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Membrane Bioreactor Treatment of Household Light Greywater: Measurement and Effects of Phosphorus Limitation

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Membrane Bioreactor Treatment of Household Light Greywater: Measurement and Effects of Phosphorus Limitation

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Dedication

For my parents.

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Membrane Bioreactor Treatment of Household Light Greywater: Measurement and Effects of Phosphorus Limitation

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As water stresses increase across the U.S., interest in household water reuse is growing. Such reuse typically focuses on light greywater, that is all wastewater generated in the house excluding toilet waste and kitchen wastewater. As this practice becomes more widespread, higher level reuse is expected to require greater greywater treatment prior to reuse. Membrane bioreactors (MBRs) are an attractive technology for this application because they offer a robust combination of treatment processes and are already used in some households in countries such as Japan.

This research sought to understand the role of phosphorus availability in determining the quality of effluent from MBR treatment of light greywater because phosphorus concentrations are expected to be low with phosphorus phased out of many consumer products. Less than 30 μg/L of dissolved orthophosphate was present in synthetic greywater made from three common household products, and no measurable amount of dissolved orthophosphate was found in real greywater, but low concentrations of particulate phosphate were detected. These concentrations were well below levels believed necessary to achieve full BOD_5 removal in biological treatment. Nevertheless, MBR performance was not adversely affected until no supplemental phosphorus was provided. Measurement of extracellular enzyme activity showed an increase in the ratio

of phosphatase activity to total glycosidase activity with declining phosphorus concentration, providing an early indication of nutrient stress before changes in effluent water quality were detected.

Removal of three xenobiotic organic compounds (XOCs) in treatment of synthetic greywater was also evaluated under conditions of phosphorous limitation and balance. Abiotic removal mechanisms were not deemed to be important, but removal of methylparaben and sodium lauryl sulfate via biodegradation responded to nutrient limitation similarly to overall COD removal while removal of diethyl phthalate was affected to a greater extent. Measurement of plasmid DNA concentrations was evaluated as a potential indicator of the effect of nutrient limitation on plasmid-mediated biodegradation of XOCs. An overall reduction in the plasmid content was observed in all cases under conditions of phosphorus limitation; however, the extent of reduction was reactor dependent.

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Chapter 1: Introduction

MOTIVATION

Although water covers much of the earth's surface, freshwater accounts for only three percent of these resources, and more than two-thirds of that freshwater is frozen in icebergs and glaciers (Shiklomanov 1998). Therefore, a limited supply of freshwater is accessible for human use and consumption. The available supply has become further stressed as water sources become contaminated with industrial and human waste, and demand for water continues to grow with increasing population on Earth. As a result, more varied water sources are being sought. Although centralized treatment and distribution of water in the U.S. and some other parts of the world has been praised as one of the engineering achievements of the twentieth century (Constable and Somerville 2003), recognition is growing that not all uses require water to be of potable quality, and highly treated potable water could be conserved by reusing water for non-potable applications.

As a result, interest in decentralized water treatment and reuse is growing, including at the household level. Household water reuse focuses primarily on greywater, which is defined as all wastewater generated in the household excluding toilet waste. Many believe that, with toilet waste excluded, greywater will be far less polluted than typical wastewater, safer from a public health standpoint, and therefore more conducive to reuse. In reality, greywater is highly variable in both quantity and quality, and both of these are dependent on location or country, quantity of water usage, and personal habits such as usage of personal care products (PCPs). As a result, greywater can be as concentrated in organic content and micropollutants as domestic wastewater is.

Greywater treatment and reuse is presently not widespread in the U.S. By some estimates, up to 13 percent of households in states such as Arizona and California – where water sources are particularly stressed – reuse some of their greywater, but greywater reuse is believed to be even less prevalent in most other states (Sheikh 2010). However, interest in and reuse of greywater are expected to grow as water stress increases (particularly if potable water becomes more expensive as a result).

As decentralized water reuse becomes more common, higher level reuse is expected to necessitate greater treatment of the greywater prior to reuse. A wide variety of greywater treatment technologies have been evaluated in the literature. Membrane bioreactors (MBRs) are among the most promising because they offer robust treatment combining both biological and physical treatment mechanisms, and they are already used in some households in Japan (Gaulke 2006). MBRs have been found to reduce the concentration of indicator organisms in greywater by several orders of magnitude even without a chlorination step; therefore, they can offer some protection of human health that other technologies do not (Fangyue *et al.* 2009, Pidou *et al.* 2007). To date, relatively little data exists on biological treatment of U.S. greywater, in general, and essentially none on MBRs in particular. Information is needed on process performance and operation to understand the role that MBRs can play in achieving effluent water quality goals for greywater reuse.

RESEARCH CHALLENGES

Biological treatment of greywater might in some circumstances prove challenging because it can be nutrient-limited when toilet waste and kitchen wastewater are segregated from the rest of the household wastewater (Li *et al.* 2009, Pidou *et al.* 2007). Nitrogen and phosphorus are both essential to microbial growth and are typically

plentiful in domestic wastewater entering a municipal wastewater treatment plant (WWTP). However, by one estimate, less than 20 percent of the nitrogen and less than 30 percent of the phosphorus in domestic wastewater is present in greywater when toilet waste is separated from it (Gunther 2000). In addition, although limited characterization of greywater in the U.S. has been performed, phosphorus concentrations in greywater are expected to be low, since phosphorus has been phased out of many consumer products including laundry detergent (in 1994) and dishwasher detergent (in 2010) (Litke 1999, McCoy 2011). Previous research has also demonstrated that direct measurements of phosphorus concentrations might not be the best indicator of a phosphorus limitation in greywater because the measured concentrations have suggested sufficient phosphorus but supplementation still improved treated water quality (Jefferson *et al.* 2001, Krishnan *et al.* 2008). An assay of extracellular enzyme activity has been used to assess nutrient limitations in surface water bodies and found to be a better indicator than direct measurements of macronutrients (Foreman *et al.* 1998, Hill *et al.* 2010a, Hill *et al.* 2006, Hill *et al.* 2010b, Sinsabaugh *et al.* 1997, Sinsabaugh and Foreman 2001). Such an assay has not been used previously to monitor nutrient limitations in wastewater treatment systems.

Typically, the performance of greywater treatment has largely been evaluated using measurements of chemical or biochemical oxygen demand (COD or BOD); however, the organic load is likely to be comprised of a wide variety of xenobiotic organic compounds (XOCs), hundreds of which have been detected in greywater, many of them originating from PCPs (Almqvist and Hanaeus 2006, Andersen *et al.* 2007, Eriksson *et al.* 2003, Eriksson *et al.* 2010, Hernández Leal *et al.* 2010, Palmquist and Hanæus 2005, Sharvelle *et al.* 2012). Furthermore, researchers have identified a number of potential consequences that cause XOCs in reused greywater to be of concern. Among

these are the potential for increased antimicrobial resistance and decreased microbial diversity in soils irrigated with greywater containing antimicrobial products (Harrow *et al.* 2011); the buildup of surfactants in soil irrigated with greywater and the detrimental effect the surfactants can have on plant growth (Gross *et al.* 2005, Sharvelle *et al.* 2012); and the estrogenic effects that XOCs in greywater can cumulatively exert (Hernández Leal *et al.* 2010). As a result, focusing on removal of COD alone might not be sufficient for greywater treatment prior to reuse. XOC removal in WWTPs has been shown to vary widely from less than 50% removal for the commonly used fragrance galaxolide to a typical removal of more than 80% for methylparaben (a common preservative in many PCPs) (Oppenheimer 2007), but far less is known about their fate in greywater systems. In particular, no investigation of the removal of XOCs in greywater under conditions of nutrient limitation has been undertaken.

Previous research suggests that the genes encoding the degradative pathways for some XOCs are extrachromosomal, and removal of such compounds might be adversely affected by nutrient limitation when these extrachromosomal DNA molecules are lost under stress (Freedman *et al.* 2005). However, monitoring of household greywater treatment systems for a wide variety of XOCs seems unlikely; therefore, an alternate means of tracking such a loss of degradative ability would be useful in these systems.

RESEARCH APPROACH

The research directly addresses each of the challenges outlined above that are anticipated in biological treatment of greywater. This research focuses on MBR treatment of greywater because MBRs are expected to achieve acceptable effluent water quality as measured by COD and suspended solids removal while also being more protective of public health from a microbiological perspective than other available

technologies. Because phosphorus content in PCPs is minimal and low phosphorus concentrations are expected in greywater, a central goal of this research was to understand the potential for phosphorus to determine the quality of treated effluent in MBR treatment of greywater and evaluate extracellular enzyme activity as an alternative method for identifying nutrient limitation in wastewater treatment. In addition, the research sought to understand how the phosphorus limitation would affect the removal of XOCs in the course of biological treatment of greywater and evaluate whether measurement of plasmid DNA concentrations in the reactor mixed liquor could serve as an indicator of loss of degradative ability for some XOCs.

DISSERTATION STRUCTURE

The remainder of this dissertation is organized as follows. Chapter 2 reviews the existing literature related to greywater usage, characteristics, and treatment technologies; nutrient limitations in greywater and their effect on biological treatment; the use of extracellular enzyme activity to measure nutrient limitations; the occurrence of XOCs in greywater, their removal in treatment, and the potential consequences of their presence in reused greywater; and the known effects of nutrient limitations on removal of XOCs as well as plasmid stability in biological treatment. Chapter 3 describes data related to the measurement of phosphorus limitation in MBR treatment of real and synthetic greywater. Chapter 4 presents data on the removal of XOCs in the course of MBR treatment of greywater under conditions of phosphorus limitation and balance. Chapter 5 summarizes experiments evaluating the use of plasmid DNA as an indicator for potential changes in XOC removal under nutrient-limited conditions. Chapter 6 draws conclusions from the data presented in Chapters 3 through 5 and suggests future work that could build on the results presented herein.

Chapter 2: Literature Review

This literature review first introduces greywater and presents its current state of usage and treatment. Subsequently, the available literature that provides the background and motivation for each of the objectives outlined above is presented. To review, these objectives are:

- to understand the potential for phosphorus to determine the quality of treated effluent in MBR treatment of greywater;
- to evaluate extracellular enzyme activity as an alternate indicator of nutrient limitation in greywater treatment;
- to establish how XOC removal will vary in biological treatment of greywater under conditions of phosphorus limitation and phosphorus balance; and
- to evaluate whether changes in plasmid DNA concentration could be monitored as an indicator of potential changes in XOC removal under nutrient-limited conditions.

OVERVIEW OF GREYWATER USAGE, CHARACTERISTICS, AND TREATMENT TECHNOLOGY

State of Greywater Usage in the U.S. and Around the World

Water reuse in the U.S. dates back more than a century to the use of untreated wastewater for agricultural irrigation (Metcalf & Eddy 2007). Currently, up to 2,600 million gallons per day (MGD) of treated wastewater treatment plant effluent are beneficially reused for a variety of applications including irrigation of golf courses, agricultural irrigation, industrial uses such as cooling towers in electric power plants, and even toilet and urinal flushing in some commercial buildings (Metcalf & Eddy 2007).

Greywater reuse is not believed to be nearly as widespread in the U.S., but its extent is difficult to quantify because of both existing practices and policy decisions. For example, in states such as Arizona, Utah, and Texas, greywater systems that reuse less than 400 gallons per day (and adhere to certain minimum standards and management practices) do not require a permit (Sheikh 2010). Nevertheless, by one estimate, 13 percent of households in Arizona are reusing at least some of their greywater (Sheikh 2010). As of 2010, up to 1.77 million households in California were estimated to divert a portion of their greywater for reuse, but only approximately 200 greywater systems were permitted in the state (Sheikh 2010). Rates in most other states are believed to be even lower. The vast majority of those households in the U.S. that are reusing a portion of their greywater are believed to do so without treatment (Sheikh 2010). The most common greywater reuse configuration in the U.S. currently entails diversion of greywater from a washing machine for use in irrigation of lawns and landscaping (Roesner *et al.* 2006). In other parts of the world, greywater is routinely treated before it is reused although the treatment methods vary (Andersen *et al.* 2007, Eriksson *et al.* 2003, Nolde 2000), and some industry experts do expect the growing influence of Leadership in Energy and Environmental Design (LEED) certification to encourage greywater treatment and reuse in the future (Sheikh 2010, U.S. EPA 2012a).

Greywater Characterization

Greywater quantity and quality both depend on the country, volume of water used, and individual choices such as personal care products (PCPs) used, making generalizations difficult.

Quantity of Greywater Generated

The most recent comprehensive data on indoor water usage in the U.S. are summarized in Table 2-1; however, these data are now more than a decade old.

Table 2-1 – Indoor Water Usage in the U.S. (AWWA 1999)

End Use	Average gallons per capita per day	Average liters per capita per day	Percentage of indoor water usage	Category
Toilet	18.5	70.0	30.9%	Blackwater
Kitchen sink	8.4	31.9	14.1%	Dark greywater
Dishwasher	1.0	3.8	1.7%	Dark greywater
Clothes washer	15.0	56.8	25.1%	Light greywater
Shower	11.6	43.9	19.4%	Light greywater
Bathroom sink	2.5	9.3	4.1%	Light greywater
Bath	1.2	4.5	2.0%	Light greywater
Other domestic	1.6	6.1	2.7%	Light greywater
Indoor Total	59.8	226.3	100.0%	

If wastewater is source-separated within a household, it can be divided into three fractions: blackwater, dark greywater, and light greywater. Blackwater is toilet waste and is estimated to comprise 18.5 gallons per capita per day (gpcd) or 31 percent of total indoor water usage. Kitchen wastewater, at 9.4 gpcd, is considered dark greywater and is often excluded from greywater reuse systems because it can contain higher loads of chemical oxygen demand (COD) and nitrogen as well as greater numbers of pathogens (Li *et al.* 2009). Lastly, the remaining portions of household wastewater constitute light greywater at approximately 31.9 gpcd or 53 percent of indoor residential water usage in the U.S.

Quality of Greywater Generated

Table 2-2 summarizes published characteristics for light greywater sources as well as those typical of raw domestic wastewater. In spite of the perception that organic loads in greywater will be lower than those in domestic wastewater, the median COD and biochemical oxygen demand (BOD) values found in greywater are very similar to those typical of domestic wastewater. In addition, the median BOD:COD ratio of 0.50 of light greywater indicates that biological treatment of greywater should be feasible.

Although the BOD and COD of greywater are comparable to those of domestic wastewater, the concentrations of nitrogen and phosphorus found in greywater can be quite different, which could have important implications for greywater treatment. The median value of total nitrogen found in light greywater is less than one-fourth of the typical value for raw wastewater (likely attributable to the exclusion of urine from the waste stream), and the median concentration of ammonia-nitrogen detected to date in light greywater is less than one-tenth of the typical value for raw wastewater.

Table 2-2 – Summary of Published Light Greywater Characteristics (Allen et al. 2012, Birks and Hills 2007, Chaillou et al. 2011, Eriksson et al. 2009, Eriksson et al. 2003, Eriksson et al. 2002, Finley et al. 2009, Friedler and Gilboa 2010, Friedler et al. 2008, Friedler et al. 2005, Laine 2001, Lamine et al. 2012, Metcalf & Eddy 2003, Pidou et al. 2008, Prathapar et al. 2005, Qasim 1999, Sharvelle et al. 2012, Winward et al. 2008b)

									Nitrogen					Phosphorus	
Reference	Year	Greywater Sources	Country	Volume L/(pd)	pH	BOD (mg/L)	COD (mg/L)	BOD: COD	Total N (mg/L)	TKN (mg/L)	NH_4-N (mg/L)	NO_3-N (mg/L)	NO_3 & NO_2 (mg/L)	Total P (mg/L)	PO_4-P (mg/L)
Laak	1974	Bath		32		192[2]	282	0.68			1.34	0.36			0.94
Siegrist *et al.*	1976	Shower/bath	US	38		170[1]			17		2	0.4		2	1
Rose *et al.*	1991	Shower/bath	US								0.11-0.37				
Burrows *et al.*	1991	Shower water	US		6.7-7.4										
Surendran and Wheatley	1998	Shower/bath	UK		7.6	216[2]	424	0.51			1.56	0.9			1.63
Almeida *et al.*	1999	Bath	England	16			210				1.1	4.2			5.3
Almeida *et al.*	1999	Shower/bath	England	12			501				1.2	6.3			19.2
Nolde	1999	Shower/bath	Germany	30-35		50-100[3]	100-200	0.50	5-10					0.2-0.6	
Nolde	1999	Shower	Germany	15-20		70-300[3]	113-633	0.50							
Laine	2001	Shower	England		7.5	146[1]	420	0.35	8.7					0.3	
Laine	2001	Bath	England		7.6	129[1]	367	0.35	6.6					0.4	
Prathapar *et al.*	2005	Shower	Oman	83	7.4	130[2]	249	0.52				6.48			
Pidou *et al.*	2008	Shower	England		7.3-7.8	166[1]	575	0.29	16.4		1.0	7.5			1.3
Lamine *et al.*	2012	Shower	Tunisia			97.3[1]	164	0.59			6.8	0.2	0.24		
Laak	1974	Bathroom sink		8		236[2]	383	0.62			1.15	0.28			48.8
Laine	2001	Hand sink	England		7.3	155[1]	587	0.26	10.4					0.4	
Surendran and Wheatley	1998	Wash basin	UK		8.1	252[2]	433	0.58			0.53	0.34			45.5
Almeida *et al.*	1999	Wash basin	England	13			298				0.3	6			13.3
Prathapar *et al.*	2005	Bathroom sink	Oman	9	7.1	42[2]	58	0.72				2.3			
Christova-Boal *et al.*	1996	Bathroom	Australia		6.4-8.1	76-200[1]				4.6-20	<0.1-15		<0.05-0.20	0.11-1.8	

Table 2-2 – Summary of Published Light Greywater Characteristics (continued)

Reference	Year	Greywater Sources	Country	Volume L/(pd)	pH	BOD (mg/L)	COD (mg/L)	BOD: COD	Nitrogen					Phosphorus	
									Total N (mg/L)	TKN (mg/L)	NH_4-N (mg/L)	NO_3-N (mg/L)	NO_3 & NO_2 (mg/L)	Total P (mg/L)	PO_4-P (mg/L)
Eriksson *et al.*	2003	Shower and hand sink	Denmark	20	7.6-8.6	26-130[2]	77-240	0.49	3.6-6.4		0.02-0.42	<0.02-0.26		0.28-0.78	
Friedler *et al.*	2005	Shower/bath and bathroom sink	Israel			59[2]	158	0.37							
Friedler *et al.*	2008	Shower/bath and bathroom sink	Israel			104[2]			4.6					0.7[4]	
Friedler and Gilboa	2010	Shower/bath and bathroom sink	Israel			95[2]	148	0.64							
Winward *et al.*	2008	Shower/bath and bathroom sink	England			8-34[1]	33-138	0.23							
Pidou *et al.*	2008	Shower/bath and bathroom sink	England		6.6-7.6	39[1]	144	0.27	7.6		0.7	3.9			0.5
Birks and Hill	2008	Shower/bath and bathroom sink	England		6.5-8.8	10-160[2]	25-420	0.48		4.6				0.86	
Eriksson *et al.*	2009	Shower and hand sink	Denmark				142	0.47							0.5
Chaillou *et al.*	2011	Shower and hand sink	France		7.34-7.71	81-670[1]	112-1001	0.47-0.69	4.3-15.9					0.20-1.12	
Laak	1974	Laundry				282[2]	725	0.39			11.3	1.26			171
Siegrist, Witt, and Boyle	1976	Clothes wash	US			380[1]			21		0.7	0.6		57	15
Siegrist, Witt, and Boyle	1976	Clothes rinse	US			150[1]			6		0.4	0.4		21	4
Rose *et al.*	1991	Laundry wash	US								0.1-3.47				
Rose *et al.*	1991	Laundry rinse	US								0.06-0.33				
Christova-Boal *et al.*	1996	Laundry	Australia		9.3-10	48-290[1]				1.0-40	<0.1-1.9		0.10-0.31	0.062-42	
Surendran and Wheatley	1998	Washing	UK		8.1	472[2]	725	0.65			10.7	1.6			101
Almeida *et al.*	1999	Washing	England				1815				2	2			21
Prathapar *et al.*	2005	Laundry	Oman	13	8.3	178[2]	231	0.77				5.83			
Finley *et al.*	2008	Shower/bath and laundry	Canada		6.7-7.6		278-435				1.2-6.2			0.24-1.02	
Sharvelle *et al.*	2012	Shower/bath, hand sink, laundry	US		6.7-7.5	178-214[1]	341-391	0.51-0.55	23.0-27.3		15.4-18.6	0.5-0.9		6.0-7.0	8.7-8.8
Allen *et al.*	2012	Shower/bath, hand sink, laundry	US		5.5-9.7										
Raw Domestic Wastewater			Minimum	8	5.5	8	33	0.23	3.6	1.0	0.02	<0.02	<0.05	0.062	0.5
			Maximum	83	10	670	1815	0.77	37.3	40.0	18.6	7.5	0.31	57	171
			Median	16	7.54	152.5	327	0.50	8.7	12.3	1.18	1.26	0.205	0.70	8.75
			Range		6.7-7.5	110-400	200-780	0.3-0.8	20-85		12-50		0-small	4-8	
			Typical		7	210	400	0.53	40		20		0	6	

[1] Reported as BOD_5

[2] Reported as BOD

[3] Reported as BOD_7

[4] Reported as dissolved P.

L/(pd) - liters per person per day

mg/L - milligrams per liter

The phosphorus data indicate a large range in measured concentrations of both total phosphorus and phosphate-phosphorus. Some of the temporal and spatial variability in the data might originate from differences in public policy in different countries and changes in those policies in recent decades. Some of the data presented in Table 2-2 are over three decades old, and, beginning in 1971, U.S. municipalities and states began to limit the amount of phosphate allowable in laundry detergent or ban it altogether (Litke 1999). Ultimately, only 27 states and the District of Columbia passed legislation limiting phosphate in laundry detergent, but manufacturers opted not to distribute both high- and low-phosphate detergents, and the patchwork bans led to a complete phase-out of phosphate from detergent in the U.S. by the 1990s (Litke 1999). Therefore, the higher phosphorus concentrations shown in Table 2-2 of up to 57 mg/L of total phosphorus or 15 mg/L phosphate-phosphorus (detected four decades ago in water from the washing machine) would no longer be expected in the U.S. However, even today, phosphate usage in laundry detergent in Europe varies by nation. As of 2007, only 55 percent of laundry detergent used in the United Kingdom was deemed phosphate-free compared to 80 percent in Denmark (Commission of the European Communities 2007). The lack of a phosphorus ban in the UK could explain concentrations as high as 101 mg/L measured in the UK even in the last fifteen years (Table 2-2). Similarly, detergent manufacturers in Australia only agreed to remove phosphates from their products in 2011, so both phosphate-containing and phosphate-free detergents can be sold until 2014 (Barlass 2011). Therefore, higher phosphorus concentrations in greywater would continue to be expected in Australia, but the same issues of phosphorus limitation are likely to be of increasing importance in the future as phosphorus is also phased out of consumer products there.

In reviewing a variety of published greywater characterization and treatment schemes, Fangyue *et al.* (2009) suggest that bathroom, laundry, and mixed greywater will all be deficient in nitrogen, and that laundry and mixed greywater will be deficient in phosphorus in locations or situations where phosphorus-free detergents are in use. As a result, the authors suggest that some kitchen greywater be mixed with other sources if the greywater is to be treated biologically (Fangyue *et al.* 2009). This approach might alleviate a nitrogen limitation, but it is unlikely to counteract phosphorus limitation in the U.S., since more than 16 states moved in 2010 to reduce the amount of phosphorus in dishwasher detergents, which resulted in its complete removal from dishwasher detergents sold in the U.S. (McCoy 2011).

Greywater Treatment in Membrane Bioreactors

A wide variety of technologies for greywater treatment have been evaluated including physical treatment (such as sedimentation, sand filtration, and membrane filtration), chemical treatment (including coagulation and disinfection), and biological treatment systems (including rotating biological contactors, sequencing batch reactors, and constructed wetlands) (Li *et al.* 2009, Pidou *et al.* 2007). A membrane bioreactor (MBR) combines biological treatment typical of the activated sludge process used in conventional wastewater treatment with filtration through a micro- or ultrafiltration membrane for separation of the microorganisms employed in biological treatment from the treatment process effluent (Stephenson *et al.* 2000). One advantage of an MBR in greywater treatment is that it combines both physical and biological treatment processes, so, while one alone might be insufficient, the combined mechanisms are more likely to achieve treatment objectives. Several studies have investigated the treatment of greywater (of varying sources and quality) in MBRs (Table 2-3).

