73.5 ± 0.5 and $74.7 \pm 2.1\%$, respectively. Corresponding partition coefficients for ^{14}C -SDZ and ^{14}C -labeled SDZ oxidation intermediates were 6.25 ± 0.14 and 6.82 ± 0.79 L kg⁻¹, respectively. Sulfonamides are polar compounds and these results are in agreement with low sulfonamide sorption to soils; e.g. partition coefficients describing sulfapyridine sorption to two sandy soils were 7.0 and 20.9 L kg⁻¹ (Thiele-Bruhn *et al.*, 2004). The similarities of the partition coefficients obtained in this study for ^{14}C -SDZ and ^{14}C -labeled oxidation intermediates show that the characteristics of ^{14}C -labeled oxidation intermediates that affect sorption (e.g., solubility, polarizability) were similar to those of the parent compound. For the bioreactors inoculated with lake sediments, the extent of the biological mineralization of spiked ^{14}C was less than the aqueous ^{14}C concentration that remained in solution once sorption equilibrium was obtained; therefore, it is unlikely that sediments interfered with the bioavailability of ^{14}C -SDZ and ^{14}C -labeled SDZ oxidation intermediates.

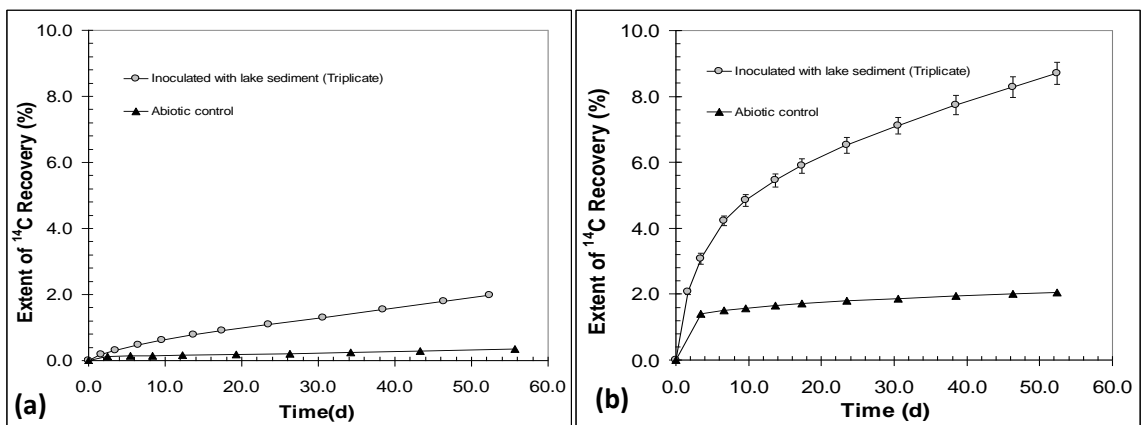


Figure 6. Mineralization of ^{14}C associated with (a) SDZ and (b) SDZ oxidation intermediates by bacteria in lake sediments. Experiments were conducted in buffered, UV/ H_2O_2 treated WWTP. UV/ H_2O_2 oxidation conditions are shown in Table 1.

CHAPTER 4 – CONCLUSIONS AND ENVIRONMENTAL SIGNIFICANCE

When BACs are oxidized by the UV/H₂O₂ process at conditions typically used in water treatment plants, mineralization does not occur, and the identity and toxicity of the oxidation product mixture is unknown. Therefore, the purpose of this study was to investigate whether UV/H₂O₂ treatment produces photooxidation intermediates that can be mineralized faster by natural microbial consortia than their respective parent compounds.

At the tested conditions, the UV/H₂O₂ advanced oxidation step transformed 94%, 88% and >99% of SDZ, BPA and DCL, respectively. Mineralization during the UV/H₂O₂ treatment was 27% for DCL and negligible for SDZ and BPA (<2%). Mineralization of ¹⁴C-labeled oxidation intermediates by lake water bacteria was extremely slow (<1.1% for SDZ, <0.8% for BPA and <0.8% for DCL after 30 days), and mineralization rates of the non-oxidized parent compounds were even slower. The use of lake sediments enhanced the biodegradation rate of SDZ and its UV/H₂O₂ oxidation intermediates, but mineralization rates were still slow (1.1% for SDZ and 5.2% for SDZ UV/H₂O₂ oxidation intermediates after 30 days).

