EFFECTS OF ORGANIC CARBON ON THE BIODEGRADATION OF ESTRONE IN MULTIPLE SUBSTRATE, MIXED-CULTURE SYSTEMS

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Abstract

This dissertation describes the study of the effect of organic carbon on the biodegradation of estrone (E1) in multiple substrate, mixed-culture systems. In exploring this topic, important degradation mechanisms related to organic carbon were tested to determine which, if any, play an important role. Additionally, the effects of organic carbon concentrations, loads, and quality on E1 degrading activity of cultures from a wastewater treatment system were determined.

Catabolic repression effects on E1 degradation was studied by adding synthetic septage to an E1 degrading culture to determine if degradation rates were affected. No differences in first-order E1 degradation rates between test and control reactors were observed in the 2 h or 8 h period following the addition of synthetic septage, ruling out catabolic repression as an important mechanism in E1 degradation in wastewater treatment-like conditions. Cultures were grown in membrane bioreactors (MBRs) with and without exposure to E1 to determine if (i) E1 exposure is necessary for E1 degrading ability, and if so (ii) whether multiple substrate utilization and/or cometabolism play an important role in the degradation of E1. These cultures were capable of degrading E1 regardless of prior exposure. Higher rates of E1 degradation were observed in cultures with prior E1 exposure, and a lag phase of 6 h was observed in cultures without prior E1 exposure. These results indicate that E1 was degraded metabolically, demonstrating that multiple substrate utilization is the key mechanism for E1 degradation.

Longer term effects of organic carbon concentrations on E1 degrading activity were explored by comparing cultures operating under starvation conditions and cultures operating on a daily feeding cycle. Cultures fed daily showed a large initial increase in E1 degradation activity, attributable to a corresponding increase in biomass. Subsequently, however, E1 degradation activity dropped substantially even though biomass continued to increase, suggesting that E1 degraders were outcompeted when subjected to repeated exposure to high organic carbon concentrations. Conversely, starvation cultures had moderate but sustained increases in E1 degradation rates. Another experiment using MBRs to distinguish organic loads from organic concentrations confirmed the positive effect of organic carbon loads on E1 degradation via biomass growth, indicating that high organic carbon concentrations rather than loads were responsible for the drop in E1 degradation rates. A follow-up study was carried out to determine if altering the duration between feeding cycles could mitigate the negative effects of high organic carbon concentrations on E1 degradation. When cultures were exposed to high organic carbon concentrations (600 mg COD/L over a 6 d period), increasing the duration between feeding cycles improved performance. Conversely, at lower organic carbon concentrations (180 mg COD/L over a 6 d period), no differences in E1 degrading activity was observed.

Effects of organic carbon quality on E1 degradation were explored using aged synthetic septage and waters from various treatment and natural sources to culture mixed communities. In these experiments, spectrophotometric methods (specific UV absorbance, spectral slope ratios, excitation-emission matrices, and fluorescence index)

were used to characterize organic carbon. Additional analyses and experiments were conducted to rule out organic carbon, nitrogen species, and trace element concentrations as complicating factors. These experiments showed that microbially-derived organic carbon was associated with E1 degrading ability, while organic carbon from natural water sources (river and lake) was not. Furthermore, the experiments with aged synthetic septage suggest that products from cell lysis and/or microbial products under stress by starvation may be important for E1 degradation.

Overall, this work shows that multiple substrate utilizing bacteria are important for E1 degradation in wastewater treatment-like systems and indicates various organic carbon parameters that are vital for the selection of these bacteria.

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Chapter 1: Introduction and Literature Review

1.1 Introduction to Human Steroid Estrogens and Endocrine Disruption

Steroid Estrogens

The endocrine system regulates organs by secreting hormones in order to control various processes including growth, physiology, and reproduction (1). These hormones function as signaling molecules, binding to receptors in order to trigger physiological responses (2). As a chemistry-based process, hormonal signaling is slow but efficient, triggering multiple systems and generating prolonged responses of hours to weeks while being effective at very low doses, in the ng/L range (3).

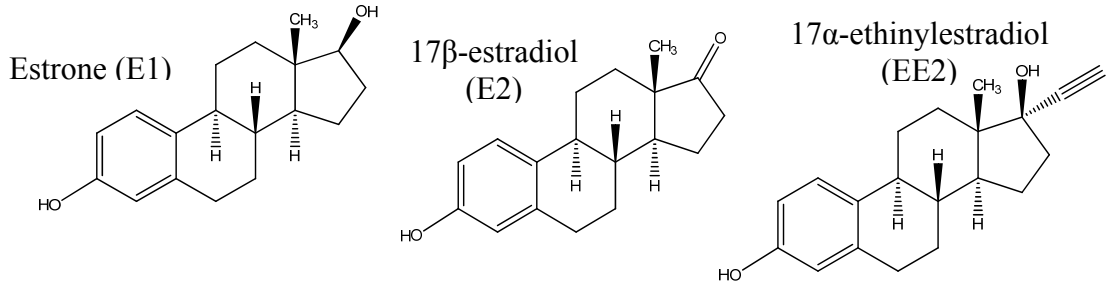

Figure 1.1: Structure of Key Steroid Estrogens

Steroid estrogens are the primary female sex hormone, and play key roles in reproduction and development. The major naturally-occurring steroid estrogens are estrone (E1), 17-β estradiol (E2), and estriol (E3), and are present in both males and females. Another important estrogen is the synthesized 17-α ethinylestradiol (EE2), the active ingredient in birth control pills. The efficacy of these hormones at low concentrations and the critical roles these compounds play make it important to understand the presence and effects estrogens have in the environment.

Steroid Estrogens as Key Endocrine Disrupters in Surface Waters

Endocrine disruption occurs when chemicals that are not normally present either trigger (agonist effect) or inhibit (antagonist effect) hormonal receptors (4). Exposure to endocrine disrupting compounds (EDCs) can have major physiological, developmental, and behavioral effects as a result of the roles hormones play. Medical science has made use of endocrine disruption, the principal example of which is the use of EE2 in the birth control pill to prevent pregnancies by preventing ovulation. As a hormone, EE2 affects a wide variety of health outcomes in addition to preventing ovulation, including blood clots, cancer, and depression (5-7). The risks and benefits of the birth control pill are well understood, and the overall effect is to slightly reduce mortality rates (8, 9). Nevertheless, the range of effects that EE2 has on humans serves as an important reminder of the broad role that hormones can play in organisms.

Indeed, unintended EDC exposure may have similar broad-spectrum consequences that are hazardous to the health of humans and other organisms. A well-known example is dichlorodiphenyltrichloroethane (DDT), a once widely used insecticide that is now known to: be carcinogenic, increase risk of premature birth, and harm infant and childhood development (10-12). Because of the effects of hormones and other EDCs on organisms at low concentrations the presence of EDCs in surface waters would is especially alarming due to the risk of unintended EDC exposure, both for aquatic life, and for humans that may use these waters as sources for drinking water.

The problem of EDC presence in surface water is not hypothetical, as steroid estrogens are present in virtually all surface waters in the United States and around the

world (13-15). While a wide range of other EDCs do exist, including halogenated chemicals, pesticides, various personal care products, metals, and phytoestrogens, (16-18), steroid estrogens are far more potent and have effects on aquatic life at far lower concentrations (16). The prevalence and potency of steroid estrogens make these critically important EDCs in surface waters. Consequently, developing a strategy to prevent steroid estrogens from entering surface waters is critical to maintaining aquatic, and potentially human, health.

Municipal wastewater is the major source of steroid estrogens to surface waters. The average person excretes 13.8 μg/d E1, 3.3 μg/d E2, and 0.89 μg/d EE2 (19). In locations with centralized sewage treatment systems, practically all of this is channeled directly to wastewater treatment plants (WWTPs). Although some of these estrogens are removed during treatment, environmentally relevant levels of steroid estrogens remain and are discharged into surface waters (20). While improving estrogen removal in wastewater treatment presents a challenge, the centralized, point-source nature of this problem means that engineering solutions can be implemented quickly once developed.

Municipal wastewater represents the largest source of estrogens to surface water, with agriculture runoff representing the next largest source. Loading from agriculture sources is difficult to calculate but it is estimated that agriculture is responsible for approximately 15% of estrogens in surface waters, primarily because sorption to soil minimizes runoff (19). Regardless of the precise numbers, treated municipal wastewater is both the primary estrogen load to surface waters and represents a point source

discharge, making municipal wastewater treatment a strategic point for preventing steroid estrogens from entering surface waters.

1.2 Implications of Endocrine Disruption for Aquatic and Human Health

Impacts on Aquatic Life

The adverse impacts of estrogen exposure on the physiology and reproductive ability of aquatic life are well-documented. These compounds induce production of vitellogenin, an egg protein, and development of female gonadal tissue in male fish (21-24), resulting in intersex fish with diminished or no reproductive ability. Furthermore, low male to female fish ratios are observed downstream of treated municipal wastewater discharge points, further compounding reproductive problems (25). Apart from these physiological changes, estrogens also cause behavioral alterations that have further effects on population viability. Larval fathead minnows exposed to E1 as embryos have lowered C-start performance, resulting in slower escape response when threatened by predators (26). Additionally, low levels of estrogen exposure reduce courtship behavior in male fish (27) and adversely affect the ability of these fish to find and maintain nesting sites (28), increasing the risk that eggs are eaten by other fathead minnows (29). Cumulatively, the physiological and behavioral disruption can result in population crashes (30).

The effects of EDC exposure on aquatic life are severe and there is growing evidence that these effects are not limited to the exposed population but also affect subsequent generations. This occurs through epigenetics, which is the addition of

functional groups to DNA that changes expression of genetic material. EDCs trigger epigenetic changes, which can be passed down to future generations (31). These effects have been observed in medaka, perch, and zebrafish on next, and even multiple generations, perpetuating some of the physiological and behavioral alterations described above (32-34).

Concerns in Water Reuse

In addition to the effects on aquatic life, EDCs present challenges to drinking water production, particularly in water reuse situations. Water shortages in various parts of the world could make direct water reuse a necessity. This raises concerns about the presence of estrogens in drinking water. Various EDCs cause aneuploidy, which is the leading cause of miscarriage, congenial defects, and mental retardation; alterations to the menstrual cycle; early puberty; and polycystic ovary syndrome, a major cause of infertility (35-40). More tenuous links have been made between EDC exposure and lowered sperm counts, increased rates of testicular cancer, and alterations in the human sex ratio (38, 41-43).

The estimated risks to human health from estrogens in drinking water are low when compared to those for aquatic life. This a result of limited water consumption by humans versus continuous exposure by aquatic organisms (44). Nonetheless, many questions remain about the effects of long-term chronic exposure and cumulative effects when steroid estrogen exposure is combined with exposure to other EDCs. Additionally, timing of exposure is critical, as EDC exposure during key stages of development (in utero, infancy, and puberty) can have permanent and irreversible effects that would not

be seen in adults with the same EDC exposure (45-46). These concerns necessitate the application of the precautionary principle. In addition, with growing public awareness of the presence of estrogens in treated wastewater, the removal of these compounds may be necessary to gain public acceptance of water reuse projects (44). Because of the effects of estrogens on aquatic life and the potential risks these compounds pose to human health, it is imperative that these compounds are removed in municipal wastewater treatment.

1.3 Steroid Estrogens in Wastewater Treatment

Steroid Estrogens in Raw and Treated Wastewater

Steroid estrogens are present in influent wastewater at tens to hundreds of nanograms per liter, with typical loads ranging from 20-180 ng/L for E1, 3-50 ng/L for E2, 29-676 ng/L for E3, and 0-7 ng/L for EE2 (20, 47-48). These compounds enter municipal wastewater through urine and feces (49) and are predominantly conjugated with glucuronide or sulfate groups (50). Much of these are converted to the free form of estrogens before reaching a WWTP, but a significant fraction may remain in a conjugated form, so measurement of free estrogens alone will underpredict the actual load (51). Because estrogens have environmental impacts at nanograms per liter concentrations, up to three-log removal is required to protect aquatic life.

Transformation pathways and degradation rates make E1 the principle estrogen in treated wastewater. Estrogen removal routinely occurs during wastewater treatment. These compounds frequently persist in treated effluent wastewater, however, at environmentally relevant concentrations. Among the estrogens present in wastewater, E2

and E3 are rapidly degraded while the degradation of E1 is slower, leaving it as the dominant estrogen in the effluent (47, 52). In spite of its recalcitrance and high potency, the low influent levels of EE2 mean that it is a minor contributor of overall estrogenicity in treated effluent, at less than 1% (53). Because effluent concentrations of E1 are still sufficient to cause endocrine disruption in wastewater-dominated effluents, it is the key target for reducing the estrogenic activity of wastewater. Improving the efficiency of existing removal processes or adding new processes to the treatment train is necessary to achieve this.

Removal Processes

There are several pathways for removal of chemicals from wastewater: partitioning to solids (sorption) which are then separated from the water, biological or chemical transformation, and straining (physical separation via membrane treatment).

Sorption

Steroid estrogens have a hydrophobic structure that suggests potential exists for removal via sorption; nevertheless repeated studies show that this is not a key removal pathway. These compounds have a moderately high affinity for solids, with K_d values for E1, E2, and EE2 are 402 L/kg, 476 L/kg, and 584 L/kg respectively, which would suggest that between 50-75% of these compounds sorb to sludge during activated sludge treatment (54). Mass balance analyses show, however, that this pathway accounts for less than 2% of the total estrogens entering the treatment system (54-56). This indicates that other removal pathways such as biological or chemical transformation dominate. Attempts to improve removal by sorption via coagulation and flocculation to remove

colloids yield removal rates of between 0 to 36 percent for the various estrogens (47). Research with activated carbon as a potential treatment step has also shown that it is is ineffective at removing estrogenic activity (57). Consequently, other strategies to improve estrogen removal are required.

Biological Removal

Biological treatment lies at the center of modern wastewater treatment and can remove a significant fraction of influent estrogens. In fact, significant biological removal of estrogens is achievable, with greater than 98 percent removal of E1 and E2, and 90 percent removal of EE2 observed in a system combining denitrification and nitrification (54). Nonetheless, wide variations in treatment efficiency occur, with studies of activated sludge treatment plants showing average removals of combined E1 and E2 between 24 and 93 percent (48, 60, 63-64). These variations are observed across a range of treatment plants and also in the same treatment plants over time. It is known that biodegradation of free estrogens in wastewater treatment plants is concentration-dependent and follows first-order kinetics (59). E2 is rapidly converted to E1 under aerobic conditions, followed by the slower degradation of E1 (60). This is consistent with studies of pure cultures, where many isolates are capable of converting E2 to E1, but few are capable of degrading E1 (61, 62). Given that E1 is the dominant estrogen in treated effluent, it is necessary to consider the combined removal of E1 and E2 for biological treatment. This presents an engineering problem but also suggests that optimization of biological treatment is possible.

Chemical and Membrane Treatment

Due to the incomplete and variable removal of estrogens through biological processes, various chemical and physical removal approaches have been proposed, principally ozonation and membrane treatment (65-67).

Chemical treatment via ozonation or other indiscriminate oxidation processes is complicated by the presence of other organic matter in wastewater effluent at relatively high concentrations. Some studies show insufficient removal (68-69), while others demonstrate very low residual estrogenic activity (70-71). Even if chemical treatment is possible, it runs the risk of producing disinfection byproducts, such as bromate in the case of ozonation, which poses other environmental risks (72-74). Additionally, aromatic compounds are formed during ozonation. While these compounds do not bind to estrogen receptors, they may induce the production of estrogens (75). Removal of estrogens via membrane treatment is possible but requires optimization of membrane material and treatment conditions (76). Furthermore, EDCs sorbed to membranes can be released at high levels during backflushing or pH variations (77). While membrane treatment can be an important part of a multiple barrier strategy, it should not be relied upon as the sole method of estrogen treatment.

Finally, the major limitation of chemical oxidation and membrane treatment for estrogens is the lack of selectivity, which drives cost and energy requirements (68, 74), and exacerbates potential drawbacks (disinfection byproducts, production of aromatic compounds, etc.). Because of these issues, it is not clear if these methods are practical or

sustainable. Biological treatment has the potential to overcome these selectivity issues and may be the better solution if optimization is possible.

1.4 Factors in Efficiency of Biological Treatment of Steroid Estrogens in Wastewater Treatment

Biological treatment holds many advantages over other solutions for estrogen removal but performance varies widely in full-scale treatment plants, particularly for the key estrogen E1. As a result numerous factors have been explored to try to explain this performance variation and optimize treatment. Solids retention time (SRT), hydraulic retention time (HRT), and nitrifying conditions are the most studied variables.

It is well-established that increased SRT is correlated with improved micropollutant and E1 removal (78). With regard to SRT, E1 degradation follows the pattern of other micropollutants, with optimal E1 degradation observed at long SRT values. In activated sludge systems, clear improvements in steroid estrogen removal are seen with increasing SRT, up to 10 days or 30 days (56, 78, 81). Extended SRTs are also beneficial in MBRs, with an optimal SRT value of 60 days (82). One explanation for long SRTs favoring micropollutant and E1 degradation is that increased SRT allows for slow-growing organisms critical to micropollutant removal to thrive. Nevertheless, it appears that many microbes capable of degrading E1 may not require long SRT values, with significant removal observed at an SRT of 3 days (83). Given that fast-growing E1 degraders exist, it is possible that these populations are enhanced at longer SRT values; alternatively, more efficient but slower growing E1 degraders may exist, which become important when SRT

is increased. It has also been suggested that increased SRT allows for a more diverse community, and hence conditions in which there is an increased likelihood of micropollutant degraders present. This explanation has been disputed, however, because long SRT appears to be have no impact (79) or may actually reduce (80) diversity.

Hydraulic Retention Time

The effects of HRT on E1 degradation are less clear and in fact, the importance of HRT on E1 removal is disputed. Indeed, in the context of Monod kinetics (pure culture, single substrate conditions), SRT rather than HRT should determine effluent concentrations of substrates. High HRT improves estrogen biodegradation when coupled with long SRT values (82) and high removal of E1 at E2 at HRT values as low as 35 min have also been observed (56). One challenge of determining the effect of HRT on E1 removal in full scale systems is that it is confounded with food to microorganism (F/M) ratios (59), which may also have an impact on E1 degradation. It is therefore likely that some other variable associated with HRT rather than HRT itself actually impacts E1 degradation.

Nitrifying Conditions

Nitrifying conditions improve steroid estrogen removal (59), but the mechanism by which this occurs is not clear. Interestingly, systems with both phosphorus and nitrogen removal have poorer estrogen removal performance than systems with nitrogen removal only (84).

It has been suggested that ammonia oxidizing bacteria degrade steroid estrogens via cometabolism with the ammonia monooxygenase enzyme (85-86). Pure cultures of

Nitrosomonas and *Nitrosospira*, however, are not capable of biologically degrading estrogens in the presence or absence of ammonia, although abiotic nitrification of estrogens was observed at very high ammonia concentrations (87). An alternate explanation is that nitrifying conditions favors selection of estrogen degrading heterotrophic bacteria (88). A key component of nitrifying conditions is low organic carbon levels, suggesting that organic carbon may influence E1 degradation.

Uncertainties

Several attempts have been made to find other correlations between operating parameters and estrogen removal in full-scale systems, but these have been unsuccessful (47, 84, 89-90). Biological oxygen demand, HRT, ammonia nitrogen, and rainfall cannot be linked to estrogen removal performance (89). Lower temperatures reduce estrogenic activity as deconjugation of conjugated estrogens is inhibited but does not appear to affect the degradation of free estrogens (47, 89). In each of these studies, the conclusion was not that these parameters do not impact estrogen removal, but rather the complexity of full-scale systems and variability in operating conditions makes it difficult to identify key parameters. Consequently, carefully controlled laboratory studies are necessary to determine the role of these parameters.

1.5 Organic Carbon Effects on Estrogen Removal

The relationship between organic carbon and biological degradation of E1 is potentially complicated. Nevertheless, organic carbon may be an important parameter to study because of its possible role in nitrifying activated sludge and because it is affected by, or affects, many other operating parameters. Three possible mechanisms can be

devised by which organic carbon could play a critical role in E1 degradation: catabolic repression, multiple substrate utilization/cometabolism, and community changes. These mechanisms and their impact on E1 degraders are illustrated in Figure 1.2 and are explained in detail below. Several of these mechanisms may be active at a time and may have synergistic or opposing effects.

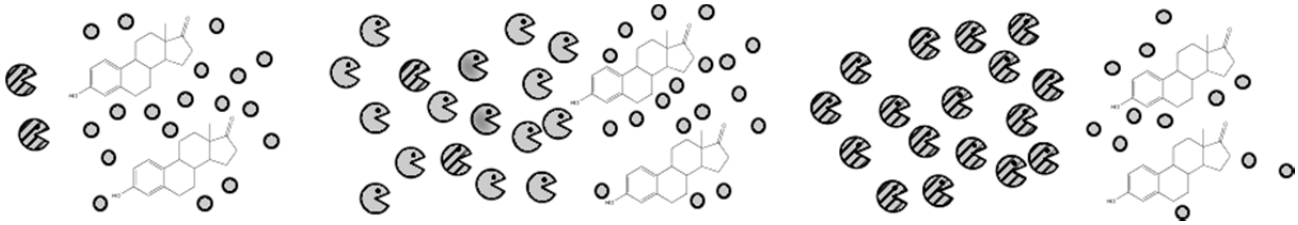

Figure 1.2: Potential Effects of Organic Carbon on Removal of Steroid Estrogens: (a) substrate competition; (b) community selection; (c) cometabolism/multiple-substrate utilization. Light circles represent general organic carbon; chemical structure is that of E1. "Pac man"-like symbols represent bacteria: light bacteria do not degrade E1, dark bacteria specialize on E1 degradation, and striped bacteria are capable of degrading both E1 and general organic carbon.

Catabolic repression

Catabolic repression is a well-known phenomenon in which bacteria target "better" carbon sources by inhibiting the production of enzymes used for the degradation of "lower quality" substrates when preferred carbon sources are present (91). This minimizes energy wasted on the production of less energy efficient enzymes, increasing the fitness of the organism. Higher organic carbon concentrations may induce catabolic repression and slow the degradation of particular compounds. Such behavior has been observed with several antibiotics in WWTPs (92) and has been incorporated into models developed to describe the biological removal of E1 (56)

Attempts to observe catabolic repression with E1 in full-scale treatment systems have yielded conflicting results, however, and have been complicated by confounding factors. The effect of higher F/M ratio is disputed, with both negative impacts (0.5 kg BOD_5/(kg MLVSS·d) vs. 0.1 kg BOD_5/(kg MLVSS·d)) (47) and no impacts (0.1 kg BOD_5/(kg MLVSS·d) vs. 0.05 kg BOD_5/(kg MLVSS·d)) (84) observed. A major difficulty in determining the impact of the F/M ratio in full scale treatment systems is that it is typically a function of HRT rather than variations in wastewater BOD loads, making it difficult to separate the two factors (59).

Whether catabolic repression is an important factor in E1 degradation depends on the alternative carbon source used and may be influenced by the identity of the key microbe or microbes. When isolated on estrogens, pure culture studies of *Novosphingobium* and *Rhodococcus* showed preferential degradation of E2 in the presence of yeast extract or glucose (61, 93). In mixed culture systems in which the identity of the key estrogen degrader was unknown, both positive and negative impacts have been observed when an additional carbon source is provided. Glucose was observed to inhibit the degradation of steroid estrogens in mixed culture systems (85, 94). Glucose is a crucial metabolic regulator, however, and is not a good representative of background organic carbon in wastewater treatment systems. Additionally, these studies did not differentiate between substrate competition and community shifts in response to glucose addition. When food sources other than glucose were present, E1-specific degradation rates have been observed to decrease, but overall E1 degradation improved (95-97). Either catabolic repression was not active in these systems, or it was offset by community

changes or multiple substrate utilization or cometabolism. Carefully designed experiments need to be developed to decouple these effects. Additionally, the effects of a complex organic carbon matrix that mimic wastewater need to be studied.

Facilitation of Multiple Substrate Utilization or Cometabolism

The low concentration of micropollutants makes these compounds poor energy sources. Organic carbon presence, therefore, may be necessary for micropollutant removal by facilitating multiple substrate utilization or cometabolism. Indeed, the presence and utilization of other carbon substrates can in fact lower utilization thresholds for certain compounds, resulting in lower final concentrations (96-97). Substrate threshold concentrations for the growth of pure cultures are in the 1-100 μg/L range (98), much higher than the concentrations of estrogens and other micropollutants in wastewater. Because of this, it has been suggested that removal of micropollutants in wastewater is likely due to "K strategists" that scavenge a wide variety of low concentration carbon sources rather than organisms that grow solely on a single micropollutant (84). Such behavior is known to occur under oligotrophic stress (99-100) and is important in the degradation of several groundwater contaminants (101-103), though the quantity and quality of organic carbon may determine whether the additional substrates have a positive or negative effect on degradation (104). Alternatively, the presence of other organic carbon sources may induce cometabolism, which is production of enzymes that fortuitously break down micropollutants of concern.

Estrogen degradation behavior consistent with multiple substrate utilization or cometabolism has been observed. Higher biodegradable dissolved organic carbon

(undiluted vs. diluted wastewater) has been seen to have a positive impact on the biodegradation rates of several micropollutants including EE2, though no measurable impact was observed for E1 (105). Conversely, experiments on the biological removal of steroid estrogens from space mission-like grey water with very low organic carbon content show no removal in spite of long hydraulic retention times (HRT) and infinite solids retention times (SRT) (106). Finally, the presence of diverse food sources, which may favor the growth of multiple substrate utilizers, yields better estrogen degradation than a single type of food (96). These observations suggest that organic carbon may have an important and beneficial role in estrogen removal.

Community Changes

Organic carbon loads and concentrations can affect microbial community structure, and this in turn could alter estrogen removal performance. It is widely known that organic carbon concentrations affect community structure (107-108). There is also evidence that organic loading rates (OLR) affect microbial community structure, and in some cases may be even more important than organic carbon concentrations (109). OLR effects have been widely studied in anaerobic wastewater treatment and hydrogen bioreactors, showing shifts in microbial community due to changing OLRs (109-110) and higher diversity at higher OLRs (111-112). Community shifts may affect estrogen removal rates by selecting for or against key microbial species, while higher diversity is associated with improved estrogen biodegradation (95).

Identifying key E1-degrading species and tracking the representation of these species in a mixed microbial community may not be practical. Numerous bacteria

capable of degrading E1 have been isolated, representing a wide range of genus including *Novosphingobium, Sphingomonas, Rhodococcus, Achromobacter, Ralstonia, Stenotrophomonas,* and *Aminobacter* (61-62, 81, 93, 113-116). Most of these belong to the phylum Proteobacteria, but *Rhodococcus* is part of the phylum Actinobacteria (Table 1.1). Further compounding the problem is the likelihood that other, yet to be isolated, E1 degrading strains exist. If multiple-substrate utilizers or co-metabolizers are the key species, it may not be possible to isolate these on E1 alone.

Predicting E1 degrading ability in particular strains is not possible given the current stage of knowledge. Although E1 degrading ability is present among such diverse genus, most species and strains within each genus do not possess such ability (61-62, 113-115). The only traits common to all known E1 degrading isolates are that these isolates are aerobic and possess the ability to transform E2 to E1. Unfortunately, this makes it difficult to predict which bacteria will have E1 degrading abilities, especially since many bacteria capable of degrading E2 do not degrade E1. It is also not possible to determine the identities of these species by tracking substrate uptake through labeled substrates. While one study has attempted to do so (117), of the two pathways suggested for the degradation of E1 (115, 118), one begins with the addition of a hydroxyl group on the phenol ring. Because the bacteria responsible for the critical first step in transforming E1 do not necessarily incorporate E1 into biomass, they cannot be tracked and identified in this way. Because of the difficulties in identifying key E1 degraders, studies on how community changes stemming from varying organic carbon additions affect E1

Table 1.1 Examples of bacterial species capable of E1 degradation

Phylum	Class	Order	Family	Genus	Species	Reference
Actinobacteria	Actinomycetales	Corynebacterineae	Nocardiaceae	Rhodococcus	*Rhodococcus equi*	61
					Rhodococcus zopfi	61
					Rhodococcus sp.	114
Proteobacteria	Alpha Proteobacteria	Rhizobiales	Phyllobacteriaceae	Aminobacter	*Aminobacter sp.*	62
					Aminovorans	62
		Sphingomonadales	Sphingomondaceae	Novosphingobium	*Novosphingobium sp.*	81
					Sphingomonas sp. KC8	62
					Tardaugens sp. nov.	93
				Sphingomonas	*Sphingomonas sp.*	114
	Beta Proteobacteria	Burkholderiales	Alcaligenaceae	Achrombacter	*Xylosoxidans*	115
			Ralstoniaceae	Ralstonia	*Picketii*	115
	Gamma proteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	*Acinetobacter sp.* BP8	115
			Pseudomonadaceae	Pseudomonas	*Aeruginosa* TJ1	115
		Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	*Maltophila*	116

degradation may have to rely on observations of broad community pattern changes to infer importance.

1.6 Summary of Research Knowledge and Needs

From the current state of knowledge of estrogens and estrogen degradation in wastewater, several conclusions can be drawn:

- E1 is a key estrogen in wastewater effluent.
- E1 removal varies widely and that variation cannot be fully explained.
- Carefully controlled laboratory studies are required to determine how factors beyond SRT, HRT, and nitrifying conditions affect E1 removal.
- Organic carbon may be an important factor in E1 removal.
- Mixed culture studies with a complex carbon source are necessary to extrapolate results to wastewater treatment.

1.7 Objectives

Based on these research needs, the following objectives were developed: (i) determine if catabolic repression, multiple substrate utilization, and cometabolism are important mechanisms in E1 degradation; (ii) determine if organic carbon exposure and loading affect growth and selection of E1 degrading bacteria; (iii) determine if organic carbon quality affects growth and selection of E1 degrading bacteria. A brief summary of each chapter is described below.

Chapter 2: Impact of Organic Carbon on the Biodegradation of Estrone in Mixed Culture Systems

Several experiments were performed to determine if organic carbon concentrations and loads affected the degradation of E1. The short-term effects of organic carbon concentrations on E1 degradation were examined in batch reactors by increasing organic carbon concentrations during E1 degradation. No difference was observed in test and control reactors, showing that catabolic repression is not a factor in the biological degradation of E1. Longer-term effects of organic carbon concentrations were studied in batch systems, comparing reactors receiving a daily feed and reactors operated under starvation conditions. Initially, reactors receiving organic carbon had improved E1 biodegradation rates attributable to biomass growth, but subsequent operation of these reactors resulted in substantially reduced E1 degradation performance. Conversely, E1 degradation in starvation reactors showed more moderate but sustained increases in E1 degradation rates over the course of the study. These results show that E1 degraders can grow on general organic carbon but are outcompeted by other organisms when repeatedly fed high concentrations of organic carbon. To decouple organic carbon concentrations from organic loads, continuous flow reactors were operated using a membrane bioreactor system (MBR). In these experiments, low levels of influent organic carbon strength was seen to be detrimental to E1 removal, confirming the positive effect general organic carbon has on E1 degradation when the negative selective pressures caused by high organic carbon concentrations are avoided.

Chapter 3: Effects of Estrone and Organic Carbon Exposure on the Degradation of Estrone

The role of multiple substrate utilizers in E1 degradation and selective pressures of organic carbon on these bacteria were examined in this study. Biomass was grown in MBRs with and without E1 exposure over a 30 d period (10 d solids retention time) and transferred to batch systems to determine if the biomass had E1 degrading capability and to measure degradation rates. The biomass was capable of E1 degradation regardless of E1 exposure, but pre-exposed biomass had higher E1 degradation rates. Additionally, unexposed biomass showed a clear lag phase prior to E1 degradation. These results clearly demonstrate metabolic degradation of E1 by multiple substrate utilizing bacteria and that the ability to utilize substrates, even at extremely low concentrations, improves microbial fitness. Feeding cycle effects on E1 degradation was studied at low and high organic carbon loads to determine if the negative selective pressures associated with high organic carbon concentrations can be mitigated in batch or plug-flow reactor systems. Increasing the interval between feeding periods improved E1 degradation at high organic carbon loads, presumably by providing an extended period of low organic carbon concentrations when E1 degraders could compete with other bacteria. No feeding cycle effects were observed at low organic carbon loads, as the lower organic carbon concentrations in this scenario did not appear to negatively affect the E1 degraders in the community. Finally, lag phases were observed prior to E1 degradation under famine conditions but were absent under feast conditions, though degradation rates were similar once E1 degradation began. This shows that general organic carbon induces the

degradation of E1. This study is the first to clearly demonstrate the role of multiple substrate utilizing bacteria in E1 degradation.

Chapter 4: Effects of Organic Carbon Quality on the Growth and Selection of Estrone Degrading Bacteria

Organic carbon quality effects on E1 degradation were studied by cultivating biomass on fresh and aged synthetic septage and waters from treatment and natural sources. Experiments with fresh and aged synthetic septage showed that microbially derived organic carbon was associated with E1 degradation, and products from cell lysis or soluble microbial products released under stress may be particularly important for improving E1 degradation. Biomass grown in in wastewater treatment plant and wetland treatment effluents degraded E1 while biomass grown in river and lake water could not. Spectrophotometric analysis showed that the waters from treatment sources had stronger microbial-origin signatures than waters from natural sources, which was consistent with the results from the first experiment. Trace elements, pH, and nitrogen concentrations were ruled out as contributors to E1 degradation. There is currently very little known about how organic carbon quality affects the degradation of micropollutants. This study showed that it can be an important parameter.

Additional Work

In addition to the work described above, additional experiments on organic carbon and E1 degradation were performed and described in Appendix A. Additional contributions were also made with respect to the photolysis of antibiotics (119) and the presence of antibiotic resistance genes in the environment (120), described in Appendix B.