Table 2-3 – Performance of Membrane Bioreactors in the Treatment of Greywater (Atasoy *et al.* 2007, Friedler and Gilboa 2010, Huelgas and Funamizu 2010, Laine 2001, Lamine *et al.* 2012, Lesjean and Gnirss 2006, Liu *et al.* 2005, Merz *et al.* 2007, Winward *et al.* 2008a, U.S. EPA 2012a)

Authors	Year	Country	Sources of Greywater	Solids Residence Time	COD			BOD			Effluent SS (mg/L)
					Influent (mg/L)	Effluent (mg/L)	Removal	Influent (mg/L)	Effluent (mg/L)	Removal	
Laine	2001	England	All (synthetic)	Infinite	128	7	92%	41	1	96%	4
Liu *et al.*	2005	China	Shower	Infinite	126-322	2-37	34-85%	99-212	<5	92-98%	ND
Lesjean & Gnirss	2006	Germany	Shower, bathroom sink, kitchen sink	4 d	493	24	85-95%	NR	NR	NR	<1
Atasoy *et al.*	2007	Turkey	All	Infinite	245	13	95%	90	<5	>95%	2
Merz *et al.*	2007	Morocco	Shower	Infinite	109	15	85%	59	4	94	NR
Winward *et al.*	2008	England	Shower/bath, bathroom sink	Infinite	87	47	46%	20	1	95%	ND
Winward *et al.*	2008	England	Shower/bath, bathroom sink (partially synthetic)	Infinite	495	53	89%	164	1	99%	1
Friedler & Gilboa	2010	Israel	Shower/bath, bathroom sink	15-20d	148	42	71%	95	1.1	99%	NR
Huelgas & Funamizu	2010	Japan	Washing machine, kitchen sink (synthetic)	Infinite	675	26	96%	NR	NR	NR	NR
Lamine *et al.*	2012	Tunisia	Shower	Infinite	164	20.8	87%	97.3	12.3	87%	ND
U.S. EPA Suggested Guidelines for Unrestricted Urban Reuse					--	--	--	--	10	--	--
NSF/ANSI Standard 350-1 Effluent Criteria for Unrestricted Outdoor Use					--	--	--	--	$10^{1,2}$/$25^{1,3}$	--	10^{2}/30^{3}
NSF/ANSI Standard 350-1 Effluent Criteria for Subsurface Discharges					--	--	--	--	25^{1}	--	30

ND - not detected

NR - not reported

[1] $CBOD_5$

[2] Test average

[3] Single sample maximum

Eight of the ten studies were performed with no sludge removal from the reactor (other than for sampling purposes), which corresponds to an infinite solids residence time (SRT). A high SRT would be expected to lead to the very low effluent BOD concentrations presented in Table 2-3. The objectives of any treatment process will vary with intended reuse, local regulations, and other factors. In Table 2-3, the effluent BOD and suspended solids concentrations measured are compared to the water reuse guidelines issued by the U.S. Environmental Protection Agency (EPA) in 2012 for unrestricted urban reuse, which includes typical current uses for greywater such as landscape irrigation and toilet flushing, along with those of the NSF/ANSI Standard 350-1 for unrestricted outdoor use for residential treatment systems and for subsurface discharges

(as is required by many greywater regulations across the country) (U.S. EPA 2012a). Some of the data presented in Table 2-3 are mean concentrations and do not represent every sample analyzed in the study, but the data nevertheless indicate that the criteria for both BOD and effluent suspended solids are achievable in MBR treatment, especially with a longer SRT.

Atasoy *et al.* (2007), Laine (2001), Lamine *et al.* (2012), Merz *et al.* (2007), Winward *et al.* (2008a), and Atasoy *et al.* (2007) also analyzed greywater influent for indicator organisms and, for total coliforms, found 10^4-10^7 colony forming units (CFU) per 100 milliliters (mL). Atasoy *et al.* (2007) and Lamine *et al.* (2012) detected no coliforms in the treated effluent, corresponding to a 4 and 5 log reduction, respectively, of total coliforms while Winward *et al.* (2008a) found on average less than 3 total coliforms per 100 mL in the effluent, corresponding to a 7 log reduction in the course of treatment. Laine (2001) detected no total coliforms per 100 mL in more than 90 percent of the samples analyzed and detected on average 2 total coliforms per 100 mL, which corresponds to a 6 log reduction in total coliforms in the treatment process. Merz *et al.* (2007) observed an initial period where more than 60 fecal coliforms per 100 mL were detected in the effluent, a value which indicated a 4 log removal of fecal coliforms but was still unexpectedly high and was attributed to biofilm growth in the permeate pump. Once this pump was disinfected, no further coliforms were detected. The U.S. EPA water reuse guidelines suggest that no sample should contain more than 14 CFU per 100 mL of total (or fecal) coliforms but that the median value of daily analyses over 7 days should be no detectable total (or fecal) coliform CFUs per 100 mL (U.S. EPA 2012a). Upon review of published greywater treatment studies, Pidou *et al.* (2007) and Fangyue *et al.* (2009) concluded that MBR treatment of greywater is the only technology that had been researched that could provide sufficient microorganism (and, it is assumed,

associated pathogen) removal from greywater without a subsequent disinfection or filtration step. Therefore, MBR treatment of greywater is believed to have some advantage over some other technologies from a public health perspective.

However, as summarized in Chapter 1, MBR treatment of greywater is expected to have additional challenges resulting from phosphorus limitation and the occurrence of a wide range of XOCs. The literature relevant to each of these challenges is outlined in the next sections.

OBJECTIVE 1 –ASSESS THE POTENTIAL FOR PHOSPHORUS LIMITATION IN MBR TREATMENT OF GREYWATER

As detailed above, a wide range of phosphorus concentrations have been measured in greywater to date, but changes in public policy in the U.S. and elsewhere are expected to result in low phosphorus concentrations in greywater in the near future. Since phosphorus is an essential nutrient for microbial growth, the low availability of phosphorus might hinder biological treatment of greywater.

The importance of phosphorus in the biological treatment of greywater has been demonstrated by Jefferson *et al.* (2001). Despite detecting 1.37 mg/L of phosphorus in real greywater collected from baths, showers, and bathroom sinks, the addition of 1.63 mg/L phosphorus to the greywater improved COD removal by more than 110 percent and increased oxygen uptake by approximately 10 percent. In a synthetic greywater that contained 0.047 mg/L of phosphorus, addition of 2.95 mg/L of phosphorus resulted in an increase in COD removal of approximately 25 percent, but oxygen uptake actually dropped 12 percent, which was interpreted to indicate improvement in COD removal by chemical reactions such as adsorption or coagulation rather than stimulation of the biomass (Laine 2001).

Similarly, other research into nutrient ratios in biological treatment of greywater found that dosing even dark greywater collected from household kitchens in Malaysia with nitrogen and phosphorus improved COD removal in a sequencing batch reactor (Table 2-4).

Table 2-4 – Impact of Nutrient Balancing on Effluent COD Concentration in SBR Treatment of Dark Greywater (Krishnan *et al.* 2008)

COD:N:P (all in mg/L)	Effluent COD Concentration (mg/L)
100:1.82:0.76 (no nutrient addition)	65
100:2.5:0.5	35
100:3.5:0.75	15
100:5:1	12

The Jefferson *et al.* (2001) study only measured the effect of one dose of phosphorus (among other nutrients) addition on COD removal and oxygen uptake but did not determine the minimum phosphorus concentration for which no evidence of phosphorus limitation was apparent. Jefferson *et al.* (2001) also found that phosphorus addition to real greywater improved COD removal even when the COD to nitrogen to phosphorus ratio prior to nutrient addition had been approximately 13 mg:4 mg:1 mg. If this ratio is normalized to a 100 mg/L COD then the ratios of COD:N:P are 100:30.8:7.7. These ratios indicate an excess of both nitrogen and phosphorus relative to the ratio of 100 mg BOD:5 mg N:1 mg P often cited as necessary for nutrient balance in wastewater treatment (Metcalf & Eddy 2003). The phosphorus concentration measured, therefore, does not indicate a phosphorus limitation, and yet addition of phosphorus more than doubled the COD removal. The existing literature does not address why greywater with a phosphorus concentration that would be stoichiometrically adequate for complete COD degradation could still be phosphorus-limited. Nor does the literature provide an

alternate method for identifying the occurrence of a phosphorus limitation when phosphorus concentrations are not indicative of such a limitation.

OBJECTIVE 2 – EVALUATE EXTRACELLULAR ENZYME ACTIVITY AS AN ALTERNATE INDICATOR OF NUTRIENT LIMITATION IN GREYWATER TREATMENT

While macronutrient concentrations might be straightforward to measure, these concentrations alone might not be indicative of nutrient limitation or nutrient balance, as demonstrated above. One possible alternate indicator could be a measurement of extracellular enzyme activity (EEA), which has been developed over the last two decades as an indicator of nutrient limitation in riverine and other natural systems but has been applied to a lesser extent thus far in wastewater treatment systems.

EEA assays typically measure hydrolytic enzymes that catalyze degradation of complex compounds in the ecosystem to the monomers or other simpler compounds that can be assimilated by microorganisms. Monomer generation is believed to be a slower process than their assimilation; therefore, these hydrolytic reactions are the rate-limiting step in macronutrient acquisition by microorganisms (Sinsabaugh *et al.* 1997). EEAs have been measured and characterized in a wide variety of ecosystems and environments, initially in soil ecosystems and later in aquatic environments including lakes, rivers, and, to a more limited extent, wastewater treatment and activated sludge systems.

The relationship between EEA and nutrient limitation is best understood in soil and riverine systems. An "optimal resource allocation strategy" has been hypothesized; that is, in essence, microorganisms have limited resources and must distribute them among various competing processes. All enzymes will therefore not be expressed or produced at constant levels at all times because microorganisms can conserve resources by not producing certain enzymes when they are not needed (Sinsabaugh *et al.* 1997, Sinsabaugh *et al.* 2009). Researchers have investigated multiple systems to determine

whether the activity of these enzymes related to monomer acquisition or production are inversely related to the availability of the nutrients themselves; that is, when phosphate, for example, is readily available and assimilable, it would be advantageous to the microorganisms to produce lower levels of phosphatase enzymes that would normally be used to free phosphate from more complex molecules.

The relationship between phosphorus availability and phosphatase activity was first studied in water bodies during phytoplankton blooms, and an inverse correlation between the two was established (Gage and Gorham 1985, Vrba *et al.* 1995). Others have shown that activity of aminopeptidases – which are involved in nitrogen acquisition from amino acids – is induced by low nitrogen concentrations (Sinsabaugh 1994). Sinsabaugh *et al.* (1993) broadened the study to look at multiple classes of hydrolases by examining nitrogen and phosphorus uptake in the course of wood decomposition. In comparing activity among different locations, the researchers found an inverse correlation between nutrient accumulation and the activity of the associated enzymes for both nitrogen and phosphorus; that is, at locations where nitrogen accumulated as mass was lost (indicating an excess of nitrogen), aminopeptidase activity was low, while, at locations where phosphorus accumulated as mass was lost, phosphatase activity was low.

More recent studies (particularly in riverine systems) have examined ratios among glycosidase activity, aminopeptidase activity, and phosphatase activity as an indication of whether carbon, nitrogen, or phosphorus is the limiting nutrient, since glycosidases are used by microorganisms for carbon acquisition, aminopeptidases in protein degradation and acquisition of nitrogen from amino acids, and phosphatases in phosphorus acquisition. Hill *et al.* (2006) studied nutrient limitation in coastal wetlands and found ratios of these categories of EEAs to be inversely correlated to the ratios of the nutrients themselves. Similarly, a study of 447 locations along five rivers found that EEA ratios

generally indicated nutrient limitations at the same locations where ratios of the nutrient concentrations also did (Hill *et al.* 2010a). In a study of nutrient limitation in forested streams, Hill *et al.* (2010b) concluded that ratios of aminopeptidases activities to phosphatase activity were a better indicator of nutrient limitation than pure stoichiometric ratios of the nutrients.

EEA assays have only been applied in a limited number of studies to wastewater treatment systems. Initially, non-specific EEA assays were employed to measure activity of broad categories of enzymes (such as cellulose-degraders, detergent-degraders, or DNA-degraders). These activities were inferred either from the number of colonies that grew on selective media or measuring clear zones around colonies as an indication of use of a component of the medium (Hankin and Sands 1974). As the ability to assay the activity of specific enzymes was developed, Boczar *et al.* (1992) measured the activity of 19 different enzymes in activated sludge collected from three different wastewater treatment plants. Both alkaline and acid phosphatase – used in the release of phosphate groups from dissolved substrates – were detected in all three activated sludge samples at an activity of at least three units (on a 1 to 5 scale) as was leucine aminopeptidase, which is used in the acquisition of the most commonly occurring amino acid (leucine). The ratio of the total phosphatase activity to the sum of the glucosyl hydrolases activity varied from approximately 0.75 to 2.0 among the three plants. Not presented, however, were the nutrient concentrations of the wastewaters treated by these plants to allow for any understanding of the relationship between nutrient concentrations and EEA in wastewater treatment. Çiçek *et al.* (1999) measured and compared EEA levels for nineteen enzymes in both a pilot-scale MBR system and a bench-scale activated sludge system treating synthetic wastewater. The influent wastewater to both reactors averaged 325 mg/L COD, 41 mg/L total Kjeldahl nitrogen, and 2.0 mg/L total phosphorus. Effluent from the MBR

averaged 3.24 mg/L COD but only 0.07 mg/L total P while effluent from the activated sludge system averaged 18 mg/L and only 0.23 mg/L total P. Total phosphatase activity measured 18.5 μmol / mg VSS in mixed liquor of the MBR and 6.5 μmol / mg VSS in the mixed liquor of the activated sludge. Total glyclosidase activity (a sum of seven total enzymes assayed) was approximately 7 μmol / mg VSS in the MBR mixed liquor and 1.5 μmol / mg VSS in the activated sludge mixed liquor. Molina-Muñoz *et al.* (2007) measured EEA in a pilot-scale MBR treating domestic wastewater. The influent domestic wastewater averaged 450 mg/L COD, 5 mg/L nitrate, and 75 mg/L ammonium, but no influent phosphorus concentrations were provided. Phosphatase activity averaged 10 to 40 mM / min-g VSS while glycosidase activity averaged only 0.5 to 1.2 mM / min-g VSS, but the activity of only a single glycosidase (α-glucosidase) was measured.

More recently, work at the University at Minnesota has examined activities of four enzymes (α-glucosidase, β-glucosidase, leucine aminopeptidase, and heptanoate esterase) in treatment of synthetic wastewater in a membrane coupled bioreactor (in which the mixed liquor bioreactor and membrane components of the reactor are in sequence and the sludge that is separated by the membrane is recycled in its entirety to the first step). The researchers assert that the complete recycle of the separated biomass imposes an "increasingly stringent nutrient limitation" as an increasing number of microbial cells will be present in the reactor with the same influent composition to sustain them. They found that the total activities of all four enzymes increased over the course of the experiment (17 days), but when the enzyme activities were "normalized to biomass concentrations (measured as particulate protein)," the results were much less clear. The normalized activities of all four enzymes were fairly consistent over the course of the experiment with α-glucosidase and β-glucosidase both decreasing slightly over the course of the experiment while leucine aminopeptidase increased slightly over the course

of the experiment (LaPara *et al.* 2002). Presumably, however, the influent wastewater composition did not change over the course of the experiment, so the ratio among carbon and nitrogen and phosphorus also would not have changed, and whichever nutrient was limiting microbial growth at the beginning of the experiment was still limiting at the end of the experiment.

No studies similar to those described for riverine and wetland systems above examining the relationship between changing macronutrient concentrations and changes in various EEAs have been performed in wastewater treatment systems. The research presented in Chapter 4 incorporates the measurement of EEA associated with phosphorus acquisition to evaluate EEA as an indicator of phosphorus availability in a greywater treatment system.

OBJECTIVE 3 –ESTABLISH HOW XOC REMOVAL WILL VARY IN MBR TREATMENT OF GREYWATER UNDER CONDITIONS OF PHOSPHORUS LIMITATION AND PHOSPHORUS BALANCE.

Since publication of a nationwide study by the U.S. Geological Survey (Kolpin *et al.* 2002), much attention has been paid to the widespread occurrence of XOCs in the environment, many of which are believed to originate from consumer use of pharmaceutical and personal care products (PPCPs). Furthermore, municipal WWTP effluent has been identified as a large point source of these chemicals in the environment because a variety of PPCPs are not completely removed in the course of wastewater treatment. Conventional WWTPs were not designed for removal of these chemicals that tend to occur in raw wastewater at concentrations of micrograms per liter (μg/L) to nanograms per liter (Clara *et al.* 2005, Joss *et al.* 2005, Oppenheimer 2007). Although a large number of publications detailing the occurrence and removal of these contaminants in raw and treated wastewater have been published in the last decade, limited data on the

occurrence of organic chemicals in greywater exist, and even fewer data have been published on the removal of specific organic chemicals in the course of greywater treatment.

Occurrence of XOCs in Greywater

Given the potential for PCP ingredients to be found in greywater, a number of studies including Chaillou *et al.* (2011), Lamine *et al.* (2012), Prathapar *et al.* (2005), and Shafran *et al.* (2005) have used the methylene blue active substances assay to measure anionic surfactants and found concentrations between 0.3 and 118.3 mg/L. The first study analyzing for specific XOCs presented a qualitative analysis of Danish greywater collected from the bathroom sinks, baths, and showers of a Copenhagen apartment building and identified 191 XOCs in the greywater using solid phase extraction followed by gas chromatography-mass spectrometry (Eriksson *et al.* 2003). The same study also included a quantitative analysis for a number of surfactants, BTEX compounds, chlorophenol compounds, fragrances and flavors, and phthalates (plasticizers). Results of the quantitative analysis found numerous surfactants detected at concentrations as high as several milligrams per liter, and many of the other compounds detected at single μg/L or tens of μg/L (Table 2-5).

Table 2-5 – Summary of Studies Investigating Occurrence of XOCs in Greywater (Almqvist and Hanaeus 2006, Andersen *et al.* 2007, Eriksson *et al.* 2003, Eriksson *et al.* 2010, Hernández Leal *et al.* 2010, Palmquist and Hanæus 2005, Sharvelle *et al.* 2012)

	Authors	Eriksson *et al.*	Palmquist & Hanaeus	Almqvist & Hanaeus	Andersen *et al.*	Eriksson *et al.*	Hernandez Leal *et al.*	Sharvelle *et al.*
	Year Published	2003	2005	2006	2007	2010	2010	2012
	Country	Denmark	Sweden	Sweden	Denmark	Denmark	The Netherlands	US
Surfactants and their derivatives	Number analyzed	10	19	21	NA	2	1	3
	Number detected	10	17	13		1	1	3
	Maximum concentration (ug/L)	15,863	61.4	9		1.4	38	12,500
	Maximum detected compound	9-octadecanoic acid	4-nonylphenol-tetra-ethoxylate	4-nonylphenol-hexa-ethoxylate		iso-nonylphenol	Nonylphenol	Linear alkylbenzene sulfonates
Preservatives	Number analyzed	NA	11	7	5	5	4	2
	Number detected		6	3	5	5	4	2
	Maximum concentration (ug/L)		3,000	89.8	40	39	35.4	14
	Maximum detected compound		Dibutyl tin	Monobutyl tin	Ethylparaben	Methylparaben	Triclosan	Triclocarban
Fragrances and flavors	Number analyzed	2	NA	NA	NA	NA	3	NA
	Number detected	1					3	
	Maximum concentration (ug/L)	11.4					19.1	
	Maximum detected compound	Camphor					Galaxolide	
Ultraviolet filters	Number analyzed	NA	NA	NA	NA	6	7	NA
	Number detected					2	7	
	Maximum concentration (ug/L)					2.0	146	
	Maximum detected compound					Oxybenzone	Octocrylene	
Plasticizers	Number analyzed	9	10	10	NA	1	1	NA
	Number detected	3	5	5		1	1	
	Maximum concentration (ug/L)	39	160	20		0.15	1.2	
	Maximum detected compound	Di-(2-ethylhexyl)-phthalate	Di-(2-ethylhexyl)-phthalate	Di-(2-ethylhexyl)-phthalate		Bis-phenol A	Bis-phenol A	
BTEX compounds	Number analyzed	17	5	NA	NA	3	NA	NA
	Number detected	4	1			3		
	Maximum concentration (ug/L)	3.6	1.6			65		
	Maximum detected compound	m,p-xylenes	Toluene			Xylenes		
Chlorophenols	Number analyzed	17	NA	NA	NA	NA	NA	NA
	Number detected	3						
	Maximum concentration (ug/L)	0.10						
	Maximum detected compound	2,4,6-trichlorophenol						
Polycyclic aromatic hydrocarbons	Number analyzed	NA	16	16	NA	NA	NA	NA
	Number detected		6	7				
	Maximum concentration (ug/L)		0.05	0.15				
	Maximum detected compound		Pyrene	Acenphtylene				
Brominated flame retardants	Number analyzed	NA	13	13	NA	NA	NA	NA
	Number detected		6	6				
	Maximum concentration (ug/L)		0.76	0.014				
	Maximum detected compound		Pentabromodiphenylether	Tetrabromodiphenylether				
Polychlorhinated biphenyls	Number analyzed	NA	NA	14	NA	NA	NA	NA
	Number detected			5				
	Maximum concentration (ug/L)			0.12				
	Maximum detected compound			PCB 118				
Trihalomethanes	Number analyzed	NA	NA	NA	NA	4	NA	NA
	Number detected					4		
	Maximum concentration (ug/L)					1.1		
	Maximum detected compound					Dibromochloromethane		
Chlorinated solvents	Number analyzed	NA	NA	NA	NA	2	NA	NA
	Number detected					2		
	Maximum concentration (ug/L)					6.6		
	Maximum detected compound					Tetrachloroethene		

Two studies of Swedish greywater (a mixture of dark and light) collected from a subdivision of single family homes present data on occurrence of surfactants, antimicrobial compounds, polycyclic aromatic hydrocarbons, phthalates, polychlorinated biphenyls, and brominated flame retardants (Almqvist and Hanaeus 2006, Palmquist and Hanæus 2005). These analyses were performed using GC-MS with the exception of one group of antimicrobial compounds (organotin compounds), which were analyzed using GC-ICP-MS. Andersen *et al.* (2007) focused solely on the alkyl esters of p-hydroxybenzoic acid (also known as parabens) in greywater collected only from showers and bathroom sinks in an apartment building in Copenhagen, Denmark. Hernández Leal *et al.* (2010) presented the most recent study of concentrations of XOCs in greywater collected from 32 homes in a subdivision in the Netherlands. The analysis focused on fragrances, ultraviolet filters (typically used in sunscreens), one plasticizer, preservatives or antimicrobial compounds, and one surfactant. A limited characterization of XOC presence in U.S. greywater has been carried out by Sharvelle *et al.* (2012), focusing on greywater collected from three houses and analyzing for only a narrow range of surfactants and two antimicrobial compounds. The surfactants were detected at concentrations up to 10 mg/L while triclosan and triclocarban were detected at concentrations up to 10 μg/L. Taken together, these studies indicate that hundreds of compounds originating from a wide variety of PCPs can be expected in real greywater at concentrations as low as single μg/L or as high as many mg/L.

Potential Consequences of XOC Occurrence in Greywater Reuse

As stated previously, the most typical current uses of greywater (both treated and untreated) are for landscape irrigation and toilet flushing. Researchers have identified several potential concerns for reuse of greywater that is treated to meet objectives for

COD removal but without regard for sufficient XOC removal. Several researchers point to untreated greywater as a source of organic pollutants to aquatic environments and to groundwater (Donner *et al.* 2010, Hernández-Leal *et al.* 2011), similar to concerns expressed about WWTP effluent being a point source to surface water bodies. The Hernández Leal *et al.* (2010) study described above also evaluated the estrogenicity of the untreated and treated greywater (based on the limited number of XOCs analyzed for in the greywater) and found the untreated greywater to be weakly estrogenic (equivalent to 0.7 ng/L of 17-β-estradiol, which is within the range – but on the lower end – of the estrogenicity found in untreated domestic wastewater). They also reported that aerobic treatment of greywater reduced its estrogenicity by up to 90 percent (Hernández Leal *et al.* 2010).

More specific effects of landscape irrigation with greywater have also been documented. Harrow *et al.* (2011) analyzed microbial communities that resulted from irrigation of soil with synthetic greywater that contained triclosan and synthetic greywater lacking triclosan. Within ten weeks, the soil irrigated with triclosan-containing greywater contained significantly lower numbers of culturable microorganisms and less diversity in the microbial community than in soil irrigated with greywater that did not contain triclosan. Furthermore, resistance of isolates from each soil to four different antibiotics (ampicillin, streptomycin, chloramphenicol, and tetracycline) was measured. They found that 40-55% of viable heterotrophs isolated from the soil treated with triclosan-containing greywater demonstrated resistance to each of the antibiotics while only 15-25% of viable heterotrophs isolated from soil irrigated with greywater not containing triclosan were resistant to each antibiotic (Harrow *et al.* 2011).

Others have also identified detrimental effects that greywater can have on soil quality. Gross *et al.* (2005) studied the effects of no irrigation, irrigation with freshwater,

and irrigation with greywater on 500 m^2 plots of land over the course of three years. Anionic surfactants were measured at 23 mg/kg in soil irrigated with greywater compared to less than 5 mg/kg in both non-irrigated soil and soil irrigated with freshwater. They also found that increasing surfactant concentration in soil was correlated to lower capillary rise in the soil, indicating greater hydrophobicity of the soil, which is not amenable to plant growth (Gross *et al.* 2005). Sharvelle *et al.* (2012) evaluated soil quality of lawns at six homes that had been irrigated with greywater for more than two years. The concentration of total surfactants in surface soil samples was found to be significantly higher at locations irrigated by greywater than at than those irrigated by freshwater at five of the six homes. The antimicrobial compounds, triclosan and triclocarban, were also detected at single μg/kg concentrations in surface soil samples at all locations irrigated with greywater (with the exception of one location where only triclocarban was detected) while concentrations were below detection limits both in deeper soil samples at locations irrigated by greywater and at all sample depths in locations irrigated by freshwater (Sharvelle *et al.* 2012). At three houses where greywater had been used for irrigation for more than five years, researchers also observed that certain plant species (including Scotch pine, Haas avocado, and lemon trees) showed signs of sensitivity to the greywater, as indicated by leaf wilting, smaller leaf sizes, and reduced fruiting, although the sensitivity was not able to be directly attributed to the presence of the surfactants, antimicrobial compounds, or any other particular characteristic of the greywater used (Sharvelle *et al.* 2012). These findings are nevertheless consistent with other investigations of the effects of surfactants on plant growth that found that elevated concentrations of either non-ionic or anionic surfactants were toxic to plants as was long-term exposure to lower concentrations (Roesner *et al.* 2006).