Results from the experiments conducted during this study provide a first approximation of the expected environmental fate of BACs and BAC photooxidation intermediates. This information is useful to estimate the degree of mineralization that can be accomplished by stream microorganisms between a WWTP discharge and a downstream water treatment plant intake or an ecologically sensitive area. For example, in North Carolina, the Neuse River basin includes rapidly expanding urban areas (Durham, Cary, Raleigh), and conventionally treated wastewater from these municipalities is discharged into the Neuse River and its tributaries. The Neuse river flows into the ecologically sensitive Pamlico Sound, which is the second largest estuary in the United States and serves as a breeding and rearing ground for many species of fish. The presence of EDCs may cause intersexuality in fish (Vajda, *et al.* 2008); hence, it is of interest to estimate the expected EDC mineralization that would occur over relevant time scales (e.g. in the Neuse River between a WWTP discharge and Pamlico Sound). The Neuse River flows in a southeasterly direction from its origins north of Durham, NC, for about 150 miles before it connects with the estuary. To determine an estimate of the time that it takes for a parcel of water to travel downstream from the city of Raleigh to the estuary, the stream velocity of the Neuse River near Fort Barnwell was used. The monthly mean stream velocities for this site (USGS-02091814 Neuse River near Fort Barnwell, NC; <http://waterdata.usgs.gov/nwis>) for the period of June 2004 to February 2007 ranged from 0.697 to 1.36 ft s⁻¹. With these data, the travel time from Raleigh to the estuary was calculated to be 7 – 13 days. Assuming an average travel time of 10 days, SDZ mineralization in aerobic sediments would be only of 0.5% for the parent compound and 3.5 % for SDZ oxidation intermediates. Mineralization of BPA and DCL or their oxidation intermediates by planktonic bacteria would be negligible. Experiments conducted in this study were conducted at a temperature of 34°C and did not consider all of the factors controlling contaminant fate and transport in a flowing river.

However, the use of natural microbial consortia (instead of pure cultures) in this study provides a first basis for the biodegradability of BACs and their oxidation intermediates in surface waters.

UV/H₂O₂ oxidation of antimicrobial compounds and EDCs reduces the level of antibiotic activity and endocrine disrupting activity in water (Baeza and Knappe, 2006, Wammer *et al.*, 2006 and Rosenfeldt *et al.*, 2007). However, the biological mineralization of the BAC oxidation intermediates was extremely slow, suggesting that oxidation products may be persistent in the environment. Toxicological studies should therefore be conducted to determine the possible effects of BAC oxidation intermediates on aquatic life.

REFERENCES

- Aguayo S., Munoz M.J., de la Torre A., Roset J., de la Pena E., and Carballo M. (2004). "Identification of organic compounds and ecotoxicological assessment of sewage treatment plants (STP) effluents." *Sci. Total Environ.* 328(1-3): 69-81.
- APHA, AWWA, and WEF. (1998). *Standard Methods for the Examination of Water and Wastewater*. 20th Edition. American Public Health Association, Washington, D.C.
- Baeza A.C. (2008). *Removal of pharmaceutical and endocrine disrupting chemicals by sequential photochemical and biological oxidation processes*. PhD Dissertation, North Carolina State University, Raleigh, NC.
- Baeza A.C. and Knappe D.R.U. (2006) "Removal of sulfonamides and associated antimicrobial activity by UV photolysis and UV/H₂O₂ processes." In *Proceedings of the American Water Works Association Annual Conference and Exposition*, San Antonio, TX, June 11-15.
- Baronti C., Curini R., D'Ascenzo G., Di Corcia A., Gentili A. and Samperi R. (2000). "Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water." *Environ. Sci. Technol.* 34(24): 5059-5066.
- Batt A.L, Kim S. and Aga D.S. (2007). "Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations." *Chemosphere* 68(3): 428-435.
- Berger C., Hammes F., and Egli T. (2005). *Bericht 1: Methoden-Standardisierung und erste Resultate*. EAWAG/WVZ AOC Project Report, EAWAG, Dübendorf, Switzerland.
- Bolton, J.R. and Linden K.G. (2003). "Standardization of methods for fluence (UV dose) determination in bench-scale UV experiments." *J. Environ. Eng.* 129(3): 209-215.
- Carballa M., Omil F., Lema J.M., Llompart M., Garcia C., Rodriguez I., Gomez M. and Ternes T. (2005). "Behaviour of pharmaceuticals and personal care products in a sewage treatment plant of northwest Spain." *Water Sci. Technol.* 52(8): 29-35.
- Charnock, C. and Kjonno O. (2000). "Assimilable organic carbon and biodegradable dissolved organic carbon in Norwegian raw and drinking waters." *Water Research*, 34(10): 2629-2642.
- Chen Y., Knappe D.R.U., and Barlaz M.A. (2004). Effect of cellulose/hemicellulose and lignin on the bioavailability of toluene sorbed to waste paper. *Environ. Sci. Technol.* 38(13): 3731-3736.
- Farenthold, D.A. (2006). "Male bass across region found to be bearing eggs." Washington Post, page A-1, September 6.
- Fry D.M. and Toone C.K. (1981). "DDT – Induced Feminization of Gull Embryos." *Science* 213(4510): 922-924.
- Fukahori S., Ichiura H., Kitaoka T. and Tanaka H. (2003). "Capturing of bisphenol A photodecomposition intermediates by composite TiO₂-zeolite sheets." *Applied Catalysis B – Environmental* 46(3): 453-462.
- Fuerhacker M. (2003). "Bisphenol A emission factors from industrial sources and elimination rates in a sewage treatment plant." *Water Sci. Technol.* 47(10): 117-122.