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Chapter 2: Impact of Organic Carbon on the Biodegradation of Estrone in Mixed Culture Systems

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The effects of organic carbon concentrations and loading on the degradation of estrone (E1) were examined under various conditions in batch reactors and membrane-coupled bioreactors (MBRs). Experiments examined effects on individual microorganisms (substrate competition and growth) and on the whole community (selection). Substrate competition with organic carbon (competitive inhibition and catabolic repression) was not a factor in E1 degradation ($P = 0.19$ and 0.29 for two different analyses). Conversely, addition of organic carbon increased E1 degradation rates, attributable to biomass growth in feast-famine reactors over a five-day period ($P = 0.016$). Subsequently, however, community dynamics controlled E1 degradation rates, with other organisms outcompeting E1 degraders. More moderate but sustained increases in E1 degradation rates were observed under starvation conditions. Low influent organic carbon strength was detrimental to E1 degradation in MBRs, where organic carbon concentration and loading were decoupled ($P = 0.018$). These results point to the importance of multiple substrate utilizers in E1 degradation. They also suggest that while initial growth of biomass depends on the presence of sufficient organic carbon, further enrichment under starvation conditions may improve E1 degradation capability via the growth and/or stimulation of multiple substrate utilizers rather than heterotrophs characterized by an r-strategist growth regime.

2.1 Introduction

The presence of endocrine disrupting compounds (EDCs) in municipal wastewater is detrimental to aquatic life downstream of discharge points (1-2). Wastewater-derived EDCs are present in surface waters throughout the US (3) and cause a wide range of effects, including inhibition of predator avoidance behavior (4), alteration of nest guarding behavior (5), and production of vitellogenin and female gonadal tissue in male fish (6-7). These effects lead to reproductive disruption that can cause population collapses (8). Of the EDCs discharged in municipal wastewater, estrone (E1) is of particular importance because it is formed rapidly via the biodegradation of 17β-estradiol (E2) and is thought to be one of the major contributors of estrogenic activity in treated effluent (9-10).

While activated sludge systems can effectively remove steroid estrogens, removal of E1,E2, and general estrogenicity from wastewater varies widely across treatment plants and within individual treatment plants over time (11-12). It is known that critical solid retention times (SRTs) must be met for the degradation of various micropollutants in municipal wastewater treatment and that increased sludge age generally correlates with enhanced removal for micropollutants (13). It is also hypothesized that slower growing organisms that scavenge a variety of carbon sources are one reason for the importance of SRT in steroid estrogen removal (14). Systems with longer hydraulic residence times (HRT) appear to have better removal of estrogenic activity as well (15). Nonetheless, these parameters are insufficient to account for the variability in the removal of combined E1 and E2 and overall estrogenicity observed in the field (11-12). It is likely that other

operating or effluent water quality parameters are important, but studying these in full-scale treatment plants has proven difficult. (10,16).

Many operational parameters in wastewater treatment, including SRT, HRT, and food to microorganism ratio, are related to the concentration, and in some cases, loading of organic compounds present, which may in turn affect E1 removal in a number of ways. First, it is possible that a low concentration of organic compounds may be inherently detrimental to E1 removal or that low biomass concentrations resulting from substrate scarcity may negatively impact removal via multiple substrate utilization and/or co-metabolism. One study suggested that this was the case, with poor or non-existent estrogen degradation in low carbon greywater representative of space waste streams, in spite of long HRT, infinite SRT, and individual estrogen concentrations ranging from tens to over a hundred μg/L (17). Conversely, high food to microorganism ratios could also result in substrate competition. Substrate competition was suggested as an explanation for discrepancies in E1 degradation rate coefficients between laboratory batch experiments and a full-scale treatment system (18). Later studies, however, failed to find evidence for substrate competition playing a role in E1 degradation (14). Lastly, wastewater organic carbon content could affect the structure and performance of the microbial community (19-21), which may in turn alter micropollutant removal via changes in the individual populations present (22).

This study was performed to determine the impact of wastewater organic compound concentration and loading on E1 removal, examining effects on individual microorganisms (substrate competition and growth) and on the whole community

(selection). E1 was specifically studied because of its contribution to effluent estrogenicity (9, 10) and because our own preliminary work showed that it formed rapidly (within 2 hours) and stoichiometrically as a result of E2 degradation under simulated activated sludge conditions (data not shown). Batch studies were conducted to examine substrate competition effects and the effects of organic carbon concentration on growth and community selection. Additional experiments with a continuous flow system using MBRs further examined the role of organic carbon loads on E1 degradation. This setup enabled decoupling of organic carbon loads (relative to E1) and the concentration of organic carbon in the reactors. Understanding the effect of organic carbon on E1 biodegradation will enable better design and operation of wastewater treatment systems and thus allow treatment plants to achieve high and consistent removal of E1 from wastewater. This research will also lead to better understanding and evaluation of alternative wastewater collection and treatment design options (e.g., separate collection and treatment of urine and feces) and may have broader implications for our understanding of the roles organic carbon plays in micropollutant removal.

2.2 Experimental Section

Chemicals and Synthetic Wastewater

E1 and deuterated or ^{13}C-labeled E1 were obtained from Sigma and Cambridge Isotopes, respectively. The recipe for synthetic wastewater was adapted from Boeije et al (23), and contained (per L): 75 mg urea, 11 mg ammonium chloride, 12 mg sodium uric acid, 25 mg magnesium phosphate dibasic trihydrate, and 20 mg potassium phosphate tribasic. The synthetic wastewater also contained a carbon source made up of the

following (per L, for 100 mg chemical oxygen demand (COD)/L nominal concentration): 6 mg bacteriological peptone, 51 mg sodium acetate, 6 mg dry meat extract, 17 mg glycerine, 21 mg potato starch, and 25 mg low fat milk powder. The carbon source was diluted or concentrated for carbon feeds of various strengths.

Sludge Seed

Biomass used to start each experiment (batch and MBR) was taken from the Metropolitan Wastewater Treatment Plant in St. Paul, Minnesota, which operates with both nitrogen and phosphorus removal. A single sample of activated sludge was triple-washed with phosphate-buffered saline, divided into 3.5 mL aliquots, and cryopreserved in 15% glycerol (v/v) at -80°C until use. A single sludge aliquot was used to seed each reactor.

Batch Systems

Batch systems were used for competition and starvation versus feast-famine experiments. Excess aeration was provided so reactors were saturated with dissolved oxygen, and pH values over the course of the experiments ranged from 7.3 to 7.8. Reactors were continuously mixed with stir bars.

Competition Experiment

A substrate competition experiment was performed to test if the amendment of organic carbon had a short-term inhibitory effect on E1 degradation via competitive inhibition or catabolic repression. A 1-L batch reactor was seeded with cryopreserved activated sludge, synthetic wastewater with 100 mg/L COD, and 5 µg/L of E1. Reactors were operated for 15 days to confirm that the biomass had E1 degradation ability.

Reactor solids were filtered with a glass fiber filter and transferred to a 14-L batch reactor containing synthetic wastewater with 100 mg/L COD and E1 at a concentration of 5 µg/L. The filtration apparatus was autoclaved prior to filtration. After a 3-d period, this 14-L reactor was split into two sets (competition and control) of triplicate 2-L batch reactors. E1 was added to each reactor at a concentration of 5 µg/L. At 8 h, competition reactors were amended with synthetic wastewater, increasing the COD in the reactor by an additional 100 mg/L. Preliminary experiments using similar conditions showed soluble COD drop from 100 mg/L COD to below 20 mg/L COD (remaining COD seemed recalcitrant) over a 4-6 hour period (data not shown). Reactors were sampled every 2 h over a 16-h period to measure E1 concentration and absorbance at 600 nm. A relationship between VSS and OD_{600} was developed and used to calculate VSS for batch reactors.

Starvation and Feast-Famine Conditions

The impact of starvation and feast-famine conditions on E1 degradation was studied in two sets of triplicate batch reactors. A 15-L batch reactor was seeded with cryopreserved activated sludge, synthetic wastewater with 100 mg/L COD, and 10 µg/L E1. After a 5-d period, this 15-L reactor was split into 2 sets (starvation and feast-famine) of triplicate 2.5-L reactors. The reactors were operated for an additional 8 d (for a total of 13 d), during which feast-famine reactors received daily synthetic wastewater amendments, increasing reactor COD by 50 mg/L, while starvation reactors received no synthetic wastewater.

E1 degradation in all reactors was monitored on Days 5, 10, and 13. A 10 μg/L spike of E1 was added to each reactor, after which reactors were sampled every 2 h over a 12-h period to measure E1 concentration. Biomass was also sampled at the beginning of each monitoring period (prior to addition of substrate for that cycle for feast-famine reactors). The biomass concentration was analyzed via absorbance at 600 nm (used for biomass normalized rate calculations) and community structure was monitored via Automated Ribosomal Intergenic Spacer Analysis (ARISA) of the DNA, as described below.

Membrane Coupled Bioreactor (MBR) Experiment

Three continuous flow membrane-coupled bioreactors were operated to test the impact of organic carbon loads on E1 degradation. The continuous flow setup allowed decoupling of the organic loading rate from organic carbon concentration within the reactors. Reactors (150 mL) were operated with an HRT of 8 h and an SRT of 10 d. Influent wastewater COD to the three reactors were 20, 75, and 375 mg/L, and all reactors were fed E1 at a concentration of 2 μg/L. All treatments were run in triplicate. An additional control reactor was fed distilled water containing sodium azide at 1% by weight to assess loss of E1 as a result of sorption to the reactor surface or the membrane.

The MBRs were operated for a period of 36 d. Reactor effluent was sampled twice weekly for E1, pH, ammonia, and COD to establish reactor performance and functional stability. The reactor solids stream was also sampled twice weekly to determine biomass concentration and perform microbial community analysis.

DNA Collection, Processing, and Analysis

All samples for DNA analysis were collected and processed in triplicate. Reactor liquor (1.5 mL) was centrifuged and decanted, after which the pellet underwent three consecutive freeze-thaw cycles and incubation at 70°C for 90 min to lyse cells. DNA was extracted from lysed cells with the FastDNA spin kit (MP Biomedicals, Solon, OH) and stored at -20°C until further processing.

Automated Ribosomal Intergenic Spacer Analysis (ARISA) was conducted as described by Nelson et al. (24). Briefly, the ribosomal intergenic spacer (ITS) regions of *Bacteria* were amplified using primers ITSF (5'-GTC GTA ACA AGG TAG CCG TA-3') and ITSReub (5'-GCC AAG GCA TCC ACC-3') (25). Fragment analysis was performed by denaturing capillary electrophoresis at the Biomedical Genomics Center at the University of Minnesota using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Fragment length was estimated using the MapMarker 1000 size standard.

E1 Sample Extraction and Cleanup

Solid phase extraction (SPE) and silica gel clean-up procedures were adapted from Ternes et al. (26). Briefly, samples of 50 mL or 100 mL were collected for E1 analysis, acidified to pH 3 with concentrated sulfuric acid, and amended with 80 ng of a labeled surrogate, (2,4,16,16-D_4-estrone). Resprep Bonded Reversed Phase SPE cartridges (6 mL, Restek) were preconditioned with two column volumes each of acetone and Milli-Q water. Samples were then loaded onto the cartridges at a flowrate of ~3 mL/min. Samples were eluted from the column with two column volumes of acetone.

Eluted samples were blown down to dryness with nitrogen and resuspended in 2 mL of hexane for silica gel cleanup. Silica gel columns were prepared by packing silica gel to a height of 3 cm (~ 1 mL) into pasture pipettes and then washing with two column volumes of hexane. Samples were then loaded onto the column and eluted with three column volumes of a 65:35 mixture of acetone and hexane (v/v), blown down to dryness with nitrogen, and resuspended in a 60:40 mixture of methanol and water (v/v) containing 100 ng (a concentration of 250 μg/L) of an internal standard (13,14,15,16,17,18-$^{13}C_6$-estrone). The sample was then stored at 4°C until analysis via liquid chromatography-mass spectrometry (LC/MS). Average sample recovery was 66% with a standard deviation of 12%.

LC-MS Analysis

E1 samples were quantified via LC/MS using an HP 1050-series LC coupled to an Agilent/HP 1100 Series G1946D mass spectrometer detector. E1 was separated on a Synergi 4u Polar-RP 80A 150 × 2.00 mm 4 μm particle size column (Phenomenex). A binary gradient consisting of a pH 4 ammonium acetate buffered solution (10 mM) in 90% water and 10% acetonitrile (A) and 100% acetonitrile (B) at a flow rate of 0.2 mL/min was used. The gradient was as follows: 35% B for 17 min, followed by a linear increase to 100% B over 3 min, held at 100% B for 5 min, and stepped down to 35% B for equilibration over 5 min.

The mass spectrometer was operated in negative ion, selected ion monitoring mode with m/z ratios of 269, 273, and 275 for the detection of estrone, the surrogate, and the internal standard, respectively. Standard curves of at least seven points were used in

sample quantification. Blanks of 40:60 methanol and water, as well as method blanks were run at the beginning of each sample analysis, as well as intermittently between samples. Typical instrument quantification limits were 10 µg/L (sample quantification limits of 100 ng/L). In-vial concentrations of E1 and surrogate were corrected by the internal standard. Sample concentrations of E1 were further corrected using surrogate recovery.

Biomass, COD, and Ammonia Determination

Biomass concentrations in reactors were measured via absorbance at 600 nm with a Beckman DU 530 UV/Vis spectrophotometer. For the membrane bioreactors, a standard curve comparing volatile suspended solids to OD_{600} was created. The range of the curve was 15-1500 mg/L, with an R^2 value of 0.99. Chemical oxygen demand was measured using accu-Test Low Range and Mid Range Micro COD vials (Bioscience) and a DR/890 colorimeter (Hach). Triplicate readings had a standard deviation of 2 mg/L COD at readings below 20 mg/L COD, and a standard deviation of 15% at higher COD concentrations. Ammonia measurements were taken using an Orion 9512HPBNWP ammonia probe (Thermo Scientific), and a 5-point standard curve ranging from 1.4 to 140 mg/L as ammonium (typical R^2 values of 0.99).

Data Analysis

Nonmetric multidimensional scaling (nMDS) was used on triplicate ARISA data to compare microbial community profiles in samples, similar to the method described in LaPara et al (27). Relative peak intensity was used in this analysis. ARISA peaks falling below 0.25% of total peak intensity and peaks that were present in only one of triplicate

extractions were excluded from analysis. nMDS was performed using the ade4 package in R, version 2.4.1.33 (28). E1 degradation rates obtained from linear regression, ANOVA analysis, and the Student t-test were performed in Microsoft Excel.

2.3 Results and Discussion

Substrate Competition

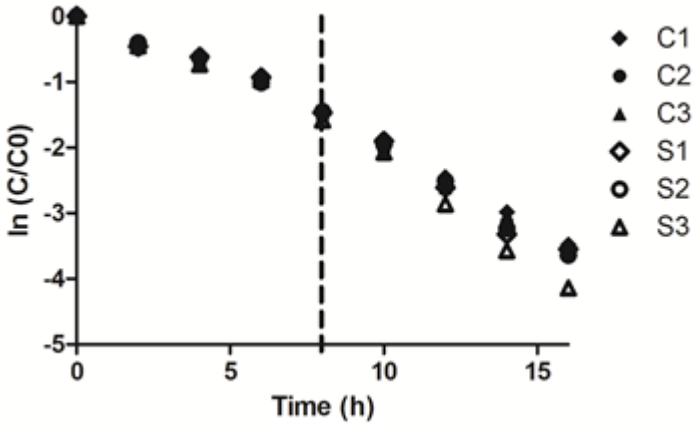

Figure 2.1: Semi-log plot of E1 in triplicate control reactors (C) and triplicate reactors amended with a synthetic septage at 8h (S). The dotted line marks the time of synthetic septage amendment. No change in E1 degradation performance was observed following the septage amendment

The effect of substrate competition was examined by amending reactors with a complex synthetic wastewater during active E1 degradation. On a short time scale (8 h after addition), the addition of synthetic wastewater did not affect E1 biodegradation rates (Figure 2.1). Neither the percentage removal of E1 in the 2-h period immediately following addition of synthetic wastewater, nor the degradation rates over the entire 16-h period (Figure C1) were statistically different between treatments ($P = 0.19$ and $P = 0.29$ respectively). This demonstrates that the organic carbon present in synthetic wastewater does not inhibit individual cells via competitive inhibition or catabolic repression with

respect to E1 degradation in mixed wastewater communities. Cells respond very rapidly to inhibitors that act via competitive inhibition or catabolic repression, so if these mechanisms were active the rate of E1 degradation would have decreased in the reactors amended with synthetic wastewater. This was not observed.

Average values of OD_{600} in the control and wastewater-amended reactors prior to addition of synthetic wastewater were 0.067 and 0. 071 (calculated VSS of 64 mg/L and 68 mg/L), respectively, and were not statistically distinguishable (P = 0.32). Following the addition of synthetic wastewater, the OD_{600} in the amended reactors increased to 0.140 (calculated VSS of 133 mg/L) by the end of the experiment, while the OD_{600} in the control reactors remained relatively constant at 0.072. This increase in biomass in the synthetic wastewater-amended reactors did not have an effect on the E1 degradation rate or percentage removal on the time-scale of the experiment (8 hours between synthetic wastewater amendment and end of experiment), as the non-biomass normalized rates of degradation were equivalent. As such, it appears that the immediate increase in biomass in the synthetic wastewater-amended batch reactors consisted of fast-growing bacteria that did not degrade E1.

The absence of substrate competition by a mixed substrate synthetic wastewater stands in contrast to previous studies using glucose as a competitive substrate (29-30) but is consistent with another study using acetonitrile (31). The study by Ren et al. took place over a 96-h period (29), which may have been long enough for community shifts to affect results, but the study by Li et al. examined degradation over an 8-h period, comparable to the time frame used in this study, and demonstrates that glucose at 10 mg/L can inhibit

the degradation of E1 (30). Glucose, however, is a key catabolite in microbial metabolism, and other organic carbon substrates may not have the same inhibitory effect. Muller et al. found that the addition of acetonitrile reduced biomass normalized E1 degradation rates but increased overall E1 removal during a 5-d study (31). This suggests that (i) acetonitrile did not competitively inhibit E1 degradation, (ii) E1 degrading biomass grew on acetonitrile, and (iii) non-E1 degrading biomass grew faster than the E1 degrading biomass when exposed to acetonitrile. In summary, while glucose may have an inhibitory effect on E1 degradation in mixed culture systems, the work by Muller et al. and the findings in this study show that the effects of glucose are very different from the bulk organic carbon that is typical of wastewater, which contains a complex mixture of biodegradable, slowly biodegradable, and recalcitrant compounds.

Starvation and Feast Famine Conditions

The effect of starvation and feast-famine conditions on E1 degradation was examined by splitting 5-d old biomass and subjecting it to continued starvation conditions or daily pulses of synthetic wastewater. Changes in E1 degradation rates in starvation and feast-famine reactors are shown in Figure 2.2, with the regressions shown in Figure C2.

E1 degradation activity in starvation reactors (both biomass-normalized and non-normalized) increased between Day 5 and Day 10 (overall rate, $P = 0.011$), even though biomass concentrations remained constant or decreased slightly. This indicates enrichment of E1 degraders in the community. In addition, degradation rates appeared to increase in starvation reactors between Days 10 and 13 as well, but this was not statistically significant at the 95% confidence interval as a result of the variability in E1

degradation rates at Day 13 (P = 0.28). It is well known that increased cell retention time improves E1 degradation (13, 18, 32), and this community selection phenomenon was observed under starvation conditions. Nevertheless, a lag phase of 6 h prior to E1 degradation was observed after 13 days of starvation, indicating that although starvation appeared to select for an E1-degrading community, the absence of organic carbon over this long period did negatively impact individual E1-degrading cells. Indeed, we hypothesize that the prolonged absence of any carbon sources may have caused individual E1 degraders to become temporarily inactive after 13 days.

No difference in biomass-normalized E1 degradation performance was observed in starvation and feast-famine reactors on Day 5 (P=0.66), when the reactors were split and synthetic septage was added to the feast-famine reactors. This was consistent with observations from the substrate competition experiment, confirming that the amendment of organic carbon does not inhibit E1 degradation via individual cells over the short-term (i.e., over a time-scale of hours). In addition, it appeared that a daily feast-famine regime caused enrichment of E1-degraders and an increase in biomass over the mid-term, increasing the rate of E1 degradation between Days 5 and 10 (P = 0.016). In fact, the change in biomass-normalized E1 degradation rate was not statistically different from that in the starvation reactors (P=0.26), even though distinct communities had formed by Day 10 (Figure 2.3). Nevertheless, the overall (non-normalized) E1 degradation rates were greater in the feast-famine reactors (P = 0.006), which must have been a result of the growth of E1-degrading biomass in response to organic carbon input, similar to that observed by Muller et al (31). This strongly suggests that the critical E1 degraders in this

study were multiple substrate utilizers, and combined with results from the competition experiment, also suggests that the growth of E1-degrading biomass occurs over a 1- to 5-d period and requires general sources of organic carbon. This is consistent with observations from Bagnall et al. (33) and from the initial cultivation of biomass prior to the competition study (data not shown) that showed E1 degradation taking place with biomass cultivated with a cell residence time of at least 3 days.

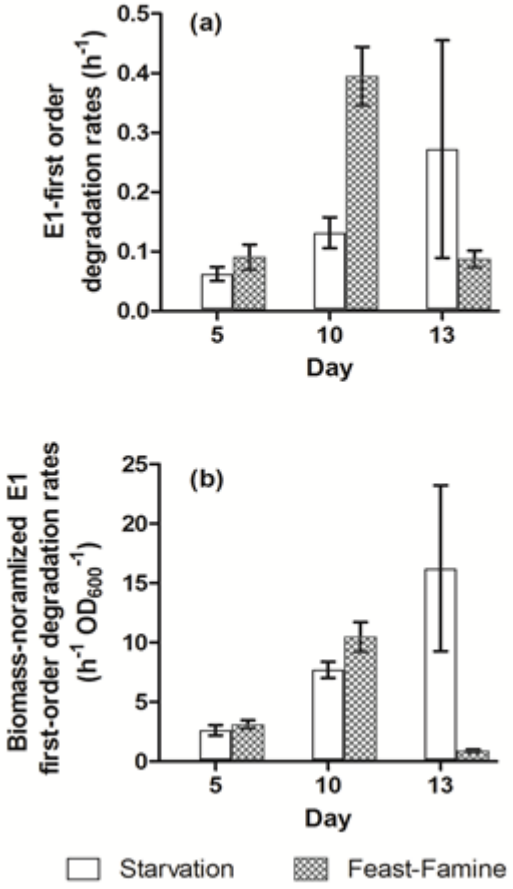

Figure 2.2: Overall (a) and biomass-normalized (b) first-order degradation rates for E1 in starvation and feast-famine reactors. A 6 hour lag phase was observed in starvation reactors on day 13; the reported degradation rate is for the subsequent period.

The subsequent decrease in E1 degradation activity in the feast-famine reactors between Day 10 to Day 13 appeared to be a result of E1 degraders being outcompeted. In spite of increased cell residence time and a continued increase in overall biomass levels, overall E1 degradation rates decreased during this time ($P = 0.014$), and biomass-normalized degradation rates were lower than at the initiation of the feast-famine cycles at Day 5 ($P = 0.027$). Although no structural shifts in community could be detected by ARISA (Figure 2.3), this suggests that community selection under repeated high organic carbon concentrations is detrimental to E1 degradation. This shows that biomass growth does not necessarily control E1 degradation rates; rather, other factors (organic carbon concentration, community structure, etc.) control degradation rates and do so differently depending on the time-scale of interest.

The absence of correlation between both the nMDS analysis of the ARISA data and individual OTUs with E1 degradation rates suggest that E1 degraders formed a very small portion (<0.25% the cutoff threshold for OTU signal/noise used in this analysis) of the microbial community in these reactors. Interestingly, all diversity indices in starvation reactors decreased over time but remained consistent in feast-famine reactors (Table 2.1). It has previously been suggested that increased SRT might improve the degradation of steroid estrogens by increasing microbial diversity (34). In this study, however, E1 degradation rates remained the same or improved in starvation reactors despite a decrease in the Shannon index ($P = 0.041$), while E1 degradation rates decreased in feast-famine reactors in which the Shannon index remained constant ($P = 0.52$). This suggests that

conditions associated with longer SRT values, rather than general diversity, are important for E1 biodegradation.

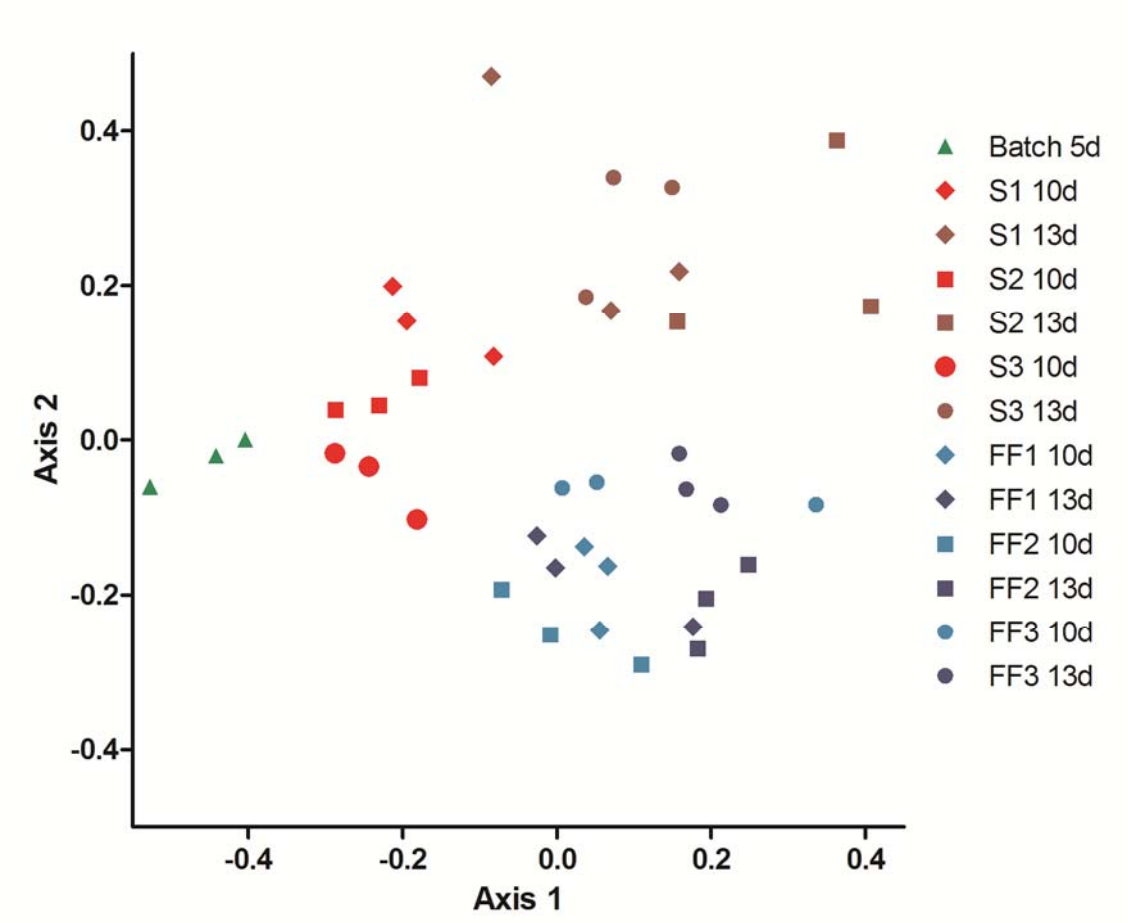

Figure 2.3: nMDS analysis of ARISA of microbial communities in starvation and feast-famine reactors. Distance between data points show relative similarity between communities.

Table 2.1: Microbial diversity analysis from ARISA of starvation and feast-famine reactors[a]

Sample	Richness	Evenness	Shannon Index
Batch 5d	33	0.703	2.46
Starvation 10 d	31±7	0.759±0.006	2.58±0.17
Starvation 13 d	17±3	0.627±0.050	1.77±0.17
Feast-Famine 10 d	30±7	0.708±0.053	2.35±0.22
Feast-Famine 13 d	25±5	0.729±0.047	2.22±0.34

[a] Values are reported as averages ± standard deviation No standard deviation is reported for the batch 5d sample as the sample was taken before biomass division into triplicate starvation and feast-famine reactors.

Organic Loading

Continuous flow MBRs were operated to achieve similar organic carbon concentrations within reactors while supplying influent wastewater at different organic carbon concentrations. This enabled the determination of how carbon loading affected the microbial community, and therefore, E1 degradation. Biological removal of E1 was observed in all reactors and was generally greater than 50%, with effluent concentrations of E1 around or below 1 µg/L (Figure C3). Sorptive loss of E1 to membrane filters was initially observed in killed-control reactors. These losses decreased over time, however, with effluent concentrations of E1 matching influent concentrations by Day 10. Similarly, E1 effluent concentrations in biologically active reactors initially increased over time, but leveled out by Day 16. As such, only E1 data on and after Day 16 (of the 36-day period). were considered in evaluating reactor performance.

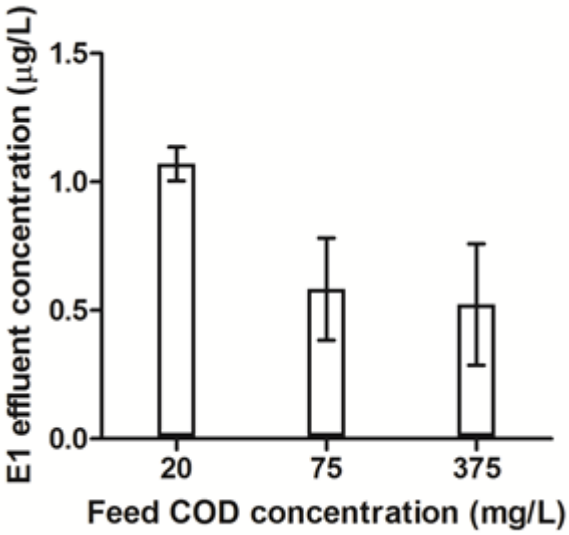

Figure 2.4: Average E1 effluent concentrations for MBRs with synthetic septage containing 20, 75, and 375 mg/L COD. Error bars represent standard deviation for triplicate reactors

In this study, degradation of E1 was slowest (resulting in the highest effluent E1 concentration) at the lowest influent wastewater organic content of 20 mg/L COD ($P = 0.018$, Figure 2.4). No difference in E1 removal was observed between reactors receiving moderate and high organic content synthetic wastewater ($P = 0.76$). All effluent and operating conditions except for influent organic content (loading) and biomass concentration (650 mg/L VSS, 140 mg/L VSS, and 80 mg/L VSS in the reactors receiving 375, 75, and 20 mg/L influent COD, respectively) were similar across reactors.

Analysis of microbial community ARISA profiles via nMDS (Figure C4 and Figure C5) show that communities in MBRs receiving synthetic wastewater with COD values of 20 and 75 mg/L tended to converge over time while MBRs receiving synthetic wastewater with a COD value of 375 mg/L developed distinct communities.

Additionally, the Shannon Index (Table C1) showed similar diversity for the MBRs receiving 20 and 75 mg/L COD (P = 0.54), but reduced diversity in the MBRs receiving 375 mg/L COD (P = 0.043). As with the starvation and feast-famine study, no OTUs appeared to be correlated with E1 removal.

These results, like those from the starvation and feast-famine reactors, suggest that broad microbial community structure and diversity are not correlated to E1 degradation. The MBR results were consistent with the results from the starvation and feast-famine reactors and point to the importance of some organic carbon for maintaining E1-degrading populations. In the MBRs, although organic carbon loading was altered, the organic carbon concentration as measured by COD in the reactors was low and relatively constant (Table C2). This prevented the non-E1-degrading heterotrophs from dominating over time as they had in the feast-famine reactors at Day 13. The higher E1 degradation rates as a result of increased organic loading (to 75 and 375 mg/L feed) was also evident in the MBRs and mirrored what was seen in the feast-famine reactors from Days 5 to 10. Finally, the lower E1 degradation rate in the MBRs fed 20 mg/L organic carbon in the form of synthetic wastewater is consistent with the results from comparing feast-famine and starvation reactors at Day 5. It is therefore likely that a reduced quantity of E1-degrading biomass developed over the 36-d MBR operating period as a result of low organic carbon loads.

Multiple Substrate Utilization

It has been suggested that multiple substrate utilizers are important in E1 degradation (14, 35) because the low levels of E1 present in wastewater are not sufficient

to sustain growth (36), and because biomass grown on more complex carbon substrates have better E1 degrading performance (37). Not much is known about the organisms responsible for E1 degradation, however. It is suggested that multiple substrate utilizers thrive under low substrate conditions and rely on continuous, broad expression of catabolic enzymes instead of specialization and strict metabolic control via mechanisms such as catabolite repression (36). Our results, namely the enhancement of E1 degradation under starvation conditions over 5-13 days and the lack of competitive inhibition over short time periods (8 h) are consistent with these hypotheses. It is known that increased SRT improves E1 degradation (13), but it has also been observed that E1 degraders grow quickly, with efficient E1 degradation taking place with SRT values as short as 3 days (33). Results from this study show both observations to be true, as E1 degrading organisms grew quickly in the feast-famine reactors, while increased cell residence time under starvation conditions (typical in wastewater treatment) enriched for E1 degrading activity relative to the overall biomass.

Potential Applications

Degradation of micropollutants can be inhibited by general organic carbon (38-39), a phenomenon that has been assumed for E1 (18). This work shows a more complicated relationship in the case of E1 degradation, driven by the relationship between organic carbon and multiple substrate utilizing E1 degraders. Increased organic carbon loads can promote the growth of these organisms, enhancing E1 degradation, and indeed, some organic carbon appears to be necessary to maintain E1-degrading activity. Nevertheless, while high organic carbon concentrations are not detrimental over the

short-term, high organic carbon concentrations (as opposed to loading) appear to favor the growth of r-strategist-type heterotrophs incapable of E1 degradation. This occurs even though changes to the overall structure of the community are minor.