Removal of XOCs in Treatment of Greywater

If XOCs are of concern in greywater reuse, then their fate in greywater treatment must be understood. Andersen *et al.* (2007), Eriksson *et al.* 2010 and Hernández Leal *et al.* (2010) are the only known studies that reported not only the occurrence of XOCs in real greywater but also the extent to which the compounds are removed in treatment. The greywater treatment system in the apartment building in Copenhagen consists of primary sedimentation, three rotating biological contactors in series, secondary sedimentation, a sand filter, and ultraviolet filtration (Andersen *et al.* 2007, Eriksson *et al.* 2010). Measurement of paraben concentrations in the primary sedimentation tank found approximately 12 percent removal of butylparaben, but otherwise on average no removal in the sedimentation tank for the other 4 parabens. However, all five were at least 98 percent removed by the outlet of the RBCs, indicating high biodegradability for the parabens (Andersen *et al.* 2007). Eriksson *et al.* (2010) studied the same system but looked at a broader suite of compounds (Table 2-5). Of the 18 compounds detected in the influent to the treatment system, 11 were found to be at least 70 percent removed. Five compounds were found to have between 20 and 70 percent removals (bromodichloromethane, bromoform, butylparaben, chloroform, and xylenes) while only the two chlorinated solvents (tri- and tetrachloroethene) were poorly removed (less than 20 percent removal) by the treatment system (Eriksson *et al.* 2010). Hernández Leal *et al.* (2010) tested removal of 17 organic contaminants in three biological treatment systems: an aerobic sequencing batch reactor, an anaerobic upflow sludge blanket reactor, and a combination of the aerobic and anaerobic systems. In both the aerobic system and combination aerobic-anaerobic system, they found that all of the compounds were removed at least 80 percent with the exception of two UV-filters and one fragrance. In the anaerobic system, removals were much more variable with only two compounds

(one fragrance and one UV-filter) removed more than 80 percent, another 6 compounds at least 50 percent removed, and the remaining six compounds less than 50 percent removed. Hernández Leal *et al.* also compared removals found in their aerobic treatment system to removals reported in the literature for WWTPs (Table 2-6). The comparison can be difficult since they are comparing a single analysis of one greywater system to multiple analyses of removal in a variety of WWTP configurations under different operating conditions. The removals were nevertheless fairly similar with two exceptions. The removal of tonalide from greywater was found to be on the very low end of the range of removals presented (from four references) for WWTPs, while the removal of the ultraviolet filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) from greywater was less than half of that presented from two different references for WWTPs. However, as the authors point out, conventional wastewater treatment typically involves multiple treatment stages and might include tertiary treatment or other additional steps that are not included in the three lab-scale greywater treatment systems evaluated.

The Hernández Leal *et al.* (2010) study points to the fact that existing data on the removal of XOCs in conventional wastewater might in many cases provide useful information as to the fate of those XOCs in greywater treatment. However, the characteristics that differentiate greywater from typical domestic wastewater (such as fluctuations in hydraulic and chemical characteristics as well as potential nutrient limitations) have not been thoroughly explored in the literature, nor have the effect that these differences might have on removal of XOCs in the course of greywater treatment. The results presented in Chapter 4 contribute to this effort by examining XOC removal under conditions of phosphorus limitation and phosphorus balance to establish how the removal of these XOCs varies with changes in nutrient availability.

Table 2-6 – Comparison of Removal of XOCs in Typical Wastewater Treatment and Aerobic Biological Treatment of Greywater (Hernández Leal *et al.* 2010)

		Removal in WWTPs	Aerobic Removal from Greywater
Surfactants	Nonylphenol	74.0-97.8	88.7
Preservatives	Triclosan	95-96	98
	Propylparaben	99.7	92.8
	Butylparaben	99.9	95.3
Fragrances and flavors	**Tonalide**	**26-89**	**32**
	Galoxolide	42-88	80
	HCA	99.5-99.9	93.2
Ultraviolet filters	PBSA	99.6	NR
	4MBC	25.0-75.6	93.5
	EHMC	**97.9-99.6**	**49.0**
	BP3	93.1	86.0
	Octocrylene	58.3-98.5	90.8
Plasticizers	Bisphenol A	80.1-94.2	90.5

Nutrient Limitations and XOC Removal

Nutrient addition could affect removal of organic contaminants in wastewater via several mechanisms. If the nutrient had been limiting microbial growth prior to the addition, its addition might allow for growth of greater cell biomass, which might contribute both to additional biodegradation as well as more biomass available to sorb the contaminants (Jefferson *et al.* 2001). Christopher *et al.* (2000) found that addition of 1.6 to 3.3 mg/L of phosphate to an influent wastewater that contained no measurable concentrations of phosphate increased COD removal only slightly but increased the rate of 2,4-dinitrotoluene (2,4-DNT) removal from 0.017 mg 2,4-DNT/mg VSS-d to 0.046 mg 2,4-DNT/mg VSS-d. The researchers could not identify the specific mechanism responsible for the improved removal of 2,4-DNT beyond noting that the absence of phosphorus in the unamended wastewater meant that addition of phosphorus "likely stimulated microbial activity, including the organisms responsible for DNT degradation"

(Christopher *et al.* 2000). However, overall COD removal was only slightly improved, so the difference between the change in overall COD removal and the improvement in 2,4-DNT removal indicates the possibility of some other mechanism at work. Breedveld and Sparrevik (2000) found that addition of nitrogen and phosphorus to soil columns improved degradation of 4-ring polycyclic aromatic hydrocarbons from less than 10 percent removal without nutrient addition to more than 80 percent degradation after nutrient addition while 2- and 3-ring PAHs showed 60 to 90 percent degradation whether or not nutrients were added. The authors could not identify a reason that the compounds were differentially removed under nutrient-limited and supplemented conditions. Granger *et al.* (1999) found that BTEX compounds were persistent in groundwater in which no phosphorus was detectable, such that more than two-thirds of the benzene and toluene were still detectable in groundwater for more than 60 days, while phosphorus addition (of only 0.11 mg/L) caused both compounds to disappear to non-detectable levels within approximately 12 days.

Freedman *et al.* (2005) investigated the removal of methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), toluene, and xylenes in nutrient-limited textile manufacturing wastewater. Batch experiments demonstrated near complete removal of MEK and MIBK in a matter of hours whether or not nutrients were added to the solution. However, only about 30 percent of the toluene and half of the p-xylenes were removed in more than 90 hours without nutrient amendments. Addition of nitrogen and phosphorus to the batch reactors resulted in near 100 percent removal of both toluene and xylenes in the same 90-hour time frame. The authors hypothesize that the differential removals might be due to different degradation pathways for MEK and MIBK than for toluene and p-xylenes, since degradation of toluene and p-xylenes requires enzymes coded for on the TOL plasmid. They theorize that perhaps the TOL plasmid is shed under the nutrient-

limiting conditions making their degradation slower or incomplete in the absence of the required nutrients. On the other hand, no change in the removal of MEK or MIBK would be expected since their degradation pathway is encoded in the chromosome, which would be unaffected by nutrient limitation.

OBJECTIVE 4 – EVALUATE WHETHER CHANGES IN PLASMID DNA CONCENTRATION COULD BE MONITORED AS AN INDICATOR OF POTENTIAL CHANGES IN XOC REMOVALS UNDER NUTRIENT-LIMITED CONDITIONS.

As documented above, hundreds of XOCs have been detected in greywater. Without studying or monitoring every possible XOC, a framework to understand which XOCs might be removed (or specifically biodegraded) under conditions of nutrient limitation – and which would not be – would be useful in developing treatment systems and understanding potential risks in various reuse applications. The role of a plasmid-encoded degradative pathway for a specific XOC might be one aspect of such a framework. Moreover, if a subset of XOCs requires retention of specific plasmids for their biodegradation, then measurement of plasmid concentrations might provide a monitoring tool for assessing the ability of a biological treatment system to degrade certain XOCs under conditions of nutrient limitation.

Since the genes responsible for degradation of camphor and octane were found to be outside of the microbial chromosome in 1971 (Sayler *et al.* 1990), plasmids, transposons, and other so-called mobile genetic elements have been recognized to be involved in the degradation of many organic contaminants that derive from human activity. Carrying genes for degrading novel or unusual compounds on plasmids can be advantageous because they can be transferred throughout a microbial community if the degradative pathway is needed and allow the community to adapt to prevailing environmental conditions (Sayler *et al.* 1990, Top *et al.* 2002). Conversely, the plasmid

and its associated degradative ability can also be lost under certain environmental conditions if not essential to the microorganisms. Under conditions of phosphorus limitation, for example, microorganisms must expend greater energy and resources for the acquisition of phosphorus. As a consequence, they might shed any non-essential plasmids in an effort to conserve that energy in other areas. In such cases, the entire plasmid can be lost at the time of cell division, as in segregational instability. Competitive instability occurs when carriage of the plasmid imposes a metabolic burden on the cells, and, as a result, plasmid-free cells will outgrow and overtake plasmid-containing cells in a non-selective medium (Love 1994). Structural instability can also occur when only a portion of the plasmid (such as those containing non-essential catabolic genes) are lost (Love 1994).

Nutrient Limitation and Plasmid Loss

Jones *et al.* (1980) investigated the persistence of four different plasmids coding for antibiotic resistance in pure culture under phosphate-limited conditions in the absence of the antibiotic for which the plasmid conferred resistance. In two of the four cultures, the resulting communities were dominated by plasmid-free cells, although small residual populations (less than 1 percent of the total microorganism cells) of plasmid-containing cells were maintained (Jones *et al.* 1980). In one culture in which plasmid-free cells did not arise within 80 generations under nutrient-limited conditions, the plasmid copy number nevertheless dropped to twenty percent of the original value over the course of the experiment while the minimum inhibitory concentration (MIC) for ampicillin also fell to twenty-five percent of its original value, indicating loss of plasmid content even in those situations where complete plasmid loss was not observed (Jones *et al.* 1980).

Noack *et al.* (1981) also studied plasmid retention under conditions of nutrient limitation in the absence of a selective pressure such as the presence of the antibiotic for which the plasmid confers resistance. Similar to Jones *et al.* (1980), they found inconsistent results in that one of the plasmids (pBR 325 – which encodes for ampicillin, tetracycline, and chloroamphenicol resistance) was lost in up to 99 percent of cells under both glucose and nitrogen limitation at multiple dilution rates and in both hosts. However, the second plasmid investigated (pBR 322 – which encodes for ampicillin and tetracycline resistance) did not appear to be lost under conditions of either glucose or nitrogen limitation in either of the two hosts that were investigated (Noack *et al.* 1981). Wouters *et al.* also investigated pBR 322 in *E. coli* culture and found that, under phosphate-limited conditions, ampicillin- and tetracycline-resistance was lost within a period of 6 to 8 days when the chemostat dilution rates were 0.1 and 0.3 h^{-1} (Wouters *et al.* 1980). However, in these experiments, plasmid loss was inferred from the loss of antibiotic resistance. As in the Jones *et al.* study, a small fraction of the cells retained the antibiotic resistance throughout the course of the experiment (Wouters *et al.* 1980).

Jenkins and Heald (1996) found that a pure culture of *Pseudomonas putida* in a nitrogen-free medium lost the ability to degrade toluene in under 150 hours. No measurements of plasmid DNA concentrations or presence of plasmids encoding toluene degradation were measured; however, the loss of degradative ability was assumed to be attributable to the loss of the TOL-plasmid. Chew *et al.* (1988) similarly investigated the impact of different limiting nutrients and found that ampicillin resistance was most stable with sulfate as the limiting nutrient followed by phosphate and then glucose. In a phosphate-limited culture, in the absence of ampicillin, more than 80 percent of cells were no longer resistant to ampicillin within 25 generations when the dilution rate was 0.15 h^{-1} and within 50 generations with a dilution rate of 0.30 h^{-1} (Chew *et al.* 1988).

This study was performed on a pure culture of *E. coli* with a single plasmid pAT153 (Chew *et al.* 1988). Modi *et al.* (1991) investigated stability of the plasmid pBR322 (coding for ampicillin and tetracycline resistance) in glucose-limited continuous culture containing ampicillin but not tetracycline and found that within 300 generations only 10 percent of remaining cells contained the plasmid. However, from that point on the fraction of the plasmid-containing cells that was sensitive to tetracycline but resistant to ampicillin increased to about 99 percent, indicating that in this subpopulation, the plasmid had been retained, but that the portion of the plasmid coding for tetracycline resistance had been deleted (Modi *et al.* 1991).

The published work related to plasmid loss under nutrient limitation has, by and large, explored losses of specific plasmids in pure microbial cultures with fixed influent compositions, conditions that are not representative of those anticipated in activated sludge or other mixed culture treatment processes with variable influent composition. In addition, as Freedman *et al.* (2005) stated, few studies have been published documenting the effects of nutrient limitations on removal of specific organic contaminants in activated sludge processes. No specific evidence was presented for the Freedman hypothesis that the loss of plasmid was correlated to the change in XOC removal with nutrient addition in their treatment process. Therefore, the research presented in Chapter 5 investigates precisely that connection. The organic compounds investigated are not those encoded by the TOL-plasmid since the BTEX compounds are not expected to be abundant in greywater. However, the compounds investigated are those expected in greywater and for which the biodegradation is known to be plasmid-mediated at least under some circumstances.

Chapter 3: Measurement and Effects of Phosphorus Limitation in Biological Treatment of Household Light Greywater

As water stresses increase across the U.S., several cities, including Austin, are contemplating rewriting their greywater regulations to both allow for and encourage greater household water reuse. The most common existing reuse systems are typically "laundry-to-landscape," in which wastewater is collected from washing machines and flows by gravity to irrigate lawns and other landscape, usually without treatment (Roesner *et al.* 2006). However, changes in regulation elsewhere have led to technological innovation and advancement in greywater treatment. For example, the State Senate in Berlin, Germany adopted greywater regulations in 1995 that allowed for indoor reuse of treated greywater, provided it meets certain water quality parameters, and, as a result, a variety of greywater treatment systems have since been installed in both single-family and multi-family residential buildings, hotels, and university dormitories. Nolde (2005) contends that, while many technologies such as those employing electrochemical disinfection have since been abandoned, the most successful systems are those that include a biological treatment step. Therefore, if greywater reuse regulations and systems develop in a similar manner in the U.S., greater understanding of the biological treatment of greywater treatment is necessary. Membrane bioreactors (MBRs) might not be thought of as appropriate for household use, but MBRs are already in use for on-site wastewater treatment in Japan (Gaulke 2006) and offer robust treatment with a combination of treatment processes. In addition, many regulators in the U.S. remain concerned about ensuring adequate protection of public health in any greywater reuse scenario (Glenn 2012). As a result, technologies able to reduce the risk to human health that might exist from exposure to greywater are of particular interest as this field develops. Among the many technologies that have been evaluated for greywater

treatment and reuse, Pidou *et al.* (2007) and Fangyue *et al.* (2009) concluded that the membrane bioreactor (MBR) is the only technology that had been researched that could provide sufficient microorganism (and, it must be assumed, associated pathogen) removal from greywater without a subsequent disinfection or filtration step. While biological treatment of domestic wastewater is well understood even for small scale systems, few studies have examined the unique characteristics of household light greywater that could lead to different treatment outcomes than are seen in traditional wastewater treatment.

Among the challenges that light greywater could present in biological treatment is the availability of macronutrients that are essential to microbial growth once blackwater (toilet waste) and dark greywater (kitchen wastewater) are excluded. In particular, previous studies of greywater present an extremely wide range of measured phosphorus concentrations measured across nearly four decades in seven countries: 0.062 – 57 mg/L of total phosphorus and 0.5 – 171 mg/L of phosphate-phosphorus (Eriksson *et al.* 2009, Eriksson *et al.* 2003, Eriksson *et al.* 2002, Laine 2001). Therefore concentrations of phosphorus would be expected to be on the low end of the reported range, particularly in light of regulatory changes to phase phosphorus out of consumer products, including from laundry detergent and, more recently, from dishwasher detergents (Litke 1999, McCoy 2011).

The initial objective of this work, therefore, was to evaluate whether U.S. greywater would be deficient in phosphorus as expected and the extent to which the quality of MBR-treated greywater would be controlled by phosphorus concentrations in the greywater. This objective was achieved by monitoring the performance of two MBRs treating synthetic greywater at varied influent phosphorus concentrations using traditional indicators such as COD removal and MLSS concentrations.

METHODS AND MATERIALS

Two lab-scale MBRs (R1 and R2) were operated in the laboratory. R1 was originally seeded with return activated sludge from the Walnut Creek Wastewater Treatment Plant in Austin, Texas, operated with no sludge wasted other than samples occasionally collected for analytical purposes [corresponding to a nearly infinite solids residence time (SRT)] for approximately 120 days, and then subsequently operated at an SRT of approximately four days (by wasting approximately 2.1L of mixed liquor from the reactor approximately every day) for more than 1150 days. R2 was seeded by mixed liquor wasted from R1, operated at a nearly infinite SRT for 30 days, and then operated at an SRT of approximately four days for more than 220 days. Other characteristics common to both reactors are summarized in Table 3-1.

Table 3-1 – Operational Characteristics of R1 and R2

Reactor volume	8.4 L
Hydraulic residence time	24 hours
Solids residence time	4 days
Membrane	Provided by Enviroquip, Inc. (Austin, Tex.)
Material	Polyethylene
Dimensions	22.5 x 32 x 0.60 cm
Nominal pore size	0.4 μm (microfiltration)
Influent flow rate	5 mL/min (total)
Synthetic greywater	4 mL/min
Supplemental phosphorus	1 mL/min
Effluent flow rate	Variable; controlled by a level switch to keep constant volume in the reactor
Vacuum applied by effluent pump	Variable; typically 20 – 100 kPa
Air flow	Continuously delivered through perforated tubing beneath the membrane

Two influent solutions were pumped into each reactor: a synthetic greywater and a supplemental phosphorus solution. The composition of the synthetic greywater

solutions is detailed in Table 3-2 and its characteristics compared to those of real greywater in Table 3-3.

Table 3-2 – Composition of Synthetic Greywater Influent Solution

Ingredient	Brand	Quantity
Deionized water	--	1 L
Hand soap	Essence of Beauty Creamy Fig	0.19 g
Shampoo	Bed Head Moisture Maniac	0.27 g
Laundry detergent	Tide HE Liquid	0.34 mL
Sodium bicarbonate	--	0.15 g

The composition of the synthetic greywater solution was developed to match the mean value of 242 mg/L of BOD in a typical light greywater (calculated from 12 of the studies presented in the literature and summarized in Table 3-3) while keeping the relative amounts of each product used consistent with available data for typical per capita daily usage (AWWA 1999, Fuls *et al.* 2008, Loretz *et al.* 2006). Sodium bicarbonate was added to match the alkalinity of approximately 70 mg/L found in City of Austin tap water (Austin Water Utility 2008a, 2008b, 2008c, 2009). Hand soap, shampoo, and laundry detergent were purchased from CVS Pharmacy (Austin, TX). The listed ingredients of the personal care products that comprise the synthetic greywater are compiled in Appendix A.

Table 3-3 – Comparison of characteristics of synthetic greywater used in this experiment to greywater characteristics reported in the literature (Allen *et al.* 2012, Birks and Hills 2007, Chaillou *et al.* 2011, Eriksson *et al.* 2009, Eriksson *et al.* 2003, Eriksson *et al.* 2002, Finley *et al.* 2009, Friedler and Gilboa 2010, Friedler *et al.* 2008, Friedler *et al.* 2005, Laine 2001, Lamine *et al.* 2012, Metcalf & Eddy 2003, Pidou *et al.* 2008, Prathapar *et al.* 2005, Qasim 1999, Sharvelle *et al.* 2012, Winward *et al.* 2008b)

	Literature		Mean Measured in Synthetic Greywater
	Range	Median	
pH	5.5 - 10	7.54	7.92
BOD (mg/L)	8 - 670	153	236
COD (mg/L)	33 - 1815	327	405
Total N (mg/L)	3.6 - 37.3	8.7	3.5
NH_4-N (mg/L)	0.02 - 18.6	1.18	0.13
NO_3-N (mg/L)	<0.02 - 7.5	1.26	0.79
Total P (mg/L)	0.062 - 57	0.70	<0.050
PO_4-P (mg/L)	0.5 - 171	8.8	0.034

Table 3-4 presents the composition of the phosphorus solution provided to the reactors throughout each phase of the experimental work.

Table 3-4 – Composition of Supplemental Phosphorus Solution

	Concentration in Deionized Water (mg/L)					
	R1	R2				
Ingredient		Phase I	II	III	IV	V
Monobasic sodium phosphate	35	35	4.2	1.7	0.56	0
Dibasic sodium phosphate	36	36	5.9	1.7	0.56	0
Resulting [PO_4-P][1]	17.9	17.9	2.5	0.82	0.27	0
Diluted [PO_4-P][2]	3.6	3.6	0.5	0.16	0.055	0
Length of Phase (days)	--	88	27	42	21	75

[1]"Resulting [PO_4-P]" is the concentration of phosphate-phosphorus that resulted from the addition of the given masses of both forms of sodium phosphate in solution.
[2] The supplemental phosphorus solution was applied at one-fifth the total flow rate to the reactor; therefore the diluted influent phosphate-phosphorus concentration is one-fifth of the "resulting [PO_4-P]" shown in the line above and was the actual concentration of phosphorus targeted to enter the reactor.

The concentration of 3.6 mg/L provided to R1 and to R2 in Phase I was expected to provide excess phosphorus to the reactor assuming complete consumption of approximately 360 mg COD per liter, a typical yield coefficient of 0.39 g of cells produced per g COD consumed (Metcalf & Eddy 2003), and 2.3 g of phosphorus per 100 grams of microbial cells assuming a biomass composition of $C_{60}H_{87}O_{23}N_{12}P$ (Metcalf & Eddy 2003).

From the initial influent phosphorus concentration of approximately 3.6 mg/L in R2, the influent phosphorus concentration was reduced step-wise. After each change in influent phosphorus concentration, approximately two weeks (or slightly longer than 3 times the SRT) was allowed for the reactor to adjust to the new operating conditions. Analytical measurements to monitor reactor performance were performed for several weeks following acclimation to each new set of operating conditions. In most cases, sampling and analysis were performed for four weeks, but in certain circumstances (*e.g.*, Phase III) where very little change was observed after a change in influent phosphorus concentration, the next step change was undertaken sooner.

COD was measured immediately upon sample collection using low-range digestion vials (Hach; Loveland, CO) with dilution of samples to reach the linear range (3 – 150 mg/L) of this method when necessary. Mixed liquor suspended solids was measured according to Standard Methods using a 0.22-μm membrane filter (AHPA *et al.* 1999). Synthetic influent, supplemental phosphorus, and reactor mixed liquor samples were also filtered using a 0.22-μm membrane filter (Pall; Port Washington, NY), acidified to a pH of less than 2 using concentrated sulfuric acid, and stored in glassware that had been acid washed and baked at 550°C for at least two hours. Filtered influent and reactor dissolved orthophosphate were routinely measured by the ascorbic acid method (Method 4500-P E.; AHPA *et al.* 1999). Influent and reactor total dissolved phosphorus were measured on a select number of occasions by persulfate digestion (Method 4500-P B.5; AHPA *et al.* 1999) and the ascorbic acid method.

Extracellular enzyme activity (EEA) of three glycosidases (α-D-glucosidase, β-D-glucosidase, β-N-acetylglucosaminidase), two aminopeptidases (L-glycine aminopeptidase and L-leucine aminopeptidase), and acid / alkaline phosphatase was measured on unfiltered reactor samples within hours of collection. An assay using fluorogenic substrates was adapted from Sinsabaugh and Foreman (1998), Montuelle and Volat (1998), Sinsabaugh and Foreman (2001), Elonen (2012), Elonen (2005), and the University of Toledo (2000).

Sterile 200 μM solutions of the six substrates (4- methylumbelliferyl (MUB)-α-D-glucoside, 4-MUB-β-D-glucoside, 4-MUB-N-acetyl-β–glucosaminide, glycine-7-amido-4-methylcoumarin (AMC), L-Leucine-AMC, and 4-MUB-phosphate) and 100 μM solutions of the standards (4-methylumbelliferone and 7-amino-4-methylcoumarin) were prepared in deionized water. Before measuring EEA, 50 mL of mixed liquor from each reactor were homogenized by blending for 45 seconds. 200 μL of the homogenized

mixed liquor from each reactor were mixed with 50 μL of each of the six substrates in triplicate in a 96-well black microtiter plate. The resulting fluorescence was measured every 5-15 minutes for at least eight hours on a Biotek Synergy HT plate reader at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. If the enzyme of interest is present, it will cleave the substrate and release the fluorescent moiety, producing either 4-methylumbelliferone or 7-amino-4-methyl-coumarin. Emission coefficients of the standards and quenching by the mixed liquor matrix were measured by mixing 50 μL of each standard with 200 μL of a 5 mM sodium bicarbonate solution and each of the homogenized reactor mixed liquors in triplicate. The measured emission and quenching coefficients were then used to convert the fluorescence measured in sample wells over time to the rate of accumulation of either 4-methylumbelliferone or 7-amino-4-methylcoumarin and determine the maximum rate associated with each enzyme over the course of the experiment. Fluorescent standards (4-methylumbelliferone and 7-amino-4-methylcoumarin) were purchased from Alfa Aesar (Ward Hill, MA). 4-MUB-β-D-glucopyranoside and 4-MUB-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). 4-MUB-α-D-glucopyranoside and 4-MUB-N-acetyl-β-D-glucosaminide were purchased from EMD Millipore Chemicals (Billerica, MA). L-leucine-AMC was purchased from Bachem Americas, Inc. (Torrance, CA). L-glycine-AMC was provided by Colleen Elonen at the U.S. Environmental Protection Agency National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division (Duluth, MN). Further details of the extracellular enzyme assays are presented in Appendix B.