- Göbel A., Thomsen A., McArdell C.S., Joss A., and Giger W. (2005). Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environ. Sci. Technol.* 39(11): 3981-3989.
- Groning J., Held C., Garten C., Claussnitzer U., Kaschabek S.R. and Schlomann M. (2007). "Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate." *Chemosphere* 69(4): 509-516.
- Guillette L.J., Gross T.S., Masson G.R., Matter J.M., Percival H.F., and Woodward A.R. (1994). "Developmental Abnormalities of the Gonad and Abnormal Sex-Hormone Concentrations in Juvenile Alligators from Contaminated and Control Lakes in Florida." *Environmental Health Perspectives* 102(8): 680-688
- Hammes F.A. and Egli T. (2005). "New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum." *Environ. Sci. Technol.* 39(9): 3289-3294.
- Hartig C., Storm, T. and Jekel M. (1999). "Detection and identification of sulphonamide drugs in municipal waste water by liquid chromatography coupled with electrospray ionisation tandem mass spectrometry." *J. Chromatography A*, 854(1-2): 163-173.
- Hayes T., Haston K., Tsui M., Hoang A., Haeffele C., and Vonk A. (2002). "Herbicides: Feminization of male frogs in the wild." *Nature* 419 (6910): 895-896
- Heberer T., Reddersen K., and Mechlinski A. (2002). "From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas." *Water Sci. Technol.* 46 (3): 81-88.
- Hirsch R., Ternes T., Haberer K., and Kratz K.L. (1999). "Occurrence of antibiotics in the aquatic environment." *Sci. Total Environ.* 225(1-2): 109-118.
- Ho P.C. (1986). "Photooxidation of 2,4-dinitrotoluene in aqueous solution in the presence of hydrogen peroxide." *Environ. Sci. Technol.* 20(3): 260-267.
- Höhne C. and Püttmann W. (2008). "Occurrence and temporal variations of the xenoestrogens bisphenol A, 4-tert-octylphenol, and tech. 4-nonylphenol in two German wastewater treatment plants." *Environ. Sci. Pollution Research* 15(5): 405-416.
- Ingerslev F. and N. Nyholm. (2000). "Shake-flask test for determination of biodegradation rates of C-14-labeled chemicals at low concentrations in surface water systems". *Ecotoxicology and Environmental Safety* 45(3): 274-283.
- Ingerslev F. and Halling-Sørensen B. (2000). "Biodegradability properties of sulfonamides in activated sludge." *Environ. Toxicol. Chem.* 19(10): 2467-2473.
- Johnson A.C. and R.J. Williams. (2004). "A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works." *Environ. Sci. Technol.* 38(13): 3649-3658.
- Jørgensen S.E. and Halling-Sørensen B. (2000). "Drugs in the environment." *Chemosphere* 40(7):691-699.
- Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B. and H.T. Buxton. (2002). "Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance." *Environ. Sci. Technol.* 36 (6): 1202-1211.