Creative designs based on these observations may improve E1 removal. Some minimum COD threshold is needed to generate adequate biomass levels to ensure good E1 degradation. In fact, the poorer removal of estrogens in low organic content wastewater suggests that chemical treatment of certain types of wastewater (i.e., source-separated urine) may be an excellent option for removal of E1 and other micropollutants if the addition of COD is not favored. While decoupling of organic carbon loads from organic carbon concentrations already takes place in continuous flow systems, it may be possible to enrich for and then grow E1 degraders under feast-famine conditions by having longer "famine" periods prior to "feasting." In existing full-scale systems, some mechanism for biomass retention under low carbon conditions, such as a biofilm system or an MBR, could facilitate enrichment of multiple substrate utilizers that seem to be particularly active in E1 degradation.

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Chapter 3: Effects of Estrone and Organic Carbon Exposure on the Degradation of Estrone

Exposure of biomass to estrone (E1) and alternate organic substrates was studied to determine if cometabolism or multiple substrate utilization are key mechanisms for the degradation of E1 and if feeding intervals affect the selection of E1 degrading bacteria. Biomass generated in membrane bioreactors (MBRs) were capable of degrading E1 regardless of E1 exposure. Pre-exposed biomass, however, had higher E1 degradation rates ($P = 0.05$) while un-exposed biomass showed a clear lag phase (6 h) prior to E1 degradation, demonstrating metabolic degradation of E1 by multiple substrate utilizing bacteria. In the feeding interval study, longer intervals between feeding periods selected for E1 degraders at high organic carbon loads (100 mg COD/L/d; $P = 0.018$)), but had no effect at low organic carbon loads (30 mg COD/L/d; $P = 0.4$). Lag phases were observed in E1 degradation during famine periods but were absent during feast periods, showing that the presence of other organic carbon substrates induces the degradation of E1. This research is the first to clearly demonstrate the role of multiple substrate utilizating bacteria in the degradation of E1 and suggests operating conditions that can improve selection for and activity of these organisms.

3.1 Introduction

The ubiquitous nature (1) and adverse ecological effects (2-4) of estrogens in surface water are well known. Municipal wastewater treatment plants (WWTPs) are one of the primary sources of estrogens and other micropollutants to the environment (5). Estrone (E1) is of particular concern because it is a major contributor to estrogenic activity in treated wastewater (6-7) and observed E1 removal rates across WWTPs vary widely (8-11). Improving and optimizing micropollutant removal in municpal WWTPs is therefore critical. A more fundamental understanding of micropollutant degradation, and in particular E1 degradation, is needed to provide the scientific underpinning for improved WWTP operation.

It is thought that cometabolism and/or multiple substrate utilization are involved in E1 degradation in WWTPs (12-14), but neither mechanism has been conclusively indicated. Indeed, as a micropollutant, E1 is present in wastewater at low concentrations. It is therefore unlikely that E1 provides sufficent energy to sustain microorganisms specialized in E1 degradation (15). Furthermore, the efficient degradation of E1 in nitrifying systems (16) is consistent with either cometabolism or multiple substrate utilization. The ammonia monooxygenase enzyme present in nitrfying systems is very non-specific, and may cometabolically degrade E1 (12-13). It is also thought, however, that multiple substrate utilization is associated with oligotrophic stress (17-18), and the low organic carbon present under nitrifying conditions may select for these bacteria, which are also capable of E1 degradation (14).

Distinguishing between these two mechanism is not merely a theoretical exercise, as either would have important, and different, implications for process optimization.

Biomass exposure to E1 and/or other organic carbon substrates will result in different E1 degradation patterns depending on the degradation mechanism. If cometabolism is the key E1 degradation mechanism in wastewater, prior exposure to estrogens will not affect degradation rates, but the presence of cometabolites is crucial. Alternatively, if multiple substrate utilization is the key mechanism, exposure to E1 provides a theoretical competitive advantage, though it may not be large enough to detect. In fact, there is suggestive evidence that prolonged exposure to estrogens can improve degradation performance (19). Additionally, organic carbon exposure would affect E1 degradation by multiple substrate utilizers. It has been theorized that multiple substrate utilizing bacteria are slower growers (20), so exposure to organic carbon may have community selection effects. A previous study showed supporting evidence for this: organic carbon has positive effects via biomass growth but that repeated high organic carbon concentrations selected against E1 degraders (21).

The purpose of this research was to determine the key mechanism for the degradation of E1, cometabolism or multiple substrate utilization, and to explore the effects of biomass exposure to E1 or other organic carbon substrates on E1 degradation upon. Two sets of studies were performed. First, biomass generated in a membrane bioreactor (MBR) with and without exposure to E1 was used in kinetic studies to determine whether cometabolism or multiple substrate utilization was the key mechanism involved in E1 degradation. Next, experiments were performed to determine if the adverse selection effects observed in a previous study conducted in our laboratory (21) could be mitigated by reducing organic carbon concentrations or varying the period

between feeding cycles. Additionally, degradation rates during feast and famine periods during these cycles were compared. Together, these studies provide a much clearer picture of E1 degradation.

3.2 Experimental Section

Chemicals and Synthetic Wastewater

E1 and deuterated or ^{13}C-labeled E1 were obtained from Sigma and Cambridge Isotopes, respectively. The recipe for synthetic wastewater was adapted from Boeije et al. (22) and contained (per L): 75 mg urea, 11 mg ammonium chloride, 12 mg sodium uric acid, 25 mg magnesium phosphate dibasic trihydrate, and 20 mg potassium phosphate tribasic. The synthetic wastewater also contained a carbon source made up of the following (per L, for 100 mg chemical oxygen demand (COD)/L nominal concentration): 6 mg bacteriological peptone, 51 mg sodium acetate, 6 mg dry meat extract, 17 mg glycerine, 21 mg potato starch, and 25 mg low fat milk powder. The carbon source was diluted or concentrated for carbon feeds of various strengths.

Sludge Seed

Biomass used to start each experiment (batch and MBR) was taken from the Metropolitan Wastewater Treatment Plant in St. Paul, Minnesota, which operates with both nitrogen and phosphorus removal. A single sample of activated sludge was triple-washed with phosphate-buffered saline, divided into 3.5 mL aliquots, and cryopreserved in 15% glycerol (v/v) at -80°C until use. A single sludge aliquot was used to seed each reactor.

Batch Systems

Batch systems were used for E1 exposure and feast-famine experiments. Excess aeration via air sparging was provided so that reactors were saturated with dissolved oxygen. pH values over the course of the experiments ranged from 7.2 to 7.6. Reactors were continuously mixed with stir bars.

E1 Exposure Experiment

Continuous flow MBRs were operated as described in a previous paper (21; Chapter 2) to culture biomass with and without exposure to E1. Briefly, reactors (150 mL) were operated with an HRT of 8 h and an SRT of 10 d and fed a synthetic wastewater with a COD of 100 mg/L for a 30-d period (3 SRTs). Reactors were either exposed or not exposed to E1 over this 30-d period (10 µg/L E1 in the influent or no E1 in the influent, respectively). Each treatment was run in triplicate. Following the 30 d period, the reactors were sacrificed and the biomass recovered by centrifuging. The biomass from each MBR was resuspended in a 1-L batch reactor, and biomass concentration in each reactor was determined via a volatile suspended solids (VSS) test. Additional biomass was collected for Illumina analysis. Previously collected effluent from the MBRs was used as reactor liquor. This effluent had pH of 7.4, ammonia concentration of 18.2 mg/L, and COD of 12 mg/L. E1 was added to each batch reactor at a concentration of 10 µg/L, and E1 degradation was monitored over a 22-h period.

Feast-Famine Experiment

The impact of the interval between COD addition on E1 degradation was examined using batch reactors. Two experiments were performed. In each experiment a

10-L batch reactor was seeded with cryopreserved activated sludge, synthetic wastewater with 100 mg/L COD, and 10 µg/L E1. After a 5-d period, this 10-L reactor was split into 3 sets of triplicate 1.0-L reactors. The reactors received 10 µg/L of E1 on Day 0 and were operated for 12 d following one of the two feeding regimes shown in Table 1, resulting in exposure to a total of either 180 or 600 mg/L COD over a 6-d experimental cycle. E1 (10 µg/L) was added periodically to each reactor (Table 3.1) and the E1 degradation rate at that time point was determined over a period of 12 h. Biomass was also collected at the time of each E1 kinetic study and quantified by VSS. The experiments were identical with the following exceptions: (1) different total COD concentrations were fed over a 6-d experimental cycle (Table 3.1) and (2) an E1 degradation study was performed on Day 11 during the low organic carbon experiment, but was not performed during the high organic carbon experiment.

Table 3.1: Feeding and Kinetic Study Schedules

	Low Organic Carbon		High Organic Carbon	
	Feeding schedule	Total organic carbon added over 6 d cycle	Feeding schedule	Total organic carbon added over 6 d cycle
1d Feed	30 mg/L COD each day	180 mg/L	100 mg/L COD each day	600 mg/L
3d Feed	90 mg/L COD on days 0, 3, 6, 9, 12	180 mg/L	300 mg/L COD on days 0, 3, 6, 9, 12	600 mg/L
6d Feed	180 mg/L COD on days 0, 6, 12	180 mg/L	600 mg/L COD on days 0, 6, 12	600 mg/L
Kinetic Studies	Days 6, 11, 12		Days 6, 12	

Sample Processing and Analysis

Detailed procedures for E1 and DNA sampling and analysis are provided in the Apendix D. Briefly, E1 samples were processed via solid phase extraction and silica gel cleanup based on the method by Ternes et al. (23). Processed samples were analyzed via LC-MS as described in Tan et al. (21) or by LC-MS-MS with methods adapted from Di Carro et al. (24).

DNA samples were extracted using the FastDNA spin kit (MP Biomedicals, Solon, OH), the 16S region amplified using primers described by Muyzer et al. (25), and prepared for Illumina sequencing as described by Bartem et al.(26). Illumina sequencing was carried out on an Illumina MiSeq platform at the University of Minnesota Genomics Center (Saint Paul, MN, USA). Illumina sequence reads were processed using Quantitative Insights Into Microbial Ecology (QIIME) (27) and clustered using uclust (28). Reference sequences for each OTU were compared to the Greengenes reference database (29). UNIFRAC was used for principle coordinate analysis of communities (30).

Data Analysis

E1 degradation rates obtained from linear regression and the Student t-test were performed in Microsoft Excel. Community analysis of Ilummina results to detect enrichment at the genus level resulting from E1 exposure was conducted in two ways: (i) ANOVA of the OTUs generated, and (ii) screening of paired reactors for OTUs that were consistently more abundant in reactors with E1 exposure followed by Student t-test of the screened OTUs.

3.3 Results and Discussion

Effect of E1 exposure on E1 degradation: Evidence for multiple substrate utilizers

The occurrence of E1 degradation was not dependent on prior biomass exposure to E1 (Figure 3.1). Prior E1 exposure, however, did affect degradation rates and whether a lag phase occurred, as shown in Figure 3.1. The degradation of E1 in all reactors clearly shows that E1 degraders do not require the presence of E1 to grow. Interestingly, a lag phase of 6 h was observed in cultures without prior E1 exposure, indicating induction of E1 degrading enzymes. This stands in contrast to typical cometabolic patterns where lag phases are not typically observed (31-32). Additionally, higher E1 degradation rates were observed in biomass with prior exposure to E1 ($P = 0.05$), showing that the ability to degrade E1 provided a competitive advantage when E1 was present in MBR influent.

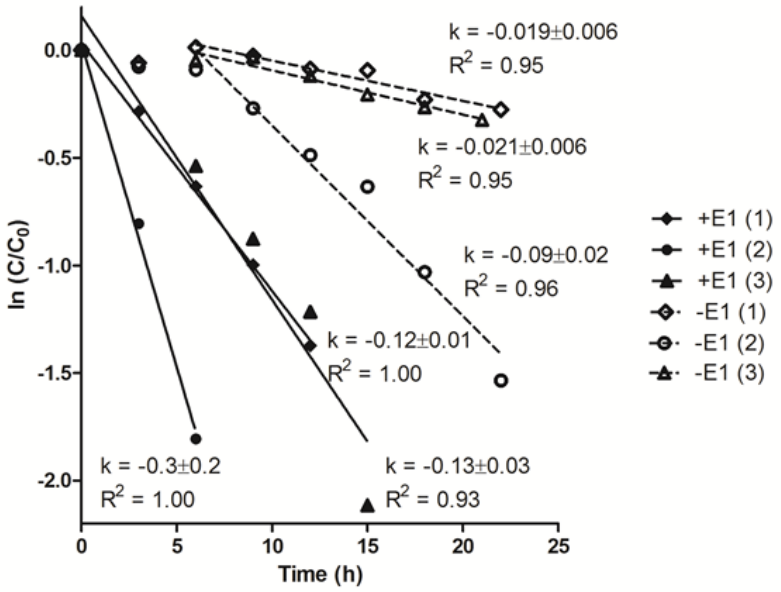

Figure 3.1: Degradation of E1 by biomass with (closed symbols) and without (open symbols) prior exposure to E1. First-order degradation rate coefficients are shown as k values. Error values on the k values represent 95% confidence intervals.

Principal coordinate analysis of Illumina sequences, shown in Figure 3.2, showed no broad community effects from E1 exposure. Instead, the initial sludge seed appeared to drive variation in communities. Analysis of operational taxonomic units at the genus level (Figure D1), and at the species level for abundant genus (data not shown), did not show enrichment resulting from E1 exposure, suggesting that (i) if E1 degraders are distinguished by specific genus/species, they may be a very small fraction of the population, or (ii) that multiple genus/species may degrade E1.

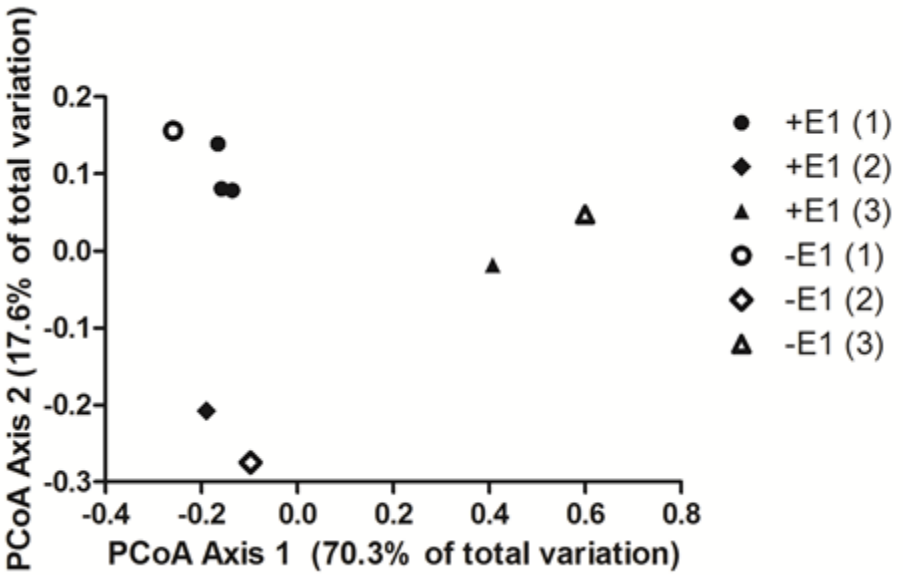

Figure 3.2: Principal coordinate analysis of Illumina sequences of microbial communities with and without prior exposure to E1. Closed symbols represent biomass with E1 exposure and open symbols represent biomass without E1 exposure. One sample (+E1 (1)) was analyzed in triplicate. Reactors were run in pairs, hence the designators (1), (2), and (3), using the same initial sludge seed.

These observations demonstrate that multiple substrate utilizers are involved in the degradation of E1. This hypothesis has been suggested by others (19-20, 33). Prior evidence for the role of multiple substrate utilization in E1 degradation includes: higher

degradation rates associated with municipal wastewater biomass compared to industrial wastewater biomass (34); improved E1 degradation with extended exposure to E1 (19); possible metabolic degradation of E1 (35); and higher rates of estrogen degradation in the presence of diverse food sources, which may favor growth of multiple substrate utilizing bacteria (33). The results shown herein, however, are the first that we are aware of that directly compare the effect of E1 exposure on similarly cultured biomass, clearly proving the role of multiple substrate utilization in the biodegradation of E1.

Effect of carbon exposure on E1 degradation

Famine conditions appeared to induce a lag phase prior to E1 degradation, suggesting that exposure to some minimal level of organic carbon was beneficial to E1 degradation. A clear 4 h lag phase was observed in E1 degradation experiments for the 6-d feed reactors on Day 11; 2-4 h lag phases seemed to occur for the 3-d feed reactors as well (Figure D4). No comparable lag phases were observed for any reactors during feast periods (Figures D2-D3). In contrast to lag phase effects, the presence of other carbon sources did not appear to affect the rate of E1 degradation, because the degradation rate for a given reactor was similar on Days 11 and 12. While the presence of a certain low level of organic carbon is beneficial for inducing the degradation of E1, there did not appear to be additional benefits, particularly in terms of degradation rates, of higher organic carbon concentrations.

Figure 3.3 shows that longer intervals between feeding cycles was beneficial for reactors receiving higher COD loads, suggesting that feeding cycles can be important for selection of E1 degraders. Among the reactors receiving higher COD loads, those fed

every 6 d were the only reactors to have a statistically significant increase in overall or biomass-normalized E1 degradation rates between Days 6 and 12 (P = 0.0065 and 0.029, respectively). The increase in biomass-normalized rates indicates that this change in performance is not a result of only biomass growth, but was also a result of community development. At Day 12, these reactors had significantly higher degradation rates than those fed every day (P = 0.018); rates were not statistically distinguishable from those fed every 3 d (P = 0.148).

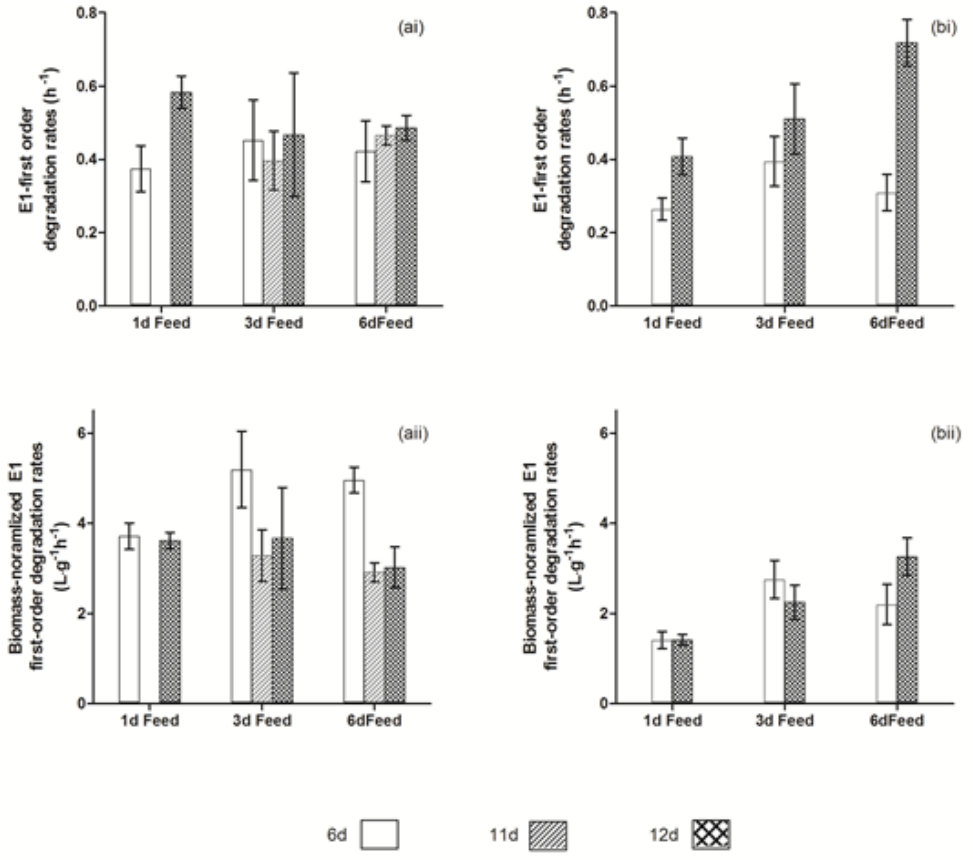

Figure 3.3: E1 degradation rates in feeding cycle experiments. Figures (ai) and (bi) show overall degradation rates for reactors receiving low and high organic carbon loads, respectively, while Figures (aii) and (bii) show degradation rates normalized to biomass concentrations (as measured by VSS) for reactors receiving low and high organic carbon loads, respectively. Error bars represent the standard deviation for triplicate reactors.

Conversely, among the reactors receiving lower COD loads, the longer interval between feeding did not confer any advantage, suggesting that the importance of feeding cycles for selection of E1 degraders depends on the abundance of food. Overall and biomass-normalized degradation rates were similar across these reactors on Day 12 ($P = 0.40$ and 0.78 respectively). For this group of reactors, only those fed daily showed an increase in overall E1 degradation rates ($P = 0.0031$). This change appears to be attributable to the increase in biomass, as biomass-normalized degradation rates did not change ($P = 0.85$). Additionally, biomass-normalized degradation rates appeared to drop between Days 6 and 12 for the 6-d feed reactors ($P = 0.058$). It is not clear why this occurred, but overall degradation rates did not change.

The difference in E1 degradation behavior in the presence and absence of exposure to other carbon sources is further evidence of multiple substrate utilization of E1 and gives us further insight into this mechanism. As in the previous experiment, if cometabolism were the mechanism of degradation, no lag phase would be expected during the famine period. Additionally, during this period, slower degradation rates or the absence of E1 degradation might be expected as a result of a lack of cometabolites. As such, the similarities in degradation rate between Day 11 and 12 are consistent with multiple substrate utilization. We had not predicted, however, a lag phase during famine conditions based on this mechanism. At least one other study has observed a similar reduced lag phase for micropollutant degradation, not attributable to microbial growth and following exposure to supplementary organic carbon (36). It is known that oligotrophic stress can cause de-repression of the catabolome (37), facilitating multiple

substrate utilization. The prevailing view of this behavior is that of multiple substrate utilizing bacteria producing a large variety of enzymes at all times to scavenge food (15, 37). We hypothesize that in addition to de-repression and constant production of a variety of enzymes, exposure to carbon sources may up-regulate the production of this suite of enzymes. Further work needs to be performed to explore and confirm this idea, which would lead to a better understanding of the metabolic activity of multiple substrate utilizers.

Recovery time from exposure to high concentrations of organic carbon may be important for the selection of E1 degraders. In a previous paper (21; Chapter 2), we observed that repeated exposure to high levels of organic carbon selected against E1 degraders. We had also observed that cultures typically took between 3-5 d to develop E1 degrading capabilities. Consequently, these microbes might be outcompeted by faster growing bacteria when subjected to daily feeds. The current study tested the hypothesis that these effects could be mitigated by longer intervals between feeding, and the improved performance of the reactors receiving the higher COD load at 6 d intervals is consistent with this idea. The reactors exposed to lower levels of organic carbon did not seem to be subject to the same selective pressures, presumably because the lower concentrations of organic carbon reduced the competitive disadvantage of the E1 degraders. Consequently, no differences in E1 degradation performance were observed as a result of changing feeding intervals across that set of reactors.

E1 and carbon exposure: Strategies for optimizing E1 degradation

Optimizing E1 degradation requires generating a sufficient quantity of biomass while maintaining a selection pressure for E1 degraders. Understanding that these bacteria are multiple substrate utilizers points us in certain directions for achieving optimization. This study illustrates that carbon and E1 exposure are critical for selecting for multiple substrate utilizing, E1 degrading bacteria. While it is not practical to control E1 exposure in wastewater treatment systems, organic carbon concentrations are a key operating parameter. In this study, we observed that reactors receiving lower COD loads tended to have higher biomass-normalized degradation rates. Conversely, the highest E1 degradation rates were observed in the 6d feed reactors receiving higher COD loads, generating more biomass. This is consistent with our previous paper (21; Chapter 2) that showed that high COD concentrations, rather than high COD loads, select against E1 degraders. Conventional continuous stirred tank reactors achieve this through continuous feeding to maintain low COD concentrations while simultaneously achieving high COD loads and therefore higher biomass growth. While CSTRs are used for industrial wastewater treatment, they are not commonly used for municipal wastewater treatment. An alternate strategy of recovery time between feeding periods was explored in this study and also appears to be a feasible option. This approach, however, may cost more in terms of sludge storage requirements. In addition to E1 and organic carbon exposure, there are further factors that may influence selection of multiple substrate utilizing bacteria that need to be explored, including the diversity of food sources, as theorized by Racz et al.

(33), and the quality of organic carbon available, which could have selection effects on seemingly slower growing multiple substrate utilizers.

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Chapter 4: Effects of Organic Carbon Quality on the Growth and Selection of Estrone Degrading Bacteria

Biomass was grown on organic matter of various qualities to explore the impact on E1 degrading ability. Synthetic septage was aged, filter-sterilized, and used as a food source for biomass to determine if growth using more recalcitrant organic carbon would enhance E1 degradation. Higher E1 degradation was observed by biomass grown on 8 d old synthetic septage compared to biomass grown on fresh synthetic septage ($P = 0.033$) despite the much lower concentration of bacteria. Minimal or no E1 degradation was observed in biomass grown on 2 d old synthetic septage. Dissolved organic carbon concentrations and other organic carbon analyses suggest that products of cell lysis or microbial products released under strarvation stress stimulate E1 degradation. Biomass was also grown in other water sources: lake, river, and effluents from a municipal wastewater treatement plant and a treatment wetland, to determine which water sources are conducive to E1 degradation. E1 degradation was only observed in biomass grown in treatment effluent. Nitrogen, dissolved organic carbon, and trace element concentrations were ruled out as causative factors for E1 degradation. In both sets of experiments, spectrophotometric analyses show that degradation of E1 is associated with microbially derrived organic carbon but not general recalcitrance (as measured by aromaticity).

4.1 Introduction

The presence of micropollutants in wastewater presents multiple challenges for treatment because of the diversity of these compounds and the target concentrations necessary to avoid environmental impacts. Advanced treatment processes such as advanced oxidation and membrane treatment can be effective at treating micropollutants (1-3). Nevertheless, the high cost and energy requirements of these processes and the generation of byproducts remain substantial issues (4-6). For biological treatment, efficacy of micropollutant removal is affected by solids retention time (SRT), hydraulic retention time, temperature, and redox conditions (4). Little is known, however, about organic carbon quality effects on the activity and growth of micropollutant-degrading bacteria (7).

Organic carbon quality may be associated with micropollutant removal. Multiple substrate utilization and cometabolism are thought to be the two most likely paths for micropollutant removal in wastewater treatment systems (4). Low organic carbon conditions are conducive to multiple substrate utilization (8-9). It is possible that the recalcitrant organic carbon present under these oligotrophic conditions may be important for stimulating activity. Likewise, conditions associated with improved removal of various micropollutants such as long SRTs or nitrification (10-12) also result in lower concentrations and changes in the quality of organic carbon. If organic carbon quality is indeed a factor in micropollutant removal, this would have important implications for the design and operation of treatment systems.

Estrone (E1) is an important micropollutant because of its prevalence (13), contribution to estrogenic activity (14-15), and ecological effects (16-19). Our previous study also showed removal of this compound by multiple substrate utilizing bacteria in mixed substrate, mixed culture systems (20; Chapter 3). For these reasons, E1 was chosen as the target compound and model micropollutant for this study.

The purpose of this research was to explore how organic carbon quality affects the activity of E1-degrading bacteria. Two sets of studies were performed. First, biomass was grown in fresh and aged synthetic septage mixtures to determine if more degraded and aged organic carbon would select for E1 degrading capability. Next, biomass was grown in various waters taken from treatment facilities and natural sources to determine if the trends observed in the first study were applicable to a wider variety of organic carbon types and to observe other trends that might emerge. Organic carbon quality was characterized through a variety of spectrophotometric methods. Together, this work increases our understanding of E1 degradation and points toward possible directions to further explore the relationship between organic carbon quality and micropollutant degradation.

4.2 Experimental Section

Chemicals and Synthetic Wastewater

E1 and deuterated or ^{13}C-labeled E1 were obtained from Sigma and Cambridge Isotopes, respectively. The recipe for synthetic wastewater was adapted from Boeije et al. and contained (per L): 75 mg urea, 11 mg ammonium chloride, 12 mg sodium uric acid, 25 mg magnesium phosphate dibasic trihydrate, and 20 mg potassium phosphate tribasic.

The synthetic wastewater also contained a carbon source made up of the following (per L, for 100 mg chemical oxygen demand (COD)/L nominal concentration): 6 mg bacteriological peptone, 51 mg sodium acetate, 6 mg dry meat extract, 17 mg glycerine, 21 mg potato starch, and 25 mg low fat milk powder. The carbon source was diluted or concentrated for carbon feeds of various strengths.

Sludge Seed

Biomass used to prepare aged synthetic septage mixtures and initial microbial cultures were taken from the Metropolitan Wastewater Treatment Plant in St. Paul, Minnesota, which operates with both nitrogen and phosphorus removal. A single sample of activated sludge was triple-washed with phosphate-buffered saline, divided into 3.5 mL aliquots, and cryopreserved in 15% glycerol (v/v) at -80°C until use. A single sludge aliquot was used to seed each reactor.

Batch Systems

Batch systems were used for E1 exposure and feast-famine experiments. Excess aeration via air sparging was provided so that reactors were saturated with dissolved oxygen. pH values over the course of the experiments ranged from 7.2 to 7.6. Reactors were continuously mixed with stir bars.

Aged Synthetic Septage Study

To determine if organic carbon quality affects the growth of E1 degrading bacteria, microbial cultures were grown in batch reactors with fresh or aged synthetic septage. Aged septage was prepared by seeding a reactor containing fresh synthetic septage with an initial COD of 100 mg/L. Reactor liquor was filter-sterilized after 2d and

8d and refridgerated until use, and water quality analysis was conducted as described below. An E1 degrading culture was also prepared in advance by seeding a reactor containing fresh synthetic septage with an initial COD of 100 mg/L and operated until E1 degrading activity was observed (5 d). This biomass was sepeated from the reactor liquor by filtration with a glass fiber filter, and 1 mg (wet weight) of biomass was added to batch reactors containing 1L of fresh, 2 d, or 8 d synthetic septage mixtures, and 10 µg/L of E1 . These reactors were then opeated for 5 d, after which an addition 10 µg/L of E1 was added and E1 degradation was monitored over a 24 h period. Biomass samples were also collected for DNA analysis. A follow-up study was carried out to check the effects of organic carbon concentration. Reactors with 8 d synthetic septage and a 1:2 diluted 8 d synthetic septage (using the synthetic septage receipe without organic carbon elements) were set up and opeated as described previously.

Water Sources Study

To further explore the effects of organic carbon quality on growth of E1 degrading bacteria, microbial cultures were grown in batch reactors with water taken from: (i) effluent from the secondary clarifier at the Metropolitan Wastewater Treatment Plant in St. Paul, Minnesota; (ii) effluent from a treatment wetland at the Fields of St Croix Wastewater Treatment Plant in Lake Elmo, Minnesota; (iii) lake water from the USGS Cottonwood Lakes Study Area in Jamestown, North Dakota; and (iv) the St Croix River upstream of Stillwater, Minnesota. Water sources were filter-sterilized and refridgerated until use, and water quality analysis was conducted as described below. Reactors were set up and operated in triplicate as described above. A follow-up study was

carried out to rule out the effects of nitrogen concentrations, using wetland water, and river and lake water supplemented with ammonium, nitrate, and nitrite to match the wetland nitrogen concentrations.

Water Quality Analysis

Filter-sterilized synthetic septage and water samples were analyzed for dissolved organic carbon (DOC), ammonium, nitrate, and nitrite. Trace element analysis was also carried out on water samples. DOC was measured using a GE Sievers 900 Portable TOC Analyzer. Ammonia measurements were taken using an Orion 9512HPBNWP ammonia probe (Thermo Scientific), and a 5-point standard curve ranging from 1.4 to 140 mg/L as ammonium (typical R^2 values of 0.99). Nitrate and nitrite were quantified by colorimetric analysis using the cadmium reduction method with a Lachet 8500 FIA and multi-element inductively coupled plasma atomic emission spectroscopy was used for trace element analysis by the Research Analytical Laboratory (St Paul, MN, USA)

Organic Carbon Analysis

Organic carbon was characterized via specific ultraviolet absorbance (SUVA), excitation-emission spectrum (EEMs), fluorescence index, and slope ratios. Filtered solutions were analyzed for ultraviolet/visible light absorbance at 254 nm, and from 275-400 nm using a Shimadzu UV-1601 PC spectrophotometer and a quartz cuvette. SUVA was calculated by normalizing UV_{254} by the DOC concentration (21). Slope ratios were calculated by using the ratio of the absorbance slope at 275-295 nm to the absorbance slope at 350-400 nm (22).

EEMs were obtained using a Jobin-Yvon Horiba Floromax 3 fluorometer with xenon lamp according to the method developed by Cory and McKnight (23). An excitation range of 240 to 600 nm (5 nm intervals) and an emission range of 350 to 550 nm (2 nm intervals) were used. Samples were first analyzed for UV_{254}, and samples with an absorbance greater than 0.3 were diluted to avoid inner filter effects. The fluorescence index (FI) was calculated from EEM measurements, using the ratio of emission intensity at 470 nm to that at 520 nm, at an excitation of 370 nm (24).

E1 and DNA Sample Processing and Analysis

Detailed procedures for E1 and DNA sampling and analysis are provided in the Supporting Information. Briefly, E1 samples were processed via solid phase extraction and silica gel cleanup based on the method by Ternes et al (25). Processed samples were analyzed via LC-MS as described in Tan et al (26; Chapter 2) or by LC-MS-MS with methods adapted from Di Carro et al (27).

DNA samples were extracted using the FastDNA spin kit (MP Biomedicals, Solon, OH), and analyzed for (i) the 16S rRNA gene; (ii) amoA gene (which was not detected); and (iii) Illumina sequencing. Quantitative PCR (qPCR) analysis was conducted using an Eppendorf Mastercycle ep *realplex* thermal cycle (Eppendorf, Westbury, NY), using primers described by Muyzer et al. and Harms et al. for the 16S rRNA and amoA genes respectively (28-29). For Illumina sequencing, the 16S region was amplified using primers described by Muyzer et al. and prepared for Illumina sequencing as described by Bartem et al (28, 30). Illumina sequencing was carried out on an Illumina MiSeq platform at the University of Minnesota Genomics Center (Saint Paul,

MN, USA). Illumina sequence reads were processed using Quantitative Insights Into Microbial Ecology (QIIME) (31) and clustered using uclust (32). Reference sequences for each OTU were compared to the Greengenes reference database (33). UNIFRAC was used for principle coordinate analysis of communities (334).