Results and Discussion

Summary of MBR Performance

When operation of the control reactor (R1) began, the influent was comprised of only the synthetic greywater (using each constituent at approximately 80 percent of the composition presented in Table 3-2) with no supplemental phosphorus solution to establish a baseline of operational performance. No measurable concentration of orthophosphate was detected (method detection limit ~0.15 mg/L) in eight samples collected from the synthetic influent and analyzed in the first two months of operation. In spite of non-detectable orthophosphate concentrations, effluent BOD_5 as low as 12 mg/L and effluent COD as low as 57 mg/L were initially detected (Figure 3-1). Over the first two months of operation, no sludge was wasted from the reactor, but the MLSS concentrations measured declined from 1458 mg/L to less than 400 mg/L indicating that cell growth might have been relying on endogenous metabolism and cell contents liberated by lysis of other microbial cells as a source of phosphorus initially. However, after two months of operation the effluent BOD and COD increased to approximately 62 mg/L and 150 mg/L, respectively. At that point, the reactor setup was modified to provide a supplemental phosphorus solution.

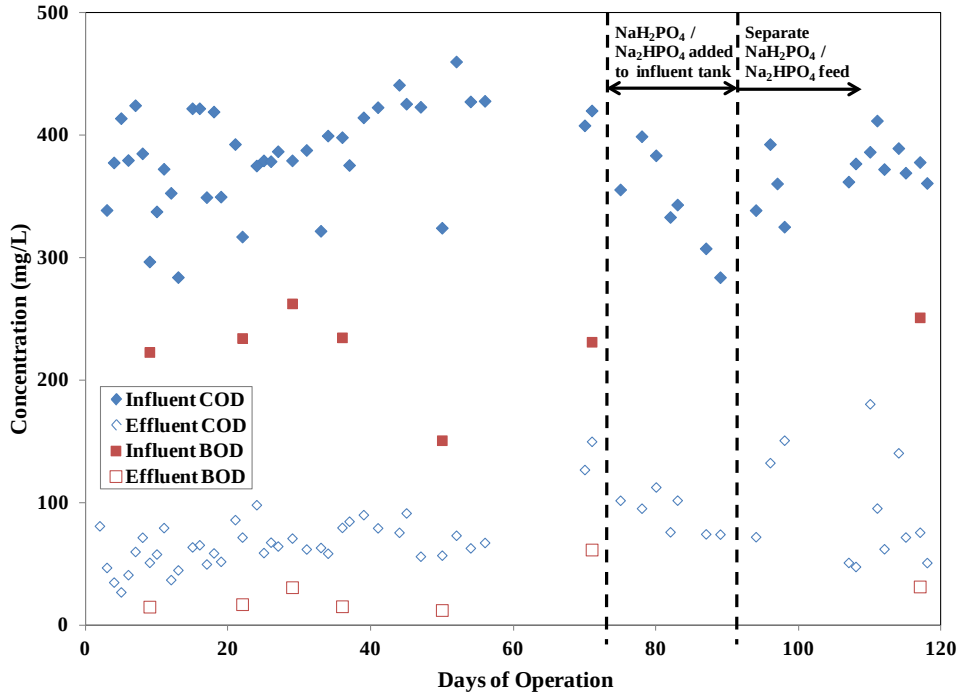


Figure 3-1 – Influent and effluent BOD and COD measured in MBR treating synthetic greywater influent at an infinite SRT upon startup and after addition of sodium phosphate to the synthetic influent

Addition of sodium phosphate to the system provided further evidence of the phosphorus limitation of the synthetic greywater influent. Both forms of sodium phosphate were initially added directly to the same influent tank as the hand soap, shampoo, detergent and sodium bicarbonate, and shortly thereafter visible bacterial growth appeared for the first time in the influent tank, and COD measured in the influent to R1 (i.e., that leaving the influent tank) declined over time (between the dashed lines in Figure 3-1). This decline indicated that biodegradation of the organics in the synthetic influent was now occurring in the influent tank, which had not occurred prior to the addition of phosphate to the influent tank. As a result, the reactor system was modified to its subsequent configuration with a second influent tank containing the supplemental phosphorus solution. Although some scatter in effluent COD was seen immediately after

the addition of the second tank, the reactor subsequently achieved very stable operation. Nevertheless, the observed behavior in R1 in response to changes in influent phosphorus concentration provided the first demonstration that the low phosphorus concentration available in the greywater influent could adversely affect treated effluent water quality. This initial result was consistent with research in England that tested supplementing both real light greywater (originally containing 1.37 mg/L) and synthetic light greywater (with 0.047 mg/L) with phosphorus up to a total influent concentration of approximately 3 mg/L. Although initial COD measured only 18-42 mg/L, COD removal increased 110 and 25 percent, respectively with the additional phosphorus (Jefferson *et al.* 2001, Laine 2001). Similarly, Krishnan *et al.* (2008) found that supplementing even dark greywater from household kitchens in Malaysia from a ratio of 100 mg COD:1.82 mg N:0.76 mg P up to a ratio of 100:5:1 reduced the effluent COD from 65 mg/L to 12 mg/L.

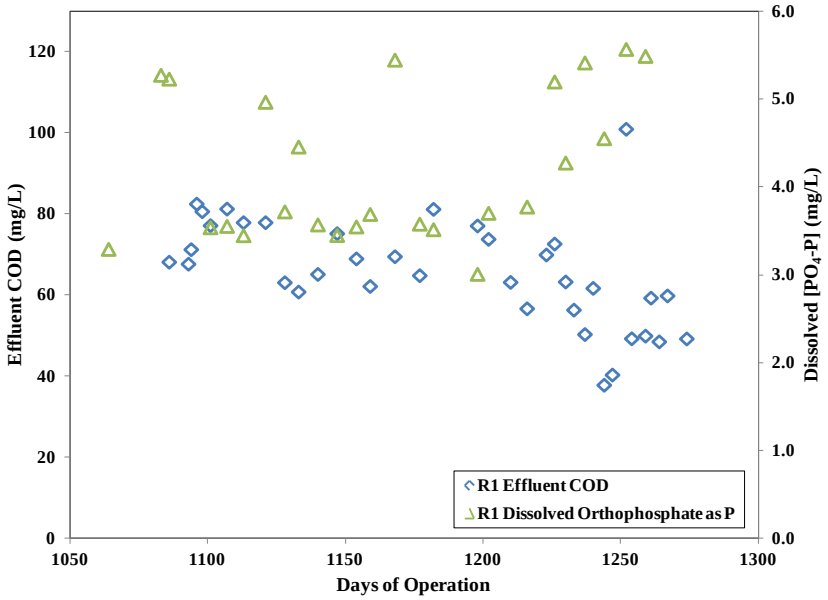
Later, the influent phosphorus concentration provided to R1 was maintained while that provided to R2 was reduced step-wise (Table 3-4), and the performance of both reactors was monitored to evaluate the impact on COD removal. Based on an assumed ratio of 100 mg BOD_5:5 mg N:1 mg P needed for successful aerobic treatment of wastewater (Metcalf & Eddy 2003), the synthetic greywater containing on average 236 mg/L BOD_5 would require approximately 2.4 mg/L of phosphorus, while less than 50 μg/L was detected in this synthetic greywater. As a result, a marked increase in effluent COD concentration was expected in R2 as the supplemental phosphorus concentration was reduced in the subsequent experiments.

The effluent COD from R1 measured 67 mg/L across Phases I – V, and the 4 mg/L of dissolved orthophosphate remaining in the reactor indicates a clear excess of phosphorus available in this reactor [Figure 3-2(a)]. Although the effluent COD concentration from R1 decreased somewhat at the end of this experimental period,

effluent concentrations between 40 and 60 mg/L were still consistent with those measured over all 1200+ days that this reactor was in operation providing a stable baseline for comparison to R2 in which the influent phosphorus concentration was decreased over Phases II-V.

In contrast to the expected performance of R2, throughout the first four phases of operation, even as the influent phosphorus concentration was reduced as low as 0.055 mg/L (equivalent to BOD_5:N:P ratio of 4291:63:1) and the residual dissolved orthophosphate concentration in the reactor was even lower, no impact on the effluent COD was apparent [Figure 3-2(b) and Figure 3-3]. Throughout Phases I through IV, effluent COD from R2 averaged 67 mg/L, nearly identical to that measured in R1 over the same time period, indicating little impact from the lack of available phosphorus, at least through these measurements of bulk organic removal.

a)



b)

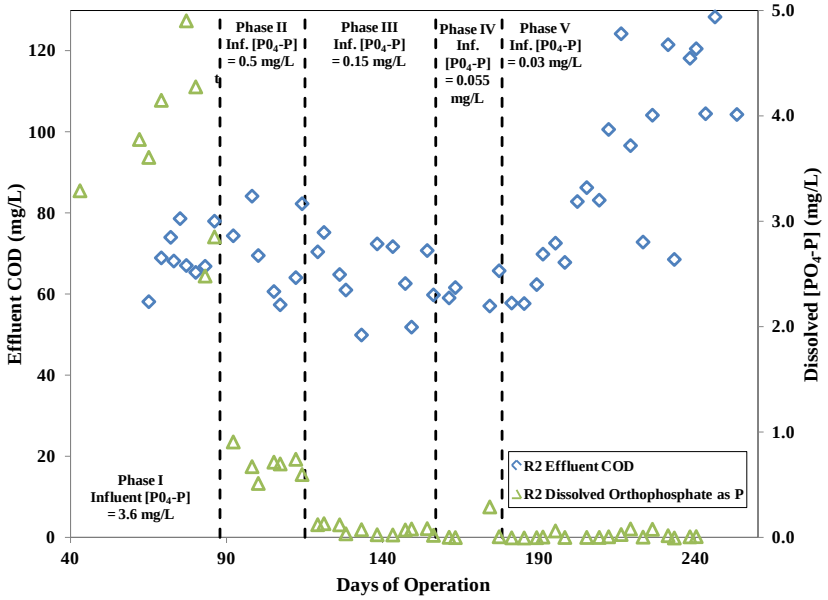


Figure 3-2 – Effluent COD (primary y-axis) and dissolved orthophosphate as phosphorus (secondary y-axis) measured in a) R1 and b) R2. Influent COD averaged 405 mg/L. Because it had been in operation longer, the values on the time scale for R1 do not match those of R2; nevertheless, the reactors were operating in parallel.

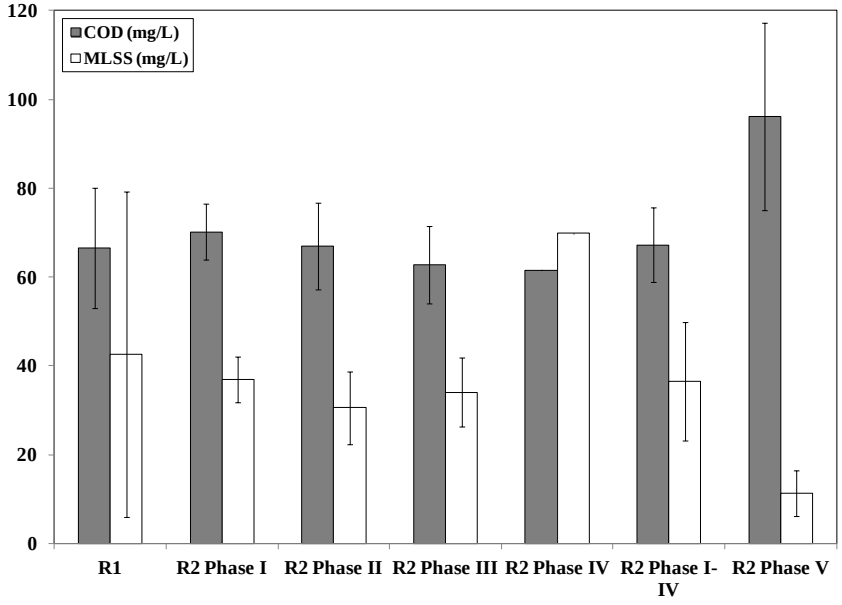


Figure 3-3 – Mean effluent COD and MLSS concentrations measured in R1 and R2 in each phase (omitting the first twelve days after each change in phosphorus concentration to allow for the reactor to achieve steady operations thereafter). Error bars shown are plus and minus one standard deviation. (No standard deviation was calculated for R2 Phase IV because it only included two data points.)

Only when absolutely no supplemental phosphorus was provided to the reactor was any effect on effluent COD concentration apparent, as the effluent concentration climbed as high as 129 mg/L and averaged 96 mg/L in Phase V. Similarly, relatively constant MLSS concentrations were observed across the first four phases in R2, and the measured MLSS across these four phases was consistent with those measured across the entire experiment in R1. With the advent of Phase V, as the phosphorus availability neared zero, a decline in MLSS concentration was apparent, indicating that at this point phosphorus availability was finally limiting both degradation of organic compounds in the reactor and microbial growth in the system. This change in reactor performance, as

measured by both effluent COD and MLSS, was observed only when in influent phosphorus concentration was nearly two orders of magnitude below the stoichiometric requirement for complete oxidation of the influent COD. The results indicate that very effective MBR treatment of greywater could be maintained with the provision of even a very small amount of phosphorus well below the 1 mg P per 100 mg BOD_5 that might be expected.

Also noteworthy among the performance data for both reactors is the low suspended solids concentrations that were measured, particularly under phosphorus-balanced conditions (*i.e.*, in R1). Given an average removal of 200 mg/L of BOD_5 and an average MLSS concentration of 43 mg/L and SRT of 4 days, the cell yield coefficient was approximately 0.054 mg TSS/mg COD (or 0.043 mg VSS/mg COD, assuming MLVSS/MLSS was approximately 0.8). This yield coefficient was approximately an order of magnitude lower than would be expected for aerobic heterotrophs. The observed yield is more consistent with anaerobic conditions when an alternate electron acceptor results in less energy liberated from catabolism of the organic material. However, in this system, excess oxygen was provided to the system. Some evidence suggests that yields in MBRs could be as much as 50 percent lower than those in comparable conventional suspended growth systems due to complete solids retention leading to greater endogenous decay, greater presence and retention of predators, and microbial growth on cell products liberated by lysed cells (Côté *et al.* 1997, Ghyoot and Verstraete 2000, Wong 2012). However, all of these phenomena are related to or most apparent at MBR operation at extended SRTs, longer than the 4d SRT employed here.

Another possible explanation of the low cell yield apparent in these systems is metabolic uncoupling of catabolic and anabolic processes, such that some of the energy expected to be diverted to anabolic processes is instead dissipated as heat. Metabolic

uncoupling can be induced by a variety of conditions, including the presence of certain chemical uncouplers that act as protonophores and transport protons across cell membranes, which can reduce the driving force for oxidative phosphorylation (Wei *et al.* 2003). As a result of metabolic uncoupling, sludge production can be reduced while not actually reducing substrate removal, and increased energy will be lost as heat instead of being converted to ATP and ultimately producing cell mass (Liu 2003). Numerous chemical uncouplers have been identified by researchers in an effort to exploit them to minimize sludge production in biological treatment systems, including paranitrophenol, 2,4-dinitrophenol, 2,4,5-trichlorophenol, and m-chlorophenol (Wei *et al.* 2003). Many of the known metabolic uncouplers are believed to be harmful to the environment and therefore not desirable or allowed to be added to a treatment system that discharges to the environment. As a result, some research has focused on 3,3',4',5-tetrachlorosalicylanilide (TCS), which, at least historically, was a known PCP ingredient and believed to be less harmful to the environment (Liu 2003). Research has shown that the presence of TCS at concentrations below 1 mg/L can reduce sludge production by more than 45 percent while still maintaining the same 95 percent substrate removal observed in reactors with no TCS present (Chen *et al.* 2002). Although this compound is not specifically known to have been in the synthetic greywater used here, the synthetic greywater used was comprised of several PCPs, and the specific ingredients of the laundry detergent in particular are not precisely known; therefore, TCS or another metabolic uncoupler could have been present causing the low solids production. From an operational perspective, reduced sludge production could be advantageous in a household membrane bioreactor where sludge growth could lead to membrane fouling or the need for solids disposal, so reduced solids generation could result in the need for less frequent maintenance or oversight.

Observations in MBR Treatment of Real Greywater

The consistent effluent water quality produced in spite of phosphorus concentrations that were lower than thought necessary could result simply from the consumer products used to produce the greywater, resulting in a synthetic greywater unrepresentative of real greywater. To determine if similar results would be observed in real greywater, R2 was operated for a separate four-month period that culminated in treating real greywater from a local household.

The real greywater was obtained from the first permitted residential greywater reuse system in Austin, Texas (Price 2012). The greywater originated from the washing machine, a bathroom sink, and a bathtub in a house occupied by two adults and one child and was intended for reuse in landscape irrigation. During collection of the greywater for purposes of this study, flow to the distribution field was stopped to allow for accumulation of the greywater in a 400 gallon holding tank (Jashinski 2010).

For this experimental period, R2 was seeded with sludge wasted from R1 and initially fed the synthetic greywater and sodium phosphate solutions at the highest concentration of 3.6 mg P/L (Tables 3-2 and 3-4). It was operated in four distinct phases:

1. synthetic influent with supplemental phosphorus at an infinite SRT;
2. synthetic influent with supplemental phosphorus at an SRT of 4 d;
3. real greywater influent with supplemental phosphorus at an SRT of 4 d;
4. real greywater influent with no supplemental phosphorus at an SRT of 4 d.

The transition from Phase II to Phase III entailed a gradual switch from all synthetic to all real greywater by increasing the fraction of greywater by 25 percent every three to four days.

The real and synthetic greywater were quite similar in characteristics including pH, nitrate-nitrogen concentration and COD concentration (Table 3-5). The most

substantial difference between the synthetic and real greywaters was the suspended solids concentrations. The influent TSS in the real greywater ranged from 8.7 to 441 mg/L, with a mean TSS of 249 mg/L, while TSS was not measured in the synthetic greywater because the constituents were dissolved. The difference in TSS might also explain the lower BOD_5 of the real greywater. A large fraction of the measured COD in the real greywater was likely particulate COD, which might be less readily biodegradable than the dissolved fraction of COD. As a result, although the COD of the two greywaters was quite similar, the BOD of the real greywater was much lower. The ammonia concentration was also substantially higher in the real greywater than in the synthetic greywater, but both values were within the range of concentrations expected in real greywater.

As with the synthetic greywater, measured dissolved orthophosphate and dissolved total phosphorus concentrations were also found to be less than the detection limit of the standard ascorbic acid method. However, 0.28 to 0.54 mg/L of orthophosphate was detected in unfiltered real greywater influent samples, indicating that some particulate phosphorus was among the particulate matter in the real greywater. Examination of the personal care products used in this household indicates that the source of the phosphate was likely the toothpaste used by the occupants. Nevertheless, the concentration of phosphorus measured in the real greywater was still less than half of that needed to provide 1 mg/L of phosphorus for every 100 mg/L of BOD_5.

Table 3-5 – Comparison of characteristics of real and synthetic greywater used in this experiment to greywater characteristics reported in the literature.

	Literature		Mean Measured in Synthetic Greywater	Mean Measured in Real Greywater
	Range	Median		
pH	5.5 - 10	7.54	7.92	7.93
BOD_5 (mg/L)	8 - 670	153	236	111
COD (mg/L)	33 - 1815	327	405	433
Total N (mg/L)	3.6 - 37.3	8.7	3.5	7.1
NH_4-N (mg/L)	0.02 - 18.6	1.18	0.13	5.0
NO_3-N (mg/L)	<0.02 - 7.5	1.26	0.79	0.76
Total P (dissolved) (mg/L)	0.062 - 57	0.70	<0.050	<0.15
PO_4-P (dissolved) (mg/L)	0.5 - 171	8.8	0.034	<0.15

Reactor performance, based on characteristics such as COD and BOD removal and reactor MLSS concentrations, during Phases I and II was similar to the performance of R1 during comparable phases of operation (synthetic greywater influent at an infinite and 4d SRT). At that point, the reactor influent was gradually changed to real greywater. Operation during Phase III (with real greywater and supplemental phosphorus as the influent solutions) resulted in effluent COD averaging approximately 31 mg/L (Figure 3-4). Removal of the supplemental phosphorus feed to the reactor (in Phase IV) caused an increase in the next two measured effluent COD values to 52 and 73 mg/L, but removal efficiencies remained at more than 90 percent on these days, and thereafter the COD concentrations declined to a mean concentration of 39 mg/L. These effluent COD concentrations were comparable to those measured during Phase III, providing no evidence of phosphorus stress.

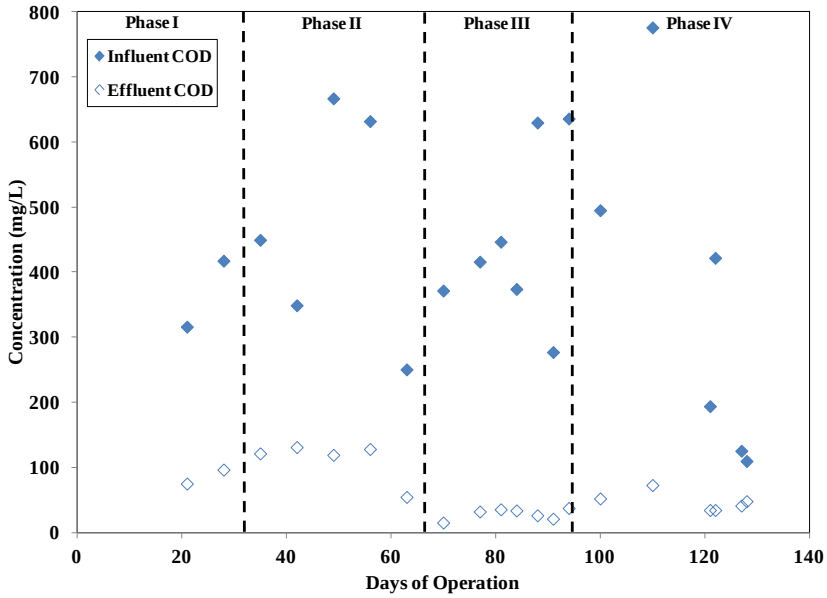


Figure 3-4 – Influent and effluent COD measured in R2 treating real greywater.

Similarly, while influent suspended solids was a large (and highly variable) contributor towards MLSS and MLVSS measured in the reactor, nearly identical mean MLSS concentrations were measured in Phases III and IV, and the mean MLVSS concentration actually increased in Phase IV (Figure 3-5). The similarity in effluent COD concentrations and MLSS and MLVSS concentrations provides little evidence of a treatment system experiencing nutrient stress, in spite of the influent containing at most 0.54 mg/L, well below the amount believed necessary to treat an influent containing 100-200 mg/L BOD_5.

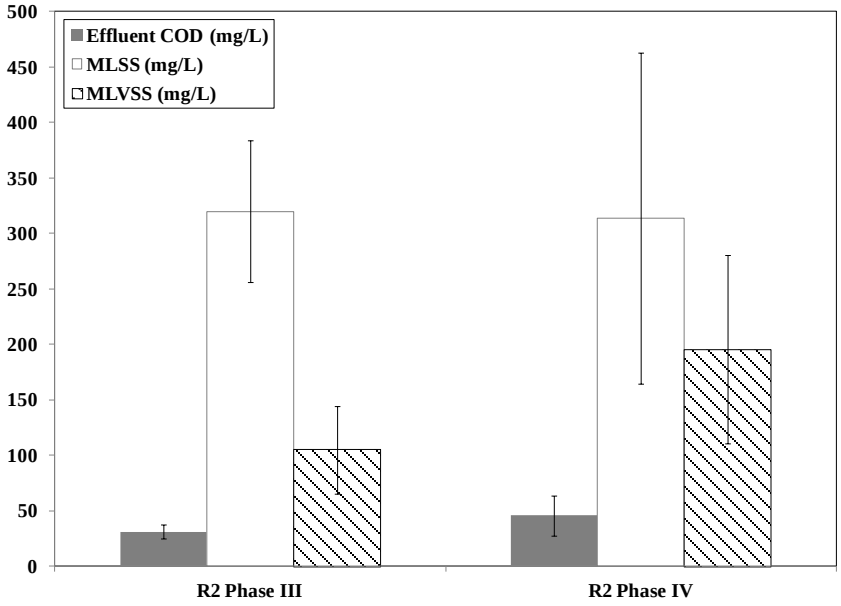


Figure 3-5 –Effluent COD and MLSS and MLVSS concentrations measured in R2 treating real greywater with (Phase III) and without (Phase IV) supplemental phosphorus.

Available phosphorus concentration still was not wholly irrelevant. On two occasions, no residual dissolved orthophosphate could be detected in the reactor, indicating that all available phosphorus had been consumed (Figure 3-6). Furthermore, although effluent concentrations in Phase IV leveled off to concentrations comparable to those measured during Phase III (when supplemental phosphorus had been provided to the reactor), measured influent COD concentrations also declined in the later part of Phase IV indicating a reduction in COD removal efficiency. COD removal averaged 93 percent in Phase III compared to an average of 83 percent in Phase IV (reaching as low as 53 percent). Given the variability seen in influent COD and TSS, the influent phosphorus concentration was also likely to be highly variable as well. These data imply that during certain periods of operation phosphorus can still be the limiting nutrient in MBR treatment of greywater.