- Kümmerer, K. (2001). "Present knowledge and need for further research." In *Pharmaceuticals in the Environment*. Springer Verlag: Heidelberg, 239-245.
- Liu, W.J., Andrews, S.A., Stefan, M.I. and J.R. Bolton. (2003). "Optimal methods for quenching H₂O₂ residuals prior to UFC testing." *Water Research* 37(15): 3697-3703.
- Lindsey M.E., Meyer M., and Thurman E.M. (2001). "Analysis of trace levels of sulfonamide and tetracycline antimicrobials, in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry." *Analytical Chem.* 73(19): 4640-4646.
- Miao X.S., Bishay F., Chen M., and Metcalfe C.D. (2004). "Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada." *Environ. Sci. Technol.* 38(13): 3533-3541.
- Nasu M., Goto M., Kato H., Oshima Y., and Tanaka H. (2001). "Study on Endocrine Disrupting Chemicals in Wastewater Treatment Plants." *Water Sci. Technol.* 43(2): 101-108
- Paxeus N. (2004) "Removal of selected non-steroidal anti-inflammatory drugs (NSAIDs), gemfibrozil, carbamazepine, beta-blockers, trimethoprim and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment." *Water Sci. Technol.* 50(5): 253-260.
- Perez S., Eichhorn P. and Aga D.S. (2005). "Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment." *Environ. Toxicol. Chem.* 24(6): 1361-1367
- Perez-Estrada L.A., Malato S., Gernjak W., Aguera A., Thurman E.M., Ferrer I. and Fernandez-Alba, A.R. (2005). "Photo-Fenton degradation of diclofenac: Identification of main intermediates and degradation pathway." *Environ. Sci. Technol.* 39(21): 8300-8306.
- Quintana J.B., Weiss S., and Reemtsma T. (2005) "Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor." *Water Research* 39: 2654-2664.
- Rosenfeldt E.J., Chen P.J., Kullman S., and Linden K.G. (2007). "Destruction of estrogenic activity in water using UV advanced oxidation." *Sci. Total Environ.* 377(1): 105-113.
- Rittmann, B.E. and McCarty P.L. (2001). *Environmental Biotechnology: Principles and Applications*. McGraw-Hill Inc., New York, NY.
- Snyder E.M., Pleus, R.C. and Snyder S.A. (2005). "Pharmaceuticals and EDCs in the US water industry - An update." *J. Am. Water Works Assoc.* 97(11): 32-36.
- Ternes (1998) "Occurrence of drugs in German sewage treatment plants and rivers." *Water Research* 32: 3245-3260.
- Thiele-Bruhn S., Seibicke T., Schulten H.R. and Leinweber P. (2004). "Sorption of sulfonamide pharmaceutical antibiotics on whole soils and particle-size fractions." *J. Environ. Quality* 33(4): 1331-1342.
- Vajda A.M., Barber L.B., Gray J.L., Lopez E.M., Woodling J.D. and D.O. Norris (2008). "Reproductive disruption in fish downstream from an Estrogenic wastewater effluent." *Environ. Sci. Technol.* 42 (9): 3407-3414.
- van der Kooij D. (1992). "Assimilable Organic-Carbon as an Indicator of Bacterial Regrowth." *J. Am. Water Works Assoc.* 84(2): 57-65.

- Wammer K.H., Lapara T.M., McNeill K., Arnold W.A. and Swackhamer D.L. (2006). "Changes in antibacterial activity of triclosan and sulfa drugs due to photochemical transformations." *Environ. Toxicol. Chem.* 25(6): 1480-1486.
- Williams R.J., Johnson A.C., Smith J.J.L., and Kanda R. (2003). "Steroid estrogens profiles along river stretches arising from sewage treatment works discharges." *Environ. Sci. Technol.* 37(9): 1744-1750.
- Zuccato E., Castiglioni S., Fanelli R., Reitano G., Bagnati R., Chiabrando C., Pomati F., Rossetti C., and Calamari D. (2006). "Pharmaceuticals in the environment in Italy: Causes, occurrence, effects and control." *Environ. Sci. Pollution Research* 13(1): 15-21.

APPENDIX A: NaOH trap effectiveness for ^{14}C recovery

Abiotic experiments were performed to determine if the NaOH trap in the bioreactor used for assessing biodegradation was effective for ^{14}C recovery. These tests employed ^{14}C -acetate to determine if low molecular weight ^{14}C -labeled organic acids (formed through chemical or biological oxidation) may be found inside the NaOH trap and interfere with the assessment of mineralization rates that are determined on the basis of ^{14}C trapping. These experiments were performed in bioreactors with 50 ml of buffered ultrapure water at pH 7.0. Two reactors were spiked with ^{14}C -sodium carbonate and two reactors were spiked with ^{14}C -acetate. Both compounds were spiked at the same concentration (30,000 DPM per reactor). After spiking, reactors were closed rapidly to minimize ^{14}C loss. The 2N NaOH trap was sampled periodically. At each sampling time, the content of the NaOH trap was removed for analysis by scintillation counting and replaced with fresh 2N NaOH solution. The content from the basic trap was divided in two equal aliquots and each was mixed with 12 mL of Hionic Fluor Scintillation cocktail (Perkin Elmer, Groningen, Netherlands). Samples were counted by a TRI-CARB 2800TR scintillation counter (Packard Instrument Company, Downers Grove, IL, USA).