Data Analysis

E1 degradation rates obtained from linear regression and the Student t-test were performed in Microsoft Excel.

4.3 Results and Discussion

Effects of Aging Synthetic Septage on Selection of E1 Degrading Communities

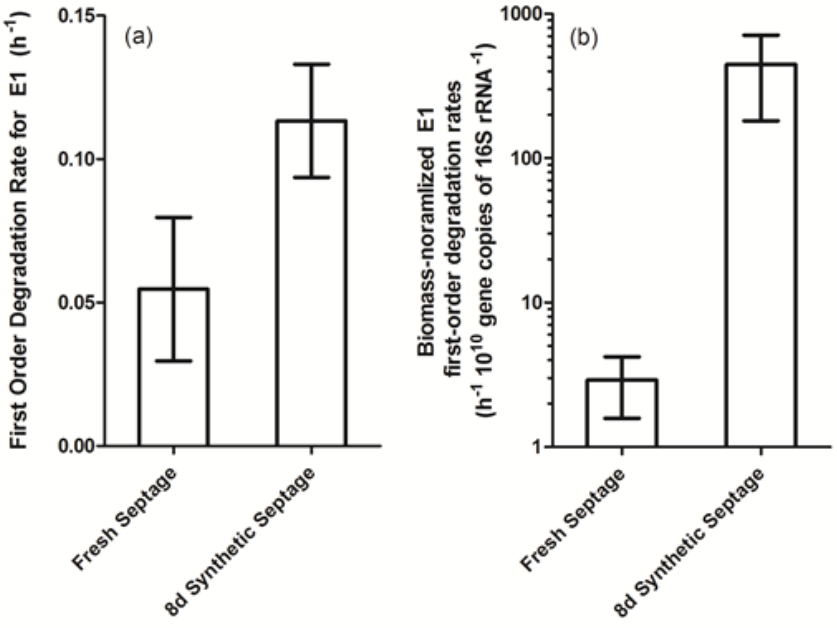

Figure 4.1: Overall (a) and biomass normalized (b) 1st order E1 degradation rates for biomass grown in fresh and aged synthetic septage. Error bars represent standard deviations for triplicate reactors. Note that biomass normalized data is shown on a log-scale for clarity.

Biomass cultured in 8d old septage had much higher E1 degradation rates than biomass cultured in fresh synthetic septage (P = 0.033), despite the lower biomass concentrations present (3.7 x 10^6 vs 1.9 x 10^8 16S rRNA gene copies per mL). In addition, E1 degradation was observed in only one of the three reactors containing biomass cultured in 2d old septage (k = 0.017 h^{-1}), following a 12 h lag phase (Figure E2). Starting pH values were similar across all reactors (7.5-7.8), while initial ammonium concentrations were somewhat lower but still abundant in the 2d old septage reactors (Table E1). No AMO genes were detected in any of the reactors, suggesting that ammonium oxidizing bacteria did not play a critical role in E1 biodegradation in our study. Initial DOC concentrations in the 2d old septage were half that of the 8d old septage (5.04 ppm vs. 11.25 ppm), both of which were lower than that in the fresh synthetic septage reactors (35.8 ppm)

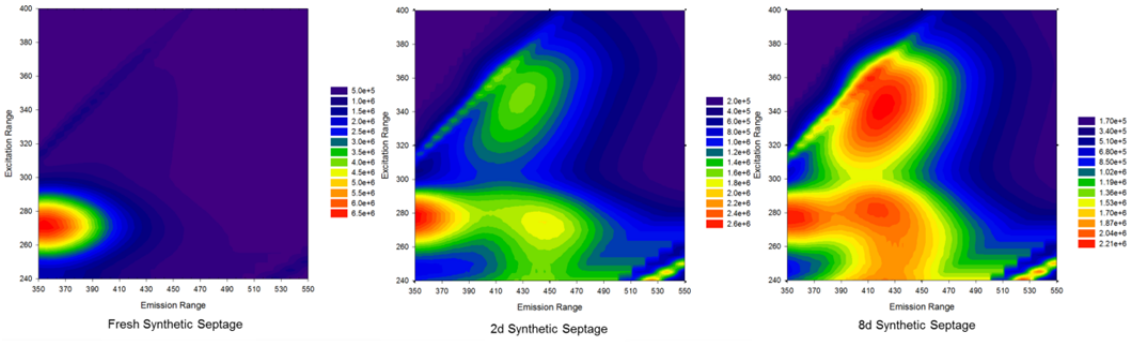

Figure 4.2: EEMs of reactor liquor at the beginning of aged septage experiments. For comparison purposes, plots have the same number of contours to accentuate differences in shape (23), so scales for individual plots vary.

The quality of organic carbon in each of the reactor liquors changed progressively over time as a result of microbial activity during aging, as seen clearly in EEMs of the

reactor liquors at the beginning of the 5d incubation period (Figure 4.2). Fresh septage had a single peak at excitation wavelength 270 nm emission wavelength 350 nm (270 → 350). 2d old septage formed new visible peaks at 350 → 420 and 270 → 445, while 8d old septage saw the 270 → 445 peak shift or become encompassed by a new peak at 280 → 420. Emissions at 350 nm are associated with amino acids (35) and were the dominant component in the fresh synthetic septage. Emissions at 420 nm are associated with fulvic acids (35) and increased with septage age, suggesting that the composition and concentration of these compounds increased over time.

In addition to EEMs, fluorescence index values (36-37) and spectral slope ratios (21) both indicated that the 8d septage contained DOC with a more microbiologically derived character than the 2d septage (Table E2). Interestingly, SUVA values, which are positively correlated with aromaticity and thought to increase with DOC weathering (21, 38), were lower for the 8d septage than the 2d septage (Table E2), indicating that the 8d septage was less aromatic than the 2d septage. The reason for this is unclear, although this may be a result of cell lysis under starvation conditions.

Microbial community analysis showed that communities in all reactors shifted over the 5d growth period (Figure 4.3). Taxonomic analysis of these communities is shown in Supporting Information (Figure E3). Communities in the 2d and 8d septage reactors developed similarly and were distinct from the communities in the fresh synthetic septage reactors, which might be expected from the initial organic carbon compositions. These results show that broad community structure is not predictive of E1 degrading ability, because distinct communities (fresh septage and 8d old septage) shared

the ability to degrade E1, while similar communities (2d old septage and 8d old septage) did not.

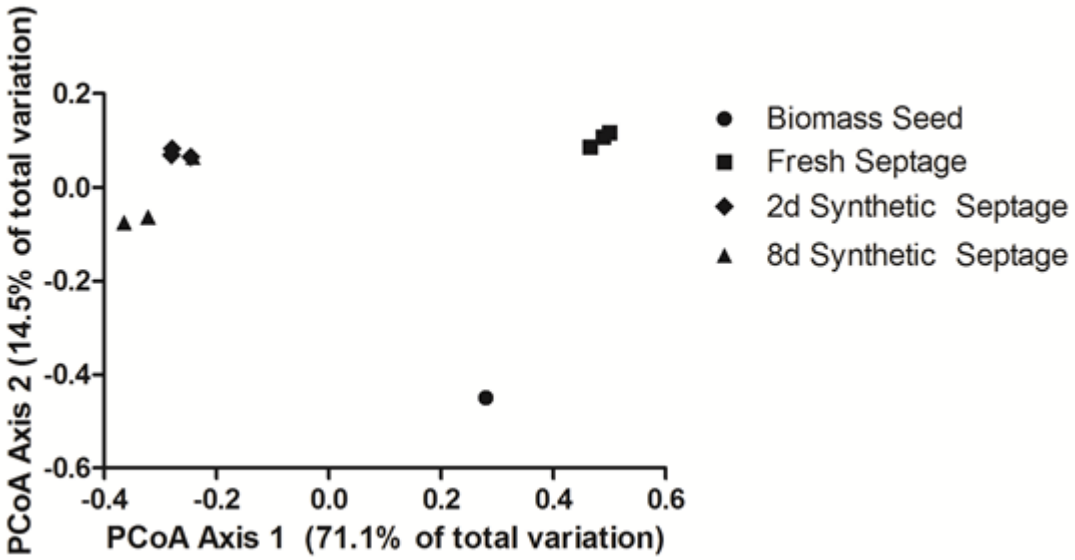

Figure 4.3: Principal coordinate analysis of Illumina sequences of microbial communities grown in synthetic septage of different ages and the initial seed. All communities diverged from the initial seed, but communities in the 2d and 8d synthetic septage cluster together while the community grown in fresh synthetic septage is distinct.

It was surprising that E1 degrading ability was strongest in communities grown on 8d septage but absent in communities grown on 2d septage given the similar patterns in community and carbon quality. To determine if E1 degradation was subject to the presence of some minimum carbon threshold, the 8d old septage study was repeated, using diluted and undiluted 8d septage (Figure E4). E1 was degraded in both sets of reactors, indicating that there was no apparent DOC concentration threshold at which E1 degradation "turned on". These results suggest that differences in organic carbon quality,

rather than concentration, were responsible for the dramatic changes observed in the E1 degradation activity of the biomass present.

Effects of Water Sources on Selection of E1 Degrading Communities

To further investigate the impact of organic carbon quality on E1 degradation, E1 degradation by biomass cultured in different water sources was investigated. E1 degradation occurred only with biomass cultured in WWTP and treatment wetland effluents (Table 4.1). Differences in reactor DOC and nitrogen (Table 4.1), did not affect E1 degradation ability, while trace element concentrations (Table E3) also did not appear to be an important factor. Furthermore, in a follow-up study wherein river and lake water was supplied with ammonia and nitrate to the levels found in the wetland samples, E1 degradation did not occur (Table 4.1). Again, these results point toward organic carbon quality as a critical determining factor in E1 degradation in these reactors

Table 4.1: E1 degradation coefficients and associated DOC and nitrogen concentrations for the water sources study.

Water Sample	1^{st} Order E1 Degradation Coefficient (h^{-1})	DOC (ppm)	Ammonia (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)
River	0	2.67	1.02	1.30	0.04
Lake	0	25.8	0.32	0.0	0.00
Wetland	0.48 ±0.14	12.9	41.27	6.5	3.50
Metro	0.38 ± 0.20	14.2	29.07	5.10	0.07
River + N	0	2.67	41.27	6.5	3.50
Lake + N	0	25.8	41.27	6.5	3.50
Wetland (2)	0.20 ± 0.06	12.9	41.27	6.5	3.50

Analysis of the organic carbon in these samples via EEMs show distinct differences between the water sources that were (Metro and wetland) or were not (river

and lake) associated with E1 degradation. In the Metro and wetland water sources EEMs (Figure E5) show emissions at 350 nm, which are associated with amino acids (35). This signal is absent in the river and lake water sources. The Metro and wetland sources had a peak at 335 → 420, while the river and lake had a peak at 310 → 415. Fulvic acid peaks at the longer excitation wavelength are associated with treated wastewater while those at the shorter wavelengths are associated with natural waters (35; 39). For comparison, septage degradation products generated in the previous experiment after 2d or 8d had a peak at 345 → 420. All samples also had a peak around 250 → 440.

Additional analyses of organic carbon quality (Table 4.2) are consistent with the EEMs observations. Higher fluorescence index values were observed in the Metro and wetland sources, which are associated with microbially derived fulvic acids (36-37). Likewise, the wetland water source had a lower slope ratio than the other sources, which is again consistent with microbially derived organic carbon (21). It was surprising that the treated wastewater effluent had a similar slope ratio to the river and lake waters, given the biological treatment origin of this water source. We hypothesize that exposure to sunlight may have elevated the slope ratio in this water source (21). As in the previous experiment, E1 degradation was not associated with aromaticity as measured by SUVA (21, 38).

Table 4.2: Organic carbon parameters for water sources study

Water Source	SUVA	Fluorescence Index	Slope Ratio
River	2.55	1.56	1.10
Lake	2.00	1.52	1.13
Metro	1.61	1.82	1.10
Wetland	1.99	1.63	0.94

Organic Carbon Quality as a Key Parameter for Selection of E1 Degrading Communities

Together, these results point to organic carbon quality as a critical determinant with respect to E1 degradation. Specifically, microbiologically derived organic matter seems to be associated with E1 degradation. In the first study, the highest E1 degradation rates were observed for biomass grown in the 8 d old septage, which also contained organic carbon that appeared to be the most microbiologically derived, as measured by fluorescence index and slope ratio. Presumably, the reactors with fresh septage generated these microbial products over the five-day incubation period prior to the kinetic study, enabling the degradation of E1 in these reactors. In the second study, water sources from biological treatment systems could be used to cultivate E1 degradation ability while the river and lake water sources were not, with both the fluorescence index and EEMs patterns showing microbially derived DOC in the former water sources.

We also hypothesize that products from cell lysis or microbial products released after prolonged starvation are important for the E1 degradation activity of organisms. The 2 d old septage reactors in the first study were unable to degrade E1 despite the presence of microbially derived DOC. It seems that the microbial products generated between Days 2 and 8 were important for the growth of E1 degraders. Given the higher DOC levels in the 8 d old septage compared to the 2 d old septage, this DOC would seem to be from cell lysis or some microbial product released under stress rather than degradation byproducts. The lower aromaticity of the 8d old septage is consistent with this hypothesis. The low amount and recalcitrant nature of the organic carbon in the 2 d old septage might have hindered cell growth and generation of these products during the 5 d

incubation period, preventing the selection of E1 degraders. If correct, this would explain the ability of E1 degraders to grow in water sources associated with biological treatment, but not in the natural water sources. In contrast, aromaticity, as measured by SUVA, was not predictive of E1 degrading ability in either study. While we do not know the specific component of organic matter driving the different E1 degrading ability of the biomass, the study clearly shows that organic carbon quality affects the growth of E1 degraders. These effects result in much faster biodegradation of E1 in treatment systems than in the environment, which should be beneficial. This study also provides information regarding how to further stimulate E1 degradation, with the design of systems that concentrate microbiologically derived DOC.

Organic Carbon Quality and Micropollutant Removal

Prior to this study, there was little known about how DOC affects the biodegradation of micropollutants. This is an important contribution because it not only improves our understanding of E1 degradation, but may also have implications for the biological treatment of other micropollutants. What little is known appears to have been focused on the effect of organic carbon quality on the degradation of the pesticide linuron (7; 40-41). In contrast to our study, humic acids and river DOC had positive effects on linuron degradation in batch systems, while a more readily degradable DOC source (maize leaf leachate) had negative effects (7). These effects, however, were not observed for biofilms in continuous-flow systems (41). Additionally, DOC effects when linuron was present at mg/L concentrations were not predictive of DOC effects when linuron was present at micropollutant (μg/L) concentrations (40-41). Evidently, DOC affects

biological degradation of micropollutants, but the specific effect may be dependent on the micropollutant and operating setup. The removal of micropollutants from wastewater is an important challenge today. To meet this challenge, additional work needs to be performed to verify the importance of DOC quality and micropollutant degradation, and to clarify the exact relationship between DOC quality and degradation of E1 and other micropollutants.

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Chapter 5: Conclusions and Recommendations

The effects of organic carbon on the biodegradation of E1 in multiple substrate, mixed-culture systems were explored in this work. The objectives were to: (i) determine if catabolic repression, multiple substrate utilization, and cometabolism are important mechanisms in E1 degradation; (ii) determine if organic carbon exposure and loading affect growth and selection of E1 degrading bacteria; and (iii) determine if organic carbon quality affects growth and selection of E1 degrading bacteria. From the results described previously, we can draw the following conclusions:

First, of the mechanisms studied, multiple substrate utilization is key in E1 degradation. Catabolic repression by general organic carbon was shown not to be in effect for E1 degradation while no evidence was found for cometabolism. The role of multiple substrate utilizers in E1 degradation can be exploited to improve E1 removal in WWTPs by determining the operational parameters that select for these organisms. As these parameters are further defined, additional work needs to be done to determine if these parameters also (i) select for known isolates that degrade E1, which will provide evidence for the key species of bacteria for E1 removal in WWTPs, and (ii) improve the degradation of other micropollutants.

Second, general organic carbon can promote the growth of E1 degrading bacteria, as might be expected of multiple substrate utilizing organisms. Repeated exposure to high concentrations of organic carbon, however, selects against these organisms. Conversely, E1 degradation capability is maintained under starvation conditions, though this can

induce lag-phases of several hours before degradation activity is observed. This suggests that E1 degrading bacteria are slower growing and capable of thriving under low organic carbon conditions. This is consistent with the efficient removal of E1 observed in WWTPs with nitrogen removal, as nitrifying systems require both long SRTs and low organic carbon concentrations. These negative selection pressures can be mitigated by extending the period between exposures to high organic concentrations, which may have implications for batch or plug-flow reactor operation.

Finally, organic carbon from microbial sources appears to select for E1 degrading bacteria, while general aromatic/recalcitrant organic carbon does not do so. This suggests that removal is most effective in WWTP systems, where these compounds are present at high concentrations. Because very little is currently known about organic carbon quality and micropollutant removal, this finding is important and suggests possible avenues for further exploration. Additional work needs to be done to determine if the positive effects of microbially derived organic carbon is unique to E1 degrading bacteria, or if it applies to other multiple substrate utilizing bacteria, particularly those that degrade other micropollutants.

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Appendix A: Additional Experiments Showing Short-Term Disruption of E1 Degrading Ability

In addition to the studies described in the main body of the dissertation, two additional sets of studies were conducted on E1 biodegradation. In each case, biomass was transferred from one reactor liquor to another. This transfer appeared to inhibit E1 degradation for up to a 24 h period, which rendered the studies unfeasible.

The first study examined the effects of extended starvation periods on biomass from continuous-flow systems on E1 degradation. It was shown in Chapter 2 that starvation of biomass improved E1 degradation performance over time. It was uncertain, however, if these benefits would be significant for biomass that already had exposure to low organic carbon concentrations and an extended SRT. Biomass was grown in triplicate MBRs as described in Chapter 2, with a 10 d SRT and an 8 h HRT. Following 30 d of operation, two 50 mL samples of reactor liquor and associated biomass was withdrawn from each reactor and incubated aerobically or anaerobically under batch conditions over a 5 d period, during which the MBRs continued normal operation. After the 5 d period, biomass was transferred into 1 L reactors containing a minimal media and an E1 kinetic study was conducted. The purpose of the experiment was to ascertain (i) if the starved biomass had better E1 degradation performance, and (ii) if this the starvation benefit occurred under anaerobic conditions (E1 degradation requires aerobic conditions). If this study had been successful, follow-up work would be done on re-introducing starved biomass into continuous culture to determine if the improved E1 degradation performance would persist. This experiment was run twice, and E1 degrading ability of

the biomass prior to transfer was confirmed. However, no E1 degradation was observed subsequent to the transfer.

The second study examined short-term effects of organic carbon quality on E1 degradation. Fresh and aged synthetic septage mixtures were prepared as described in Chapter 4. A large quantity of E1 degrading biomass (up to 5 g wet weight) was transferred to 1 L reactors with the synthetic septage mixtures, and an E1 kinetic study was conducted. The purpose of this experiment was to determine if organic carbon quality had short-term effects of E1 degrading activity, and would be a companion to the growth study described in Chapter 4. This experiment was conducted three times, using biomass grown in (i) 100 mg COD/L synthetic septage over a 5 d period; (ii) 500 mg COD/L synthetic septage over a 5 d period (in order to generate higher biomass concentrations to facilitate separation by centrifuging); (iii) biomass grown in MBRs with an influent strength of 100 mg COD/L. In each case, no E1 biodegradation was observed at up to 24 h, by which time microbial growth and changes in organic carbon quality would convolute short-term effect results.

There was one study involving the transfer of biomass from one reactor liquor to another in which E1 degrading ability was not disrupted: the E1 exposure study described in Chapter 3. In that study, biomass was grown in MBRs, and the reactor liquor the biomass was transferred into was filter-sterilized effluent from those MBRs. This suggests that the disruption in E1 degrading ability observed in the previous two studies is due to the abrupt change in conditions. Interestingly, the abrupt increase in organic

carbon concentrations in the catabolic repression study in Chapter 2 did not affect E1 degradation, indicating that another, undetermined, factor or factors are involved.

Appendix B: Other Contributions

In addition to my dissertation work, I made contributions to the two papers attached in this appendix: "Direct and indirect photolysis of sulfamethoxazole and trimethoprim in wastewater treatment plant effluent" and "Tertiary-Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes into Duluth-Superior Harbor."

In the first paper, I ran experiments examining the photodegradation of trimethoprim under air-saturated and deoxygenated conditions at various pH values. These experiments indicated that the excited state of trimethoprim is quenched by oxygen, reducing photodegradation rates. I confirmed these results in a follow-up study where cimetidine, a compound only susceptible to indirect photolysis, was photodegraded together with trimethoprim under oxygenated and deoxygenated conditions. Cimetidine degraded only under oxygenated conditions, showing that trimethoprim acted as a singlet oxygen sensitizer.

The second paper was the result of the study done as part of the class CE 8551, Environmental Microbiology: Molecular Theory and Methods. I was involved in sample collection and processing, some of the quantitative real-time polymerase chain reaction preparation, and analysis of bacterial community composition from automated ribosomal intergenic spacer analysis (ARISA) data via nonmetric multidimensional scaling (nMDS).

Direct and Indirect Photolysis of Sulfamethoxazole and Trimethoprim in Wastewater Treatment Plant Effluent

This paper was published in *Water Research* and is cited as:

Ryan, C.C.; Tan, D.T.; Arnold, W.A. Direct and indirect photolysis of sulfamethoxazole and trimethoprim in wastewater treatment plant effluent. *Water Res.* **2011**, *45*(3), 1280-1286. DOI: 10.1016/j.watres.2010.10.005

The photolysis of two antibacterial compounds, sulfamethoxazole and trimethoprim, was studied in wastewater effluent. The rate of loss of sulfamethoxazole was enhanced in wastewater effluent due to indirect photolysis reactions, specifically reactions with hydroxyl radicals and triplet excited state effluent organic matter. Photolysis in the presence of natural organic matter, however, did not lead to enhanced degradation of sulfamethoxazole. Trimethoprim was also found to be susceptible to indirect photolysis in wastewater effluents, with hydroxyl radical and triplet excited effluent organic matter being the responsible species. Deoxygenation of solutions led to more rapid direct photolysis of sulfamethoxazole and trimethoprim, indicating that direct photolysis proceeds through a triplet excited state, which was verified by demonstrating that trimethoprim is a singlet oxygen sensitizer. In the wastewater effluents tested, photolysis could be apportioned into direct photolysis (48% for sulfamethoxazole, 18% for trimethoprim), reaction with hydroxyl radicals (36% and 62%, respectively) and reaction with triplet excited effluent organic matter (16% and 20%, respectively). These results indicate that allowing photolysis in wastewater stabilization ponds or wastewater treatment wetlands may lead to enhanced pharmaceutical removal prior to discharge and that effluent organic matter has different photoreactivity than natural organic matter.

Introduction

Sulfamethoxazole and trimethoprim are two human-use antibacterial compounds that are often prescribed together to treat various bacterial infections. Sulfamethoxazole belongs to the sulfonamide class of antibacterial compounds, while trimethoprim does not belong to any specific class. Antibiotics/antibacterials that are used by humans are not

entirely metabolized by the digestive system and pass into the sanitary sewer system. At wastewater treatment plants, some fraction of the drugs entering the plants are degraded, but a portion may pass through, either sorbed to the waste solids or dissolved in the liquid effluent (1-4). The discharge of effluent or the application of solids to the land surface leads to the contamination of environmental systems with the residual pharmaceuticals (5-7). Concerns about sulfamethoxazole and trimethoprim are related to the potential for resistance to be developed to this drug combination because of its widespread use. Since these compounds first began to be used in combination in 1968, the frequency of bacterial isolates showing resistance to the combination has gradually increased (8). Besides resistance developed through normal use, concerns exist about resistance developing due to bacteria being exposed to the drugs at low concentrations in the environment (9).

When sulfamethoxazole and trimethoprim reach environmental systems, there are multiple routes for their possible removal, including photodegradation, biodegradation, and partitioning to sediments. Focusing on photodegradation, previous work has found sulfamethoxazole to degrade predominantly by direct photolysis (10-11). Reported solar quantum yields (F) for photolysis of sulfamethoxazole are 0, 0.5, and 0.09 for the three protonation states, with the 0.09 value being relevant for most environmental conditions ($pH > 7$) (10). Indirect photolysis was found to be important for sulfamethoxazole in one study, for adding nitrate or humic acids to solutions increased the degradation rates above what was observed for distilled water solutions (12). Sulfamethoxazole was also found to act as a photosensitizer (13), producing singlet oxygen and radical species. For

trimethoprim, direct photolysis proceeds at slow rates under environmentally relevant conditions when compared to other pharmaceutical compounds (13-14). A quantum yield of 3×10^4 (in methanol) was found, and trimethoprim degradation was sensitized by aromatic ketones (15). Zhou and Moore (13) found that trimethoprim did not generate singlet oxygen or radicals in solution upon photolysis.

Photolysis is a potential means to limit the release of pharmaceuticals carried by wastewater effluents into the environment. An engineered ultraviolet light photolysis system could be used to photodegrade the compounds. Alternatively, photolysis in sunlight may occur in stabilization ponds or treatment wetlands. The photochemistry of pharmaceutical compounds in a wastewater matrix, however, has not yet been thoroughly evaluated. Natural surface waters are dominated by natural organic matter (NOM) as a photosensitizer, whereas wastewater effluents contain effluent organic matter (EfOM). EfOM has different characteristics than NOM (16), which may affect its photoreactivity. Additionally, wastewater effluents that have gone through a nitrification process will have potentially high levels of nitrate, which is a photosensitizer for the production of hydroxyl radicals (17). In effluent dominated streams, the dissolved constituents in the effluent (organic matter, nitrate) may impact photolysismore so than the dissolved constituents in the upstream waters (e.g., natural organic matter), pointing to the need to understand photolysis in the wastewater matrix. The goal of this study was to examine various aspects of the direct and indirect photolysis of sulfamethoxazole and trimethoprim in wastewater effluents. The photolysis rates in ultrapure water, natural

water, and wastewater effluent were compared to determine the important processes in each matrix.

Materials and Methods

Chemicals

Sulfamethoxazole (98%), trimethoprim (98%), 4-chlorobenzoic acid (pCBA; 99%), perinaphthenone (97%), cimetidine (99%), 2-propanol (IPA; 99.5%), 4-nitroacetophenone (PNAP; 98%) and pyridine (99%) were purchased from Sigma-Aldrich. Isoprene (99%), 4-nitroanisole (PNA; 99%) and 30-methoxyacetophenone (98%) were purchased from Acros Organics. Nitrogen (zero grade) and oxygen (ultrapure grade) were purchased from Minneapolis Oxygen Company. Argon (zero grade) was obtained from Airgas. All solvents were high-performance liquid chromatography (HPLC) grade. Chemicals were used as received except for PNA, which was recrystallized before use. Ultrapure (Milli-Q) water was obtained from a Millipore Simplicity UV purification system. Samples of final effluent from wastewater treatment plants were collected in 1-L glass bottles, filtered through 0.2 mm filters, acidified with sulfuric acid to pH 2, and stored at 4 °C until use in experiments. The pH was readjusted to 8.0 with sodium hydroxide before initiating photolysis experiments.

Analytical Methods

Concentrations were quantified using an Agilent Technologies 1200 Series HPLC equipped with UV/visible and photodiode array detectors. All compounds were analyzed on a Supelco Ascentis RP Amide 150mm 4.6 mm, 5 mm column. For sulfamethoxazole

and trimethoprim, a methanol:pH 3 phosphate buffer gradient method was used, starting at 20:80 and changing to 50:50 over 1 min and then holding for 4 min, with a 1 ml/min flow rate and a detection wavelength of 274 nm. For PNAP and PNA, a 50:50 acetonitrile:pH 3 phosphate buffer mobile phase at a flow rate of 1 ml/min was used, with detection wavelengths of 254 nm and 280 nm respectively. Cimetidine analysis was carried out with a 5:10:85 methanol:acetonitrile:pH 3 phosphate buffer mobile phase at a flow rate of 1 ml/min with a detection wavelength of 219 nm. Analysis for pCBA used an isocratic 75:25 methanol: pH 3 phosphate buffer mobile phase at a flow rate of 1 ml/min with a detection wavelength of 240 nm. Dissolved organic carbon concentrations of the wastewater effluents were determined using a Sievers 900 portable TOC analyzer and nitrate concentrations were measured with a Metrohm 761 compact ion chromatograph. UV-visible light absorption spectra of trimethoprim were obtained with a Shimadzu 1601-PC spectrophotometer. UV-visible light absorption spectra for sulfamethoxazole have been reported previously (10).

Photolysis

Laboratory photolysis experiments were conducted using a Suntest CPS þ solar simulator with a UV-Suprax optical filter (Atlas Materials Testing Solutions) with the light intensity set at 765 W/m2. Samples were held in quartz test tubes (o.d. ¼ 1.3 cm, i.d. ¼ 1.1 cm, V ¼ 10 ml) set at an angle of 30° from horizontal. Tubes were filled with approximately 7 ml of solution of the desired composition. Deoxygenated samples had nitrogen or argon gas bubbled through them for 5 min and were subsequently capped and sealed. As subsamples were taken from the tubes, the appropriate gas, either nitrogen or

argon, was injected into the headspace of the vials to replace the lost volume. In determination of the quantum yield of trimethoprim, PNA/pyridine and PNAP/pyridine actinometers were used (18), and the quantum yield was determined by comparison of the first order rate constant for trimethoprim to that of PNA or PNAP, which have known quantum yields. The ratio of rate constants and the spectral overlap integrals (determined from the molar absorptivities determined from UVevisible spectra of trimethoprim and the actinometers and the manufacturer-reported lamp output spectrum) were used to calculate the quantum yield as described by Leifer (19). Quantum yields were determined in pH 5 Milli-Q water adjusted with phosphoric acid and in 10 mM phosphate pH 8 buffered Milli-Q water under both air saturated and deoxygenated conditions. These pH values were selected to evaluate the photolysis of the protonated (positively charged) and deprotonated (neutral) forms of trimethoprim (pKa of 6.7) (13).

Experiments investigating indirect photolysis compared the behavior of 1 mM solutions of either sulfamethoxazole or trimethoprim in a 10 mM phosphate pH 8 buffered Milli-Q water, wastewater effluent from the Blue Lake treatment plant (42 MGD, advanced secondary treatment of domestic and industrial wastewater, Shakopee, MN; final effluent DOC = 7.49mg/L, NO_3^- = 16.5mg/L as N, pH= 8.0), effluent from the Metro wastewater treatment plant (250 MGD, advanced secondary treatment of domestic and industrial wastewater, St. Paul, MN, DOC = 8.12 mg/L, NO_3^- = 11.8 mg/L as N, pH = 8.0), and Lake Josephine water (Roseville, MN, DOC = 6.03 mg/L, NO_3^- = 0.4 mg/L as N, pH = 8.0). To verify the roles of different photochemically produced reactive intermediates, quencher and sensitizer experiments were also performed. Solutions of 1%

isopropyl alcohol were used to scavenge (hydroxyl) radicals. Isoprene, at a concentration of 0.1%, was added to selected experiments with sulfamethoxazole, as a scavenger of triplet excited states. Deoxygenation of solutions was also used to explore the role of triplet excited states. To verify susceptibility to reaction with triplet excited states, perinapthenone and 30-methoxyacetophenone (20) were used in the Milli-Q water as model triplet sensitizers, at a concentration of approximately 1 mM. The involvement of a triplet excited state in the degradation of trimethoprim was also examined using 40 mM trimethoprim and 2.5 mM cimetidine (which only reacts via singlet oxygenation) (21) in 10 mM phosphate pH 8 buffered Milli-Q water solutions.

Experiments quantifying the relative importance of direct and indirect photolysis of sulfamethoxazole and trimethoprim were conducted outdoors on August 3, 2009 in Minneapolis, MN, USA (~45 °N latitude). These experiments involved comparing the degradation rates of trimethoprim, sulfamethoxazole, and pCBA in Milli-Q water, effluent from the Blue Lake plant, and solutions containing 1 mM potassium nitrate and 1 mg/L octanol (22). pCBA was used as a hydroxyl radical probe to quantify hydroxyl radical steady state concentrations. Photolyses were performed on individual compounds in duplicate for each water sample/set of conditions.

Results and Discussion

Sulfamethoxazole

As shown in Fig. 1, sulfamethoxazole is susceptible to photolysis in wastewater effluent from the Blue Lake treatment plant. Adding a radical quencher (IPA) suppressed

the reaction rate, indicating that reaction with photogenerated radicals (an indirect photolysis process) also occurred. Addition of isoprene suppressed the reaction rate, and an increase in the reaction rate was observed in the absence of oxygen (Fig. 1; Table 1). Oxygen (a ground state triplet because of its unpaired electrons) is a quencher of triplet excited states, and thus its removal decreases the total quenching rate of triplets in the system. These results taken together provide strong evidence for the involvement of triplet excited species in the indirect photolysis of sulfamethoxazole. Such effects have previously been used to substantiate a role for triplet excited organic matter in the degradation of pharmaceuticals (23). Photolysis in Milli-Q water in the presence of 1 mM perinapthenone led to rapid loss of sulfamethoxazole ($45 \pm 12\ h^{-1}$; all reported errors are 95% confidence intervals), indicating that sulfamethoxazole reacts with triplet excited states. (While perinapthenone is also a singlet oxygen sensitizer, sulfamethoxazole has a relatively small singlet oxygenation rate constant) (10). Because the IPA and isoprene quenchers did not completely suppress reaction, direct photolysis is also important in the wastewater matrix.