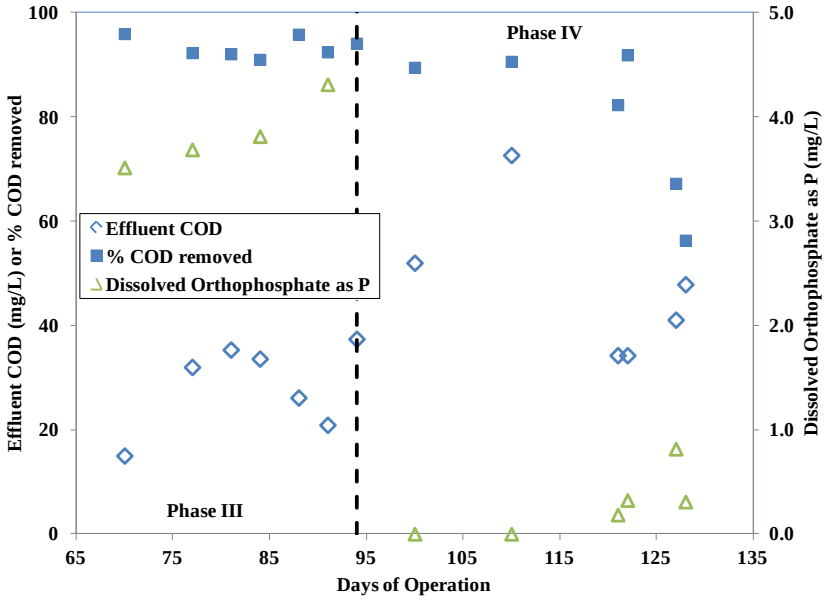


Figure 3-6 – Effluent COD, percent COD removed, and dissolved phosphorus concentrations measured in mixed liquor with (Phase III) and without (Phase IV) addition of sodium phosphate in MBR treating real light greywater at an SRT of 4 d.

The difficulty remains, however, that in the data presented here for real and synthetic greywater, influent phosphorus concentration does not seem to be an adequate indicator of the potential for phosphorus limitation in greywater treatment. Here, the accepted ratios of BOD_5 to phosphorus over-predicted the amount of phosphorus required to produce consistent effluent water quality by more than an order of magnitude. In contrast, Jefferson *et al.* (2001) found that phosphorus supplementation of real greywater improved COD removal by 110 percent in spite of the real greywater already having a ratio of COD:N:P ratio of 13:4:1 while phosphorus supplementation of synthetic greywater only improved COD removal by 25 percent even though the synthetic greywater had a COD:N:P ratio of 894:106:1. For both economic and environmental reasons, extra phosphorus should not be added to a treatment system if it will not be

taken up by a flourishing microbial community. On the other hand, if insufficient phosphorus is supplied to a treatment system, requirements for treated water quality might not be met. Therefore, an alternate measurement that might provide a better indication of when a treatment system is suffering from a nutrient limitation could ensure that this balance between under- and overdosing is achieved.

Use of Extracellular Enzyme Assay as an Indicator of Nutrient Limitation

One alternate indicator of nutrient limitation could be a measurement of extracellular enzyme activity (EEA), which has been developed over the last two decades as an indicator of nutrient limitation in riverine and other natural systems but has only been applied to a limited extent thus far in wastewater treatment systems. EEA assays can be used to measure activity of hydrolytic enzymes that catalyze degradation of complex compounds in the ecosystem to simpler compounds that can be assimilated by microbes (Sinsabaugh *et al.* 1997). Hill *et al.* (2010b), in particular, concluded in a study of nutrient limitation in forested streams that ratios of aminopeptidases activities to phosphatase activity were a better indicator of nutrient limitation than were the pure stoichiometric ratios of the nutrients. EEA assays have been applied in a limited number of studies to wastewater treatment systems but not to monitor the relationship between changing macronutrient concentrations and changes in EEA. The EEA assay employed here focused on three groups of enzymes: glycosidases, which are utilized in carbon acquisition; aminopeptidases, for nitrogen acquisition; and the esterases (acid and alkaline phosphatase) related to phosphorus acquisition (Table 3-6). In general, others have asserted that the relative activity of each group of enzymes should be in proportion to the relative abundance of each nutrient in the microbial biomass (Hill *et al.* 2006); that is, if the microbial biomass is assumed to be of the formula $C_{60}H_{87}O_{23}N_{12}P$ (as might be

expected in activated sludge), then glycosidase activity should be approximately five times greater than aminopeptidase activity, and total glycosidase activity should be sixty times that of phosphatase activity. Because only a subset of enzymes in each category is being assayed, these exact proportions cannot be expected. Hill *et al.* (2006) investigated nutrient limitations in coastal wetlands but interpreted any stretch in which the aminopeptidase activity exceeded both of the other categories to be nitrogen-limited, while stretches in which the phosphatase activity exceeded the other two categories to be phosphorus-limited.

Table 3-6 – Summary of Extracellular Enzymes Assayed

Enzyme	Function	Substrate
Glycosidases		
α-D-glucosidase	Starch degradation	4-methylumbelliferyl (MUB)-α-D-glucoside
β-D-glucosidase	Cellulose degradation	4-MUB- β-D-glucoside
β-N-acetylglucosaminidase	Chitin and peptidoglycan degradation	4-MUB-N-acetyl-β-glucosaminide
Aminopeptidases		
L-glycine aminopeptidase	Protein degradation	Glycine-7-amido-4-methylcoumarin
L-leucine aminopeptidase	Protein degradation	L-Leucine 7-amido-4-methylcoumarin
Esterases		
Acid / alkaline phosphatase	Acquisition of phosphate	4-MUB-phosphate

The three glycosidases were chosen because they had either previously been detected in activated sludge or had been found to spike in samples collected from a river downstream of WWTP outfall (Boczar *et al.* 1992, Chappell and Goulder 1994). The aminopeptidases chosen are used in acquisition of two of the three most common amino

acids that occur in nature (Voet and Voet 1995). Phosphatase occurs in both acid and alkaline forms that are active or functional only in their relevant pH ranges.

For baseline purposes, the ratios of activity of the different classes of enzymes in R1 and in phase I in R2 were evaluated and compared because the reactors were believed to be phosphorus-balanced during these periods. The average ratio of phosphatase activity to total glycosidase activity measured in Phase I in R1 and R2 was 0.54 and 0.21, respectively. The average ratio of the total aminopeptidase activity to the total glycosidase activity measured in Phase I in R1 and R2 was 0.22 and 0.21, respectively.

The measured phosphatase activity and ratio of phosphatase to total glycosidase activity were both vary stable over time in R1 where a consistent influent phosphorus source was provided (Figure 3-7). In particular, the ratio of phosphatase activity to total glycosidase activity was nearly always less than 0.5 (dimensionless), consistent with the expectation of Hill *et al.* (2006) that in a phosphorus-balanced system, glycosidase activity should well exceed phosphatase activity. The measured aminopeptidase activities were in all cases quite low in R1 (less than 1.0 μmol 7-AMC/mg TSS/hr), and the ratio of total aminopeptidase activity to total glycosidase activity always measured less than 1.0 and averaged 0.29 in R1, again consistent with a system in which nitrogen is not the limiting nutrient. These values can be then considered as baseline values for a phosphorus-balanced reactor, to which the results of R2 can be compared.

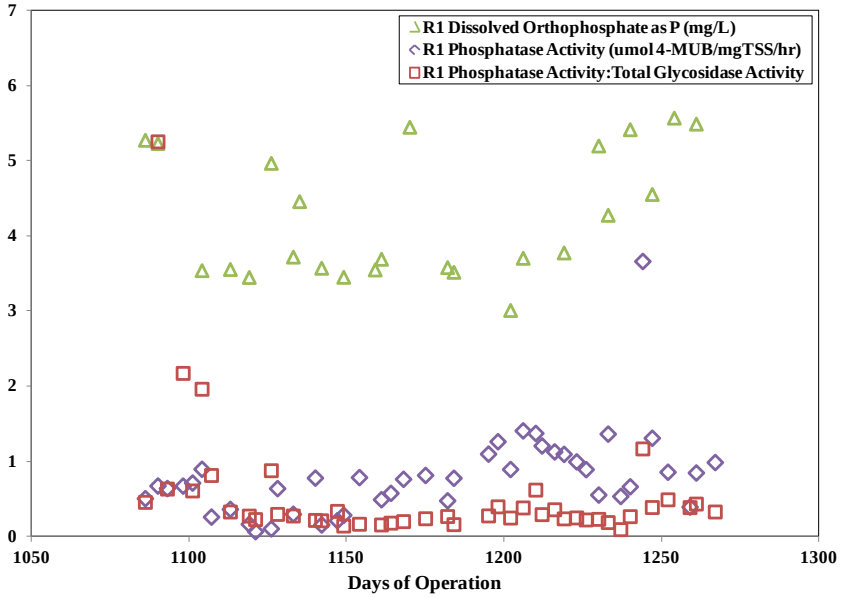


Figure 3-7 – Dissolved orthophosphatase concentration, phosphatase activity, and ratio of phosphatase activity to total glycosidase activity measured in R1. Excess phosphorus was provided to this reactor throughout the experimental period. Influent COD concentrations averaged 405 mg/L, and effluent COD averaged 67 mg/L.

In contrast to R1 where the ratio of phosphatase activity to total glycosidase activity remained under 1.0 throughout the course of the experiment, changes in enzyme activity were apparent in R2 as the supplemental phosphorus concentration declined (Figure 3-8).

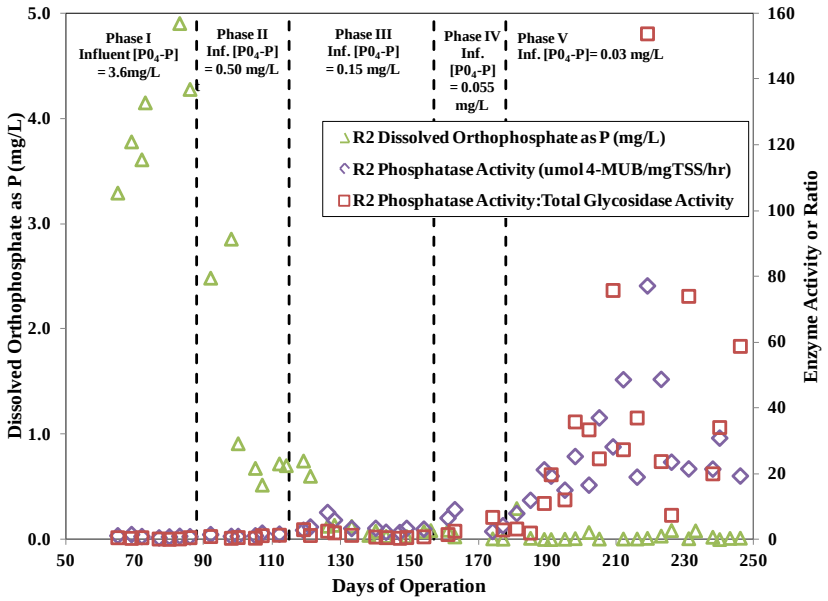
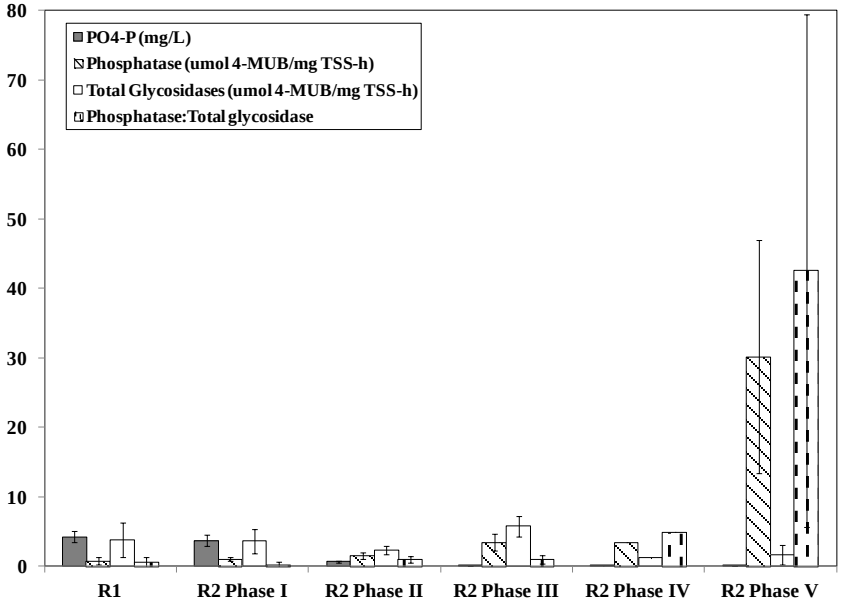


Figure 3-8 – Dissolved orthophosphatase concentration, phosphatase activity, and ratio of phosphatase activity to total glycosidase activity measured as influent phosphorus concentration was reduced in R2.

Most apparent from these data is that in Phase V (no supplemental phosphorus provided to the reactor), both the phosphatase activity and the ratio of phosphatase to total glycosidase activity increased by one to two orders of magnitude from averages of 1.04 and 0.21, respectively, in Phase I to averages of 30 and 43, respectively, in Phase V (Figure 3-9). In particular, the lowest ratio of phosphatase activity to total glycosidase activity measured in Phase V was 7. In all cases in Phase V this ratio indicated that phosphatase activity exceeded the glycosidase activity by at least seven times, indicating a clear phosphorus limitation by the standard of Hill *et al.* (2006).

a)



b)

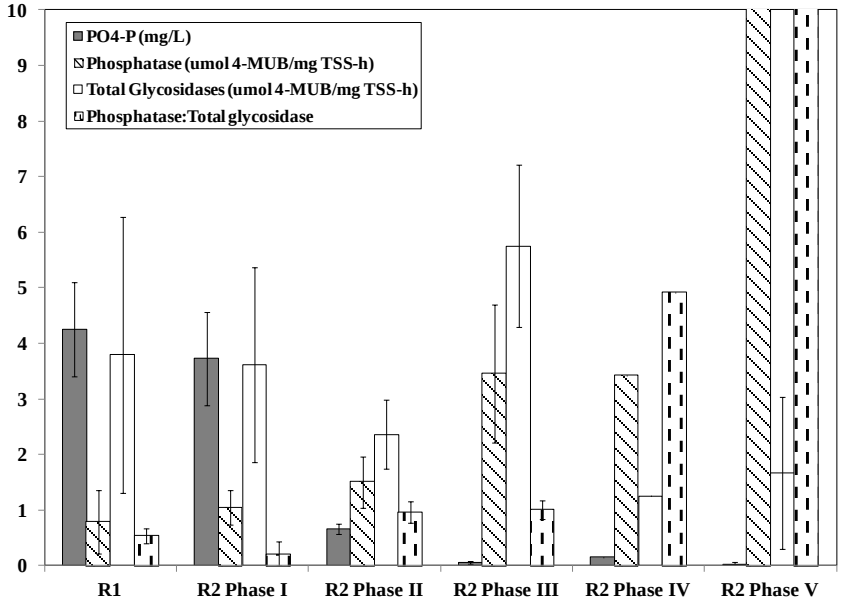


Figure 3-9 – Mean dissolved orthophosphate concentration, phosphatase activity, total glycosidase activity, and ratio of phosphatase to total glycosidase activity measured in R1 and R2 in each phase. Error bars shown are plus and minus one standard deviation. (No standard deviation was calculated for R2 Phase IV because it only included two data points.)

The substantial increase in the ratio of phosphatase activity to total glycosidase activity from Phase III to Phase IV appears to be attributable to the decline in total glycosidase activity (rather than change in phosphatase activity) which continued into Phase V. The first indication of a phosphorus limitation appears, then, to be a steady increase in phosphatase activity. These data indicate that eventually as phosphatase limitation persists the glycosidase activity might collapse. Since the glycosidases are involved in the acquisition of carbon through degradation of starch, cellulose, chitin, and peptidoglycan, a decline in their activity could over time translate into reduced biodegradation of the microorganisms' carbon source, which could over time translate into the spike in effluent COD that was observed in Phase V.

Also of note is the ratio of total aminopeptidase activity to total glycosidase activity in R2, which remained quite stable across the first four phases in R2, varying from an average of 0.11 in Phase I to 0.53 in Phase IV (Figure 3-10). However, in Phase V, this ratio rose to an average of 2.70. The total aminopeptidase activity was still be more than an order of a magnitude less than the total phosphatase activity, a situation that is still indicative of a phosphorus limitation in Phase V in R2, but the peak in aminopeptidase activity might still be a signal of the stress that the microbial community is experiencing.

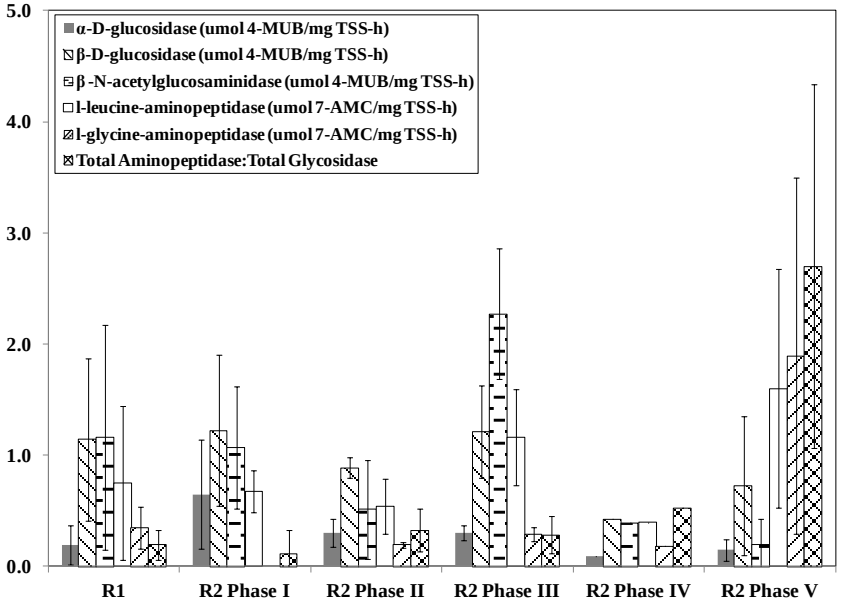


Figure 3-10 – Individual glycosidase activities, individual aminopeptidase activities, and ratio of total aminopeptidase activity to total glycosidase activity measured in R1 and R2 in each phase. Error bars shown are plus and minus one standard deviation. (No standard deviation was calculated for R2 Phase IV because it only included two data points.)

If EEA is to be of interest as a potential indicator of nutrient limitations, spikes in enzyme activity are less illuminating if they only co-occur with the spike in COD or drop in TSS, since the stress will already be apparent by a drop in effluent water quality. Both phosphatase activity and the ratio of phosphatase to total glycosidase activity generally increased with each decline in influent phosphate concentration [Figure 3-9(b)]. However, as early as Phase II, the average ratio of phosphatase activity to total glycosidase activity is nearing the threshold of 1.0 even though no drop in TSS or increase in effluent COD was yet apparent in the reactor, and half of the individual measurements of the ratio in Phases II and III exceeded 1.0. In Phase IV, the ratio had already reached nearly 5.0, well in excess of the 1.0 threshold. Therefore, the ratio of phosphatase activity to total glycosidase activity was providing an indication of nutrient

stress in the reactor in advance of the changes in effluent COD and reactor TSS concentrations.

The data presented support the idea that the ratio of the phosphatase activity to total glycosidase activity provides an indication of a microbial community under stress resulting from a phosphorus limitation. Further work might be necessary to establish whether the 1.0 threshold is the most appropriate benchmark against which to measure the ratio in treatment systems, since, as was done here, most reactors would not be operated through a controlled period of nutrient balance followed by normal, less controlled operations.

CONCLUSIONS

Results in both real and synthetic greywater indicate that greywater has the potential to be phosphorus-limited on at least an intermittent basis. However, in both synthetic and real greywater, any indications of the effects of the limitation were only limited to situations where the total available phosphorus concentration was less than 0.5 mg/L, far less than the phosphorus requirement that would be calculated from stoichiometric conversions from measured oxygen demand. Nevertheless, the data presented here indicate that phosphorus supplementation, even at low levels of less than a milligram per liter, in both real and synthetic greywater would result in more consistent, higher effluent water quality.

For wastewater systems, an assay of enzyme activity focusing on the ratio of phosphatase activity to total glycosidase activity can be used to identify a microbial community under stress before changes in bulk measurements of effluent water quality are evident. Further work is likely needed to identify a consistent basis for interpreting these data and understanding when the assay signals the need for phosphorus

supplementation. Also needed is an understanding of what other changes in reactor behavior or effluent water quality might be apparent during these initial stages of phosphorus limitation as the microorganisms expend greater energy and resources for the acquisition of phosphorus even if the effects are not yet seen in effluent COD.

Finally, synthetic greywater was shown in the course of this work to be an imperfect surrogate for real greywater. In particular, the substantial suspended solids concentration present in real greywater leads to important differences between the two greywaters including a lower biochemical oxygen demand relative to the total COD but also a higher measurable phosphorus concentration originating from that particulate fraction in the real greywater. The synthetic greywater composition could be modified to include particulate matter, COD, and phosphorus to more closely mimic the behavior of real greywater.

Chapter 4: XOC Removal During Phosphorus-Limited MBR Treatment of Household Light Greywater

Ever since a team of USGS researchers surveyed the nation's waterways and suggested that the presence of an array of organic micropollutants was due in part to incomplete or inadequate removal of these contaminants in wastewater treatment plants (Kolpin *et al.* 2002), much attention has been paid to the fate and transport of organic contaminants within water and wastewater treatment systems and effluent receiving waters to which they are discharged. Many of these contaminants found in wastewater effluent are believed to originate from household use of pharmaceuticals and personal care products (PCPs) and, as such, would be expected to be present in greywater, perhaps at even higher concentrations than in domestic wastewater. In addition, as outlined previously, current greywater practice typically involves direct discharge of greywater to landscape without treatment. Thus, while potable water can be saved through greywater reuse, these systems might also directly discharge PCP ingredients to the environment, including chemicals with a variety of known and unknown environmental and toxicological effects along with some chemicals for which the fate and transport is well characterized but others for which they are poorly understood (Donner *et al.* 2010, Hernández-Leal *et al.* 2011).

Specific environmental consequences of irrigation with greywater containing personal care product ingredients have been documented including accumulation of surfactants in soil, greater hydrophobicity of the soil, weak estrogenicity of the discharged water, reduction in the number and diversity of culturable microorganisms along with greater antimicrobial resistance (if triclosan is present in greywater) (Gross *et al.* 2005, Harrow *et al.* 2011, Hernández Leal *et al.* 2010, Roesner *et al.* 2006). The desire for greater control over discharge of these xenobiotic organic compounds (XOCs)

might provide further incentive for increased greywater treatment. At the same time, an increase in the intensity of greywater treatment also requires understanding of the fate and transport of the same XOCs in the course of that treatment. Since the Kolpin *et al.* (2002) study, a large number of publications detailing the occurrence and removal of these contaminants in raw and treated wastewater have emerged, but extremely limited data on the occurrence of organic chemicals in greywater exists. Organic contaminants in light greywater have been investigated, identified, and to some extent quantified in Denmark, Sweden, the Netherlands, and (to a very limited extent) the U.S. (Almqvist and Hanaeus 2006, Andersen *et al.* 2007, Eriksson *et al.* 2003, Eriksson *et al.* 2010, Hernández Leal *et al.* 2010, Palmquist and Hanæus 2005, Sharvelle *et al.* 2012). A wide variety of compounds were identified, including milligram per liter (mg/L) concentrations of some surfactants and microgram per liter (μg/L) concentrations of plasticizers, fragrances, and antimicrobial compounds including triclosan. Perhaps more unexpected are the detections of BTEX compounds, polycyclic aromatic hydrocarbons, or polychlorinated biphenyls. Even less data is available on the removal of organic chemicals in the course of greywater treatment.

Andersen *et al.* (2007) found five paraben compounds (common preservatives in personal care products) to be at least 98 percent removed in a greywater treatment system in Denmark consisting of primary sedimentation, three RBCs in series, secondary sedimentation, a sand filter, and ultraviolet filtration. Hernández Leal *et al.* (2010) evaluated the removal of 17 organic compounds in an aerobic sequencing batch reactor, an anaerobic upflow sludge blanket reactor, and a combination of the aerobic and anaerobic systems. The aerobic and combination aerobic-anaerobic systems were found to be the most consistent and effective in achieving at least 80 percent removal for 14 of the 17 compounds. Hernández Leal *et al.* (2010) also compared removals found in their

aerobic treatment system to removals reported in the literature for wastewater treatment plants (WWTPs). The removals were largely comparable to those in WWTPs with the exception of those of the fragrance tonalide, the removal of which was on the very low end of the removals in WWTPs, and the sunscreen ingredient 2-ethylhexyl p-methoxycinnamate (EHMC), the removal of which was less than half of that in WWTPs.

The Hernández Leal, et al. (2010) study points to the fact that existing data on the removal of XOCs in conventional wastewater might, in many cases, provide useful information as to the fate of those XOCs in greywater treatment. However, the characteristics that differentiate greywater from typical domestic wastewater (such as fluctuations in hydraulic and chemical variability and potential nutrient limitations) have not been thoroughly explored in the literature, nor have the effects that these differences might have on XOC removal in greywater treatment. The research described herein is intended to bridge this gap by examining the removal of three different PCP ingredients in MBR treatment of household light greywater under conditions of phosphorus limitation and phosphorus balance. The results from Chapter 4 suggest that phosphorus requirements in this treatment system were lower than would be dictated by conventional guidelines; only with no phosphorus supplementation were the effects of a phosphorus limitation seen on bulk measurements such as COD removal and MLSS concentrations. The work herein evaluates how the removal of individual organic contaminants comprising some fraction of the total organic load will be affected by a phosphorus limitation in the course of MBR treatment.