Figure 1 shows the ^{14}C recovery in the NaOH trap as a function of time.

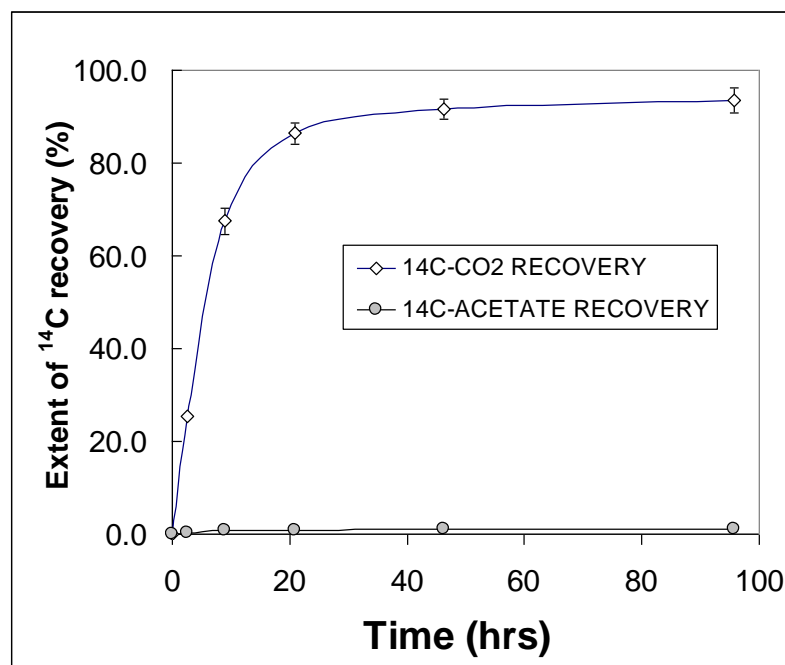


Figure A.1. ^{14}C recovery in the NaOH trap for ^{14}C CO₂ and ^{14}C -acetate experiments as a function of time.

APPENDIX B: Assimilable organic carbon (AOC) method

Introduction

The conventional AOC method was first developed by the van der Kooij research group (1982). This method was later adapted and simplified by Kaplan *et al.* (1993) and serves as the basis for *Standard Method 9217* for AOC measurements. The AOC bioassay is based on the growth of *Pseudomonas fluorescens* P17 and *Spirillum* NOX until a stationary phase is reached. *Pseudomonas fluorescens* P17 is able to take up a broad range of low molecular weight compounds at low concentrations. *Spirillum* NOX grows only on carboxylic acids, such as formate, oxalate, glycollate and glyoxalates, compounds that are not utilized by the *Pseudomonas fluorescens* P17. AOC samples are incubated with the pure culture mixture for 9 days at 15°C. Microbial growth is measured on days 7, 8 and 9 by preparing three sample dilutions and plating each dilution in triplicate on nutrient agar. Plates are incubated for 3 – 5 days at 25°C. Overall, the AOC method will require approximately 10 - 14 days and a minimum of 27 enumeration plates to obtain a result. LeChevallier *et al.* (1993) suggested reducing the incubation time by increasing the temperature and the density of the inocula. In addition, this author used the adenosine triphosphate (ATP) luciferin-luciferase technique to quantify organism growth. As a result the AOC concentration could be obtained in 2 – 4 days. Even though the ATP enumeration methodology is faster and simpler than the plate count procedure, plating is still more commonly used as an enumeration technique (Hammes and Egli, 2005).

A novel method for AOC determination was published by Hammes and Egli (2005) and utilized in this study. This new method utilizes flow cytometry in combination with a fluorescent dye for organisms enumeration. The AOC flow-cytometric method permits a more rapid AOC analysis, producing results in 2-3 days. An additional advantage of the flow cytometric method is the use of a natural consortium for inoculation; therefore, the AOC measurement is based on microbial communities that more appropriately describe environmental growth/re-growth.

Methodology

AOC was measured according to the protocol described by Hammes and Egli (2005). The following section is a summary of the AOC methodology that was used.