The reaction of sulfamethoxazole via indirect photolysis was unexpected, given that our previous results (10) in Milli-Q and natural (Lake Josephine) water had indicated that direct photolysis was the primary process. Thus, experiments in Milli-Q water and Lake Josephine water were repeated and compared to the results in wastewater effluent (Fig. 2; Table 1). Sulfamethoxazole was found to degrade with a rate constant of 0.40 ± 0.01 h in pH 8 buffered Milli-Q water. Similar to previous results (10), the rate constant in Lake Josephine water was identical, pointing to direct photolysis as the dominant

process in natural waters (Table 1). Both of these rate constants, however, are significantly slower than that in the Blue Lake wastewater effluent (Table 1). Photolysis in the Metro Plant effluent gave similar results ($0.61 \pm 0.05\ h^{-1}$) to the Blue Lake wastewater, verifying that indirect photolysis was an important loss process for sulfamethoxazole in both the wastewater effluents.

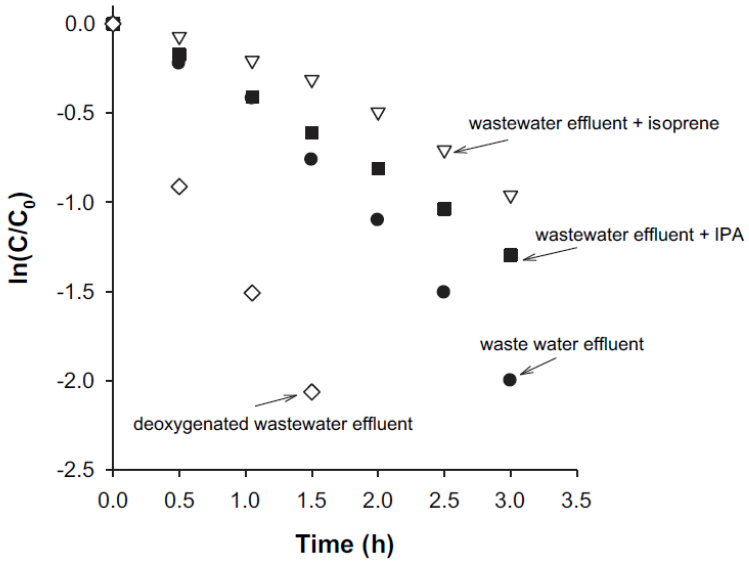

Figure 1: Photolysis of sulfamethoxazole in Blue Lake wastewater effluent with and without quenchers for photochemically produced reactive intermediates. Isopropanol (IPA) is a radical quencher. Isoprene is a quencher of excited triplet states, and deoxygenation removes the triplet quencher, oxygen.

Table 1: Photolysis rate constants (k_{obs}) for sulfamethoxazole and trimethoprim

Matrix	Quencher/alteration[a]	k_{obs} (h^{-1})
Sulfamethoxazole		
Wastewater effluent	None	0.68 ± 0.07[b]
	Isopropyl alcohol	0.43 ± 0.04
	Deoxygenation	1.37 ± 0.29
	Isoprene	0.32 ± 0.07
Ultrapurified water	None	0.40 ± 0.01
	Isopropyl alcohol	0.40 ± 0.006
	Deoxygenation	0.66 ± 0.10
Lake water	None	0.43 ± 0.02
	Deoxygenation	0.63 ± 0.23
Trimethoprim		
Wastewater effluent	None	0.18 ± 0.01
	Isopropyl alcohol	0.016 ± 0.019
	Deoxygenation	0.23 ± 0.01
Ultrapurified water	None	0.03 ± 0.01
	Deoxygenation	0.36 ± 0.03
Lake water	None	0.06 ± 0.02

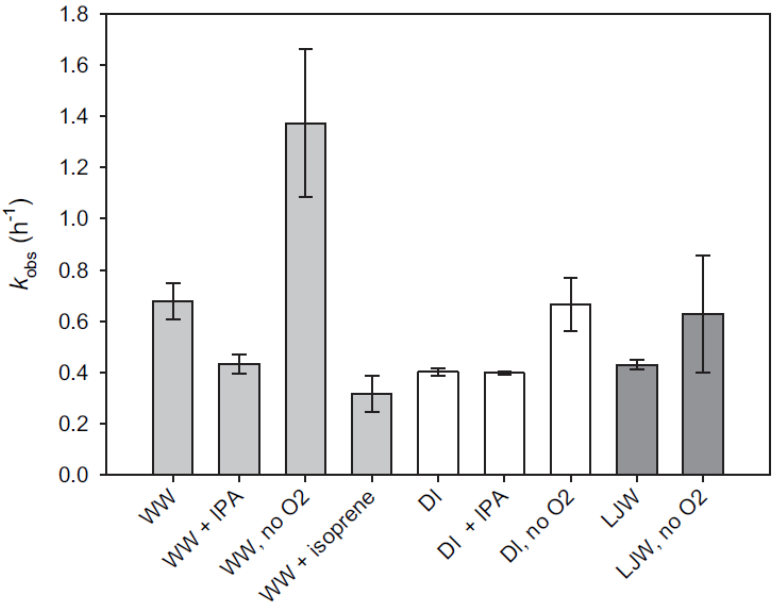

Figure 2: Comparison of observed first order rate constants for the photolysis of sulfamethoxazole in Blue Lake wastewater effluent (WW), Milli-Q water (DI), and Lake Josephine water (LJW). Results with quenchers (isoprene, isopropyl alcohol) and under deoxygenated conditions are also shown. Errors are 95% confidence intervals (n =1-3).

The suppression of the sulfamethoxazole photolysis rate constant in wastewater effluent by either IPA or isoprene to values lower than that of unaltered wastewater (Table 1) indicates that radicals and triplet excited states are both active as indirect photolysis process. As shown in Fig. 2, adding IPA to the Milli-Q water had no effect, and similar results with Lake Josephine water were seen previously (10). Deoxygenation of both Milli-Q water and Lake Josephine water leads to an increase in the loss rate (Table 1, Fig. 2). The fact that the magnitude of the change is the same in both of these matrices, however, indicates that the excited state triplet NOM in the natural water is not serving as a photosensitizer. Rather, the direct photolysis of sulfamethoxazole proceeds through a triplet excited state, and the removal of oxygen increases the lifetime (and decreases the quenching) of this excited state (SterneVolmer quenching), allowing a greater fraction of the photo-excited molecules to be transformed (i.e., the quantum yield increases in the absence of oxygen). This explanation is consistent with previous findings that sulfamethoxazole is a singlet oxygen sensitizer (13). The effect of deoxygenation further demonstrates that singlet oxygen is not important in the transformation of sulfamethoxazole. If singlet oxygen were the major reactive species responsible for the indirect photolysis, deoxygenating samples would dramatically decrease degradation rates, which was not observed.

Trimethoprim

Like sulfamethoxazole, trimethoprim was photolyzed much more rapidly in Blue Lake wastewater effluent than in pH 8 buffered Milli-Q water or Lake Josephine water (Table 1; Fig. 3). Quenching with IPA dramatically lowered the rate constant in

wastewater effluent, whereas deoxygenation led to a slight, but statistically significant, increase (Table 1; Fig. 3). The difference between the wastewater effluent and the natural water again indicates that the photosensitizing ability of the wastewater matrix is greater than that of the natural water.

Deoxygenating the pH 8 buffered Milli-Q also dramatically increased the rate constant, indicating that the excited triplet state trimethoprim is effectively quenched by oxygen. This is borne out by the quantum yields measured for trimethoprim which are an order of magnitude higher in deoxygenated solutions (Table 2). The quantum yield reported here at pH 5 is similar to that previously reported in methanol (15). The difference is likely due to the solvent. Trimethoprim has a pKa of 6.7 (13) and the absorbance spectrum is influenced by pH (Supplementary Data). Thus, the quantum yield is pH, as well as oxygen, dependent.

If trimethoprim in an excited triplet state is quenched by oxygen, singlet oxygen should be produced. This was tested by conducting experiments in pH 8 buffered Milli-Q water containing both trimethoprim (40 mM) and cimetidine (2.5 mM), a compound that is only susceptible to indirect photolysis via singlet oxygenation (21). In the presence of trimethoprim and oxygen, cimetidine degraded (first order rate constant of 0.49 ± 0.04 h^{-1} when sparged with oxygen and 0.21 ± 0.05 h^{-1} when sparged with air), whereas in deoxygenated solutions, trimethoprim degraded, but cimetidine loss was negligible, with a rate constant that was not statistically different than zero (0.04 ± 0.09 h^{-1}). This indicates that trimethoprim is a singlet oxygen sensitizer, contrary to the findings of Zhou and Moore (13).

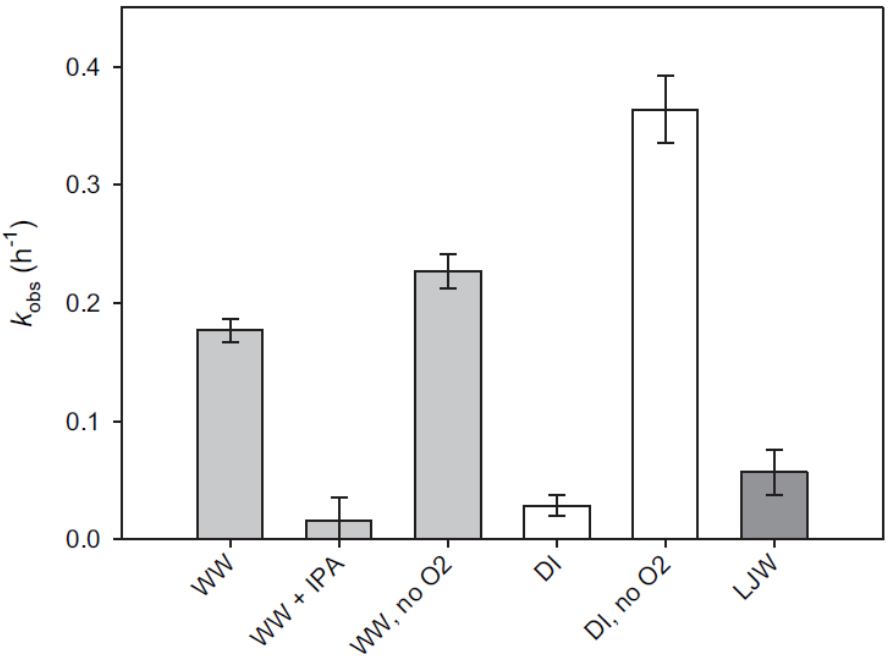

Figure 3: Comparison of observed first order rate constants for the photolysis of trimethoprim in Blue Lake wastewater effluent (WW), Milli-Q water (DI), and Lake Josephine water (LJW). Results with isopropyl alcohol and under deoxygenated conditions are also shown. Errors are 95% confidence intervals (n = 1-4).

Table 2: Quantum yields for direct photolysis of trimethoprim.[a]

pH	Air saturated	Deoxygenated
5	6.2×10^{-4}	7.9×10^{-3}
8	1.2×10^{-3}	7.0×10^{-2}

a Quantum yields are for the simulated solar spectrum of the lamp. See Supplementary data.

The more rapid reaction in deoxygenated wastewater could either be caused by a decrease in the rate of quenching of the trimethoprim itself or a decrease in the quenching rate of triplet excited effluent organic matter. Experiments performed with the triplet sensitizers perinapthenone and 30-methoxyacetophenone in pH 8 buffered water gave the

rate constants of 1.2 ± 0.2 h^{-1} and 0.12 ± 0.01 h^{-1} respectively, confirming the finding of Dedola et al. (15) that trimethoprim is susceptible to reactions with triplet excited states. Again, the observed increase in rate upon deoxygenation rules out a major role for singlet oxygen in the photolysis of trimethoprim.

Contribution of indirect photolysis processes

To quantify the fraction of reaction occurring via direct and indirect photolysis processes, a series of experiments were performed in sunlight in air saturated solutions. To determine the steady state concentration of hydroxyl radicals, pCBA was used in Blue Lake wastewater effluent and in an aqueous solution containing nitrate (a sensitizer for hydroxyl radical) and 1-octanol (to serve as a hydroxyl radical quencher). pCBA degrades exclusively by interaction with hydroxyl radicals, with a known second order rate constant of 5×10^{9} M^{-1} s^{-1} (24). When exposed to natural sunlight in a Blue Lake wastewater effluent solution, pCBA was found to degrade with a first order rate constant of 7.3×10^{-6} s^{-1}. By dividing the observed first order constant by the second order rate constant for reaction with hydroxyl radicals, the steady state hydroxyl radical concentration was determined to be 1.5×10^{-15} M, which is higher than normally reported for natural waters (25), but reasonable given the nitrate concentration of the water (16.5 mg/L as N). The solution containing 1 mM nitrate (14 mg/L as N) and 1 mg/L octanol had a steady state hydroxyl radical concentration of 1.8×10^{-15} M.

For sulfamethoxazole, the overall rate constant in the sunlit wastewater effluent was 2.35×10^{-5} s^{-1} (0.085 h^{-1}). Boreen et al. (10) determined a second order rate constant

of 5.8×10^9 M^{-1} s^{-1} for sulfamethoxazole reaction with hydroxyl radicals. Multiplying this rate constant by the steady state hydroxyl rate constant found in the effluent, gives a pseudo-first order reaction rate constant of 8.5×10^{-6} s^{-1}, which accounts for 36% of the total degradation of sulfamethoxazole. Direct photolysis of sulfamethoxazole in a parallel test tube containing pH 8 buffered Milli-Q water was found to proceed with a rate constant of 1.1×10^{-5} s^{-1}, which should be the same in Blue Lake effluent, ignoring the effect of screening by EfOM (calculated to be <10% of the incident light for wavelengths 290 nm). Thus, direct photolysis accounts for 48% of the degradation of sulfamethoxazole in the wastewater effluent. It is assumed that triplet excited EfOM is responsible for the remaining sulfamethoxazole degradation. This gives a pseudo-first order rate constant of 3.7×10^{-6} s^{-1} for the reaction mediated by triplet excited states, which accounts for 16% of the degradation. This assumes singlet oxygenation is negligible, which is consistent with the laboratory findings in this work and previous studies (10).

The overall rate constant for trimethoprim was 1.62×10^{-5} s^{-1} (0.058 h^{-1}) Trimethoprim was found by Dodd et al. (2006) to have a second order reaction rate constant with hydroxyl radicals of 6.9×10^9 M^{-1} s^{-1}, giving a pseudo-first order rate constant of 1.0×10^{-5} s^{-1} at the calculated steady state hydroxyl radical concentration. Hydroxyl radicals thus account for 62% of the observed degradation. The rate constant for direct photolysis was found to be 2.8×10^{-6} s^{-1}, which accounts for 18% of the degradation, and the remaining 20% of the degradation is attributed to trimethoprim interacting with triplet excited EfOM with a pseudo-first order rate constant of 3.3×10^{-6}

s^{-1}. Again, this assumes no role for singlet oxygen, which is consistent with the laboratory results presented above.

For the test tubes containing nitrate/octanol solution in water, the rate constant was predicted using the measured direct photolysis rate constant in pH 8 buffered Milli-Q water and the calculated pseudo-first order rate constant for reaction with hydroxyl radical based on the known second order rate constants and the steady state hydroxyl radical concentration determined with pCBA. These predictions were within a factor of two of the measured rate for sulfamethoxazole and within 15% for trimethoprim, indicating that parsing the reaction rate into direct and indirect photolysis pathways in this manner is reasonable.

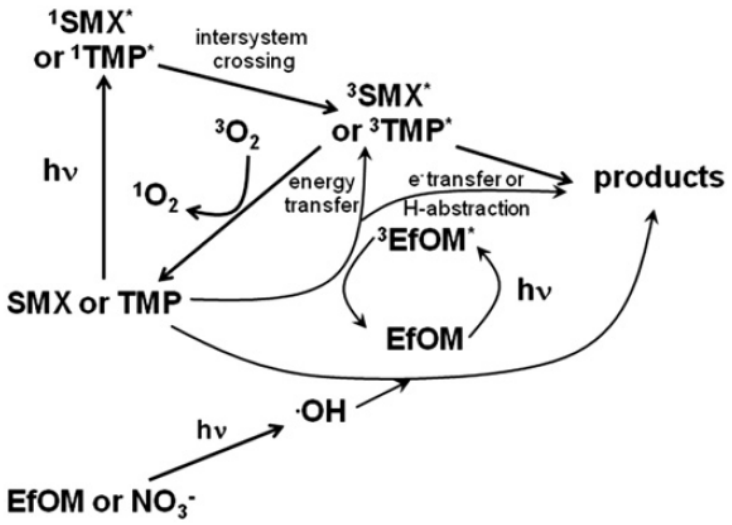

Figure 4: Hypothesized reaction scheme for the direct and indirect photolysis of sulfamethoxazole (SMX) and trimethoprim (TMP) in wastewater effluent.

The data presented thus far point to triplet excited states and hydroxyl radicals as being important for the degradation of sulfamethoxazole in wastewater effluent that is exposed to light. A schematic for the hypothesized pathways of the degradation is given in Fig. 4. The proposed route for direct photolysis proceeds as follows: absorbed sunlight excites sulfamethoxazole or trimethoprim to a singlet-excited state, which then undergoes intersystem crosses to a triplet excited state. From this point the molecule either reacts to form products or the dissolved oxygen that is present in the solution quenches the triplet excited state, returning the sulfamethoxazole or trimethoprim to the ground state. This hypothesis is consistent with the observation that the rate of photodegradation increases when dissolved oxygen is removed from Milli-Q water. For indirect photolysis there are two important possible degradation pathways. The first involves EfOM that is excited to a triplet state when it absorbs sunlight. This excited EfOM can then interact with the target to bring it directly to a triplet excited state via energy transfer, where again, it is either quenched by oxygen and returns to the ground state or proceeds to break apart to form products. Such energy transfer reactions are only possible if the triplet energy of the sulfamethoxazole or trimethoprim is lower than that of the triplet excited EfOM or the model sensitizers used in this work. We note, however, that perinapthenone (triplet energy 186 kJ mol^{-1}) reacted faster with trimethoprim than 3'-methoxyacetophenone (triplet energy 303 kJ mol^{-1}). This indicates that electron transfer and/or H-atom abstraction reactions between the excited EfOM and sulfamethoxazole or trimethoprim may be responsible for the observed reactivity (Fig. 4). The second indirect photolysis

pathway involves hydroxyl radicals that are produced by sunlight interacting with various dissolved species, principally nitrate, in the effluent water.

The finding that indirect photolysis occurred in wastewater effluents (but not in Lake Josephine water) suggests that effluents from these wastewater treatment plants contain different indirect photolysis sensitizers than local natural waters (e.g., nitrate, organic carbon levels). The concentration of nitrate (a sensitizer for hydroxyl radicals) is higher in the effluent sample than in the Lake Josephine water sample. The different composition of EfOM compared to NOM (16) may also result in different or higher energy triplet sensitizers being present. Those in EfOM may have triplet energies more conducive to reaction (via energy transfer or electron transfer/H-abstraction) with the compounds targeted in this study than does NOM. This suggests an inherent difference in the photochemistry of NOM and EfOM. Further work will need to be performed to evaluate seasonal variations in the photosensitizing ability of EfOM and NOM and the effects of pH on these processes.

Similar results demonstrating the role of triplet excited states being dependent upon the organic matter source were recently found for sulfamethoxine (26). A eutrophic water from an aquaculture pond containing autochthonous (i.e., microbially derived) organic matter was able to photosensitize the destruction of sulfamethoxine via formation of triplet excited states, but dissolved organic matter of allochthonous origin was unable to do so (26). Because EfOM is also autochthonous, the results in this work further support that there is a difference in the photosensitizing ability of autochthonous and allochthonous organic matter. Nitrate and organic matter are both sensitizers of hydroxyl

radical production. The differences in nitrate concentration are more likely to be responsible for the differences in radical production between the wastewater effluents and natural waters. The DOC levels are similar, but it cannot be ruled out that the EfOM is more efficient than NOM at producing hydroxyl radicals. This work also indicates that dissolved oxygen levels must also be considered when assessing the photolysis rates of sulfamethoxazole and trimethoprim.

Conclusions

- Both direct and indirect photolysis of sulfamethoxazole and trimethoprim occur in wastewater effluents exposed to sunlight.
- The indirect photolysis is attributable to the production of hydroxyl radicals and triplet excited state organic matter.
- Triplet excited state EfOM is able to react with sulfamethoxazole and trimethoprim, but the NOM present in a nearby surface water does not.
- Wastewater treatment wetlands/stabilizations ponds are environments in which photolysis of pharmaceuticals should be encouraged due to the presence of sensitizers not found (or found at lower concentrations) in receiving waters. According to the results of this work, the half-lives of sulfamethoxazole and trimethoprim will be 1 h and 2.3 h, respectively, in the oxygenated photic zone of a midsummer sunlit wetland/stabilization pond. (Compared to 1.7 h for sulfamethoxazole and 24 h for trimethoprim in sunlit non-wastewater matrices).
- In wastewater impacted surface waters, EfOM and nitrate are likely to be more important sensitizers than NOM.

- In engineered systems, a photolysis step performed under deoxygenated conditions (e.g., post denitrification) will be advantageous if pharmaceutical removal is desired, because photolysis rates are enhanced (up to a factor of two) under such conditions.

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Tertiary-Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes into Duluth-Superior Harbor

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LaPara, T.M.; Burch, T.R.; McNamara, P.J.; Tan, D.T.; Yan, M.; Eichmiller, J.J. Tertiary-Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes into Duluth-Superior Harbor. *Environ. Sci. Technol.* **2011**, *45*(22), 9543-9549. DOI: 10.1021/es202775r

In this study, the impact of tertiary-treated municipal wastewater on the quantity of several antibiotic resistance determinants in Duluth-Superior Harbor was investigated by collecting surface water and sediment samples from 13 locations in Duluth-Superior Harbor, the St. Louis River, and Lake Superior. Quantitative PCR (qPCR) was used to target three different genes encoding resistance to tetracycline (tet(A), tet(X), and tet(W)), the gene encoding the integrase of class 1 integrons (*intI1*), and total bacterial abundance (16S rRNA genes) as well as total and human fecal contamination levels (16S rRNA genes specific to the genus Bacteroides). The quantities of tet(A), tet(X), tet(W), *intI1*, total Bacteroides, and human-specific Bacteroides were typically 20-fold higher in the tertiary-treated wastewater than in nearby surface water samples. In contrast, the quantities of these genes in the St. Louis River and Lake Superior were typically below detection. Analysis of sequences of tet(W) gene fragments from four different samples collected throughout the study site supported the conclusion that tertiary-treated municipal wastewater is a point source of resistance genes into Duluth-Superior Harbor. This study demonstrates that the discharge of exceptionally treated municipal wastewater can have a statistically significant effect on the quantities of antibiotic resistance genes in otherwise pristine surface waters.

Introduction

Over the past several decades, antibiotic-resistant bacterial infections have become increasingly prevalent, increasing morbidity and mortality as well as the cost of treatment (1-3). In response to these clinical concerns, there has been increasing focus on environmental reservoirs of antibiotic resistance over the past several years (4-8).

Antibiotic use in agriculture, for example, has been heavily scrutinized (9-10) and recently banned in the European Union. In contrast, the role of treated municipal wastewater has received relatively little attention as a reservoir of resistance, in spite of numerous reports suggesting that bacteria resistant to multiple antibiotics (11-13) and antibiotic resistance genes (14-21) are abundant in municipal wastewater.

Determining the relative importance of treated municipal wastewater as a reservoir of antibiotic resistance is a potentially difficult task. The first challenge is to enumerate "antibiotic resistance" in some meaningful way. Historically, antibiotic resistance would have been quantified by cultivating bacteria based on their phenotypic resistance to a specific antibiotic or set of antibiotics. This approach, however, is insufficient because cultivation-based methods are well-known to underestimate the quantities and diversity of bacteria (22-23). The second challenge is to distinguish the impact of treated municipal wastewater from the background level of resistance because antibiotic resistant bacteria and antibiotic resistance genes are natural phenomena (5, 24) and because other human activities (i.e., other than the release of municipal wastewater) have presumably perturbed the majority of surface waters to some extent.

In this study, we examined the impact of tertiary-treated municipal wastewater on the quantities of three tetracycline resistance genes (tet(A), tet(X), and tet(W)) and the integrase gene of class 1 integrons (*intI1*) in the St. Louis River, Duluth- Superior Harbor, and Lake Superior. This ecosystem represents an ideal locale for studying the importance of treated municipal wastewater as a reservoir of antibiotic resistance because the St. Louis River and Lake Superior are surprisingly pristine surface waters with very

low background levels of bacteria (25), which suggests that the levels of antibiotic resistant bacteria also should be very low. Furthermore, the quality of treatment at the Western Lake Superior Sanitary District (WLSSD), which operates the municipal wastewater treatment facility in Duluth, MN, is exemplary. The WLSSD facility treats approximately 40 million gallons of residential, commercial, and industrial wastewater each day via a conventional system consisting of bar screens, grit removal, and a state-of-the-art, high-purity oxygen activated sludge process. The WLSSD wastewater treatment facility, however, is unique in that it further treats the wastewater by passing it through a mixed media filter (consisting of anthracite coal, silica sand, and garnet) before disinfecting (using sodium hypochlorite) the wastewater and discharging it to Duluth-Superior Harbor.

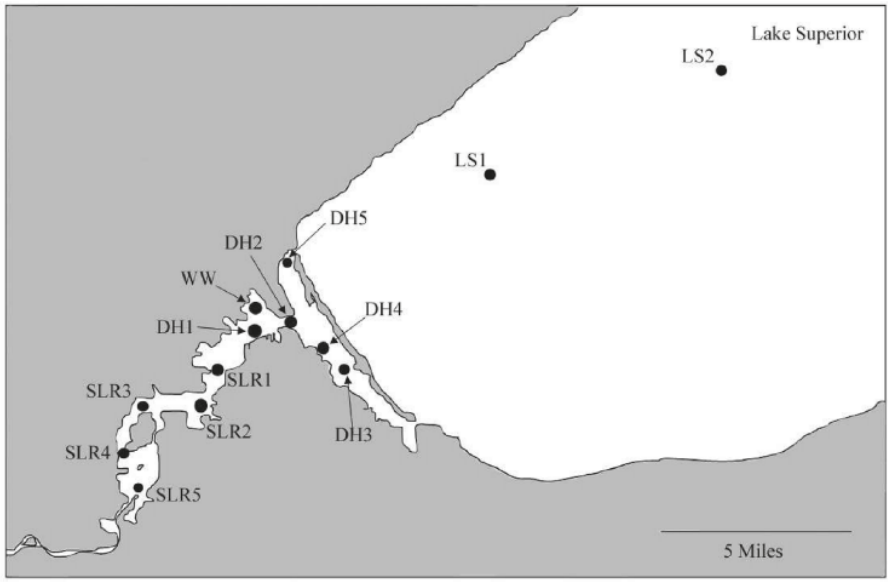

Figure 1: Map of the St. Louis River, Duluth-Superior Harbor, and LakeSuperior, identifying the locations from which samples were collected.

Materials and Methods

Sample Collection

Surface water (sample volume = 250 mL) and sediment (sample mass = ~0.75 g wet sediment) samples were collected on October 1, 2010 from the St. Louis River, Duluth-Superior Harbor, and Lake Superior while aboard the R/V Blue Heron (Figure 1). Most of the surface water samples were collected manually at a distance of 0.5 m below the water surface using sterile polystyrene bottles. A small fraction of the samples (those from Lake Superior) were collected using an SBE 32 Carousel Water Sampler (Sea-Bird Electronics, Inc., Bellevue, WA) at a depth of 5 m below the water surface. Sediment samples were collected using either a multicorer (Ocean Instruments, San Diego, CA) or a gravity-corer (HTH Teknik; Lulea, Sweden). Sediment samples represent a composite sample of the top 2.5 cm of sediment.

As soon as possible after collection (typically less than 30 min; always less than 2 h), surface water samples were passed through a 47 mm diameter nitrocellulose filter (pore size = 0.22 μm) to concentrate microbial biomass. Filters were then immersed in 0.5 mL of lysis buffer (120 mM phosphate buffer, pH = 8.0, 5% sodium dodecyl sulfate) to preserve the sample until genomic DNA could be extracted and purified. All samples were stored on ice while they were transported to the University of Minnesota (within 12 h), after which they were stored at - 20 °C until processed further.

Genomic DNA Extraction

Water samples (preserved in lysis buffer) underwent three consecutive freeze thaw cycles and an incubation of 90 min at 70 °C to lyse cells. Genomic DNA was then extracted and purified from these samples using the FastDNA Spin Kit (MP Biomedicals, Solon, OH) according to manufacturer's instructions. Genomic DNA was also extracted from sediment samples (~500 mg of wet weight per sample) using a bead beater to lyse cells. Genomic DNA was then extracted and purified from sediment samples using a FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH). All genomic DNA extractions were performed in triplicate and stored at - 20 °C until needed.

Community Analysis

The composition of the bacterial communities in the aquatic samples was compared by automated ribosomal intergenic spacer analysis (ARISA). The ribosomal intergenic spacer (ITS) regions of Bacteria were amplified using primers ITSF (50-GTC GTA ACA AGG TAG CCG TA-30) and ITSReub (50-GCC AAG GCA TCC ACC-30)26 as described previously (27). Fragment analysis was performed by denaturing capillary electrophoresis at the Biomedical Genomics Center at the University of Minnesota using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The length of the fragments was estimated using the Map Marker 1000 size standard.

Quantitative PCR

Quantitative real-time PCR (qPCR) was used to quantify the presence of three genes encoding tetracycline resistance (*tet*(A), *tet*(W), and *tet*(X)) and the integrase gene of class 1 integrons (*intI1*) as described previously (15). These genes were targeted in this study because our prior work demonstrated that these genes were easily detectable in untreated wastewater solids (15-16) and because these genes encode proteins that confer tetracycline resistance via each of the three known mechanisms of resistance.28 qPCR was also used to quantify the 16S rRNA genes of all members of the domain *Bacteria* as well as total and human-specific *Bacteroides* spp. as described previously (29-31).

The qPCR analysis was conducted using an Eppendorf Mastercycler ep *realplex* thermal cycler (Eppendorf, Westbury, NY) or an ABI Prism7000 Sequence Detection System (Applied Biosystems). Each qPCR run consisted of initial denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and anneal and extension at 60 °C (most targets) or at 56 °C (human-specific Bacteroides) for 1 min. A 25 μL reaction mixture contained 12.5 μL of iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), 25 μg bovine serum albumin (Roche Applied Science, Indianapolis, IN), optimized quantities of forward and reverse primers, and a specified volume of template DNA (usually 0.5 μL). The precise volume and concentration of template DNA were empirically optimized for each sample to generate the lowest detection limit while minimizing inhibition of PCR. Additional information on the qPCR primers, their quantification limits, and their associated products are provided in the Supporting Information.

The quantity of target DNA in unknown samples was calculated based on a standard curve generated using known quantities of template DNA. Standards for qPCR were prepared by PCR amplification of genes from positive controls, followed by ligation into a cloning vector (either the StrataClone PCR kit (Stratagene, Santa Clara, CA) or pGEM-T Easy (Promega, Madison, WI)), and transformation into E. coli JM109. Plasmids were purified using the alkaline lysis procedure (32). Plasmid DNA was quantified by staining with Hoechst 33258 dye and measured on a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using calf thymus as a DNA standard. Tenfold serial dilutions of plasmid DNA were prepared and run on the thermal cycler to generate standard curves (r2 > 0.99). Following qPCR, melting curves were generated and analyzed to verify that nonspecific amplification did not occur.

Clone Libraries

Fragments of *tet*(W) genes from four different surface water samples (samples SLR5, DH2, WW, and LS2) were amplified by PCR, purified, ligated into the pGEM-T Easy cloning vector, transformed into *Escherichia coli* JM109, and plated onto LB agar plates supplemented with 40 μg/mL of ampicillin. This resulted in libraries of *tet*(W) gene fragments from each of these samples, allowing their nucleotide sequences to be determined. Approximately 30 colonies from each library were randomly picked so that plasmids could be extracted and purified using the alkaline lysis method. Extracted plasmids were then used as template for nucleotide sequence analysis using M13F and M13R as sequencing primers. Bidirectional sequence information was then used to produce a consensus sequence. Approximately 20% of the plasmids contained primer

dimer rather than a genuine *tet*(W) gene fragment; these sequences were excluded from further analysis.

Data Analysis

Nonmetric multidimensional scaling (nMDS) was used on triplicate ARISA profiles to evaluate differences in bacterial community composition based on the presence and intensity of peaks in the electropherograms. The relative intensity of peaks, obtained by dividing the individual intensities by total intensity of all the peaks, was used in the analysis. Peaks falling below 1% of the total intensity were excluded from the analysis. nMDS was performed using the ade4 package in R, version 2.4.1 (33).

Prior to statistical analysis, samples with gene concentrations below the method detection limit were assigned a value equal to half the detection limit. All gene concentrations were then log-transformed, and this log-transformed data set was used for all subsequent statistical analysis. One-way analysis of variance (ANOVA) was performed with R version 2.12.0 for all gene targets. An F-test was conducted to determine if results from a specific surface water sample location exhibited gene concentrations that were significantly different from results at the other sample locations. Tukey's honestly significant difference (HSD) test was conducted for each gene target to determine the difference in mean gene concentrations between each possible pair of surface water samples sites. Pearson correlation coefficients of gene concentrations were also calculated using R version 2.12.0 for all possible pairs of gene targets. The detailed

results of all statistical analyses (i.e., P values and/or Pearson correlation coefficients) are provided in the Supporting Information.

All nucleotide sequences were initially compared with sequences in theGenBank database34 to verify that the cloned fragments were genuine *tet*(W) gene fragments. Sequences were then aligned using the ClustalW algorithm (35) using DNAMan version 7.0 software (Lynnon Biosoft, Vaudreuil-Dorion, Quebec, Canada). To avoid artifacts stemming from misamplification during PCR and nucleotide sequencing error (36), all sequences for which there was not a replicate were excluded from further analysis.