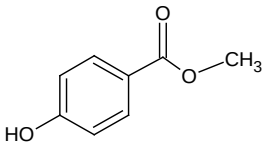
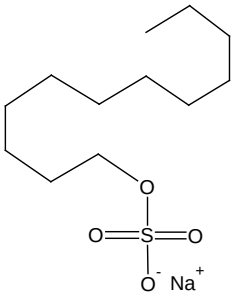
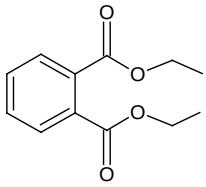
MATERIALS AND METHODS

This work was carried out concurrently with the work described in Chapter 3, with two 8.4 L MBRs operated in parallel (R1 and R2), both being fed a synthetic

greywater influent solution and a supplemental phosphorus solution. As the phosphate concentration provided as a supplement to R2 was lowered, the concentration of three XOCs in both R1 and R2 was measured in addition to COD, MLSS, and dissolved orthophosphate.

Three XOCs were selected for study based on their known presence in personal care products and household greywater and the likely location of the genes (plasmid or chromosomal) that encode the enzymes for their biodegradation. The latter criterion was chosen because bacteria can shed plasmids when under environmental stress, so treatment process performance for XOC removal might depend on whether the requisite genes are plasmid-associated or chromosomal. The preservative methyl parahydroxybenzoate (or methylparaben), the surfactant sodium lauryl sulfate (SDS), and the plasticizer diethyl phthalate (DEP) were chosen for study. Relevant properties of these chemicals are summarized in Table 4-1.

Table 4-1 – Properties of Measured XOCs (ECSCCPNFPIC 2002, U.S. National Library of Medicine 2013, Vamsee-Krishna and Phale 2008, Yeldho *et al.* 2011)

Compound	Methyl paraben	Sodium lauryl sulfate	Diethyl phthalate
Chemical formula	$C_8H_8O_3$	$C_{12}H_{25}NaO_4S$	$C_{12}H_{14}O_4$
Chemical structure			
Molecular weight	152.15 g/mol	288.38 g/mol	222.24 g/mol
log K_{ow}	1.96	1.6	2.42
Henry's law constant	$2.23x10^{-9}$ atm-m^3/mol at 25°C	$1.8X10^{-7}$ atm-m^3/mol	7.8×10^{-7} atm-m^3/mol
Expected location of degradative genes	Chromosome	Plasmid	Plasmid

Methylparaben (MP) has been detected in real greywater in Denmark at concentrations as high as 39 μg/L (Table 4-2). It is also listed as an ingredient in more than 24,721 PCPs sold in the U.S. including moisturizer, sunscreen, body wash, and hand cream according to Environmental Working Group (EWG)'s Skin Deep Cosmetics Database (2013) and 678 personal care, pet, and cleaning products (that could reasonably be expected to end up in household light greywater) according to the U.S. Department of Health and Human Services (HHS) Household Products Database (2013). MP was chosen for study as a compound for which the genes encoding its degradative pathway are expected to occur on the chromosome. SDS has not, to date, been analyzed for in real greywater, but anionic surfactants (a category that includes SDS) have been detected in greywater at concentrations between 0.3 and 118.3 mg/L (Chaillou *et al.* 2011, Lamine *et al.* 2012, Prathapar *et al.* 2005, Shafran *et al.* 2005). SDS is also listed as an ingredient in

2,609 PCPs sold in the U.S. such as toothpaste, body wash, hand soap, facial cleanser, moisturizer, and shaving cream in EWG's database (2013) and 361 personal care, pet, and cleaning products in the HHS database (2013). SDS was selected for study because its degradative genes are expected to occur on a plasmid (Yeldho *et al.* 2011). DEP is only listed as an ingredient in 25 consumer products in EWG's database (2013) and 25 PCPs in the HHS database (2013); however, it has been detected in multiple real greywater sources at concentrations as high as 38 μg/L. The genes encoding the degradative pathway for DEP are also expected to be carried on plasmids (Vamsee-Krishna and Phale 2008).

Table 4-2 – Measured Concentrations of XOCs of Interest in Greywater

	Andersen *et al.* 2007 Denmark	Eriksson *et al.* 2010 Denmark	Hernandez Leal *et al.* 2010 The Netherlands	Eriksson *et al.* 2003 Denmark	Palmquist & Hanaeus 2005 Sweden	Almqvist & Hanaeus 2006 Sweden
	Maximum Concentration Detected (μg/L)					
Methylparaben	35	39	ND	NA	NA	NA
Sodium lauryl sulfate	NA	NA	NA	NA	NA	NA
Diethylphthalate	NA	13	NA	13	38	9.4

ND - not detected
NA - not analyzed

Liquid Chromatography – Mass Spectrometry Analysis

Influent and effluent MP, SDS, and DEP were measured in both R1 and R2 over the course of nearly seven months of reactor operation. MP and DEP were analyzed by LC-ESI-MS-MS using methods adapted from González-Mariño *et al.* (2009) and Zafra-Gómez *et al.* (2008), respectively, using a Shimadzu 150×4.6 mm C18 column with a particle size of 5 μm and water and methanol to elute the compounds of interest. LC-MS grade methanol and water were purchased from J.T. Baker (Phillipsburg, NJ).

SDS was analyzed by LC-ESI-MS as described in Thermo Scientific (2012), using a Dionex Acclaim Surfactant Plus 150×4.6 mm column with a particle size of 3 μm, using an eluent solution of 75 percent acetonitrile and 25 percent 100 mM

ammonium acetate buffer (pH 5.0). LC-MS grade acetonitrile was purchased from EMD Millipore (Billerica, MA).

All LC-MS work was performed using a Finnigan Surveyor autosampler, a Finnigan Surveyor mass spectrometry pump, and a TSQuantum mass spectrometer (Thermo Electron Corporation, Waltham, MA) with electrospray ionization (Table 4-3).

Table 4-3 – LC-MS Analytical Parameters

Compound	Methylparaben		Diethyl phthalate		Sodium Lauryl Sulfate	
Chromatography						
Injection volume (mL)	10		25		10	
Flow rate (μL/min)	400		400		400	
Run time	30 min		30 min		20 min	
Gradient	5% methanol 95% water	6 min	5% methanol 95% water	6 min	75% acetonitrile 25% 100 mM ammonium acetate	20 min
	Increases linearly to 80% methanol 20% water	12 min	Increases linearly to 80% methanol 20% water	12 min		
	80% methanol 20% water	4 min	80% methanol 20% water	4 min		
	100% methanol 0% water	6 min	100% methanol 0% water	6 min		
	5% methanol 95% water	2 min	5% methanol 95% water	2 min		
Mass Spectrometry						
Polarity	Negative		Positive		Negative	
Collision energy (V)	24		26		--	
Collision pressure (mTorr)	1.2		0.5		--	
Ion spray voltage (V)	4000		3200		3600	
Capillary temperature (°C)	375		270		340	
Mass to charge ratio						
Parent ion	151		223		265	
Product ion	92		149		--	
Method						
Detection limit (μg/L)	13		23		66	

Sorption experiments

In order to deduce the relative portion of the XOC removal attributable to different removal mechanisms, experiments measuring the sorption of each XOC to biomass wasted from the reactors were performed. Sludge wasted from R1 was concentrated by centrifugation, rinsed three times with a solution of sodium bicarbonate

(147 mg/L), monobasic sodium phosphate (6.9 mg/L), and dibasic sodium phosphate (7.2 mg/L), and then reconstituted to the desired solids concentration using the same buffer solution. One hour prior to the start of the sorption experiment, the solids were mixed with a mercuric chloride solution such that the resulting mercuric chloride concentration was 1 g/L, sufficient to inactivate the microorganisms so that biodegradation of the compound would be minimized. Five 30-mL aliquots of the resulting culture was then mixed with increasing concentrations of the XOC of interest and placed on a shaker plate at 100 rpm to mix the solution. Samples were withdrawn 0.1 hr after mixing of the XOC with the solids and then again after 6 hr of mixing. Samples were also collected at 1, 2, and 4 hrs from an intermediate concentration sample to help determine whether equilibrium had been reached. At each sample point, the samples were filtered using a 0.2-μm syringe filter (VWR; Radnor, PA) and stored at 4°C until LC-MS analysis. Controls of sludge only and XOC only were also carried out to evaluate any desorption from the sludge itself along with determining the dissolved XOC concentration in the absence of any solids.

Because the MBR system is almost entirely made out of plastic and could potentially sorb some amount of each XOC entering the system, an estimate of this amount was also measured. First, upon cessation of these experiments, the reactors were rinsed with deionized water, and the rinse water collected. Next, the reactors were soaked with a solution containing monobasic sodium phosphate (6.9 mg/L) and dibasic sodium phosphate (7.2 mg/L) for one week, and samples were then collected for three straight days and these samples along with the rinse water were analyzed for each of the XOCs. No measureable concentrations of any of the XOCs were detected in any of rinse or desorption samples collected from either reactor.

Next, one at a time, a solution of each XOC [buffered with monobasic sodium phosphate (6.9 mg/L) and dibasic sodium phosphate (7.2 mg/L)] at a concentration typical of that in the synthetic greywater influent was prepared and placed in the reactor (Table 4-4).

Table 4-4 – Solutions Prepared to Test Sorption to Reactor System

XOC	Concentration
MP	300 μg/L
SDS	25 mg/L
DEP	90 μg/L

The mixture was allowed to sit for at least one week, and then samples were then collected for three straight days and analyzed for the XOC of interest. The reactor was then soaked in deionized water containing 6.9 mg/L monobasic sodium phosphate and 7.2 mg/L dibasic sodium phosphate for another week before repeating the same series of steps for the next XOC. Measurements performed thereafter indicated no detectable change in the concentrations of the XOCs remaining in the reactor after this equilibration period, indicating that sorption to the reactor itself was insignificant.

RESULTS AND DISCUSSION

Abiotic removal

Each sorption experiment resulted in a sorption isotherm (example shown in Figure 4-1) and calculation of a sorption coefficient (K_d) to describe sorption of each XOC to biomass isolated from the phosphorus-balanced reactor (Table 4-5). As expected, the calculated sorption coefficients increase with increasing hydrophobicity of the XOC, as assessed by their octanol-water partition coefficients.

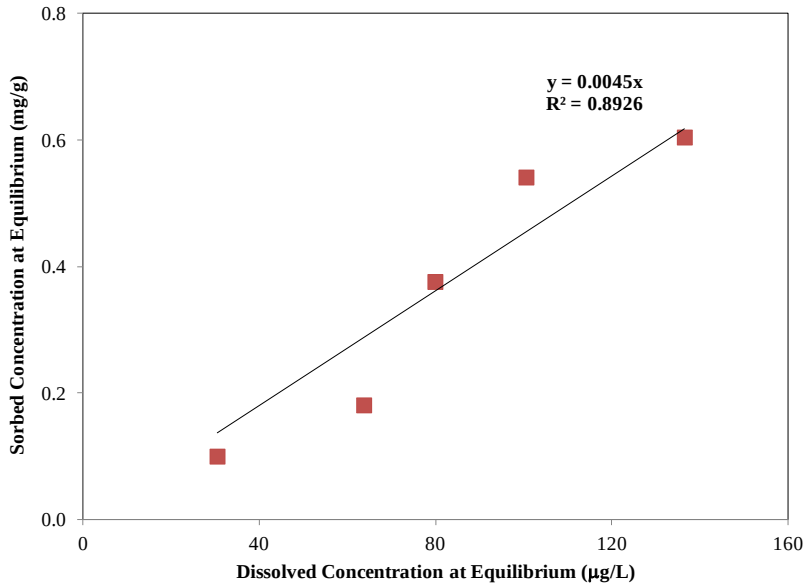


Figure 4-1 – Sorption of methylparaben to biomass concentrated from sludge wasted from R1 to 150 mg/L TSS.

Table 4-5 – Calculated sorption coefficients

XOC	K_d	log K_{ow}
MP	0.0045 L/mg	1.96
SDS	0.0016 L/mg	1.6
DEP	0.0123 L/mg	2.42

The sorption coefficients for MP and SDS indicate that less than one percent of the influent MP and SDS were removed via sorption to reactor biomass. With the highest sorption coefficient, sorption appeared to have had a slightly larger impact on DEP entering the reactors, accounting for removal of up to eight percent of the influent DEP. However, still more than 90 percent of the influent DEP remained in the dissolved phase and was available for biodegradation.

The Henry's constants for these three chemicals (Table 4-1) are one to three orders of magnitude below the U.S. EPA's threshold for volatile compounds of $1x10^{-5}$ atm-m^3/mol (U.S. EPA 2002). Modeling using the U.S. EPA's STP WIN program (2012b), which estimates removal of chemicals in activated sludge processes, indicates that less than 0.01 percent of all three chemicals would be expected to be lost due to volatilization. Therefore, volatilization was not considered to be a significant removal mechanism and its effects on the system were not assessed.

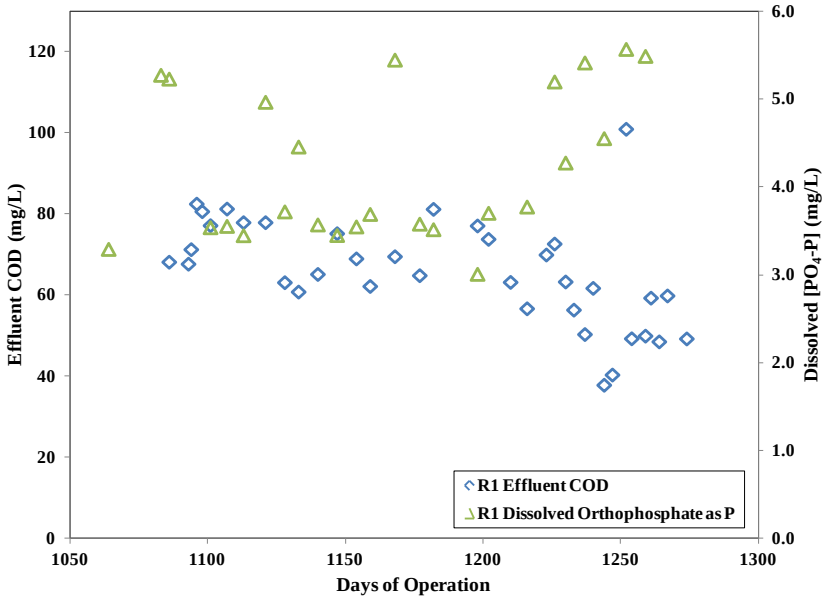
Taken together, abiotic processes did not contribute significantly to removal of these three XOCs in the treatment system.

Biodegradation

COD

COD removal in the phosphorus-balanced reactor (R1) averaged approximately 80 percent, and, as phosphorus concentrations were lowered in R2, COD removal also averaged approximately 80 percent as long as the influent phosphorus concentration remained above approximately 50 μg/L (Figure 4-2). Only when no supplemental phosphorus was provided to R2 were reductions in COD removal apparent, with removal dropping to as low as 60 percent. Not readily apparent in these data is whether all organic components in greywater that comprise the COD were affected in an identical manner, experiencing comparable stability in effluent concentrations throughout the first four phases of declining phosphorus concentrations, and decreased removal upon withdrawal of all supplemental phosphorus. Thus, COD removal provides a baseline context for overall reactor performance in examining the removal of the three XOCs presented below.

a)



b)

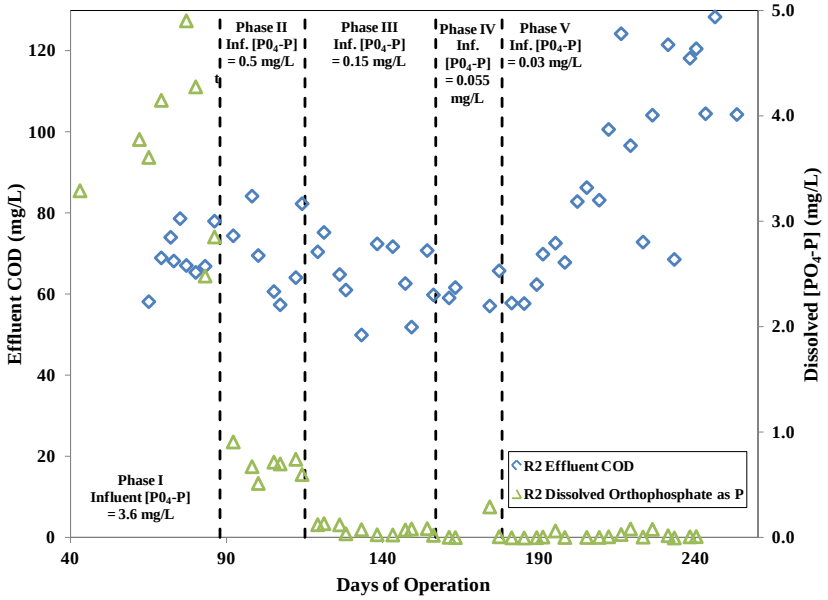


Figure 4-2 – Effluent COD (primary y-axis) and dissolved orthophosphate as phosphorus (secondary y-axis) measured in a) R1 and b) R2. (Because it had been in operation longer, the values on the time scale for R1 do not match those of R2; nevertheless, the reactors were operating in parallel.)

Methylparaben

In a survey of eight different WWTPs (both activated sludge and MBRs), MP was nearly always at least 80 percent removed in the course of treatment regardless of the SRT of the treatment plant (Oppenheimer 2007). Similarly, Andersen *et al.* (2007) found more than 98 percent removal of MP from real greywater across the biological treatment portion of the multi-stage reactor that they studied. These studies suggest that MP is readily biodegradable, but the effect of a phosphorus limitation on its biodegradability had not been previously assessed.

MP influent concentrations entering R1 varied between 200 and 390 μg/L; the average concentration was 247 μg/L (with a standard deviation of 42 μg/L). Entering R2, influent MP concentrations varied between 153 and 413 μg/L, averaging 242 μg/L (±30 μg/L). (These influent solutions originated from the same influent tank, and variability in measured concentrations could reflect heterogeneity in the influent solution or a small loss due to sorption or biodegradation in the several feet of tubing between the influent tank and the reactors themselves.) The measured concentrations are higher than the 38 μg/L measured in real greywater to date and likely a consequence of using only three products (one of which contained MP) to constitute the entire organic load of household light greywater. Nevertheless, at these concentrations, MP comprised approximately 450 μg/L of theoretical oxygen demand (ThOD) and was thus a relatively minor (0.125%) component of the overall COD entering the reactors.

Throughout operation of R1, effluent MP concentrations were in the single μg/L concentration range (Figure 4-3), below or near the detection limit of the analytical method, indicating that 97 to 100 percent of the influent MP had been removed from the greywater.

In R2, near complete MP removal was also observed in Phases I through IV even as the supplemental phosphorus concentration declined. As with COD removal, consistent effluent MP concentrations were seen, and no effect of a phosphorus limitation on MP was apparent until Phase V when no supplemental phosphorus was provided to the reactor. Much as effluent COD concentrations rose over the course of Phase V, effluent MP concentrations from R2 increased to 47 µg/L, still corresponding to 85 percent removal of the influent MP but less than the 97-100 percent removal seen in R1. These changes indicate that high levels of MP removal are sustained even at phosphorus concentrations where diminished reactor performance might be expected, but with a stringent enough limitation, eventually MP removal will decline.

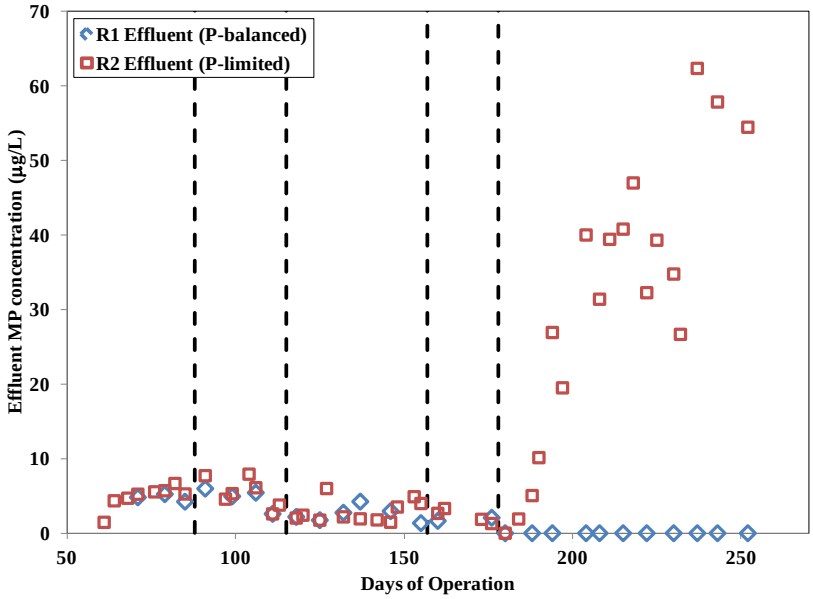


Figure 4-3 – Effluent MP from R1 (with steady influent PO_4-P concentration) and R2 (with declining influent PO_4-P concentration). Influent MP averaged 247 µg/L to R1 and 242 µg/L to R2.

Sodium Lauryl Sulfate

Although SDS removal has not previously been evaluated in greywater specifically, approximately 90 percent of influent SDS was found to be removed in a typical WWTP (Fendinger *et al.* 1992). Other screening tests also showed that SDS was readily biodegradable under conditions mimicking those of aerobic treatment in a WWTP (Berger 1997), and it was thus expected to be readily biodegradable in this aerobic treatment system although the potential impact of the phosphorus limitation was unknown.

SDS influent concentrations entering R1 varied between 18 and 30 mg/L; the average concentration was 23 mg/L (with a standard deviation of 2.8 mg/L). Entering R2, influent SDS concentrations also varied between 18 and 30 mg/L, averaging 23 mg/L (±2.7 mg/L). At these concentrations, SDS comprised approximately 46 mg/L of ThOD or more than 11 percent of the overall COD.

Effluent concentrations from R1 throughout the course of the experiment ranged from 0.7 to 3.6 mg/L (Figure 4-4), averaging 1.4 mg/L, corresponding to 85 to 98 percent removal from greywater in MBR treatment. Over the course of the first four phases in R2, effluent SDS concentrations ranged from 0.83 to 2.3 mg/L, with an average of 1.5 mg/L. These data indicated 88 to 97 percent removal in phases I to IV in R2 or nearly identical removal as was seen in R1, with little to no effect of the phosphorus limitation evident even as available phosphorus concentrations declined. With the last step down in phosphorus concentration provided to R2 in phase V, the effluent SDS concentrations rose as high as 9.4 mg/L, averaging 6.8 mg/L over that phase. Removal in this phase declined to as low as 61 percent and averaged 71 percent. Similar to results seen for MP, high levels of SDS removal were sustained throughout the first four phases where diminished reactor performance (and XOC removal) might have been expected, but SDS

removal was only adversely impacted once the supplemental phosphorus was completely withdrawn.

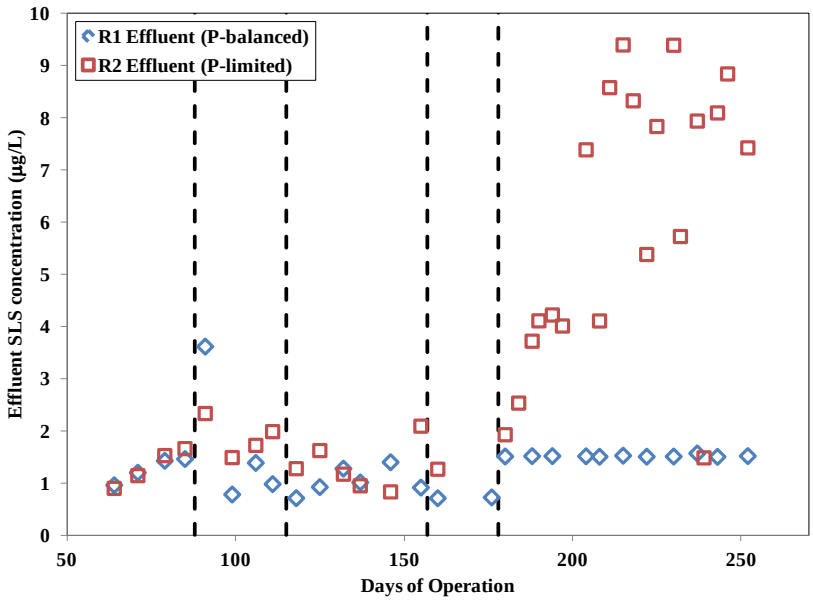


Figure 4-4 – Effluent SDS from R1 (with steady influent PO_4-P concentration) and R2 (with declining influent PO_4-P concentration). Mean influent SDS concentration was 23 mg/L to both reactors.

Diethyl phthalate

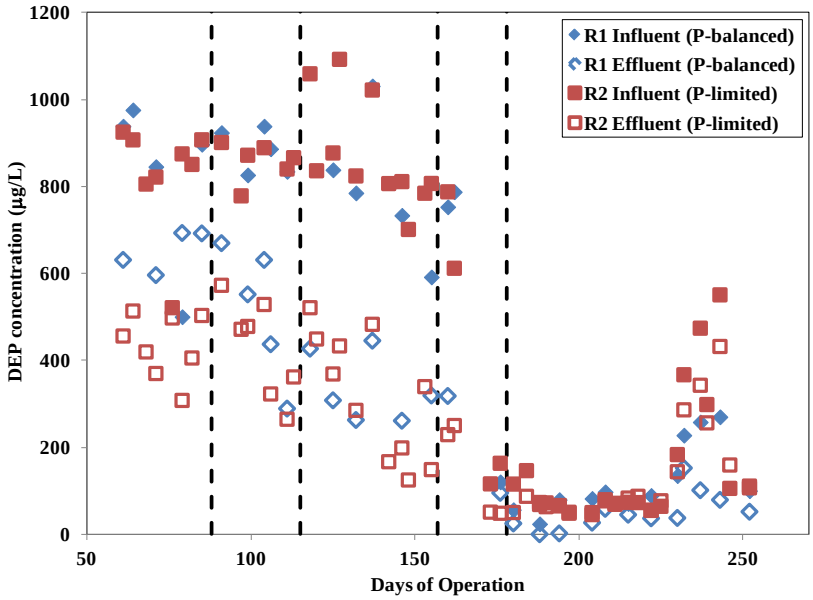
DEP has been shown to be 60 to 95% removed in WWTPs of different configurations and operating conditions (Cases *et al.* 2011; Clara *et al.* 2010, Dargnat *et al.* 2009) and thus was not necessarily expected to be fully biodegraded even under phosphorus-balanced conditions.