AOC free material. All glassware and screw caps were cleaned according to the procedure described in *Standard Methods 9217* and Charnock and Kjonno (2000). Borosilicate glass was washed with detergent, rinse thrice in ultrapure deionized water, submerged overnight in 0.2 N HCl and again rinse thrice with ultrapure deionized water. Removal of trace carbon from glassware was achieved by baking all glassware at 550°C for 6 hours. Baked glassware covered with aluminum foil was stored for a maximum of one week. Screw caps had teflon-lined silicone septa and were washed with detergent, rinse thrice in ultrapure deionized water and submerged in 10% sodium persulphate solution at 60°C for at least 1 h, rinse thrice in ultrapure deionized water and twice with 0.22 µm filtered ultrapure deionized water. AOC free pipette tips were prepared by rinsed 5 times with 0.22 µm filtered ultrapure deionized water.

AOC free gloves. Use either latex or nitrile gloves and rinsed in ultrapure deionized water for all steps to minimize carbon contamination. Change gloves between samples to minimize cross contamination.

Filter conditioning. All filters used (0.22 μm filter, Millex-GV - PVDF, Millipore) in this study were previously rinsed by passing 2 L of ultrapure deionized water overnight. (Berger *et al.*, 2005).

Mineral buffer. Prepared with sterilized ultrapure deionized water filtered through a condition PVDF membrane filters (0.22 μm filter, Millex-GV, Millipore) For 1L; 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 3.0 g KH_2PO_4 , 7.0 g K_2HPO_4 , 0.2 g KCl , 0.1 g NaCl , 50 mg MgSO_4 , 4.1 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 5.4 mg $\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$, 5.0 mg $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.1 mg ZnCl_2 , 1.3 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. (Berger *et al.*, 2005 and LeChevallier 1991). Kept refrigerated at 4°C.

Natural Microbial Consortium. The natural microbial consortium was obtained from Jordan Lake, North Carolina, USA. To obtain the inoculum, 40 mL of lake water was filtered through PVDF filter membranes (0.22- μm pore size, Millex-GV, Millipore). Prior to use, filters were conditioned as described above. The filtrate was inoculated with ~100 μL of unfiltered water and incubated at 34°C for 14 days. The cells were harvested by centrifugation (3000 rpm, 30 minutes), and subsequently re-suspended in HPLC water amended with mineral buffer. The re-suspended cells were incubated for 7 additional days to assure that no residual AOC is present in the inoculum. To obtain a cell count of the inoculum prepared, a sub-sample was taken and analyzed by flow cytometry. The inoculum can be stored for at least eight months at 4°C.

Calibration of cell counts to $\mu\text{g L}^{-1}$. The natural consortium growth is converted to AOC concentration by a yield factor, which represents the number of cells produced per μg organic carbon used. To obtain the yield factor for the inoculum a calibration was done with acetate as the organic source. The same mineral buffer used in 2.3.1. was added to all samples to assure that minerals were not limiting. The mineral buffer was added at ratio of 1 μL of mineral buffer per mL of sample. Two different inoculum were prepared in the course of this study, and the calibration method (0 – 300 $\mu\text{g L}^{-1}$ C-acetate) yielded a growth factor of (1) 6.35×10^6 cells / μg C-acetate for consortium A and (2) 4.36×10^6 cells / μg C-acetate for consortium B, showing the reproducibility of the method. Figure 1 shows the

Preparation of AOC sample. AOC samples were collected in baked, AOC-free glass bottles. Samples containing hydrogen peroxide were quenched with sodium thiosulfate (Liu *et al.*, 2003). Liu *et al.*, 2003 found that no peroxide was detected after 5-10 minutes when a 10 mg/L H_2O_2 concentration quenched with a solution containing 97.5 mg/L sodium thiosulfite. Samples were placed in the dark until no detectable peroxide concentration remains. Then, samples were filtered with pre-condition 0.22 μm filters into AOC free 40ml EPA vials and capped. The bacteria consortium (corresponding to approximately 1×10^5 organisms/ml) was then spiked into each 40 ml sample. The samples were mixed and incubated at 34°C, and enumerated using the above described flow cytometer method on the fourth day of inoculation. AOC measurements were performed in duplicate or

triplicate, and uninoculated samples were included for each AOC measurement to account for any growth of background organisms. These uninoculated samples never yielded organism growth.