Results

Bacterial Community Composition

The composition of the bacterial communities in surface water samples collected along a length of the St. Louis River, Duluth-Superior Harbor, and Lake Superior was assayed by automated ribosomal intergenic spacer analysis (ARISA) (Figure 2). The bacterial community composition gradually transitioned along the length of the St. Louis River, into Duluth-Superior Harbor, and out into Lake Superior. In contrast, the composition of bacteria in the treated municipal wastewater from the Western Lake Superior Sanitary District (WLSSD) was significantly different than all of the surface water samples.

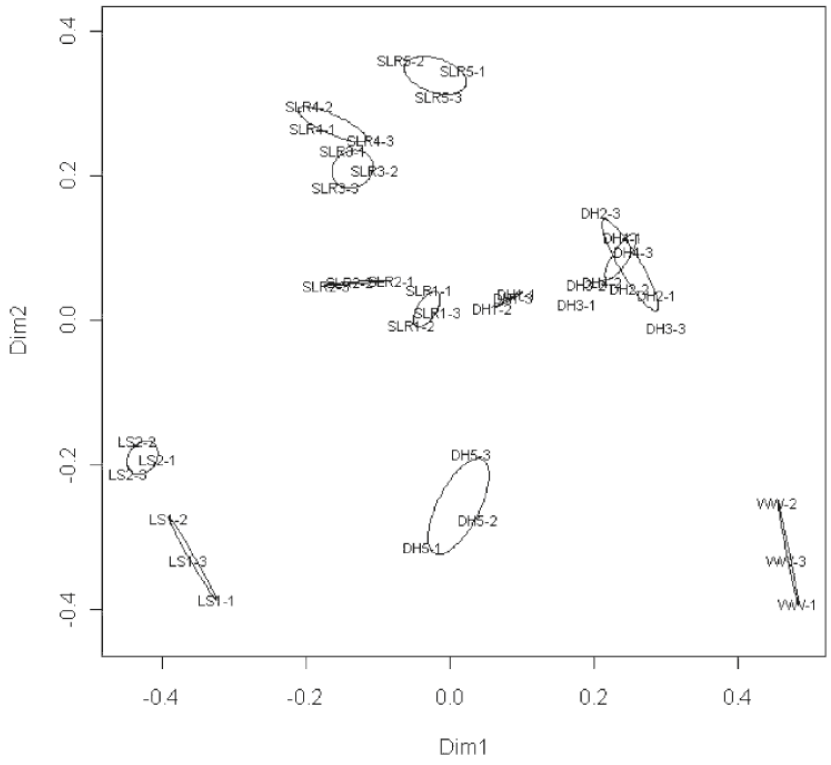

Figure 2: Results of nonmetric multidimensional scaling (nMDS) analysis of bacterial community composition as determined by automated ribosomal intergenic spacer analysis. Ellipses show the 95% confidence limit of triplicate water samples. Samples were collected from the St. Louis River (identified as "SLR"), Duluth-Superior Harbor (identified as "DH"), and Lake Superior (identified as "LS"); the precise locations from which samples were collected are shown in Figure 1.

Quantitative PCR

The amount of bacterial biomass was quantified in the surface water samples by real-time PCR of 16S rRNA gene fragments (Figure 3). Bacterial biomass in the different surface water samples varied substantially from as high as 3.6×10^6 gene copies per mL (sample location = SLR2) to as low as 2.1×10^5 gene copies per mL (sample location = LS2). These quantifications of 16S rRNA gene copies are substantially lower than that previously reported from the Haihe River in China (10^8-10^9 copies per mL) (37) and from

a drinking water source in Michigan (3.4×10^9 copies per mL) (38), but are consistent with previously reported direct cell counts from Lake Superior (1×10^5 cells/mL) (25). The quantity of bacterial biomass in the treated WLSSD effluent was 5.4×10^6 gene copies per mL, which was higher than any surface water sample.

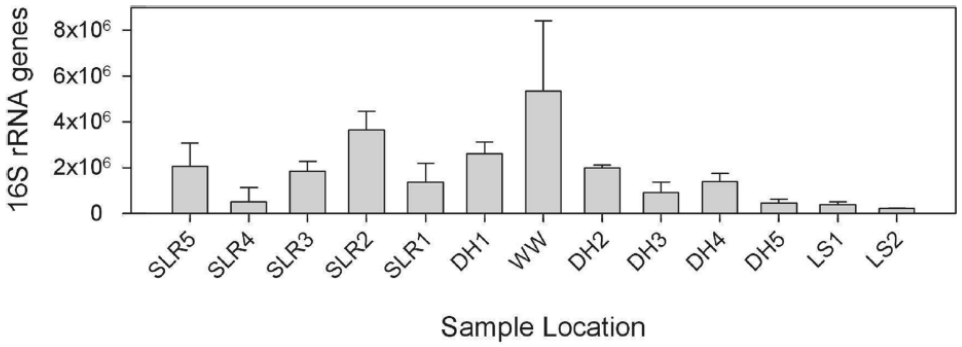

Figure 3: Quantities (gene copies per mL) of 16S rRNA genes in water samples collected from the St. Louis River, Duluth-Superior Harbor, the outfall from the Western Lake Superior Sanitary District, and Lake Superior. Values shown are the arithmetic means; error bars show the standard deviation of the mean. The locations from which samples were collected are shown in Figure 1.

The quantities of three different genes that encode resistance to tetracycline (*tet*(A), *tet*(X), and *tet*(W)) as well as the quantity of the integrase gene (*intI1*) of class 1 integrons were also determined along the St. Louis River, in Duluth-Superior Harbor, and in Lake Superior (Figure 4; for the same data normalized to 16S rRNA genes, see Supporting Information). The quantities of *tet*(A) and *tet*(X) followed similar patterns in the aquatic samples; both of these genes were at relatively high concentrations in the WLSSD effluent (*tet*(A): 6.3×10^2 copies per mL; *tet*(X): 1.2×10^3 copies per mL), slightly above the detection limit at several locations within Duluth-Superior Harbor, and below the detection limit in the St. Louis River and in Lake Superior. The pattern of *intI1*

genes was somewhat similar to that observed with *tet*(A) and *tet*(X), except that a more distinct hump-shaped profile, albeit slightly skewed into Duluth-Superior Harbor, was observed; this hump-shaped profile began in the St. Louis River and encompassed all but one sample collected from Duluth-Superior Harbor. An entirely different profile was observed with respect to the quantity of *tet*(W) genes, which were quantifiable in every aquatic sample with only the WLSSD effluent (1.8×10^4 gene copies per mL) and one sample from Duluth-Superior Harbor (sample DH4: 5.3×10^3 gene copies per mL) being statistically greater ($P < 0.05$) than the other samples. Because the quantities of 16S rRNA genes were relatively constant among the different water samples (i.e., within an order of magnitude), the quantities of *tet*(A), *tet*(X), *tet*(W), and *intI1* normalized to 16S rRNA genes follow similar patterns to those described above (see Supporting Information for more details).

The quantities of 16S rRNA genes from all *Bacteroides* spp. in the aquatic samples followed a trend similar to that observed with the *tet*(W) quantities (Figure 5A). The highest quantity of *Bacteroides* spp. was found in the WLSSD effluent (6.8×10^3 gene copies per mL), but otherwise most of the samples had relatively low concentrations that were similar. In contrast, the quantities of human-specific *Bacteriodes* spp. followed a trend like that of *tet*(A) and *tet*(X), in which a relatively high concentration was detected in the WLSSD effluent (1.0×10^2 gene copies per mL); two samples from Duluth-Superior Harbor had quantities slightly higher than the detection limit, but then all other samples were below the detection limit.

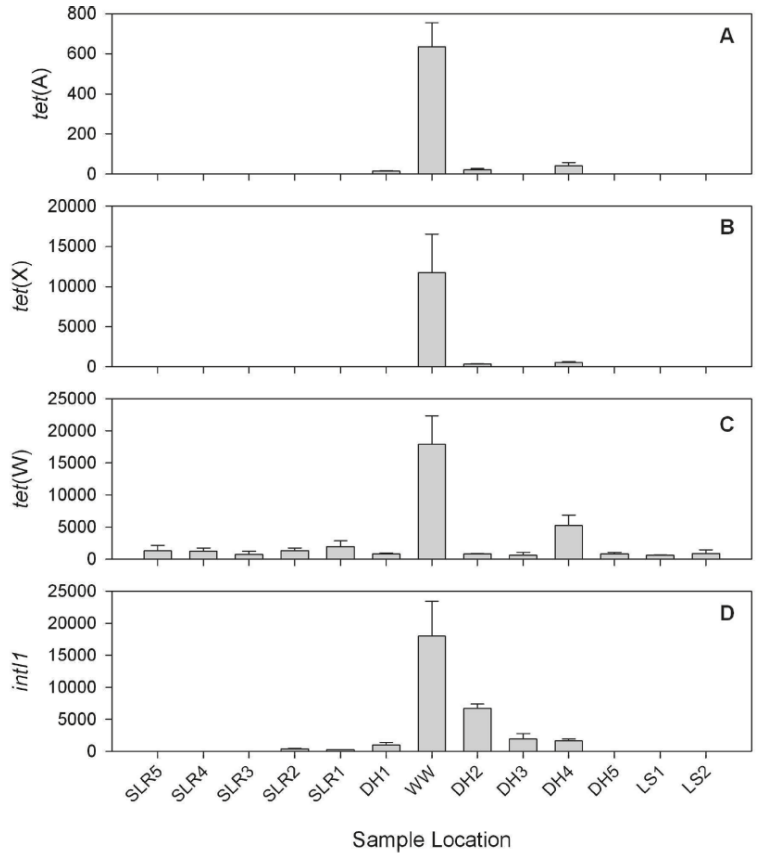

Figure 4: Quantities (gene copies per mL of water) of tet(A), tet(W), tet(X), and the integrase gene of class 1 integrons (intI1) in samples collected from the St. Louis River, Duluth-Superior Harbor, the outfall from the Western Lake Superior Sanitary District, and Lake Superior. Values shown are the arithmetic means; error bars show the standard deviation of the mean. The locations from which samples were collected are shown in Figure 1.

Four sediment samples were also collected from Duluth- Superior Harbor and Lake Superior (Table 1). Each of these samples had similar concentrations of total bacteria, as measured by qPCR of 16S rRNA genes. The quantities of the other genetic markers tracked in this study, however, varied significantly depending on sample location (except for human-specific *Bacteroides* spp., which were not detected in any of the sediment samples). The highest concentrations of these genetic markers were detected in sediment samples collected from near the WLSSD outfall (samples WW and DH1)

compared to the samples collected from the Duluth-Superior Harbor channel (sample DH3) and from Lake Superior (sample LS2).

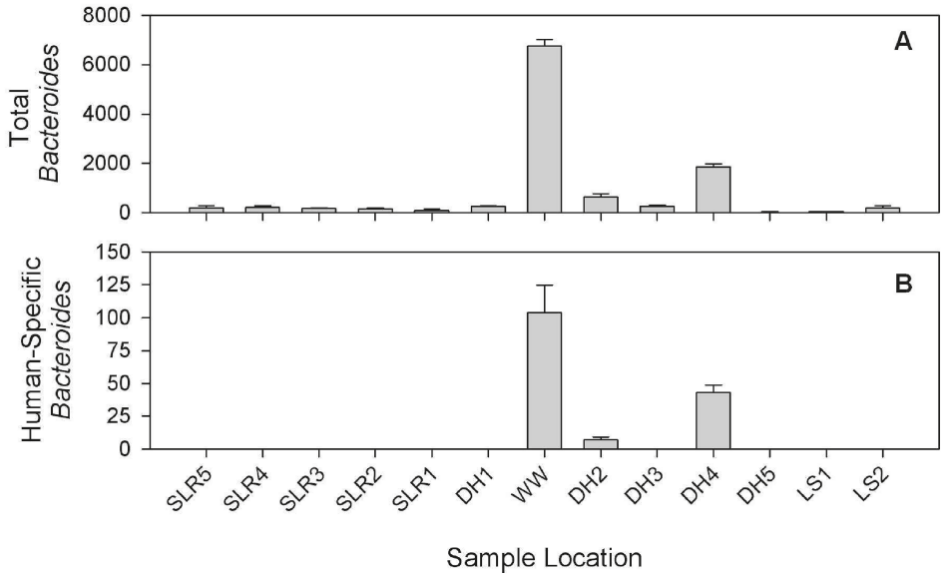

Figure 5: Quantities (gene copies per mL of water) of 16S rRNA genes from all Bacteriodes spp. and from human-specific Bacteroides spp. in water samples collected from the St. Louis River, Duluth-Superior Harbor, the outfall from the Western Lake Superior Sanitary District, and Lake Superior. Values shown are the arithmetic means; error bars show the standard deviation of the mean. The locations from which samples were collected are shown in Figure 1.

Table 1: Arithmetic Means (Units = gene copies per wet gram of sediment; n = 3) of Various Genes Detected in Sediment Samples Collected near the WLSSD Outfall, from Duluth-Superior Harbor, and from Lake Superior (See Figure 1 for Actual Locations)[a]

sample location	all 16S rRNA	all *Bacteroides* spp.	*tet*(A)	*tet*(X)	*tet*(W)	*intI1*
WW	1.9×10^{10} (6.5×10^{9})	6.9×10^{4} (3.9×10^{4})	2.1×10^{6} (6.8×10^{5})	2.9×10^{4} (5.4×10^{3})	1.9×10^{4} (9.3×10^{3})	2.4×10^{6} (6.6×10^{5})
DH1	2.1×10^{10} (5.1×10^{9})	2.3×10^{5} (1.4×10^{5})	7.4×10^{5} (1.9×10^{5})	1.2×10^{4} (2.3×10^{3})	6.5×10^{4} (3.7×10^{4})	2.5×10^{6} (5.5×10^{5})
DH3	1.7×10^{10} (5.1×10^{8})	4.9×10^{4} (2.8×10^{4})	3.7×10^{5} (6.8×10^{4})	b.d.[b]	2.6×10^{4} (1.4×10^{4})	7.7×10^{5} (4.3×10^{4})
LS2	1.9×10^{10} (5.7×10^{9})	2.3×10^{4} (3.2×10^{3})	1.2×10^{5} (1.2×10^{4})	b.d.	1.1×10^{4} (4.1×10^{3})	4.9×10^{5} (8.7×10^{4})

[a] Human-specific *Bacteroides* spp. were also targeted by real-time PCR, but were below the quantification limit in all four sample locations. The numbers in parentheses represent the standard deviation of the mean. [b] b.d., below detection.

PCR Cloning of tet(W) Gene Fragments

In a previous study, *tet*(W) gene sequences corresponded to the location from which they originated (i.e., from agriculture, from municipal wastewater, etc.) (39). Nucleotide sequences, therefore, were determined from four different clone libraries (from samples SLR5, WW, DH2, and LS2) of *tet*(W) gene fragments to determine whether or not the type of tet(W) genes varied in the St. Louis River, Duluth-Superior Harbor, and Lake Superior. Comparing only nucleotide sequences for which a matching nucleotide sequence was detected (i.e., singletons were excluded from consideration), only two distinct clones were detected. The first of these clone types (100% sequence identity to GenBank accession no. GU116971) comprised 100% of the clone library from the St. Louis River sample (sample = SLR5; n = 17), slightly less than half of the clone library from the Duluth-Superior Harbor sample (sample = DH2; 8 out of 17 clones), and the majority of the clones from the Lake Superior sample (sample = LS2; 17 out of 20 clones). In contrast, the second clone type (100% sequence identity to GenBank accession no. AP012212) represented 100% of the library from the sample collected from the tertiary-treated wastewater (sample = WW; n = 14), slightly more than half of the Duluth-Superior Harbor clone library (9 out of 17 clones), and a small fraction of the LS2 library (3 out of 20 clones).

Discussion

The importance of municipal wastewater treatment as a necessary component of modern society is without question (40-41). The primary goal of municipal wastewater

treatment is to protect surface water quality from the adverse effects of the relatively high concentration of nutrients (biodegradable carbon, nitrogen, and phosphorus) in the sewage; the secondary goal of municipal wastewater treatment is to protect public health from direct exposure to pathogens (usually via accidental ingestion of surface water) (41). An unintended consequence of municipal wastewater treatment, however, is the creation of a centralized location where bacteria from the microflora of healthy and unhealthy humans coalesce. Municipal wastewater and municipal wastewater treatment, therefore, simultaneously represent a pertinent reservoir of resistance and a potential opportunity to ameliorate this reservoir of resistance, respectively.

The present study demonstrates that treated municipal wastewater is a statistically significant point source of three tetracycline resistance determinants as well as the integrase gene of class 1 integrons into Duluth-Superior Harbor. The tertiary-treated wastewater had approximately 20-fold higher concentrations of various antibiotic resistance determinants than the local background levels in the St. Louis River and Lake Superior. Furthermore, the concentrations of antibiotic resistance genes generally correlated to either all *Bacteroides* spp. (a measure of total fecal material) or human-specific *Bacteroides* spp. (a measure of human-generated fecal material) (see Supporting Information for more details). Finally, the sequence of *tet*(W) gene fragments in tertiary-treated wastewater was unique compared to that compared to that found in the St. Louis River and Lake Superior, again suggesting that the tertiary-treated municipal wastewater was a significant source of antibiotic resistant determinants into Duluth-Superior Harbor where approximately equal amounts of these two gene sequences were detected.

The present study is unique and novel because of its ability to clearly identify tertiary-treated municipal wastewater as a point source of antibiotic resistance genes, which have been identified as an emerging pollutant of concern (42). Previous studies in which treated municipal wastewater was implicated as a source of antibiotic resistance determinants were substantially more convoluted because multiple sources of antibiotic resistance genes existed, such as agricultural activity and industrial wastewater discharges (39, 43). In contrast, the current study is considerably more straightforward to interpret because of the general transition from pristine (St. Louis River) to relatively perturbed (Duluth-Superior Harbor) back to pristine (Lake Superior), with virtually no known anthropogenic sources of antibiotic resistance genes other than a large input of tertiary-treated municipal wastewater from WLSSD (flow rate = 40 million gallons per day) and a small input of secondary-treated municipal wastewater from Superior, Wisconsin (flow rate = 5 million gallons per day; near sample location DH4).

In conclusion, municipal wastewater treatment operations need to be more carefully considered as an important factor in the global ecology of antibiotic resistance. Municipal wastewater contains numerous types of waste, of which human fecal material is known to have substantial concentrations of both antibiotic resistant bacteria and antibiotic resistance genes (44). Municipal wastewater treatment operations undoubtedly remove a very large fraction of the antibiotic resistance genes in untreated sewage prior to discharging the treated effluent. This study demonstrates that even tertiary-treated municipal wastewater is a statistically significant source of antibiotic resistance genes in otherwise pristine surface waters; additional research is needed to determine the

importance of treated municipal wastewater in the overall proliferation of antibiotic resistance.

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Appendix C: Supporting Information for Chapter 2

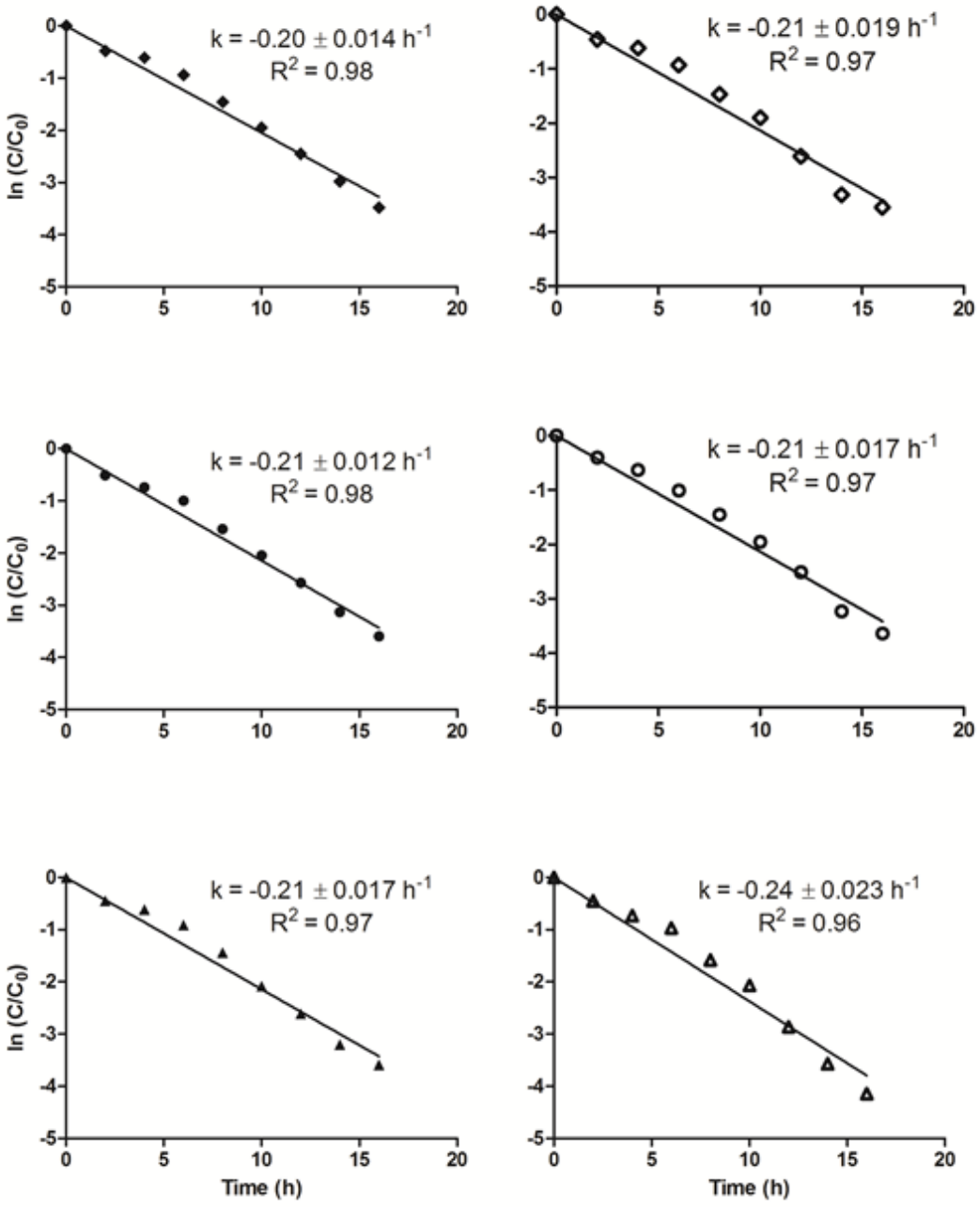

Figure C1: First-order degradation rates of E1 in control reactors (closed symbols) and reactors receiving a wastewater spike at 8 h (open symbols). Error terms represent 95% confidence intervals from regression.

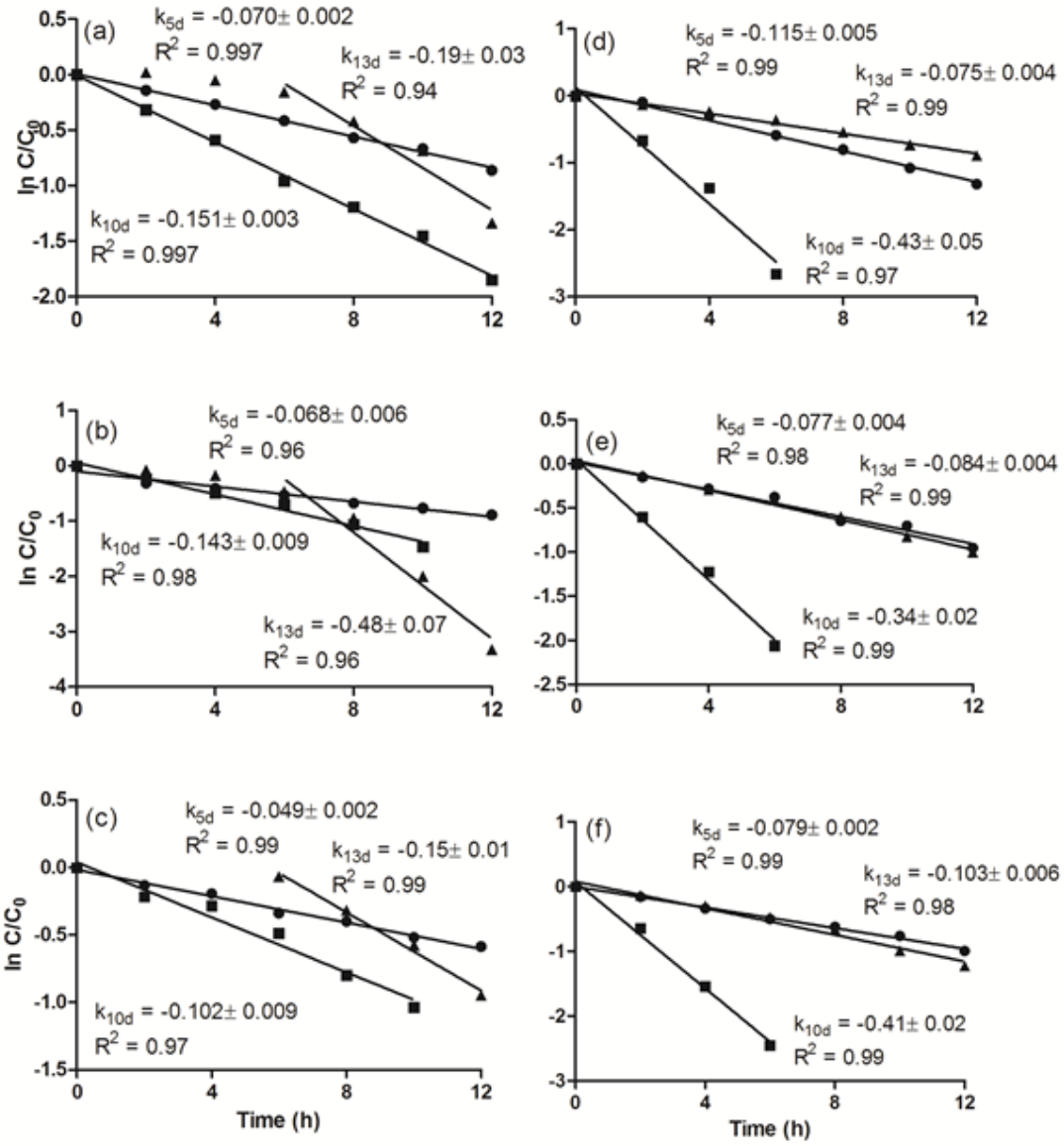

Figure C2: First-order degradation rates of E1 in starvation reactors (a-c) and feast – famine reactors (d-f). Circles, squares, and triangles correspond to Day 5, 10 and 13 respectively. Error terms represent 95% confidence intervals from regression. A lag phase of 6 h was observed in starvation reactors on Day 13. The lag phase was not taken into account when performing regression analysis.

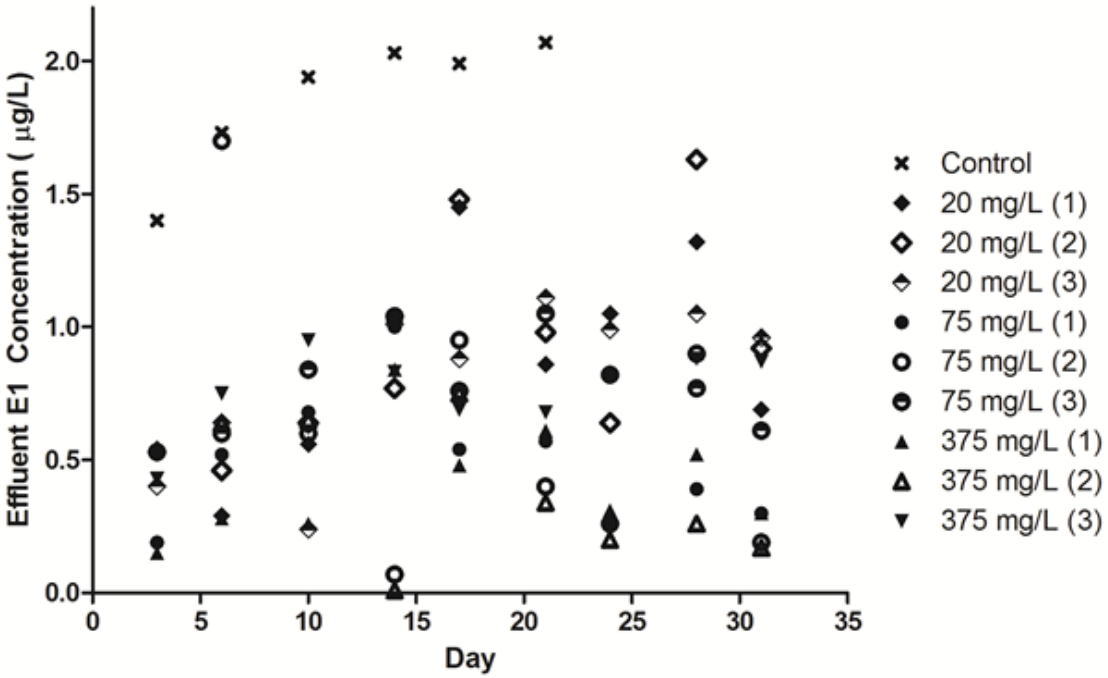

Figure C3: E1 effluent concentrations from MBRs fed with synthetic septage containing 20, 75, and 375 mg/L COD. Reactor E1 removal performance stabilized by day 17.

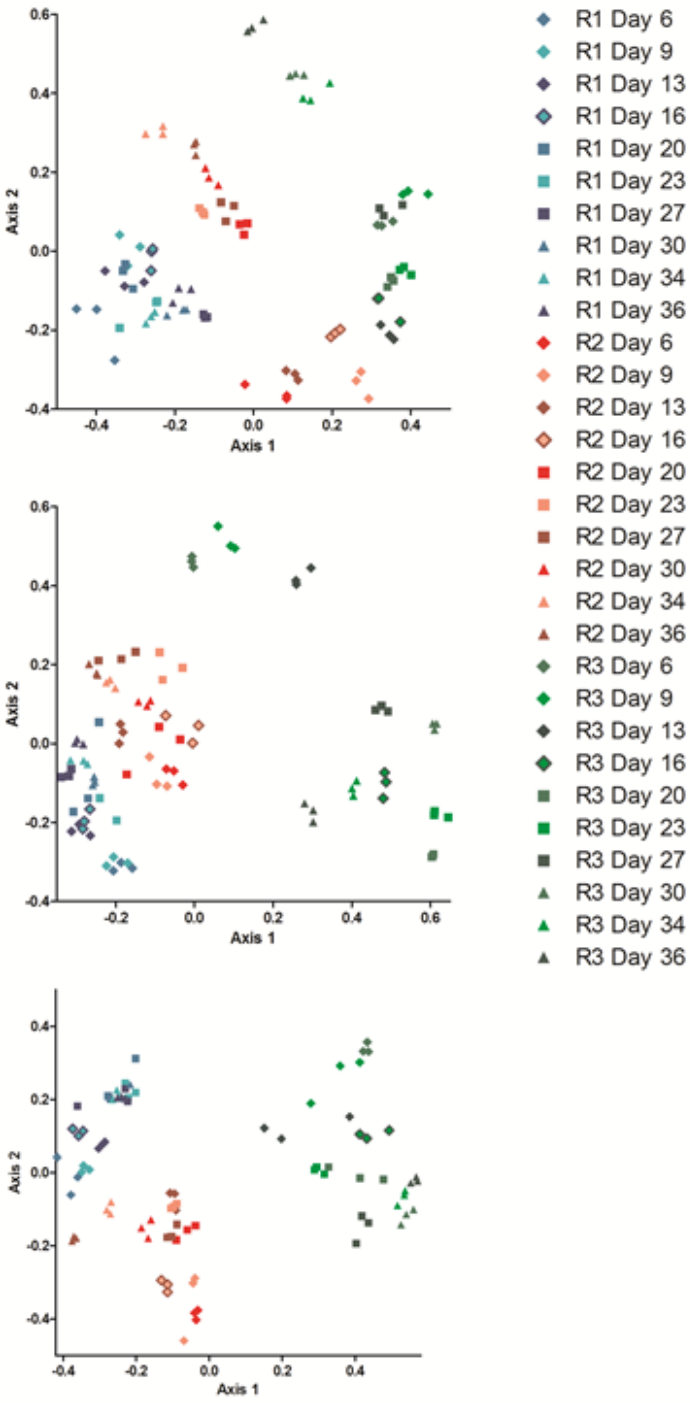

Figure C4: nMDS analysis of ARISA of microbial communities in reactor sets 1, 2, and 3 in (a), (b), and (c) show formation of distinct communities at varying influent COD concentrations. R1, R2, and R3 are reactors with influent COD loads of 20, 75, and 375 mg/L respectively.