Influent DEP concentrations varied quite widely over the course of the experiment (Figure 4-5(a)). Since the component products and chemicals that comprised the synthetic greywater influent did not change over the course of the experimental period, and, considering the relative consistency of influent concentrations measured for the other two studied XOCs, these fluctuations are unlikely to have originated from

changes to or from inconsistency in preparation of the influent solution. In addition to its uses in PCPs, DEP is also used as a plasticizer in some plastic polymers (Autian 1973, ECSCCPNFPIC 2002). Since the entire reactor system including the tanks in which the influent solutions were stored, the tubing that transported the solutions from storage tank to reactor, and the reactors themselves were made out of plastic, all of these reactor components served as a potential source of DEP and other phthalates in the treatment system. Although the tanks and the reactors were not changed over the course of the experimental period, the tubing was replaced periodically throughout the experiment as a result of tubing failure, clogging, or an observation of microbial growth in the tubing. The relatively large drop off in influent DEP concentrations in the middle of phase IV also corresponded to some challenges in reactor operations that resulted in minor changes in reactor configurations; therefore, the drop off might be attributable to a change in tubing during troubleshooting.

Because three seemingly distinct periods of operation based on influent DEP concentrations are apparent, the data can be considered in three groups: phases I through III taken together, phase V(a) during which low influent concentrations to both reactors were measured and during which time no supplemental phosphorus was provided to R2, and phase V(b) during which influent concentrations again exceeded 100 μg/L but no supplemental phosphorus was supplied to R2. Influent and effluent concentrations in each of these three experimental periods are summarized in Table 5-6. At all concentrations measured, DEP could be considered a trace chemical, comprising up to 1.5 mg/L of ThOD, which is less than 0.5 percent of the overall COD entering the reactor.

a)



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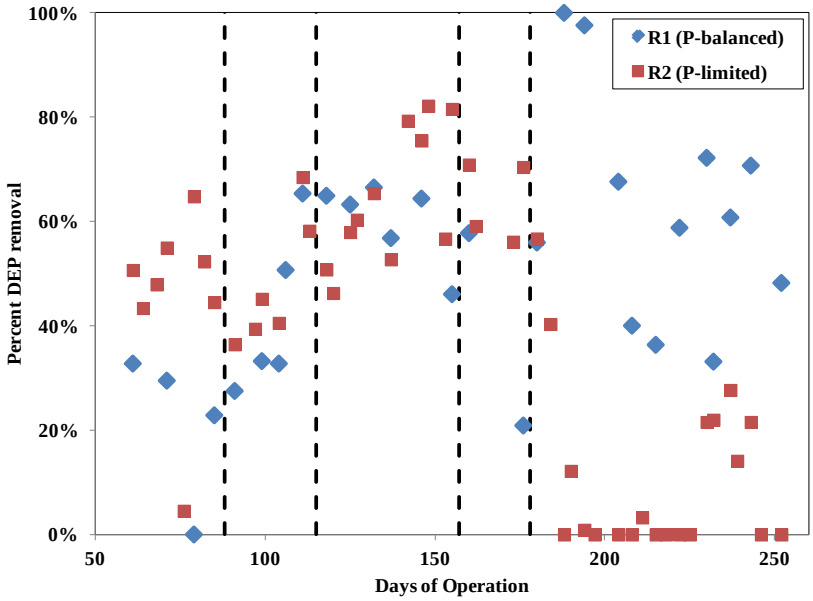


Figure 4-5 – (a) Influent and effluent DEP concentrations and (b) percent DEP removal from R1 (with steady influent PO_4-P concentration) and R2 (with declining influent PO_4-P concentration).

Table 4-6 – Summary of DEP removal

	R1					R2				
	Influent µg/L	Effluent µg/L	Total Removal µg/L	Sorption to Biomass µg/L	Calculated Biodegradation µg/L	Influent µg/L	Effluent µg/L	Total Removal µg/L	Sorption to Biomass µg/L	Calculated Biodegradation µg/L
Phases I - III	860	481	379	68	311	855	384	471	35	436
Phase Va	71	34	38	2.9	35	75	68	7	2.5	4.0
Phase Vb	198	84	114	8.2	106	298	247	51	5.6	45

In the phosphorus-balanced conditions of R1, removal of 36 to 54 percent of the influent DEP was attributed to biodegradation across all phases. Similarly, in the early phases of operation in R2, 51 percent removal was attributed to biodegradation, indicating little difference in performance to that point in spite of the declining phosphorus concentration in R2.

In Phase V(a), when no supplemental phosphorus was provided to R2, the difference in removal between the two reactors was pronounced even with similar influent concentrations, with the removal attributable to biodegradation in R2 declining to approximately five percent of the influent DEP compared to 49 percent of the influent to R1. In fact, during phase V(a) seven of 11 measurements in R2 showed no removal of DEP at all. In phase V(b), removal in R2, increased slightly to 17 percent (with two samples showing no removal) compared to 53 percent of the influent to R1. In this final phase, the DEP removal and biodegradation in the two reactors were quite different at both high and low influent DEP concentrations.

Specific Substrate Utilization Rates

While removal calculations illustrate that COD along with all three chemicals were better removed under conditions of nutrient balance than under conditions of nutrient limitation, comparisons among the chemicals might help illuminate which components (if any) of the overall organic load are disproportionately affected by the advent of a phosphorus limitation. Thus, specific substrate utilization rates were

calculated for overall COD and for each of the XOCs in each phase of reactor performance (Table 4-7).

Table 4-7 – Summary of specific substrate utilization rates

	COD				MP			
	R1		R2		R1		R2	
	Effluent Concentration mg/L	Specific Substrate Utilization Rate mg COD / mg TSS-d	Effluent Concentration mg/L	Specific Substrate Utilization Rate mg COD / mg TSS-d	Effluent Concentration µg/L	Specific Substrate Utilization Rate mg ThOD / mg TSS-d	Effluent Concentration µg/L	Specific Substrate Utilization Rate mg ThOD / mg TSS-d
	Average	Average	Average	Average	Average	Average	Average	Average
Phases I - V	76	11	--	--	6.5	0.015	--	--
Phase I	78	11	70	10	6.5	0.012	6.5	0.011
Phase II	70	13	66	12	6.5	0.015	6.9	0.016
Phase III	111	9.0	63	9.0	6.5	0.014	6.5	0.012
Phase IV	79	21	62	4.7	6.5	0.029	6.5	0.0074
Phase I - IV	89	11	67	10	6.5	0.016	6.6	0.014
Phase V	59	11	99	31	6.5	0.014	39	0.036
	SDS				DEP			
	R1		R2		R1		R2	
	Effluent Concentration mg/L	Specific Substrate Utilization Rate mg ThOD / mg TSS-d	Effluent Concentration mg/L	Specific Substrate Utilization Rate mg ThOD / mg TSS-d	Effluent Concentration µg/L	Specific Substrate Utilization Rate mg ThOD / mg TSS-d	Effluent Concentration µg/L	Specific Substrate Utilization Rate mg ThOD / mg TSS-d
	Average	Average	Average	Average	Average	Average	Average	Average
Phases I - V	1.34	1.5	--	--	285	0.016	--	--
Phase I	1.3	1.3	1.3	1.3	653	0.0059	434	0.021
Phase II	1.7	1.6	1.9	1.6	516	0.026	369	0.036
Phase III	1.0	1.2	1.3	1.2	337	0.029	249	0.031
Phase IV	0.72	2.7	1.3	0.8	207	0.043	50	0.0029
Phase I - IV	1.2	1.5	1.5	1.4	449	0.024	352	0.027
Phase V	1.5	1.4	6.8	3.3	--	--	--	--
Phase V(a)					36	0.0033	68	0.00014
Phase V(b)					84	0.0068	247	0.014

First, for Phases I - IV, the specific substrate utilization rate calculated for each chemical (and overall COD) in R1 is quite similar to that calculated in R2 (within 12.5 percent in all cases), indicating little effect of the phosphorus limitation on the removal of these individual chemicals prior to the most stringent phosphorus limitation being imposed. In Chapter 4, the data demonstrated that relatively high quality effluent could be produced even with a relatively small supplemental phosphorus concentration. These data indicate that, at least for the subset of XOCs analyzed here, the treatment system was

able to sustain relatively consistent levels of removal across the first four experimental phases, even as the phosphorus concentration provided declined well below levels expected to induce a phosphorus limitation.

Comparing the change in substrate utilization rate in Phase V from R2 to R1 allows a basis of understanding what the effect of phosphorus supplementation on XOC removal in a reactor would be (Table 4-8).

Table 4-8 – Specific substrate utilization rates in R1 compared to R2 in Phase V

Chemical	Change
COD	65% lower
MP	61% lower
SDS	58% lower
DEP Phase V(a) (Low concentrations)	225% higher
DEP Phase V(b) (High concentrations)	51% lower

From these data, phosphorus supplementation appears to have relatively similar effects on the bulk COD removal as it does on the removal of MP, SDS, and even DEP at higher concentrations. MP and SDS removal seems to change in a relatively similar manner in response to phosphorus supplementation in spite of the fact that MP would represent a trace contaminant in this system and SDS a substantial fraction of the overall organic load.

At low concentrations, the removal of DEP sees the greatest effect from phosphorus supplementation, actually having a higher specific substrate utilization rate in R1 than in R2 (unlike COD and the other XOCs). At higher concentrations, the specific substrate utilization rate is lower in R1 than in R2, but only by 51 percent, the smallest such difference of the three studied XOCs. Taken together, these results suggest that the effect of phosphorus limitation and supplementation on DEP removal is unlike the effect on the removal of either of the other compounds evaluated.

Very little data exists in the literature to compare these values to and to evaluate the effect that nutrient deficiency has on the removal of individual organic contaminants. Christopher *et al.* (2000) found that addition of 1.6 to 3.3 mg/L of phosphate to an influent wastewater that contained no measurable concentrations of phosphate increased COD removal only slightly but increased the specific utilization rate of 2,4-dinitrotoluene (2,4-DNT) by 171 percent.

Similarly here, the differences in DEP removal between R1 and R2 compared to the differences in COD removal indicate that phosphorus supplementation is even more beneficial to removal of DEP than to overall COD removal. These differences in response between overall COD removal and removal of DEP seem to indicate some other mechanism beyond simply increased microbial growth with phosphorus supplementation at work. Furthermore, seven of ten samples collected during phase V(a) of R2 exhibited absolutely no removal of DEP, a result indicating that under conditions of nutrient limitation (or a combination of nutrient limitation and low XOC concentration), the ability to degrade certain micropollutants might be lost or at least the necessary degradative pathways not induced.

Freedman *et al.* (2005) investigated the removal of methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), toluene, and xylenes in nutrient-limited textile manufacturing wastewater. Batch experiments demonstrated near complete removal of MEK and MIBK in a matter of hours whether or not nutrients were added to the solution. However, addition of nitrogen and phosphorus improved removal of toluene from 30 to near 100 percent and of p-xylenes from approximately 50 percent to nearly 100 percent within 90 hours. The authors hypothesized that the differential removals might be due to different degradation pathways for MEK and MIBK than for toluene and p-xylenes, since degradation of toluene and p-xylenes requires enzymes coded for on the TOL plasmid.

They theorize that, microorganisms might, in an effort to conserve resources, not all retain the TOL plasmid under nutrient-limiting conditions making their degradation slower or incomplete in the absence of the required nutrients. Very often the genes encoding degradative pathways for specific XOCs are carried by microorganisms on plasmids (Top *et al.* 2002), and the biodegradation of both DEP and 2,4-DNT has been shown to be plasmid-mediated by at least certain microbial strains (Suen and Spain 1993, Vamsee-Krishna and Phale 2008). [Interestingly, the biodegradation of SDS is also known in some cases to be plasmid-mediated, and yet its removal pattern in response to a phosphorus limitation was not dissimilar from that of COD. Much as changes in DEP with phosphorus supplementation were most pronounced at low concentrations, the relatively large contribution of SDS to the organic load (and thus as a carbon and energy source for the microorganisms in the reactor) in the reactor might mean that SDS is essential in these reactors even under conditions of nutrient limitation.] Nevertheless, the unusual differences in removal of DEP under conditions of phosphorus limitation and balance indicate that the removal of certain XOCs will be disproportionately affected by nutrient limitation. As a result, if XOC removal during greywater treatment is of concern, particular attention would need to be paid to this class of compounds, and monitoring of overall COD removal would be inadequate to predict removal of DEP and such chemicals under conditions of phosphorus limitation.

CONCLUSIONS

Consistent with results seen for COD removal (discussed in depth in Chapter 3), little effect of phosphorus limitation on the removal of these three XOCs was discernible prior to the very last operational phase in R2 in which no supplemental phosphorus was provided to the reactors. Consequently, these data indicate that supplementation of the

greywater influent with even 50 μg/L provides a substantial improvement in effluent water quality, not only in overall lower organic content but also likely in improved removal of many of the organic micropollutants in household light greywater.

However, the improvement in XOC removal with phosphorus supplementation is uneven among the tested compounds. The removal of MP and SDS both appeared to vary in response to phosphorus supplementation similarly to the manner in which COD removal changed with supplementation. DEP, on the other hand, experienced a disproportionate improvement in removal with phosphorus supplementation. DEP also had a unique pattern of removal, in that removal was reduced under the most extreme conditions of phosphorus limitation, but in addition when influent DEP concentrations dropped, removal nearly stopped altogether. This result might be of particular interest in considering real greywater systems in which influent XOC concentrations could be expected to vary widely over time as particular PCPs are used over the course of a day or a week.

The removal data alone do not point directly to a mechanism by which these differences among XOCs can be explained. However, taken together, the results of Freedman *et al.* (2005) and Christopher *et al.* (2000) and the results presented here suggest that a number of chemicals that are especially responsive to phosphorus supplementation are those whose biodegradation is plasmid-mediated. In mixed culture treatment systems experiencing nutrient stress, these catabolic plasmids could be shed by the microorganisms to conserve resources, thus diminishing the capacity for the treatment system to remove the XOC. Therefore, if removal of micropollutants prior to greywater reuse becomes necessary or desirable, the implications of nutrient limitation on the removal of XOCs will require further study and a means for monitoring these effects should be developed.

Chapter 5: XOC Removal and Plasmid Loss Under Conditions of Phosphorus Limitation

Results presented in Chapter 3 indicate that an MBR system can produce treated effluent of consistent quality, as measured by bulk measurements such as COD, even when supplied with relatively low phosphorus concentrations. However, data presented in Chapter 4 indicated that the removal of all three XOCs studied in this research did not respond to phosphorus limitations in the same manner as did the overall COD. In particular, diethyl phthalate (DEP) appeared to be more sensitive to phosphorus limitations than were the bulk COD or the two other XOCs, methylparaben and sodium lauryl sulfate. One hypothesis for the differences among the response of DEP and the other organic compounds relates to differences between compounds whose degradation is dependent on the presence of plasmids or other so-called mobile genetic elements that carry the genes encoding their degradation. For these compounds, predicting effluent quality and responses to nutrient limitations would require either individual monitoring of these specific compounds or identifying a surrogate parameter that signals that the microorganisms are experiencing stress and likely to lose the degradative capacity.

In spite of concern about discharge of pharmaceutical and personal care product ingredients to the environment, monitoring of XOC removal in municipal wastewater treatment plants is not widely performed in the United States; monitoring of individual greywater treatment systems for a large number of organic contaminants seems even more unlikely. Development of an analytical tool that could provide an indication of reactor operating conditions where XOC removal might be less than expected would prove more useful in greywater systems than expecting measurements of innumerable XOCs to be performed.

One possibility, evaluated here, is the ability to monitor plasmid content among a microbial community in a treatment system, since mobile genetic elements have been found to carry the genes encoding the degradation of DEP and many other organic contaminants that derive from human activity. Carrying genes for degrading novel or unusual compounds on plasmids can be advantageous to microorganisms because they can be transferred throughout a microbial community if the degradative pathway is needed and allow the community to adapt to prevailing environmental conditions (Sayler *et al.* 1990, Top *et al.* 2002). Conversely, the plasmid and its associated degradative ability can also be lost under certain environmental conditions, including those of nutrient limitation, if not essential to the microorganisms. Plasmid content in the cell could be reduced under conditions of nutrient limitation through total plasmid loss, reduction in copy number, or deletion of non-essential portions of the plasmids, as demonstrated by Jones *et al.* (1980), Wouters *et al.* (1980), and Modi *et al.* (1991). These researchers investigated the persistence of plasmids encoding antibiotic resistance in various pure cultures under nutrient-limited conditions in the absence of the antibiotic for which the plasmid conferred resistance.

These studies focused nearly entirely on specific plasmids in pure microbial cultures with fixed influent compositions, conditions that are not representative of activated sludge or other mixed culture treatment processes with variable influent composition, including greywater treatment. Freedman *et al.* (2005) inferred a connection between plasmid loss and XOC removal in mixed culture wastewater treatment, having found that no change in removal of methyl ethyl ketone and methyl isobutyl ketone occurred under all conditions while addition of nitrogen and phosphorus more than doubled the removal of toluene and p-xylenes. The authors hypothesized that the differential removals were attributable to the degradation of toluene and p-xylenes

requiring enzymes coded for on the TOL plasmid, which would be lost under the nutrient-limiting conditions making their degradation slower or incomplete in the absence of the nutrients.

The results of Freedman *et al.* (2005) are not directly relevant to greywater treatment systems since BTEX compounds should not be abundant in greywater. However, a similar effect could be expected for any XOCs found or expected in greywater for which the biodegradation pathway is known to be plasmid-mediated under some circumstances. Among these compounds is DEP, a plasticizer which is an ingredient in at least 25 consumer products (EWG 2013; HHS 2013) and has been detected in multiple real greywater sources at concentrations as high as 38 μg/L (Almqvist and Hanaeus 2006, Palmquist and Hanæus 2005). DEP was shown in Chapter 5 to be disproportionately affected by phosphorus limitation with removal of at most 17 percent under condition of phosphorus limitation increasing to 50 to 60 percent under conditions of phosphorus balance. Along with other phthalates, the biodegradation of DEP has also been shown to be plasmid-mediated by at least three different microbial strains (Vamsee-Krishna and Phale 2008).

Therefore, XOC-associated plasmids might be both necessary for the biodegradation of some XOCs but also not stable under phosphorus-limited conditions. The work herein evaluated whether these facts can be exploited to use plasmid loss as an indicator in biological treatment systems of the ability to degrade DEP under conditions of phosphorous limitation.

MATERIALS AND METHODS

To complete this evaluation, four chemostats were operated in parallel and their DEP removal and plasmid content were assessed. In two of the chemostats, DEP was the

sole carbon source available to the microorganisms (at both high and low phosphorus concentrations), and in the other two DEP comprised approximately one percent of the overall COD in a synthetic greywater influent (at both high and low phosphorus concentrations) (Table 5-1).

Table 5-1 – Summary of Experimental Conditions Tested in Chemostat Experiments

	Matrix	Phosphorus	DEP concentration
1	Inorganic salt solution	High	60 mg/L
2	Inorganic salt solution	Low	60 mg/L
3	Synthetic greywater	High	600 μg/L
4	Synthetic greywater	Low	600 μg/L

Before operation of the chemostats could begin, a consortium of microorganisms capable of degrading DEP was grown that would be used to seed each of the four chemostats. To grow this consortium, an aliquot of 10 mL of activated sludge from Walnut Creek Wastewater Treatment Plant (Austin, Texas) was transferred to approximately 200 mL of sterile inorganic salt solution (ISS) supplemented with 30 mg/L of DEP and incubated at 30°C and 200 rpm. The composition of the ISS was adapted from Lu *et al.* (2009) and is presented in Table 5-2.

Table 5-2 – Composition of Inorganic Salt Solution Used to Grow Consortium of DEP Degrading Microorganisms

Compound	Mass in 1L Deionized Water
NaCl	0.62g
NH_4Cl	0.32g
$FeCl_3 \cdot 6H_2O$	0.018g
$K_2HPO_4 \cdot 3H_2O$	1.0g
$MgSO_4 \cdot 7H_2O$	0.40g
$NaNO_3$	0.52g
$CaCl_2 \cdot 2H_2O$	0.10g

Every three days, an aliquot of the incubated consortium growing in the ISS was transferred to approximately 10 times as much fresh sterile ISS spiked with DEP. On the second transfer, the DEP concentration was increased to 60 mg/L and kept constant at 60 mg/L thereafter. [The MIC for DEP has been reported to vary between 127 mg/L and 157 mg/L in three different microbial strains (Gogra *et al.* 2010). Therefore, the culture was started at a DEP concentration of 30 mg/L and increased over time as in Lu *et al.* (2012) to limit toxic effects of DEP and allow the consortium to acclimate itself to the presence of the XOC.]

After approximately 10 transfers, when the consortium was presumably stable, 50 mL was transferred of to each of four 250-mL filter flasks (used as the chemostat vessels) containing the varying influent solutions (Table 5-1). The microorganisms were allowed to acclimate to their new solutions for three days before pumps were turned on to make these continuous-flow systems. Once the influent solutions were flowing to the chemostats, the inorganic salt solution (ISS) or synthetic greywater (SGW) comprised approximately 60 percent of the influent to each reactor, and the phosphorus solutions comprised the remaining 40 percent of the influent to each reactor. The compositions of the inorganic salt solution, the synthetic greywater, and high and low phosphorus solutions are summarized in Tables 5-3, 5-4, and 5-5, respectively. The synthetic

greywater was designed to be comparable in composition to that used in membrane bioreactor experiments in Chapter 3, but with lower chemical oxygen demand (COD) to match the theoretical oxygen demand (ThOD) of a 60 mg/L DEP solution (or approximately 116 mg ThOD/L). The listed ingredients of the personal care products that comprise the synthetic greywater are compiled in Appendix A.

Table 5-3 – Composition of Inorganic Salt Solution Used as Influent during Chemostat Operation

Compound	Mass in 1L Deionized Water
NaCl	1.0 g
NH_4Cl	0.54 g
$FeCl_3 \cdot 6H_2O$	0.028 g
$MgSO_4 \cdot 7H_2O$	0.66 g
$NaNO_3$	0.88 g
$CaCl_2 \cdot 2H_2O$	0.17 g
$NaHCO_3$	0.14 g

Table 5-4 – Composition of Synthetic Greywater Influent Solution

Ingredient	Brand	Mass in 1L Deionized Water
Hand soap	Essence of Beauty Creamy Fig	85 mg
Shampoo	Bed Head Moisture Maniac	113 mg
Laundry detergent	Tide HE Liquid	0.15 mL
Sodium bicarbonate	--	0.20 g

Table 5-5 – Composition of Supplemental Phosphorus Influent Solution

	Mass in 1L Deionized Water	
Ingredient	High Phosphorus Solution	Low Phosphorus Solution
K_2HPO_4	1.21 g	0.70 mg
KH_2PO_4	0.95 g	0.55 mg

Effluent flowed out of each chemostat by gravity through the side-arm of the filter flask, ensuring a constant volume of approximately 270 mL in each reactor. The chemostats were stirred throughout the course of the experiment and operated at a dilution rate of $0.11h^{-1}$, a rate that previous experimental work had demonstrated was sufficiently low so as not to cause washout.

Every three to five days, aliquots were removed from each reactor for analysis. COD was measured for SGW samples immediately upon sample collection by low-range digestion vial (Hach; Loveland, CO) with dilution of samples to reach the linear range (3 to 150 mg/L) of the method when necessary. (COD could not be measured for ISS samples presumably as a result of interference from high chloride concentrations in the ISS.) Optical density was measured at 600 nm. Samples were then filtered using a 0.2-μm filter and subsequently analyzed for dissolved orthophosphate and DEP concentrations. Dissolved orthophosphate was measured by the ascorbic acid method (AHPA *et al*. 1999), and DEP was measured by LC-ESI-MS-MS as described in Chapter 5. A separate aliquot was also removed for extracting plasmid DNA (pDNA) using a Qiagen Spin Miniprep kit (Qiagen, Inc.; Valencia, CA), and DNA concentrations were subsequently measured by absorbance at 260 nm using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Inc.; Wilmington, DE).

To compare the measurements in the chemostats to a well-characterized treatment system operated for an extended length of time, measurements of pDNA concentration

were also made during operation of the membrane bioreactors (MBRs) described in Chapter 4. Two 8.4 L MBRs were operated for more than seven months. Both were fed the same synthetic greywater solution described in Table 5-4 but at three times the concentration. Throughout the entire course of operation R1 was also fed a monobasic sodium phosphate / dibasic sodium phosphate solution with a target influent concentration of 3.6 mg P/L while over the last two months of operation R2 was fed only DI water with no supplemental phosphorus, thus inducing a phosphorus limitation in R2. At the end of the two months of operation, 300 mL of mixed liquor were extracted from each of the MBRs and pDNA extracted from these samples.

RESULTS AND DISCUSSION

Initially the two low phosphorus chemostats were operated with a target influent concentration of approximately 100 μg P/L, but sampling over the first two weeks of operation indicated very little difference in reactor performance on the basis of effluent COD and DEP concentrations between the high and low phosphorus conditions (Figure 5-1). The comparable level of performance was in spite of a ratio of ThOD to P of more than 1200:1 in the influent to the ISS Low P chemostat and COD to P of more than 1200:1 in the SGW Low P chemostat. Similar to results presented in Chapter 3, no evidence of nutrient limitation was apparent even at a ratio well in excess of the 100 mg/L BOD_5 to 1 mg of P believed necessary for successful biological wastewater treatment (Metcalf & Eddy 2003).