Flow Cytometry analysis. Natural consortium bacteria enumeration was performed using a Beckman-Coulter EPICS Altra flow cytometer (Fullerton, CA). Samples were prepared as described in Hammes and Egli (2005). Samples were stained with 10 $\mu\text{L mL}^{-1}$ of SYBR green I (Invitrogen, Molecular Probes. 10,000X concentrate in DMSO) previously diluted in DMSO to a concentration of 1:100. SYBR green stains total nucleic acids and emits a bright fluorescent signal at 530nm +/- 30nm (Green Fluorescence) upon excitation with a laser at 488nm. The samples are left in the dark for at least 20 minutes before analysis. Another signal is also detected above 590nm (Red Fluorescence). Utilizing these two fluorescent signals, bacteria was enumerated by gating the flow cytometer counts. Data was analyzed by a compatible shareware software called WinMDI Version 2.8 (Windows Multiple Document Interface for Flow Cytometry) developed by Joe Trotter, Scripps Institute. Figure 2 displays the gates utilized in this work, applied to a sample of pure *e. coli* and a natural consortia (A and B) harvested from Jordan Lake, in North Carolina.

References

- APHA, AWWA, and WEF. (1998). *Standard Methods for the Examination of Water and Wastewater*. 20th Edition. American Public Health Association, Washington, D.C.
- Berger C., Hammes F., and Egli T. (2005). *Bericht 1: Methoden-Standardisierung und erste Resultate*. EAWAG/WVZ AOC Project Report, EAWAG, Dübendorf, Switzerland.
- Charnock, C. and Kjonno O. (2000). "Assimilable organic carbon and biodegradable dissolved organic carbon in Norwegian raw and drinking waters." *Water Research*, 34(10): 2629-2642.
- Hammes F.A. and Egli T. (2005). "New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum." *Environ. Sci. Technol.* 39(9): 3289-3294.
- Kaplan L.A., Bott T.L. and Reasoner D.J. (1993). "Evaluation and Simplification of the Assimilable Organic-Carbon Nutrient Bioassay for Bacterial-Growth In Drinking-Water." *Applied Environ. Microbiol.* 59(5): 1532-1539.
- LeChevallier M.W., Shaw N.E., Kaplan L.A. and Bott T.L. (1993). "Development of a Rapid Assimilable Organic-Carbon Method for Water." *Applied Environ. Microbiol.* 59(5): 1526-1531.
- LeChevallier M.W., Schulz W. and Lee R.G. (1991). "Bacterial Nutrients In Drinking-Water." *Applied Environ. Microbiol.* 57(3): 857-862.
- Liu W.J., Andrews S.A., Stefan M.I. and Bolton J.R.. (2003). "Optimal methods for quenching H₂O₂ residuals prior to UFC testing." *Water Research* 37 (15): 3697-3703.
- van der Kooij D., Visser A. and Hijnen W.A.M. (1982). "Determining the Concentration of Easily Assimilable Organic-Carbon in Drinking-Water." *J. Am. Water Works Assoc.* 74(10): 540-545.

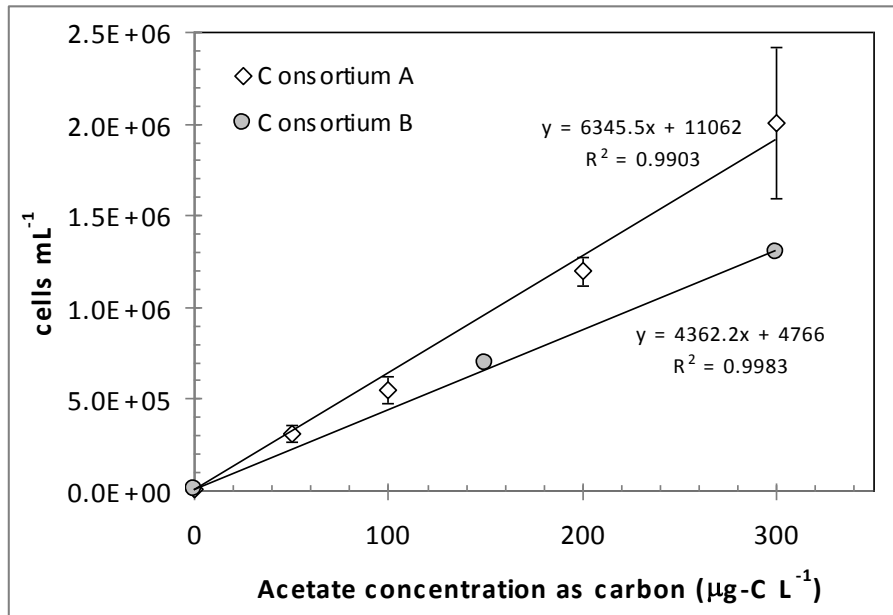


Figure B.1. Calibration relating cell counts to acetate concentration (μg-C L⁻¹) for consortia A and B. Acetate was the sole carbon source.