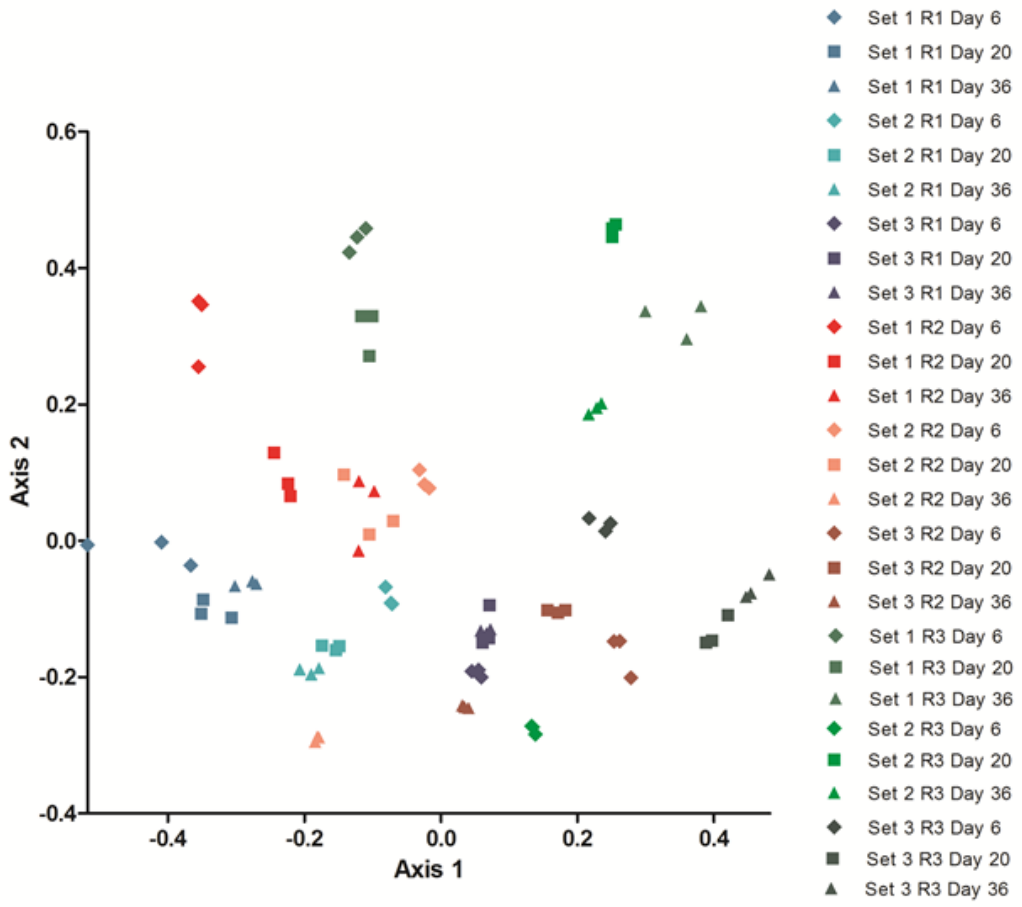

Figure C5: nMDS analysis of ARISA of microbial communities in reactor sets 1-3 on days 6, 20, and 36. These days were chosen from a larger dataset (Figure S.4) for clarity. These data points were representative of major community shifts observed over the course of the experiment, and selecting other representative subsets yield similar plots. R1, R2, and R3 and reactors with influent COD loads of 20, 75, and 375 mg/L respectively.

Table C1: Microbial diversity analysis from ARISA of MBRs show lower diversity in reactors with the highest feed concentration (375 mg/L COD). Error terms represent standard deviation from triplicate reactors.

Sample	Richness	Evenness	Shannon Index
20 mg/L COD	30±3	0.763±0.031	2.58±0.20
75 mg/L COD	29±3	0.747±0.021	2.50±0.04
375 mg/L COD	23±2	0.683±0.049	2.14±0.22

Table C2: Key reactor parameters show similar performance of reactors receiving 20 and 75 mg/L COD. Reactors receiving 375 mg/L COD had slightly higher pH and slightly higher effluent ammonia and effluent COD concentrations. Error terms represent standard deviation from triplicate reactors.

Sample	pH	Effluent Ammonia (mg/L)	Effluent COD (mg/L)
20 mg/L COD	7.48±0.16	12.8±1.3	9±2
75 mg/L COD	7.36±0.08	15.8±2.2	10±3
375 mg/L COD	7.99±0.05	18.5±2.1	28±4

Appendix D: Supporting Information for Chapter 3

Detailed Procedures for DNA and E1 Analysis

DNA Collection, Processing, and Analysis

All samples for DNA analysis were collected in triplicate and subsequently processed. Reactor liquor (1.5 mL) was centrifuged and decanted, after which the pellet underwent three consecutive freeze-thaw cycles and incubation at 70°C for 90 min to lyse cells. DNA was extracted from lysed cells with the FastDNA spin kit (MP Biomedicals, Solon, OH) and stored at -20°C until further processing.

Illumina

The V3 region of the 16S rDNA was amplified via PCR using the primers described by Muyzer et al (1), according to the method described by Bartram et al (2). The primer sequences were as follows: forward primer: 5'-ID-CCTACGGGAGGCAGCAG-3'; and reverse primer: 5'-ID-ATTACCGCGGCTGCTGG-3', where ID is the Illumina adapter sequence containing a six base identification barcode for sample identification (2). PCR products were screened using 2% agarose gels, pooled from duplicate PCR reactions, and purified using the PCR Purification kit (Qiagen; Valencia, Calif.) per manufacturer's instructions. Purified DNA was run on gels for quantification, and equal amounts of each sample were pooled and paired-end sequenced (2 × 150) on an Illumina MiSeq platform at the University of Minnesota Genomics Center (UMGC; Saint Paul, MN, USA).

DNA sequences from Illumina were binned based on the barcode sequencing by UMGC. Illumina sequence reads were processed using Quantitative Insights Into Microbial Ecology (QIIME) (3), generating paired reads of 125 base-pairs. Sequences were screened to exclude the following: sequences containing more than one mismatch in the barcoded primer, sequence lengths of less than 50 bp or more than 125 bp, and sequences with a Q-score of less than 35. Primers and barcodes were trimmed from the screened sequence reads. Sequences were clustered into de novo operational taxonomic units (OTUs) using uclust (4) at 97% similarity. Reference sequences for each OTU cluster were selected based on the most numerous sequence and compared to the Greengenes reference database (5) to determine taxonomy. Reference sequences were aligned using PyNAST (9) and a phylogenetic tree was constructed using FastTree (10). UNIFRAC was used for principle coordinate analysis of communities (6).

E1 Sample Extraction and Cleanup

Solid phase extraction (SPE) and silica gel clean-up procedures were adapted from Ternes et al (7). Briefly, samples of 50 mL or 100 mL were collected for E1 analysis, acidified to pH 3 with concentrated sulfuric acid, and amended with 80 ng of a labeled surrogate, (2,4,16,16-D_4-estrone). Resprep Bonded Reversed Phase SPE cartridges (6 mL, Restek) were preconditioned with two column volumes each of acetone and Milli-Q water. Samples were then loaded onto the cartridges at a flowrate of ~3 mL/min. Samples were eluted from the column with two column volumes of acetone.

Eluted samples were blown down to dryness with nitrogen and resuspended in 2 mL of hexane for silica gel cleanup. Silica gel columns were prepared by packing silica gel to a height of 3 cm (~ 1 mL) into pasture pipettes and then washing with two column volumes of hexane. Samples were then loaded onto the column and eluted with three column volumes of a 65:35 mixture of acetone and hexane (v/v), blown down to dryness with nitrogen, and resuspended in a 60:40 mixture of methanol and water (v/v) containing 100 ng (a concentration of 250 μg/L) of an internal standard (13,14,15,16,17,18-$^{13}C_6$-estrone). The sample was then stored at 4°C until analysis via liquid chromatography-mass spectrometry (LC-MS) (described in the Supporting Information). Average sample recovery was 52% with a standard deviation of 19%.

LC-MS Analysis

E1 samples were quantified via LC-MS using an HP 1050-series LC coupled to an Agilent/HP 1100 Series G1946D mass spectrometer detector or an Agilent 1100 series LC coupled to a 4000 QTRAP triple quadrupole mass spectrometer. E1 was separated on a Synergi 4u Polar-RP 80A 150 × 2.00 mm 4 μm particle size column (Phenomenex).

For LC-MS analysis, a binary gradient consisting of a pH 4 ammonium acetate buffered solution (10 mM) in 90% water and 10% acetonitrile (A) and 100% acetonitrile (B) at a flow rate of 0.2 mL/min was used. The gradient was as follows: 35% B for 17 min, followed by a linear increase to 100% B over 3 min, held at 100% B for 5 min, and stepped down to 35% B for equilibration over 5 min. The mass spectrometer was

operated in negative ion, selected ion monitoring mode with m/z ratios of 269, 273, and 275 for the detection of estrone, the surrogate, and the internal standard, respectively.

Tandem mass spectrometry analysis methods were adapted from Di Carro et al (8). A binary gradient consisting of a pH 5.5 ammonium acetate buffered solution (10 mM) in 90% water and 10% acetonitrile (A) and 100% acetonitrile (B) at a flow rate of 0.2 mL/min was used. The gradient was as follows: 30% B for 2 min, followed by a linear increase to 95% B over 8 min, held at 95% B for 1 min, and stepped down to 30% B for equilibration over 4 min. The mass spectrometer was operated in negative ion, selected reaction monitoring mode, using the two most abundant ion pairs for quantification and confirmation, respectively. The *m/z* pairs were: 269→145 and 269→143 for estrone; 273→147 and 273→187 for the surrogate; and 275→145 and 275→186 for the internal standard.

Standard curves of at least seven points were used in sample quantification. Blanks of 40:60 methanol and water, as well as method blanks were run at the beginning of each sample analysis, as well as intermittently between samples. Typical instrument quantification limits were 10 µg/L (sample quantification limits of 100 ng/L) for the HP 1100 Series G1946D mass spectrometer and 1 µg/L (sample quantification limits of 10 ng/L) for the 4000 QTRAP model. In-vial concentrations of E1 and surrogate were corrected by the internal standard. Sample concentrations of E1 were further corrected using surrogate recovery.

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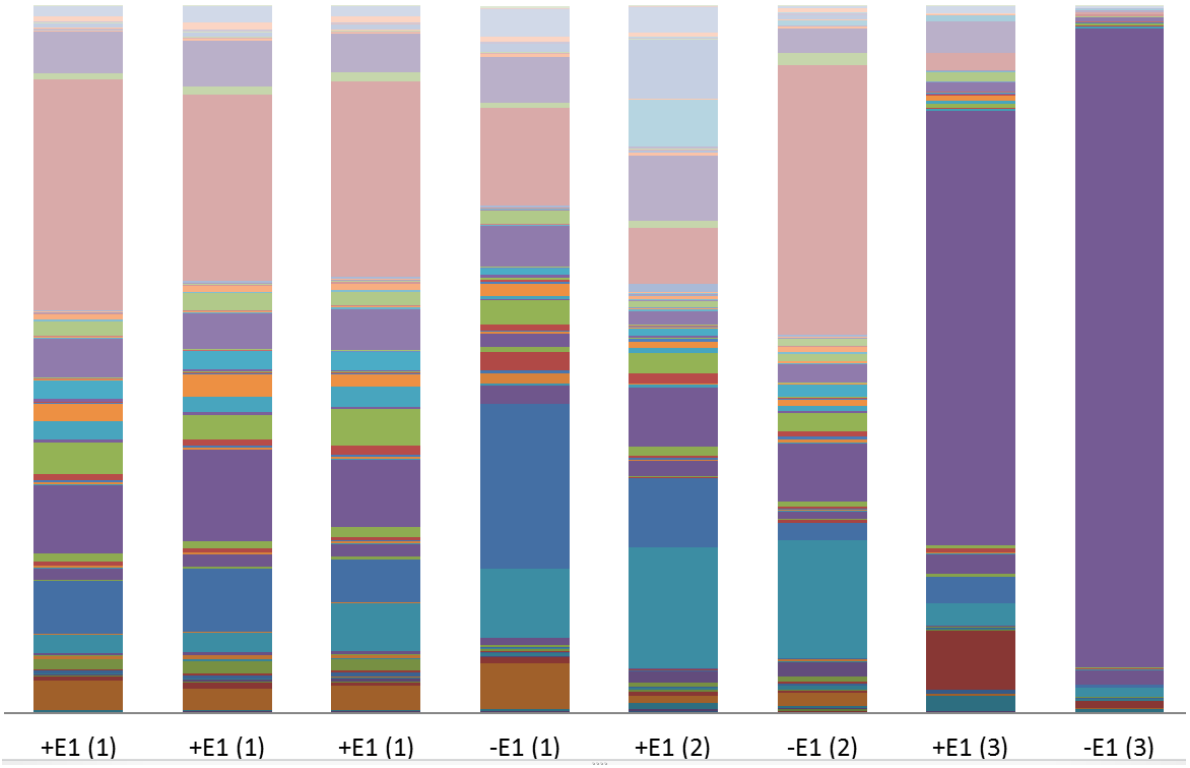

Figure D1: Visual representation of taxonomy for reactors with and without exposure to E1 as analyzed by Illumina, with details reported in the table below. Analysis was done at the genus level. Only genera comprising more than 0.1% of at least one reactor population are shown here for brevity.

	+E1 (1)	+E1 (1)	+E1 (1)	-E1 (1)	+E1 (2)	-E1 (2)	+E1 (3)	-E1 (3)
k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__RB41;f__Ellin6075;g__	0.0002	0.0002	0.0002	0.0041	0.0002	0.0000	0.0001	0.0000
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__	0.0006	0.0007	0.0006	0.0007	0.0011	0.0010	0.0000	0.0000
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Agromyces	0.0143	0.0227	0.0143	0.0392	0.0359	0.0024	0.0110	0.0032
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Microbacterium	0.0071	0.0098	0.0074	0.0060	0.0058	0.0058	0.0003	0.0001

k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;Other	0.0003	0.0005	0.0004	0.0005	0.0013	0.0006	0.0001	0.0001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__	0.0015	0.0018	0.0014	0.0022	0.0013	0.0037	0.0000	0.0000
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Arthrobacter	0.0006	0.0012	0.0006	0.0004	0.0009	0.0004	0.0000	0.0000
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium	0.0000	0.0000	0.0015	0.0000	0.0001	0.0000	0.0000	0.0005
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__	0.0062	0.0059	0.0067	0.0113	0.0827	0.0052	0.0004	0.0002
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Niabella	0.0007	0.0005	0.0007	0.0005	0.0005	0.0017	0.0000	0.0001
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Emticicia	0.0000	0.0000	0.0000	0.0000	0.0656	0.0071	0.0000	0.0000
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Larkinella	0.0002	0.0002	0.0005	0.0005	0.0030	0.0004	0.0000	0.0000
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Rudanella	0.0008	0.0013	0.0015	0.0014	0.0002	0.0000	0.0000	0.0000
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Spirosoma	0.0009	0.0010	0.0011	0.0003	0.0021	0.0003	0.0000	0.0000
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;Other	0.0008	0.0006	0.0007	0.0010	0.0029	0.0006	0.0004	0.0000
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Chryseobacterium	0.0015	0.0020	0.0016	0.0043	0.0046	0.0025	0.0002	0.0000
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Wautersiella	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0099	0.0022
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium	0.0587	0.0646	0.0529	0.0632	0.0899	0.0343	0.0432	0.0026
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__	0.0094	0.0113	0.0129	0.0072	0.0088	0.0161	0.0007	0.0003
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter	0.3223	0.2599	0.2723	0.1376	0.0780	0.3753	0.0243	0.0027
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Sphingobacterium	0.0023	0.0026	0.0028	0.0020	0.0112	0.0033	0.0003	0.0001
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;Other	0.0013	0.0009	0.0014	0.0007	0.0011	0.0018	0.0001	0.0000
k__Bacteria;p__Cyanobacteria;c__ML635J-21;o__;f__;g__	0.0000	0.0000	0.0001	0.0000	0.0013	0.0001	0.0000	0.0000
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	0.0007	0.0008	0.0011	0.0002	0.0012	0.0004	0.0002	0.0002
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Cohnella	0.0009	0.0023	0.0015	0.0000	0.0010	0.0102	0.0001	0.0000
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__	0.0010	0.0008	0.0026	0.0008	0.0000	0.0012	0.0008	0.0017
k__Bacteria;p__Firmicutes;c__Clostridia;o__Thermoanaerobacterales;f__Thermoanaerobacteraceae;g__Thermacetogenium	0.0001	0.0000	0.0001	0.0001	0.0014	0.0000	0.0001	0.0000
k__Bacteria;p__Gemmatimonadetes;c__Gemmatimonadetes;o__Gemmatimonadales;f__Gemmatimonadaceae;g__Gemmatimonas	0.0076	0.0087	0.0084	0.0000	0.0037	0.0075	0.0000	0.0000
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Gemmatales;f__Isosphaeraceae;g__	0.0024	0.0018	0.0020	0.0020	0.0019	0.0025	0.0004	0.0001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__;f__;g__	0.0003	0.0002	0.0008	0.0006	0.0012	0.0004	0.0004	0.0008
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__	0.0203	0.0239	0.0180	0.0197	0.0077	0.0094	0.0135	0.0010
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Brevundimonas	0.0004	0.0006	0.0010	0.0005	0.0013	0.0006	0.0003	0.0001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Caulobacter	0.0001	0.0004	0.0011	0.0003	0.0008	0.0005	0.0001	0.0001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Phenylobacterium	0.0020	0.0016	0.0013	0.0004	0.0012	0.0027	0.0002	0.0000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;Other	0.0016	0.0013	0.0029	0.0013	0.0026	0.0012	0.0007	0.0003
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__;g__	0.0543	0.0501	0.0559	0.0575	0.0184	0.0251	0.0127	0.0058
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bartonellaceae;g__	0.0016	0.0013	0.0015	0.0009	0.0019	0.0018	0.0001	0.0002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__	0.0008	0.0005	0.0005	0.0005	0.0012	0.0003	0.0009	0.0002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;g__Ochrobactrum	0.0003	0.0003	0.0006	0.0003	0.0012	0.0002	0.0001	0.0001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;Other	0.0007	0.0006	0.0010	0.0005	0.0013	0.0004	0.0001	0.0001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia	0.0269	0.0254	0.0251	0.0085	0.0098	0.0174	0.0018	0.0003
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Hyphomicrobium	0.0031	0.0034	0.0017	0.0046	0.0037	0.0003	0.0008	0.0003
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__	0.0010	0.0012	0.0010	0.0027	0.0011	0.0013	0.0008	0.0002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__Mesorhizobium	0.0009	0.0009	0.0008	0.0033	0.0004	0.0012	0.0012	0.0003
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;Other	0.0013	0.0013	0.0019	0.0016	0.0036	0.0013	0.0004	0.0003
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__	0.0249	0.0314	0.0171	0.0172	0.0089	0.0086	0.0071	0.0008
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Agrobacterium	0.0251	0.0216	0.0278	0.0052	0.0062	0.0071	0.0033	0.0002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Kaistia	0.0050	0.0050	0.0039	0.0010	0.0009	0.0032	0.0006	0.0000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;Other	0.0431	0.0340	0.0497	0.0348	0.0280	0.0252	0.0059	0.0019
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other;Other	0.0084	0.0080	0.0132	0.0086	0.0137	0.0066	0.0011	0.0012
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__	0.0035	0.0028	0.0031	0.0010	0.0004	0.0052	0.0002	0.0001

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__Roseococcus	0.0025	0.0022	0.0024	0.0022	0.0016	0.0038	0.0002	0.0000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__	0.0010	0.0008	0.0019	0.0011	0.0041	0.0011	0.0021	0.0040
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Azospirillum	0.0958	0.1278	0.0934	0.0180	0.0809	0.0810	0.6104	0.8947
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__;g__	0.0124	0.0096	0.0145	0.0066	0.0135	0.0066	0.0046	0.0022
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__	0.0048	0.0055	0.0037	0.0259	0.0027	0.0035	0.0053	0.0010
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas	0.0009	0.0010	0.0017	0.0044	0.0019	0.0013	0.0007	0.0005
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;Other	0.0020	0.0019	0.0023	0.0137	0.0018	0.0020	0.0018	0.0005
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;Other;Other	0.0015	0.0008	0.0009	0.0031	0.0006	0.0007	0.0007	0.0001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other;Other	0.0155	0.0167	0.0179	0.0259	0.0214	0.0094	0.0279	0.0201
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Achromobacter	0.0022	0.0030	0.0036	0.0006	0.0012	0.0022	0.0036	0.0001
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Pigmentiphaga	0.0001	0.0002	0.0002	0.0000	0.0005	0.0042	0.0002	0.0000
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__	0.0739	0.0884	0.0590	0.2300	0.0958	0.0233	0.0368	0.0045
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Comamonas	0.0007	0.0011	0.0018	0.0000	0.0002	0.0000	0.0000	0.0000
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Delftia	0.0253	0.0269	0.0666	0.0983	0.1683	0.1646	0.0321	0.0128
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Variovorax	0.0040	0.0043	0.0045	0.0087	0.0002	0.0006	0.0003	0.0005
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__	0.0003	0.0003	0.0005	0.0033	0.0007	0.0003	0.0001	0.0002
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;Other;Other	0.0000	0.0000	0.0000	0.0001	0.0018	0.0000	0.0000	0.0000
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bacteriovoracaceae;g__	0.0002	0.0003	0.0002	0.0011	0.0004	0.0005	0.0000	0.0001
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__;g__	0.0042	0.0053	0.0044	0.0001	0.0003	0.0035	0.0002	0.0001
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Myxococcaceae;g__Myxococcus	0.0008	0.0029	0.0010	0.0011	0.0002	0.0004	0.0000	0.0000
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Nannocystaceae;g__Nannocystis	0.0000	0.0000	0.0000	0.0000	0.0155	0.0200	0.0000	0.0000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__	0.0134	0.0173	0.0163	0.0042	0.0051	0.0080	0.0020	0.0003
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Enterobacter	0.0021	0.0024	0.0033	0.0004	0.0007	0.0019	0.0004	0.0008
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other	0.0039	0.0042	0.0054	0.0014	0.0007	0.0031	0.0006	0.0005
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__Halomonas	0.0007	0.0007	0.0017	0.0005	0.0001	0.0008	0.0005	0.0012
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter	0.0007	0.0013	0.0015	0.0047	0.0028	0.0045	0.0012	0.0016
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	0.0012	0.0023	0.0029	0.0007	0.0010	0.0009	0.0004	0.0001
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__	0.0017	0.0020	0.0015	0.0009	0.0022	0.0011	0.0007	0.0002
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__	0.0062	0.0086	0.0054	0.0078	0.0049	0.0024	0.0836	0.0107
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Dokdonella	0.0000	0.0000	0.0000	0.0004	0.0006	0.0003	0.0052	0.0001
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Pseudoxanthomonas	0.0413	0.0288	0.0326	0.0633	0.0094	0.0186	0.0029	0.0005
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas	0.0021	0.0022	0.0022	0.0031	0.0087	0.0026	0.0216	0.0043
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;Other	0.0005	0.0009	0.0014	0.0016	0.0027	0.0018	0.0019	0.0012
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Luteolibacter	0.0008	0.0008	0.0007	0.0005	0.0001	0.0028	0.0002	0.0000
k__Bacteria;p__WPS-2;c__;o__;f__;g__	0.0000	0.0000	0.0000	0.0000	0.0000	0.0014	0.0000	0.0000
Unassigned;Other;Other;Other;Other;Other	0.0005	0.0007	0.0004	0.0009	0.0029	0.0011	0.0003	0.0004

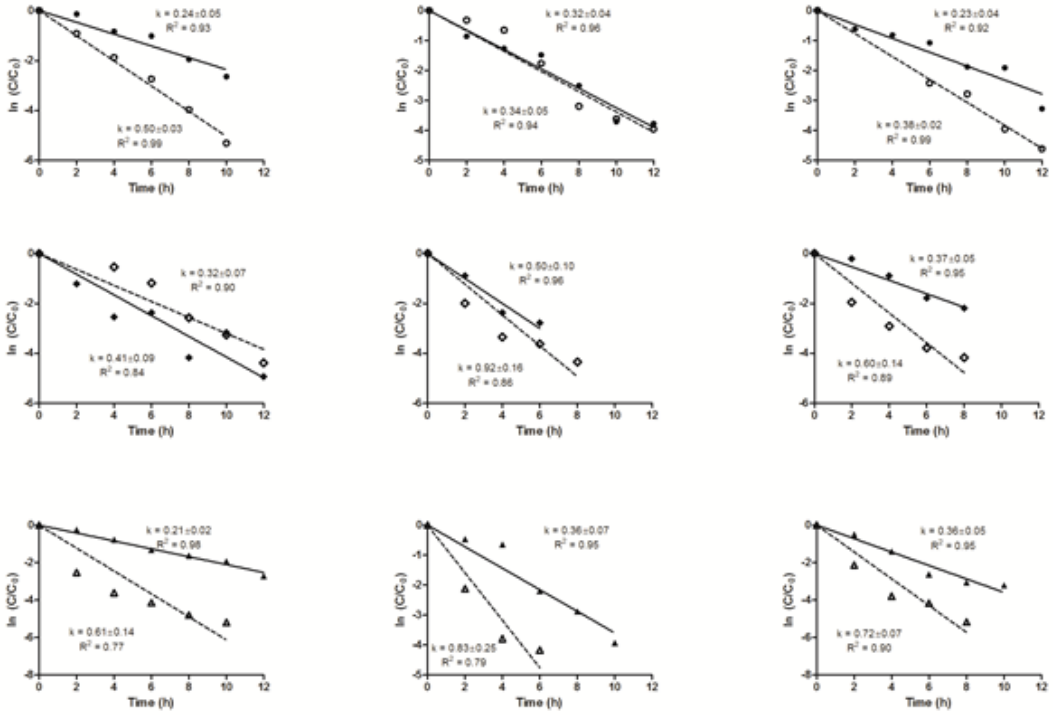

Figure D2: First-order degradation rates of E1 on Days 6 and 12 in feeding cycle experiments with high carbon loads. Closed symbols represent degradation at Day 6 and open symbols represent degradation at Day 12. Circles represent reactors with a daily feed cycle, diamonds represent reactors fed every 3 d, and triangles represent reactors fed every 6 d. Error terms represent 95% confidence intervals from regression.

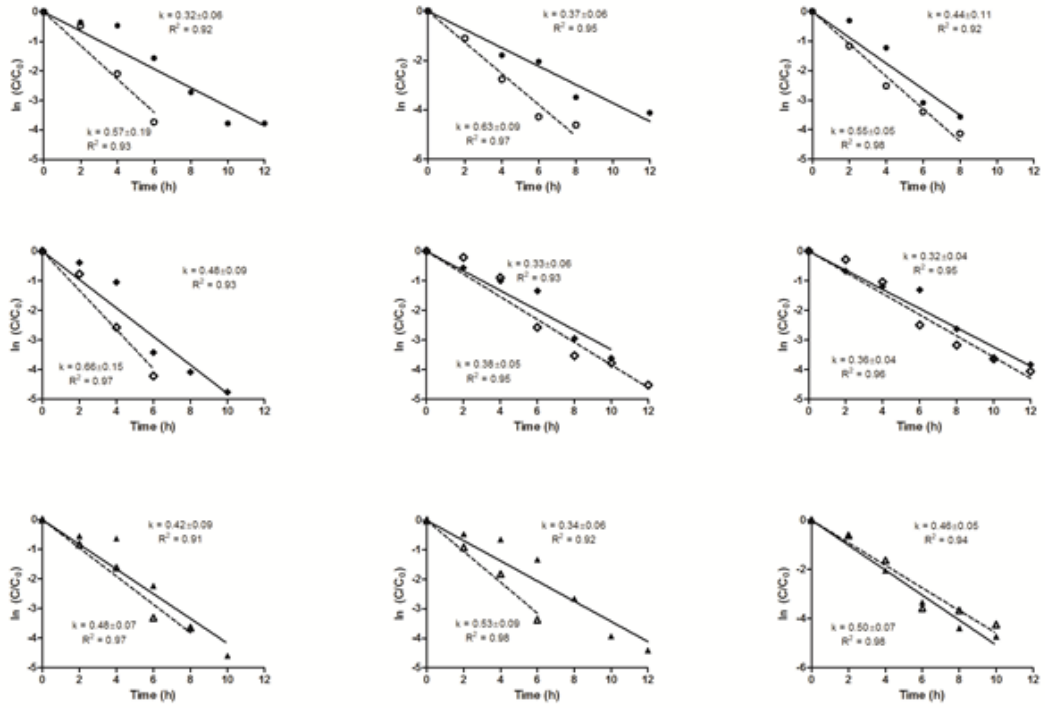

Figure D3: First-order degradation rates of E1 on Days 6 and 12 in feeding cycle experiments with low carbon loads. Closed symbols represent degradation at Day 6 and open symbols represent degradation at Day 12. Circles represent reactors with a daily feed cycle, diamonds represent reactors fed every 3 d, and triangles represent reactors fed every 6 d. Error terms represent 95% confidence intervals from regression.

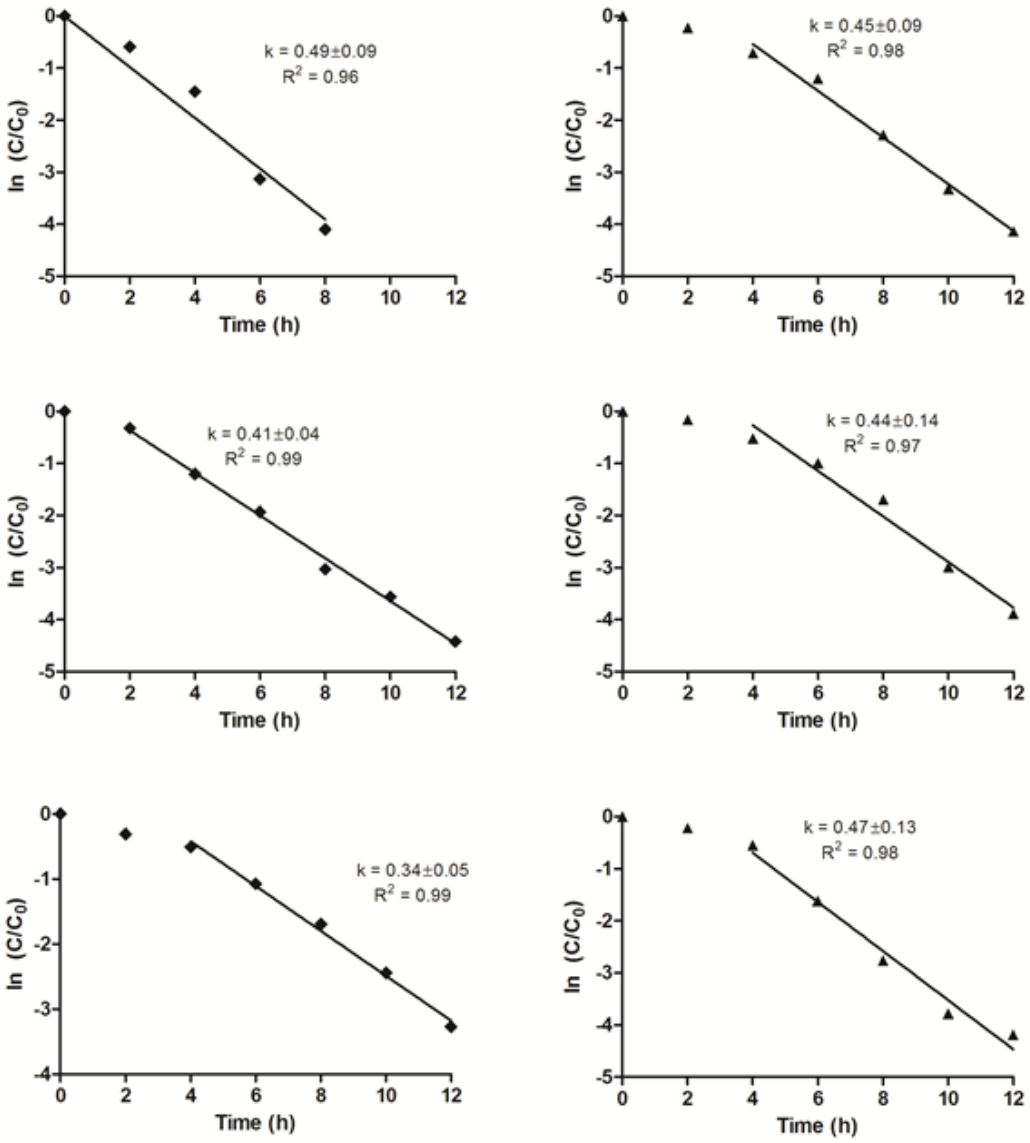

Figure D4: First-order degradation rates of E1 on Day 11 in feeding cycle experiments with low carbon loads. Data is plotted separately from Figure S3 to clearly show lag phase effects. Diamonds represent reactors fed every 3 d, and triangles represent reactors fed every 6 d. Error terms represent 95% confidence intervals from regression.

Appendix E: Supporting Information for Chapter 4

Detailed Procedures for DNA and E1 Analysis

DNA Collection, Processing, and Analysis

All samples for DNA analysis were collected in triplicate and subsequently processed. Reactor liquor (1.5 mL) was centrifuged and decanted, after which the pellet underwent three consecutive freeze-thaw cycles and incubation at 70°C for 90 min to lyse cells. DNA was extracted from lysed cells with the FastDNA spin kit (MP Biomedicals, Solon, OH) and stored at -20°C until further processing.

qPCR Analysis

The qPCR analysis was conducted using an Eppendorf Mastercycle ep *realplex* thermal cycle (Eppendorf, Westbury, NY), using primers described by Muyzer et al. and Harms et al., respectively for the 16S and AMO genes (1-2). The primer sequences were as follows: forward primer: 5'-CCTACGGGAGGCAGCAG-3', and reverse primer: 5'-ATTACCGCGGCTGCTGG-3' for 16S analysis; and forward primer: 5'-TCAGTAGCYGACTACACMGG-3' and reverse primer: 5'-CTTTAACATAGTAGAAAGCGG-3' for AMO analysis. For 16S analysis, a 12.5 μL reaction mixture containing 10 μL of iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), 1 μL bovine serum albumin (Roche Applied Science, Indianapolis, IN), and 1 μL of template was used. For AMO analysis, a 25 μL reaction mixture containing 12.5 μL of iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), 1.25 μL bovine serum albumin (Roche Applied Science, Indianapolis, IN), and 0.5 μL of template were used. Each qPCR run consisted of initial denaturation for 10 min at 95 °C, followed

by 40 cycles of denaturation at 95 °C for 15 s, and anneal and extension at 60 °C (16S) or at 55 °C (AMO gene) for 1 min.

Illumina

The V3 region of the 16S rDNA was amplified via PCR using the primers described by Muyzer et al (1), according to the method described by Bartram et al (2). The primer sequences were as follows: forward primer: 5'-ID-CCTACGGGAGGCAGCAG-3'; and reverse primer: 5'-ID-ATTACCGCGGCTGCTGG-3', where ID is the Illumina adapter sequence containing a six base identification barcode for sample identification (2). PCR products were screened using 2% agarose gels, pooled from duplicate PCR reactions, and purified using the PCR Purification kit (Qiagen; Valencia, Calif.) per manufacturer's instructions. Purified DNA was run on gels for quantification, and equal amounts of each sample were pooled and paired-end sequenced (2 × 150) on an Illumina MiSeq platform at the University of Minnesota Genomics Center (UMGC; Saint Paul, MN, USA).

DNA sequences from Illumina were binned based on the barcode sequencing by UMGC. Illumina sequence reads were processed using Quantitative Insights Into Microbial Ecology (QIIME) (3), generating paired reads of 125 base-pairs. Sequences were screened to exclude the following: sequences containing more than one mismatch in the barcoded primer, sequence lengths of less than 50 bp or more than 125 bp, and sequences with a Q-score of less than 35. Primers and barcodes were trimmed from the screened sequence reads. Sequences were clustered into de novo operational taxonomic units (OTUs) using uclust (4) at 97% similarity. Reference sequences for each OTU

cluster were selected based on the most numerous sequence and compared to the Greengenes reference database (5) to determine taxonomy. Reference sequences were aligned using PyNAST (10) and a phylogenetic tree was constructed using FastTree (11). UNIFRAC was used for principle coordinate analysis of communities (6).

E1 Sample Extraction and Cleanup

Solid phase extraction (SPE) and silica gel clean-up procedures were adapted from Ternes et al (8). Briefly, samples of 50 mL or 100 mL were collected for E1 analysis, acidified to pH 3 with concentrated sulfuric acid, and amended with 80 ng of a labeled surrogate, (2,4,16,16-D_4-estrone). Resprep Bonded Reversed Phase SPE cartridges (6 mL, Restek) were preconditioned with two column volumes each of acetone and Milli-Q water. Samples were then loaded onto the cartridges at a flowrate of ~3 mL/min. Samples were eluted from the column with two column volumes of acetone.

Eluted samples were blown down to dryness with nitrogen and resuspended in 2 mL of hexane for silica gel cleanup. Silica gel columns were prepared by packing silica gel to a height of 3 cm (~ 1 mL) into pasture pipettes and then washing with two column volumes of hexane. Samples were then loaded onto the column and eluted with three column volumes of a 65:35 mixture of acetone and hexane (v/v), blown down to dryness with nitrogen, and resuspended in a 60:40 mixture of methanol and water (v/v) containing 100 ng (a concentration of 250 μg/L) of an internal standard (13,14,15,16,17,18-$^{13}C_6$-estrone). The sample was then stored at 4°C until analysis via liquid chromatography-mass spectrometry (LC-MS) (described in the Supporting Information). Average sample recovery was 52% with a standard deviation of 19%.

LC-MS Analysis

E1 samples were quantified via LC-MS using an HP 1050-series LC coupled to an Agilent/HP 1100 Series G1946D mass spectrometer detector or an Agilent 1100 series LC coupled to a 4000 QTRAP triple quadrupole mass spectrometer. E1 was separated on a Synergi 4u Polar-RP 80A 150 × 2.00 mm 4 μm particle size column (Phenomenex).

For LC-MS analysis, a binary gradient consisting of a pH 4 ammonium acetate buffered solution (10 mM) in 90% water and 10% acetonitrile (A) and 100% acetonitrile (B) at a flow rate of 0.2 mL/min was used. The gradient was as follows: 35% B for 17 min, followed by a linear increase to 100% B over 3 min, held at 100% B for 5 min, and stepped down to 35% B for equilibration over 5 min. The mass spectrometer was operated in negative ion, selected ion monitoring mode with m/z ratios of 269, 273, and 275 for the detection of estrone, the surrogate, and the internal standard, respectively.

Tandem mass spectrometry analysis methods were adapted from Di Carro et al (9). A binary gradient consisting of a pH 5.5 ammonium acetate buffered solution (10 mM) in 90% water and 10% acetonitrile (A) and 100% acetonitrile (B) at a flow rate of 0.2 mL/min was used. The gradient was as follows: 30% B for 2 min, followed by a linear increase to 95% B over 8 min, held at 95% B for 1 min, and stepped down to 30% B for equilibration over 4 min. The mass spectrometer was operated in negative ion, selected reaction monitoring mode, using the two most abundant ion pairs for quantification and confirmation, respectively. The *m/z* pairs were: 269→145 and 269→143 for estrone; 273→147 and 273→187 for the surrogate; and 275→145 and 275→186 for the internal standard.

Standard curves of at least seven points were used in sample quantification. Blanks of 40:60 methanol and water, as well as method blanks were run at the beginning of each sample analysis, as well as intermittently between samples. Typical instrument quantification limits were 10 µg/L (sample quantification limits of 100 ng/L) for the HP 1100 Series G1946D mass spectrometer and 1 µg/L (sample quantification limits of 10 ng/L) for the 4000 QTRAP model. In-vial concentrations of E1 and surrogate were corrected by the internal standard. Sample concentrations of E1 were further corrected using surrogate recovery.

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Aged Synthetic Septage Experiments

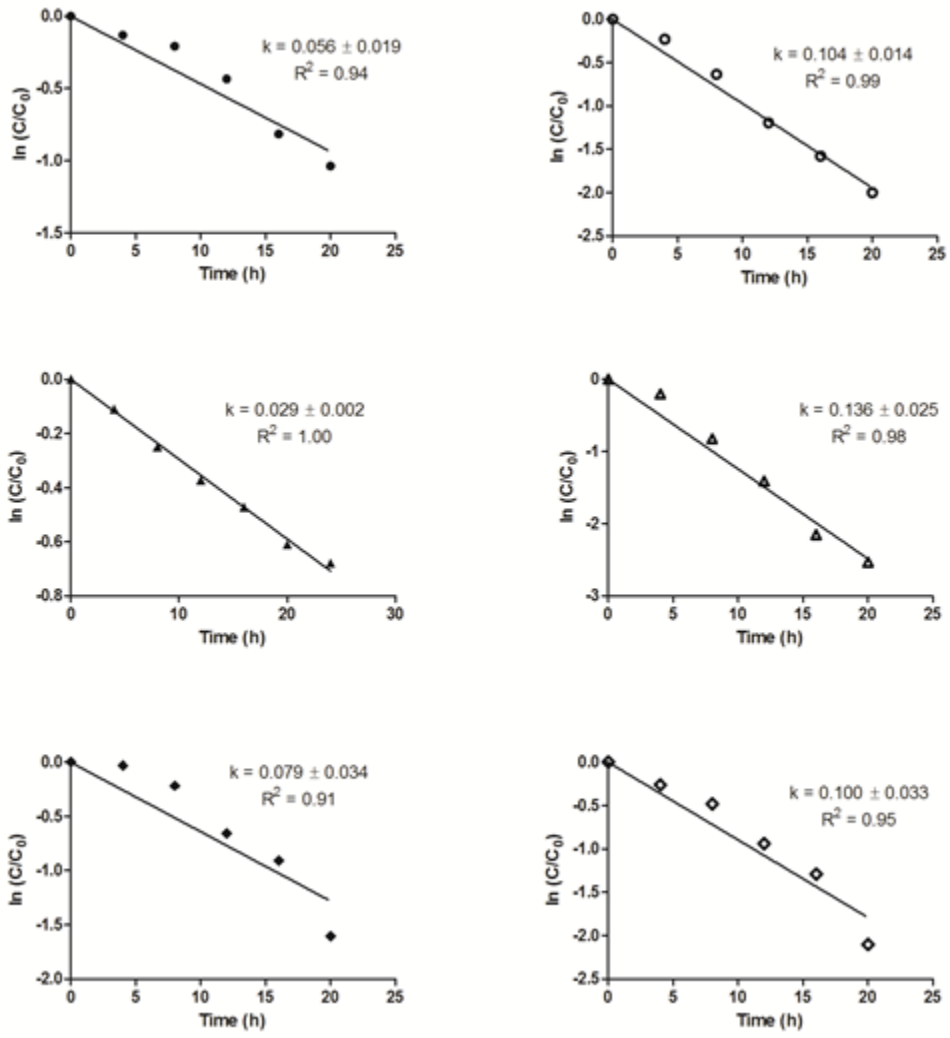

Figure E1: 1st-order degradation of E1 by biomass cultured in fresh and aged synthetic septage. Closed symbols refer to biomass grown in fresh septage while open symbols refer to biomass grown in 8d old septage. Error terms represent 95% confidence intervals.

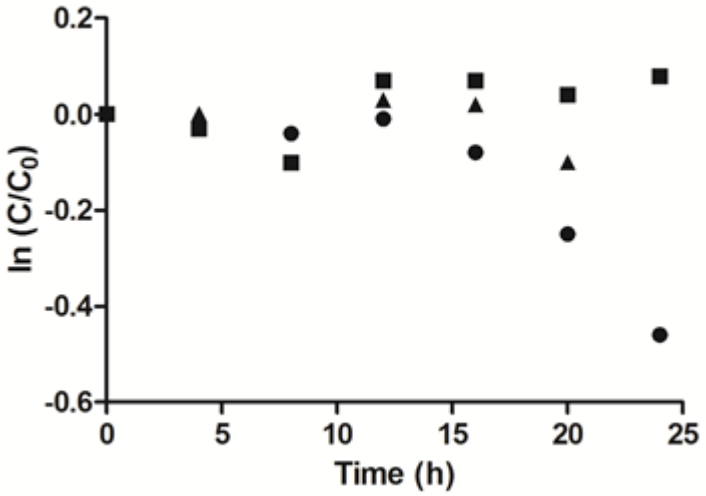

Figure E2: Degradation of E1 by biomass cultured in 2d synthetic septage. Degradation occurred in only one of three replicate reactors, following a 12 h lag phase.

Table E1: Water quality parameters for fresh and aged synthetic septage mixtures. Error term for DOC represents standard deviation from triplicate measurements.

Septage Age	pH	Ammonium (mg/L)	DOC (ppm)
Fresh	7.8	48	35.8 ± 0.9
2d	7.5	17	5.0 ± 0.1
8d	7.7	32	11.3 ± 0.9

Table E2: Spectrophotometric analysis of organic carbon parameters in fresh and aged synthetic septage mixtures. Slope ratio is not reported for fresh septage as the absorbance curve in the 275-295 nm region was erratic.

Septage Age	SUVA	Fluorescence Index	Slope Ratio
Fresh	0.64	1.89	-
2d	0.83	2.16	1.38
8d	0.52	2.54	0.31

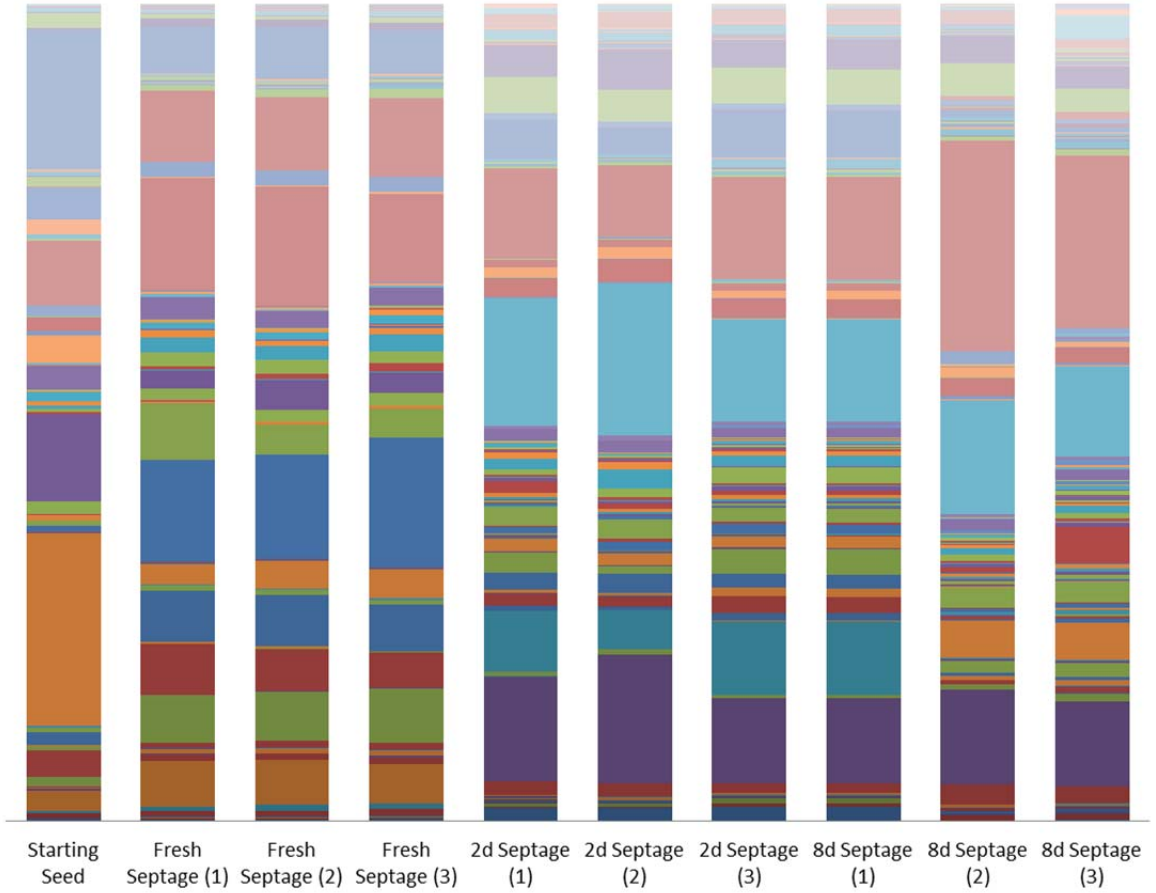

Figure E3: Visual representation of taxonomy for biomass grown on septage of different ages as analyzed by Illumina, with details reported in the table below. Analysis was done at the genus level. Only genera comprising more than 0.1% of at least one reactor population are shown here for brevity.

	Starting Seed	Fresh Septage (1)	Fresh Septage (2)	Fresh Septage (3)	2d Septage (1)	2d Septage (2)	2d Septage (3)	8d Septage (1)	8d Septage (2)	8d Septage (3)
k__Bacteria;Other;Other;Other;Other;Other	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.001
k__Bacteria;p__[Thermi];c__Deinococci;o__Deinococcales;f__Deinococcaceae;g__Deinococcus	0.002	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.001
k__Bacteria;p__[Thermi];c__Deinococci;o__Thermales;f__Thermaceae;g__Thermus	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__C111;g__	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.000	0.004
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__;g__	0.000	0.000	0.000	0.000	0.003	0.002	0.001	0.001	0.001	0.008
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__ACK-M1;g__	0.001	0.000	0.000	0.000	0.008	0.006	0.003	0.003	0.004	0.028
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinosynnemataceae;g__Lentzea	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Cellulomonadaceae;g__Cellulomonas	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium	0.000	0.000	0.000	0.000	0.016	0.019	0.016	0.016	0.014	0.011
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dietziaceae;g__Dietzia	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.001	0.000
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Intrasporangiaceae;g__	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Agromyces	0.001	0.005	0.005	0.005	0.000	0.000	0.000	0.001	0.000	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Candidatus Aquiluna	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Microbacterium	0.001	0.002	0.003	0.003	0.001	0.000	0.001	0.001	0.002	0.003
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Mycetocola	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;Other	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__	0.006	0.004	0.005	0.006	0.011	0.011	0.012	0.012	0.004	0.002
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Arthrobacter	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Mycobacteriaceae;g__Mycobacterium	0.000	0.000	0.000	0.000	0.002	0.001	0.001	0.001	0.001	0.002
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae;g__Rhodococcus	0.000	0.000	0.000	0.000	0.001	0.002	0.001	0.001	0.001	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium	0.000	0.000	0.000	0.000	0.002	0.005	0.002	0.002	0.002	0.004
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Streptomycetaceae;g__Streptomyces	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.001
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Yaniellaceae;g__Yaniella	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other;Other	0.001	0.001	0.001	0.001	0.038	0.049	0.034	0.035	0.034	0.028
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__	0.018	0.005	0.005	0.006	0.044	0.038	0.043	0.042	0.040	0.027
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Chitinophaga	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.009
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Flavisolibacter	0.000	0.000	0.000	0.000	0.006	0.007	0.008	0.008	0.006	0.004
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Sediminibacterium	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter	0.078	0.087	0.090	0.095	0.108	0.086	0.123	0.123	0.254	0.207
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cyclobacteriaceae;g__	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.001
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__	0.002	0.010	0.008	0.010	0.002	0.002	0.002	0.002	0.001	0.001
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Dyadobacter	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Emticicia	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.002
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Hymenobacter	0.170	0.057	0.064	0.052	0.045	0.030	0.055	0.055	0.008	0.007
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Larkinella	0.002	0.002	0.002	0.003	0.000	0.000	0.001	0.001	0.001	0.001
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Rudanella	0.006	0.002	0.001	0.003	0.004	0.004	0.011	0.011	0.003	0.003
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Spirosoma	0.001	0.000	0.000	0.001	0.001	0.001	0.002	0.002	0.002	0.002
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;Other	0.012	0.004	0.003	0.004	0.002	0.002	0.003	0.003	0.002	0.001
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Chryseobacterium	0.039	0.003	0.003	0.003	0.001	0.001	0.000	0.000	0.004	0.004
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];Other	0.017	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.002	0.001
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium	0.006	0.003	0.003	0.003	0.001	0.002	0.003	0.003	0.008	0.007
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__;g__	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.001
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__	0.003	0.006	0.008	0.012	0.002	0.003	0.003	0.003	0.006	0.008
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Sphingobacterium	0.013	0.018	0.018	0.019	0.000	0.001	0.000	0.000	0.015	0.007
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;Other	0.000	0.001	0.001	0.002	0.000	0.000	0.000	0.000	0.001	0.000
k__Bacteria;p__Chloroflexi;c__Dehalococcoidetes;o__Dehalococcoidales;f__Dehalococcoidaceae;g__Dehalogenimonas	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.003	0.001	0.004
k__Bacteria;p__Chloroflexi;c__SL56;o__;f__;g__	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.003
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__	0.000	0.000	0.000	0.000	0.001	0.002	0.001	0.001	0.001	0.001
k__Bacteria;p__Cyanobacteria;c__ML635J-21;o__;f__;g__	0.000	0.135	0.144	0.107	0.009	0.009	0.009	0.009	0.001	0.000
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Synechococcales;f__Synechococcaceae;g__Synechococcus	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__	0.000	0.000	0.000	0.000	0.011	0.014	0.010	0.010	0.011	0.007
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__[Exiguobacteraceae];g__Exiguobacterium	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.000
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	0.017	0.001	0.001	0.001	0.024	0.027	0.021	0.021	0.022	0.018
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Cohnella	0.006	0.001	0.002	0.001	0.001	0.001	0.002	0.002	0.004	0.002
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus	0.033	0.002	0.002	0.002	0.000	0.000	0.001	0.001	0.001	0.001
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__	0.003	0.004	0.002	0.003	0.153	0.183	0.122	0.122	0.137	0.109
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;Other	0.000	0.000	0.000	0.000	0.004	0.006	0.004	0.004	0.004	0.004
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__Desulfosporosinus	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.004	0.001	0.007
k__Bacteria;p__Firmicutes;c__Clostridia;o__Thermoanaerobacterales;f__Thermoanaerobacteraceae;g__Thermacetogenium	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
k__Bacteria;p__Gemmatimonadetes;c__Gemmatimonadetes;o__Gemmatimonadales;f__Gemmatimonadaceae;g__Gemmatimonas	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__	0.028	0.028	0.020	0.022	0.014	0.014	0.012	0.012	0.012	0.014
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Brevundimonas	0.001	0.001	0.001	0.003	0.001	0.000	0.001	0.001	0.000	0.001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Caulobacter	0.000	0.000	0.001	0.002	0.000	0.000	0.001	0.001	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Mycoplana	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.004
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;Other	0.002	0.002	0.003	0.007	0.001	0.001	0.001	0.001	0.001	0.001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__;g__	0.011	0.008	0.009	0.010	0.005	0.003	0.004	0.004	0.004	0.005
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bartonellaceae;g__	0.000	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.000	0.001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__	0.000	0.000	0.000	0.000	0.003	0.003	0.003	0.003	0.004	0.005
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Bradyrhizobium	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.001	0.002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;Other	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;g__Ochrobactrum	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;Other	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia	0.000	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.004
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.002	0.001	0.001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;Other	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__	0.005	0.009	0.006	0.008	0.008	0.010	0.005	0.005	0.004	0.003
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Agrobacterium	0.004	0.018	0.017	0.020	0.012	0.022	0.013	0.013	0.008	0.009
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Kaistia	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;Other	0.004	0.016	0.017	0.015	0.006	0.011	0.019	0.019	0.008	0.007
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other;Other	0.002	0.004	0.006	0.010	0.002	0.004	0.003	0.003	0.002	0.002

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Paracoccus	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__	0.000	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Azospirillum	0.106	0.022	0.036	0.025	0.004	0.002	0.005	0.005	0.004	0.005
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__;g__	0.016	0.013	0.015	0.015	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Pelagibacteraceae;g__	0.001	0.001	0.000	0.000	0.013	0.007	0.006	0.006	0.007	0.043
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__;g__	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__	0.001	0.001	0.001	0.001	0.006	0.004	0.004	0.004	0.004	0.006
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas	0.000	0.000	0.000	0.000	0.002	0.002	0.003	0.003	0.002	0.003
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;Other	0.000	0.001	0.000	0.001	0.002	0.002	0.003	0.003	0.003	0.004
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;Other;Other	0.000	0.000	0.000	0.000	0.002	0.001	0.002	0.002	0.004	0.005
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other;Other	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.001
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__	0.000	0.000	0.000	0.000	0.003	0.004	0.002	0.003	0.002	0.002
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Achromobacter	0.005	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.001	0.001
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Pigmentiphaga	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Burkholderia	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__	0.007	0.069	0.037	0.035	0.023	0.023	0.017	0.017	0.024	0.024
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Comamonas	0.000	0.000	0.000	0.000	0.001	0.003	0.002	0.002	0.001	0.002
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Delftia	0.008	0.124	0.126	0.158	0.008	0.010	0.011	0.011	0.004	0.005
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Hydrogenophaga	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.002
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Methylibium	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.002	0.004	0.005
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Paucibacter	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Ramlibacter	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Variovorax	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.003	0.003
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other	0.000	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.002	0.005
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__	0.234	0.025	0.034	0.035	0.016	0.014	0.013	0.013	0.045	0.045
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Janthinobacterium	0.002	0.001	0.001	0.002	0.000	0.000	0.000	0.000	0.001	0.001
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Oxalobacter	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.002	0.002	0.003
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia	0.005	0.007	0.007	0.006	0.024	0.010	0.030	0.030	0.014	0.016
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Telluria	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;Other	0.015	0.062	0.062	0.056	0.021	0.022	0.017	0.017	0.004	0.004
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;Other;Other	0.001	0.002	0.002	0.001	0.004	0.003	0.010	0.010	0.005	0.006
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales;f__Methylophilaceae;g__	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Nitrosomonadales;f__Nitrosomonadaceae;g__	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;Other;Other;Other	0.006	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bacteriovoracaceae;g__	0.032	0.061	0.051	0.043	0.014	0.013	0.020	0.020	0.005	0.008
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__;g__	0.000	0.000	0.000	0.000	0.006	0.004	0.009	0.009	0.001	0.002
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__0319-6G20;g__	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Myxococcaceae;g__	0.000	0.000	0.000	0.000	0.073	0.047	0.088	0.088	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;Other;Other	0.000	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__	0.011	0.057	0.059	0.066	0.005	0.007	0.004	0.004	0.006	0.009
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Enterobacter	0.002	0.007	0.008	0.007	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Erwinia	0.000	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other	0.001	0.005	0.006	0.007	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__Halomonas	0.002	0.002	0.001	0.002	0.126	0.154	0.102	0.102	0.114	0.102
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter	0.001	0.007	0.007	0.007	0.016	0.016	0.012	0.012	0.025	0.019
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	0.024	0.056	0.055	0.048	0.002	0.003	0.002	0.002	0.004	0.003
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;Other	0.003	0.005	0.007	0.006	0.000	0.000	0.001	0.001	0.000	0.001
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.001	0.002
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Lysobacter	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Pseudoxanthomonas	0.001	0.007	0.007	0.009	0.001	0.000	0.000	0.000	0.002	0.001
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas	0.000	0.001	0.001	0.001	0.002	0.003	0.001	0.001	0.004	0.004
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;Other;Other;Other	0.000	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__Proteobacteria;Other;Other;Other;Other	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
k__Bacteria;p__TM7;c__TM7-3;o__;f__;g__	0.000	0.000	0.000	0.000	0.003	0.002	0.002	0.002	0.001	0.002
k__Bacteria;p__Verrucomicrobia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__	0.000	0.000	0.000	0.000	0.003	0.003	0.007	0.007	0.000	0.000
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Luteolibacter	0.005	0.002	0.002	0.002	0.001	0.002	0.003	0.003	0.007	0.006
Unassigned;Other;Other;Other;Other;Other	0.004	0.002	0.002	0.002	0.017	0.016	0.018	0.018	0.001	0.003

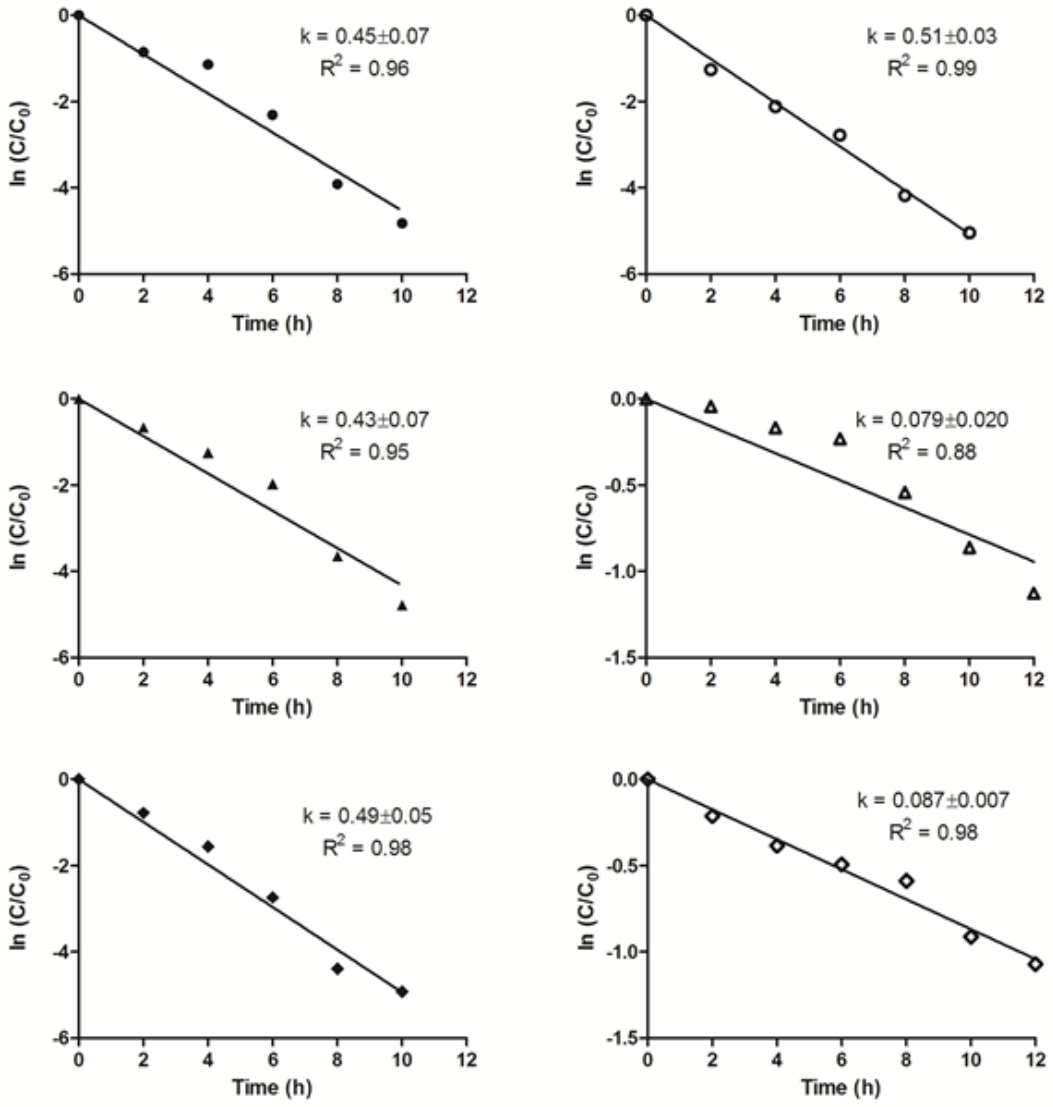

Figure E4: 1st-order degradation of E1 by biomass cultured in undiluted (closed symbols) and 1:2 diluted (open symbols) synthetic septage. Error terms represent 95% confidence intervals.

Water Sources Experiments

Table E3: Trace element concentrations in water sources. All concentrations are listed as mg/L.

	River	Lake	Wetland	Metro
Al	0.099	0.121	0.179	0.211
As	< 0.010	0.031	< 0.010	< 0.010
B	0.018	0.179	0.201	0.257
Ba	0.096	0.265	0.193	0.182
Be	< 0.010	< 0.010	< 0.010	< 0.010
Ca	4.296	9.292	8.184	8.118
Cd	< 0.010	< 0.010	< 0.010	< 0.010
Co	< 0.010	< 0.010	< 0.010	< 0.010
Cr	< 0.010	< 0.010	< 0.010	< 0.010
Cu	< 0.010	< 0.010	< 0.010	< 0.010
Fe	0.096	0.042	0.217	0.062
K	0.125	1.596	1.827	1.851
Li	0.001	0.178	0.015	0.019
Mg	15.940	136.600	29.460	26.170
Mn	0.036	< 0.010	0.191	0.012
Mo	0.004	0.007	0.006	0.019
Na	0.267	3.585	11.680	8.923
Ni	0.018	< 0.010	< 0.010	< 0.010
P	< 0.370	< 0.370	< 0.370	< 0.370
Pb	< 0.180	< 0.180	< 0.180	< 0.180
Rb	< 0.080	< 0.080	< 0.080	< 0.080
S	2.838	215.400	14.010	28.750
Sb	< 0.010	< 0.010	< 0.010	< 0.010
Se	< 0.010	0.048	0.023	0.032
Si	1.088	0.243	1.255	0.994
Sr	0.063	0.493	0.095	0.168
Ti	< 0.010	< 0.010	< 0.010	< 0.010
Tl	< 0.010	0.014	< 0.010	0.014
V	0.001	0.000	0.001	0.001
Zn	0.105	0.115	0.052	0.178

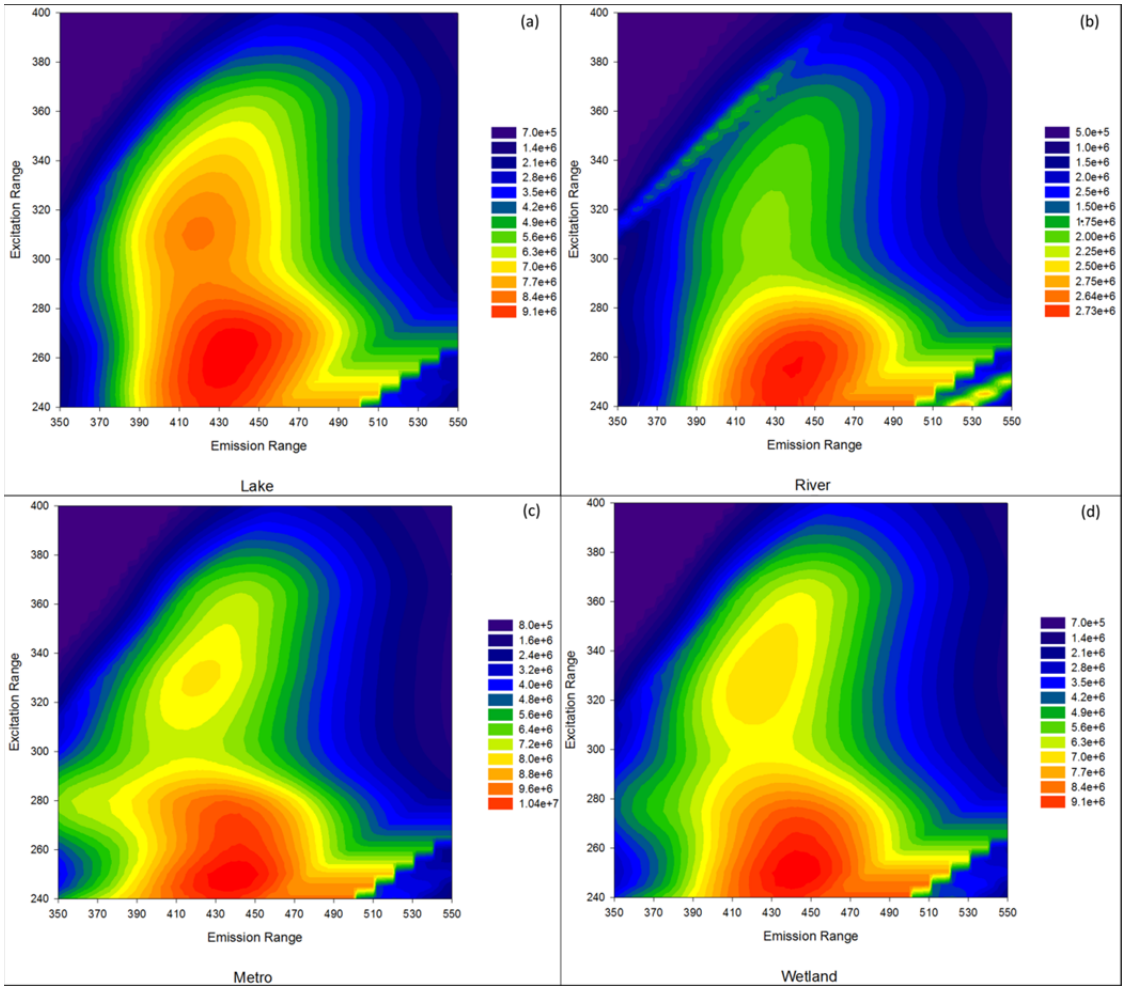

Figure E5: EEMs of water sources at the start of the 5d incubation period.