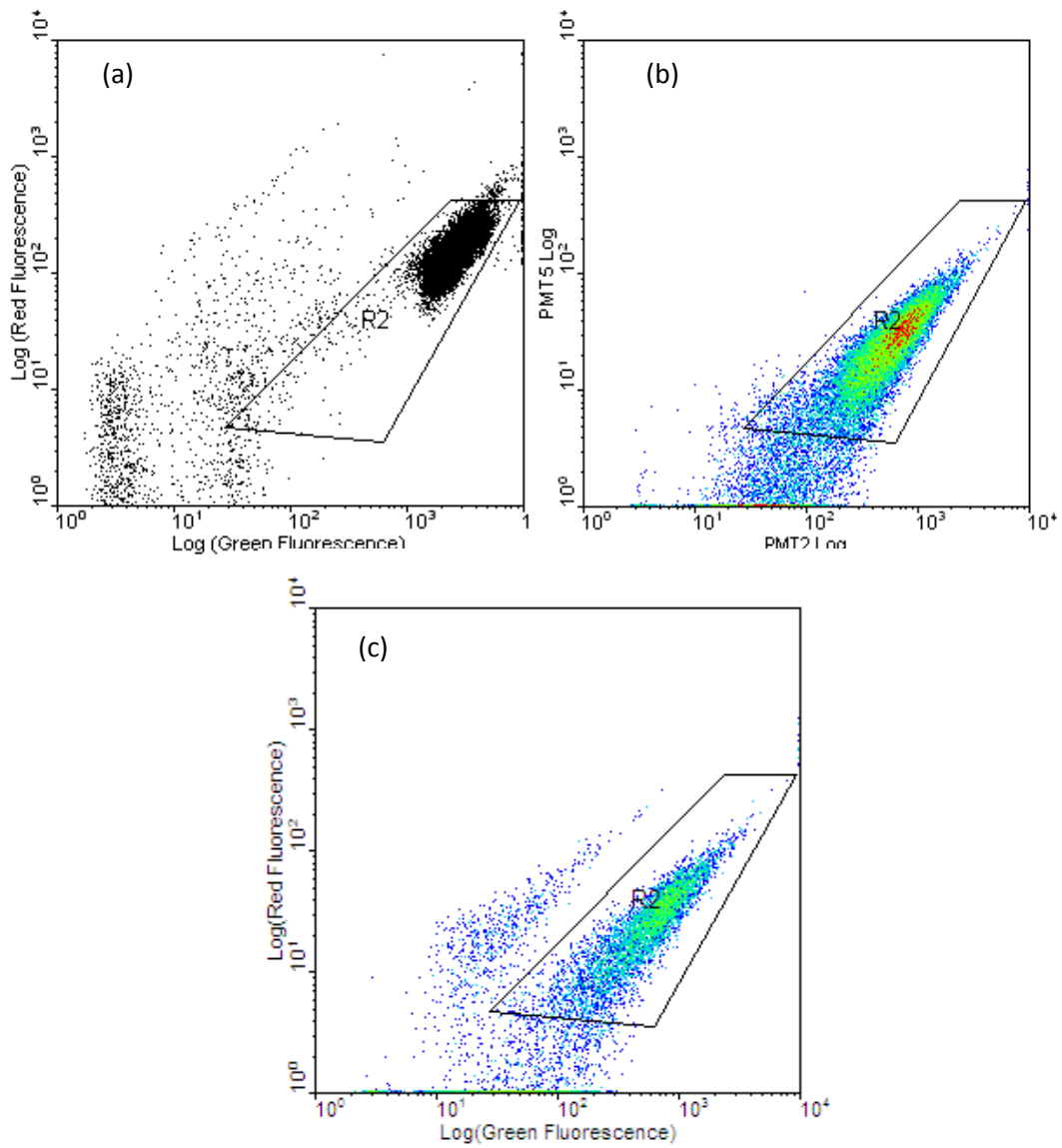


Figure B.2. Flow cytometer output, with gating, for (a) pure culture of *E. coli* ATCC® 25922, (b) Jordan Lake natural consortium A and (c) Jordan Lake natural consortium B.