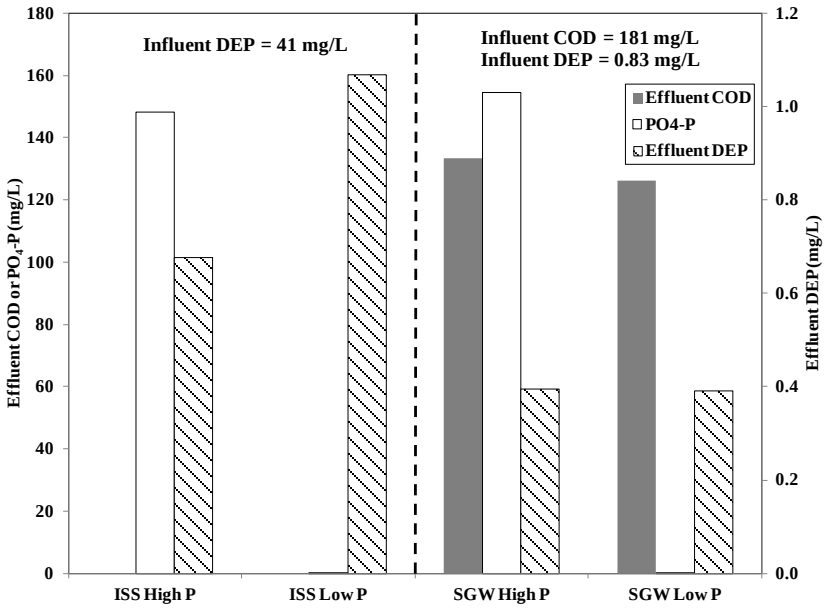


Figure 5-1 –Average chemostat performance during first two weeks of operation where little evidence of phosphorus limitation was evident in the Low P chemostats.

At that point, the mass in the low phosphorus solution was reduced to 0.35 mg/L K_2HPO_4 and 0.28 mg/L KH_2PO_4 which corresponded to approximately 50 µg P/L in the influent to each Low P chemostat. Chemostat operation was then continued for another seven weeks.

Inorganic Salt Solution Chemostats

After the reduction in the phosphorus concentration to 50 µg/L, nearly all of the DEP entering the ISS High P chemostat was removed, while 1.0 to 14 mg/L of DEP remained in the ISS Low P effluent (Table 5-6 and Figure 5-2). The higher effluent DEP concentration in the ISS Low P chemostat corresponds to a 153 percent higher specific substrate utilization rate in the ISS Low P chemostat. The differences in DEP removal and specific substrate utilization rate between the two chemostats indicated at this point that a phosphorus limitation had been induced.

Table 5-6 – Average chemostat performance in chemostats with ISS as the influent solution at high and low phosphorus concentrations.

	DEP			Plasmid DNA concentration (ng/mL)	Normalized Plasmid DNA concentration (ng-cm/mL)
	Inf (mg/L)	Eff (mg/L)	Specific substrate utilization rate (mg-cm/min)		
ISS High P	46	0.024	1.3	13	141
ISS Low P	46	6.6	3.3	1.7	72

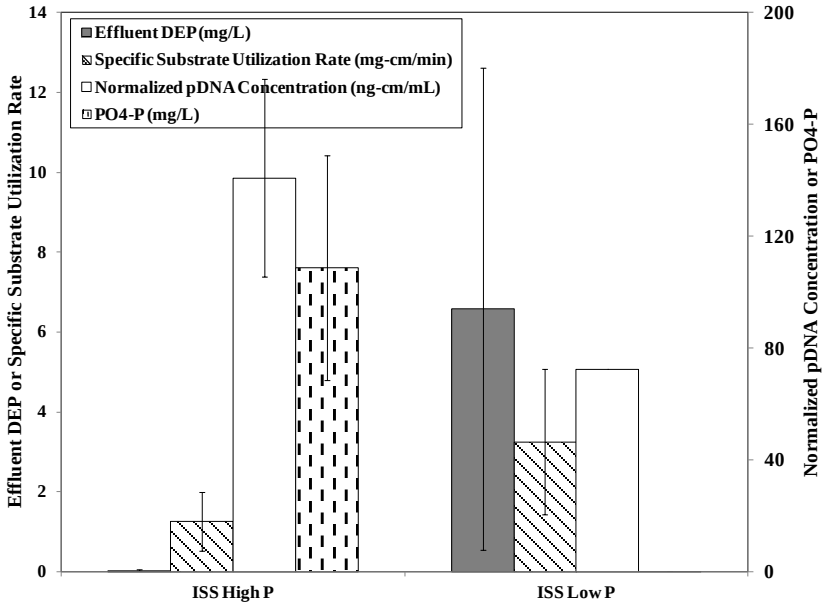


Figure 5-2 – Comparison of chemostat performance in chemostats with ISS and 46 mg/L DEP as the influent solutions at high and low phosphorus concentrations.

The measured pDNA concentration normalized by optical density (as a proxy for TSS or other measurement of cell growth) was nearly 50 percent lower in the ISS Low P chemostat than in the ISS High P chemostat. (Only one value is presented in Figure 5-2 for the normalized pDNA concentration in the ISS Low P reactor because all of the measurements while the reactor continued operation indicated a measured pDNA value

below the detection limit of the spectrophotometer used. Only when the reactors were shut down and nearly the entire reactor volume used for the DNA extraction could a measurement be achieved that was above the detection limit, resulting in the value on Figure 5-2.) This result demonstrated that plasmid loss under nutrient-limited conditions was not limited to the pure cultures previously studied but could also be successfully detected as a bulk measurement in a mixed culture, continuous-flow system. Furthermore, the lower normalized pDNA concentration evident in the ISS Low P chemostat was inversely correlated with a higher effluent DEP concentration, in support of the idea that a measurement of pDNA concentration might provide an indication of capacity for DEP (and other similar XOC) removal. Furthermore, in spite of the differences in effluent DEP concentration and specific substrate utilization rate between the two chemostats, removal in the ISS Low P chemostat continued to average approximately 85 percent. In addition, the supplemental phosphorus concentration was approximately 50 μg/L with influent ThOD of 90 mg/L compared to approximately 30 μg/L in the synthetic greywater influent with 360 mg/L COD entering R2 in Phase V of the MBR experiments in Chapter 3. As a result, supplemental phosphorus could likely have been reduced further and driven the effluent DEP concentration from the ISS Low P chemostat even further. As such, pDNA might in fact be a leading indicator of the decline in degradative capacity retained by the microbial community.

Synthetic Greywater Chemostats

After the reduction in the phosphorus concentration to the SGW Low P chemostat to 50 μg/L, effluent COD measurements from the SGW Low P chemostat consistently exceeded those measured in the effluent of the SGW High P chemostat (Table 5-7 and Figure 5-3). This difference in effluent COD concentrations taken together with the

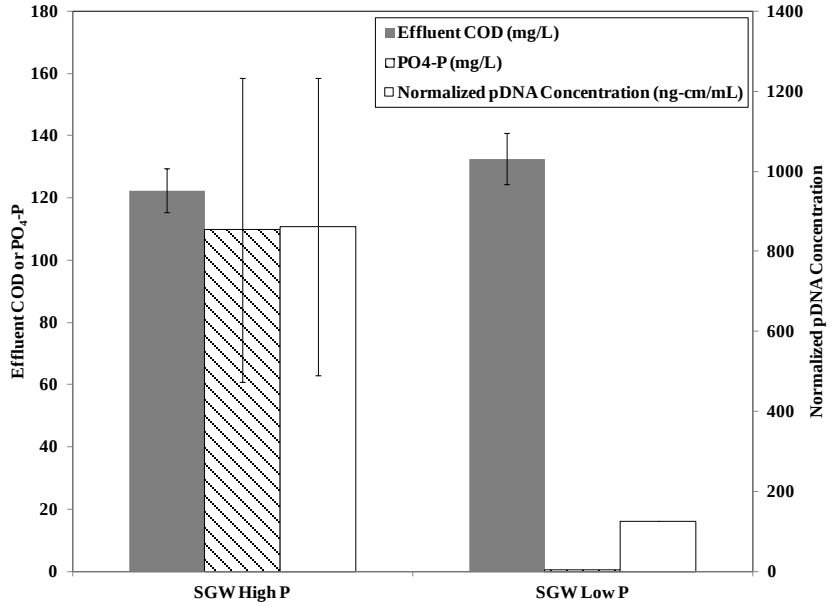
lower optical density measured in the SGW Low P chemostat indicated that phosphorus-limited conditions had been achieved. Interestingly, however, the DEP concentrations measured continued to be quite similar in the two reactors with both averaging 0.55 mg/L, indicating removal of 20 to 40 percent of the influent DEP in each reactor. These removal rates were lower than those seen in MBR operations described in Chapter 5, where under conditions of phosphorus balance and at influent DEP concentrations comparable to those used here, DEP removal averaged approximately 60 percent.

Table 5-7 – Average chemostat performance in chemostats with SGW as the influent solution at both high and low phosphorus concentrations.

	COD			DEP			Plasmid DNA concentration (ng/mL)	Normalized Plasmid DNA concentration (ng-cm/mL)
	Inf (mg/L)	Eff (mg/L)	Specific substrate utilization rate (mg-cm/min)	Inf (mg/L)	Eff (mg/L)	Specific substrate utilization rate (mg-cm/min)		
SGW High P	154	122	1.5	0.79	0.55	0.013	31	861
SGW Low P	154	133	2.1	0.79	0.55	0.024	2.1	126

Nevertheless, differences in specific substrate utilization rates between the Low and High P chemostats were apparent on both a COD and DEP basis. Much like the results presented in Chapter 4, the specific substrate utilization rate of DEP did not seem to change in response to a phosphorus limitation in a way that would be indicated by simply monitoring COD removal. Specific substrate utilization rate for COD was approximately 40 percent higher in the Low P chemostat than in the High P chemostat, while that of DEP was 85 percent higher in the Low P chemostat compared to the High P chemostat. This result only underscores the need for alternatives to monitoring only COD if concern about XOC discharge in treated greywater exists.

a)



b)

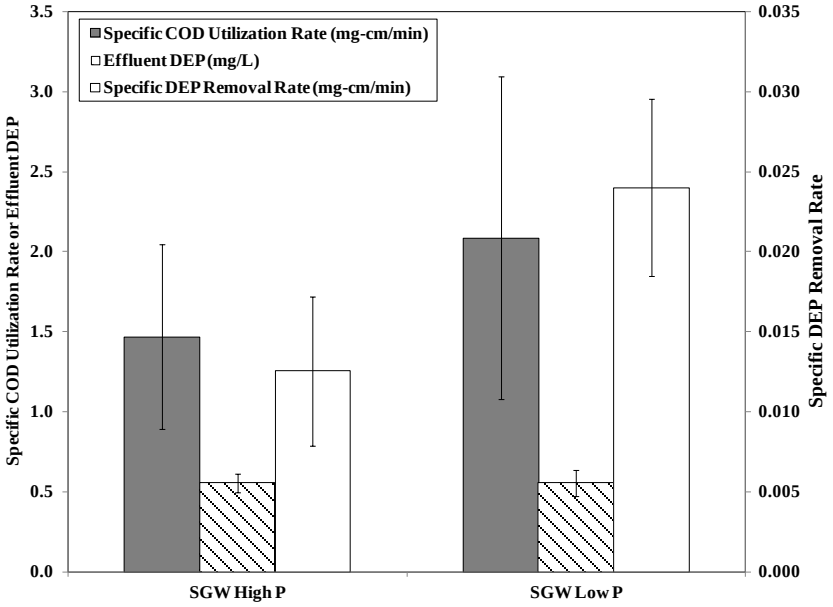


Figure 5-3 – Comparison of chemostat performance in chemostats with synthetic greywater (COD = 154 mg/L) and 600 μg/L of DEP as the influent solution at high and low phosphorus concentrations.

In spite of the consistency in effluent DEP concentrations, the normalized pDNA concentration measured in the SGW Low P chemostat was 85 percent lower than that measured in the SGW High P chemostat. As discussed for the ISS chemostats above, the supplemental phosphorus concentration could have been reduced further and exacerbated the phosphorus limitation further thereby driving the effluent DEP concentration from the SGW Low P chemostat up. This result could be an indication that pDNA really is a leading indicator of the decline in degradative capacity of the microbial community given that evidence of nutrient limitation is apparent but the effluent DEP concentrations were not different. Furthermore, in a synthetic greywater with a variety of carbon sources available to the microbial community, the much lower plasmid concentration could represent loss of degradative ability for numerous XOCs and not DEP alone.

Membrane Bioreactor Experiments

To further evaluate the ability of these measurements to be useful indicators in treatment processes, samples were also collected and analyzed from lab-scale membrane bioreactors treating synthetic household light greywater.

DEP removal was different between these two reactors; DEP was typically 50 to 60 percent removed in R1 while its removal was limited to at most 17 percent in R2. pDNA extraction and measurement from R1 (the phosphorus-balanced reactor) yielded 6.25 ng of pDNA per mL of mixed liquor, or 184 ng of pDNA per mg of suspended solids. Extraction and measurement of pDNA from R2 (the phosphorus-limited MBR) contained 1.9 ng/mL of pDNA or 171 ng per mg of MLSS. Thus, the difference between the pDNA content of the phosphorus-balanced and phosphorus-limited reactors was only seven percent, smaller than the differences observed in the SGW and ISS-fed reactors described earlier. Several possible explanations exist for the relative similarity in these

measurements to one another. Difficulty was encountered in dispersing flocs in samples collected from the MBRs and subsequently homogenizing these samples, thus raising the possibility that low extraction efficiencies resulted. Pyrosequencing results (Appendix C) indicate that the microbial community structures were different in the MBRs and the chemostats, and thus they might respond to prevailing environmental conditions differently. Lastly, the applicability of this measurement could also be process-specific and, thus, if it were intended for use in MBR treatment systems, greater understanding of process differences and their effects on this measurement and further study in an MBR would be necessary.

SUMMARY AND IMPLICATIONS

The research presented here demonstrates generalized plasmid loss under conditions of nutrient limitation in mixed cultures with both single and varied carbon sources as had been previously demonstrated in studies examining the stability of specific plasmids in pure microbial cultures.

Measurements of pDNA were carried out to evaluate their potential to identify situations where DEP removal would be affected by a phosphorus limitation to a greater extent than COD removal. In a chemostat provided a single carbon source, whose biodegradation is believed to be plasmid-mediated, a difference in pDNA content between the high and low phosphorus conditions of 34 percent was observed. In a culture provided a more complex influent, the pDNA concentration under phosphorus limitation was 85 percent lower than that under phosphorus balance. While this difference in plasmid concentration could not be directly correlated to a difference in effluent DEP concentrations, the large difference in plasmid content in a reactor with a diversity of carbon sources might only underscore the broader applicability of this kind of

an indicator to a variety of XOCs. Furthermore, data from both sets of chemostats suggest that pDNA might actually be a leading indicator of potential loss of degradative capacity under a nutrient limitation.

Measurements in two MBRs treating synthetic greywater under phosphorus-balanced and phosphorus-limited conditions yielded only a seven percent difference in normalized plasmid DNA concentration. Given the differences seen between the chemostats and MBRs, greater study would be needed in the treatment system in which the indicator would be deployed prior to implementing measurement pDNA as a monitoring tool.

Chapter 6: Conclusions and Recommendations

Reuse of household light greywater has the potential to help conserve highly treated potable water and thereby alleviate water stresses around the world. As greywater reuse becomes more common, greater treatment of the household greywater prior to reuse is expected, and membrane bioreactors (MBRs) are a promising technology for such applications. However, biological treatment of greywater could prove challenging because it can be nutrient-limited due to segregation of toilet waste and kitchen wastewater as well as the elimination of phosphorus from many consumer products in the U.S. In addition, the organic load in greywater is likely to be comprised of a wide variety of xenobiotic organic compounds, many of which originate from personal care products. As a result, focusing on removal of COD alone might not be sufficient for greywater treatment prior to reuse.

To review, the objectives of this work were to:

- to understand the potential for phosphorus to determine the quality of treated effluent in MBR treatment of greywater;
- to evaluate extracellular enzyme activity as an alternate indicator of nutrient limitation in greywater treatment;
- to establish how XOC removal might vary in biological treatment of greywater under conditions of phosphorus limitation and phosphorus balance; and
- to evaluate whether changes in plasmid DNA concentrations could be monitored as an indicator of potential changes in XOC removals under nutrient-limited conditions.

Less than 30 μg/L of dissolved orthophosphate was present in synthetic greywater made from three common household products, and no measurable amount of dissolved orthophosphate was found in real greywater, but low (less than 0.5 mg/L) concentrations of particulate phosphate were detected. These concentrations were well below levels believed necessary to achieve full BOD_5 removal in the course of biological treatment. Nevertheless, MBR performance (treating either real or synthetic greywater) was adversely affected only when the most extreme nutrient limitation (with no supplemental phosphorus) was imposed. Supplementation with as little as 55 μg/L phosphate-phosphorus resulted in effluent water of consistent quality.

An assay of enzyme activity that had previously been used to assess nutrient limitations in surface waters was adapted for the first time to an operating treatment system. Relatively stable enzyme activities were found in the control reactor (with constant influent phosphorus concentrations), but the ratio of phosphatase activity to total glycosidase activity increased with declining influent phosphorus concentration. That the enzyme ratio increased prior to increases in effluent COD concentrations suggests that these assays can signal a microbial community under nutrient stress and serve as a predictor of reactor performance.

Consistent with results seen for COD removal, little effect of phosphorus limitation on the removal of the three studied XOCs was discernible prior to imposing the most stringent phosphorus limitation (with no supplemental phosphorus provided to the reactor). Consequently, these data indicate that supplementation of the influent greywater with even 50 μg/L would be likely to provide a substantial improvement in effluent water quality, not only in overall lower organic content but also in improved removal of many of the organic micropollutants in household light greywater.

However, changes in XOC removal with phosphorus limitation were not identical for all XOCs. The removal of methylparaben (MP) and sodium lauryl sulfate (SDS) both seemed to improve with phosphorus supplementation to a similar extent as COD removal. Diethyl phthalate (DEP), on the other hand, experienced a disproportionate improvement in removal with phosphorus supplementation. DEP also had a unique pattern of removal, in that removal was reduced under the most extreme conditions of phosphorus limitation, and at lower influent DEP concentrations removal nearly stopped altogether. This result could be of particular interest in considering real greywater systems in which influent XOC concentrations are expected to vary widely over time as particular PCPs are used over the course of a day or a week.

The removal data alone do not point directly to a mechanism by which these differences among XOCs can be explained, but measurement of pDNA was evaluated as a potential indicator for situations where DEP removal would be affected by a phosphorus limitation to a greater extent than COD removal. Generalized plasmid loss under conditions of nutrient limitation was observed in mixed cultures with both single and varied carbon sources, as had been previously demonstrated for specific plasmids in nutrient-limited pure microbial cultures. A larger difference in pDNA content between the high and low phosphorus conditions was observed in a culture provided a more complex influent with multiple carbon sources, but this difference could not be directly correlated to a difference in effluent DEP concentrations. Nevertheless, the results suggest that pDNA concentrations might be a leading indicator of the effects of nutrient limitation on XOCs, for which the biodegradation is plasmid-mediated. Measurements in MBRs treating synthetic greywater under phosphorus-balanced and phosphorus-limited conditions yielded only a seven percent difference in normalized pDNA concentrations.

Given the differences seen between the chemostats and MBRs, greater study would be needed in the treatment system in which the indicator would be deployed.

IMPLICATIONS

With a small amount of phosphorus supplementation, the effluent greywater (both real and synthetic) met the NSF/ANSI Standard 350-1 effluent criteria (for BOD_5 and effluent suspended solids) for subsurface discharges. The effluent water also easily met the effluent suspended solids requirement for unrestricted outdoor reuse (by the same NSF/ANSI standard). The BOD_5 of the treated synthetic greywater typically exceeded the criteria for unrestricted outdoor reuse; however, lower effluent BOD_5 would be expected if the reactors were operated with a longer solids residence time as is likely in household use where homeowners are likely to perform minimal oversight or maintenance.

From a practical perspective, the small phosphorus deficiency seen in household light greywater could be addressed in a number of ways, including changing operational requirements, the scale at which greywater is treated and reused, or even, in extreme cases, the treatment technology. The data presented here indicate that phosphorus supplementation, even at levels of less than a milligram per liter, would result in more consistent, higher effluent water quality that are more likely to meet any standards for greywater reuse. Given that phosphorus measured in the real greywater was believed to originate from a single consumer product, biological greywater treatment systems might benefit from treating water from multiple households, such as in apartment buildings, dormitories, or even subdivisions, all of which would increase the likelihood that a source of phosphorus exists in one of the connected households. Lastly, a persistent or severe phosphorus limitation which requires phosphorus supplementation could ultimately result

in enough complexity and required maintenance and oversight that biological treatment of greywater no longer makes sense for either operational or economic reasons.

Synthetic greywater was shown in the course of this work to be an imperfect surrogate for real greywater. In particular, the substantial suspended solids concentration present in real greywater leads to important differences between the two greywaters including a lower BOD relative to total COD and also a higher measurable phosphorus concentration originating from the particulate fraction. Furthermore, the synthetic greywater contained higher than expected concentrations of at least one of the XOCs. Lastly, both greywater quantity and quality are also expected to vary in households, and this variability could have implications for treatment in the case of constituents such as phosphorus or XOC removal where treatment systems might respond differently to a chemical that is only present intermittently compared to one that is always present.

Both greywater treatment and research are in their relative infancy compared conventional wastewater treatment. If trends in other countries such as Germany are predictive of what the U.S. experience with greywater will be, greater deployment of biological treatment of U.S. greywater should be expected. The phosphorus results, in particular, are encouraging given the relative stability and robustness of reactor performance even with very low concentrations of phosphorus. At present, little concern about discharge of XOCs at the household level is encountered, but given the concurrent growth of both greywater reuse and concern about the implications of widespread XOC occurrence in the environment, this research can help inform decisions about operation and monitoring of such treatment systems.

FUTURE WORK

Further work would be necessary to develop both indicators evaluated here. For the extracellular enzyme assay, further research should be undertaken toward identifying a consistent basis for interpreting this data in treatment systems and understanding the threshold at which the assay signals the need for phosphorus supplementation.

Also needed is an understanding of what other changes in reactor behavior or effluent water quality might be apparent during these initial stages of phosphorus limitation (when the assay signals a limitation) as the microorganisms expend greater energy and resources for the acquisition of phosphorus even if the effects are not yet seen in effluent COD.

As stated previously, the removal data alone do not point directly to a mechanism by which these differences in XOC removal under conditions of nutrient limitation can be explained. Analytical scans of treated greywater under conditions of nutrient limitation and nutrient balance could identify classes of compounds that respond more like DEP and those that respond more like MP and could help further the understanding of whether the pDNA measurements are a useful indicator beyond a single XOC. Furthermore, the chemostat experiments could be repeated focusing on another XOC, such as MP, whose removal pattern under conditions of nutrient limitation was more similar to COD. These data could bolster the case for both the plasmid loss hypothesis and the need to develop pDNA measurements as an indicator. The effect of the intermittent presence of DEP on plasmid stability and DEP removal could be studied in order to mimic conditions that might be encountered by microorganisms in a real greywater treatment system in which an XOC is discharged to the system only intermittently thus providing an inconsistent selective pressure on the microbial community.

Appendix A: Ingredients of Personal Care Products Used to Produce Synthetic Greywater

	Essence of Beauty Creamy Fig Hand Soap	Bed Head Moisture Maniac Shampoo	Tide HE Liquid
Water	x	x	x
Sodium Laureth Sulfate	x	x	
Ammonium Laureth Sulfate	x		
Cocamide MEA	x		
Fragrance (Parfum)	x	x	x
Cocamidopropyl Betaine	x		
Lauryl Glucoside	x	x	
Aloe Barbadensis Leaf Juice	x		
Prunus Amygdalus Dulcis (Sweet Almond) Oil	x		
Honey	x		
PEG-40 Hydrogenated Castor Oil	x		
Polyquaternium-10	x	x	
Distearеth-75 IPDI	x		
Benzophenone-4	x		
Sodium Chloride	x	x	
Citric Acid	x		
DMDM Hydantoin	x	x	
Disodium EDTA	x		
Laureth-12	x		
Ext. Violet 2 (CI 60730)	x		
Orange 4 (CI 15510)	x		
Red 40 (CI 16035)	x		
Cocamidopropyl Betaine		x	
Alcohol Denat. (SD Alcohol 40/Alcool Specialement Denature 40)		x	
Glycol Stearate		x	
Hydroxypropyl Methylcellulose		x	
Isostearoyl Hydrolyzed Collagen		x	
Panthenol		x	
Tetrasodium EDTA		x	
Methylparaben		x	
Propylparaben		x	

	Essence of Beauty Creamy Fig Hand Soap	Bed Head Moisture Maniac Shampoo	Tide HE Liquid
CI 15985 (Yellow 6)		x	
CI 20170 (Brown 1)		x	
CI 17200 (Red 33)		x	
Sodium alcoholethoxy sulfate			x
MEA citrate			x
Sodium alkyl sulfate			x
Alcohol ethoxylate			x
Linear alkylbenzene sulfonate, MEA salt			x
Sodium fatty acids			x
Polyethyleneimine ethoxylate			x
Diethylene glycol			x
Propylene glycol			x
Diquaternium ethoxysulfate			x
Borax			x
Polyethyleneimine ethoxylate propoxylate			x
Ethanol			x
Sodium cumenesulfonate			x
DTPA			x
Disodium diaminostilbene disulfonate			x
Mannanase			x
Cellulase			x
Amylase			x
Sodium formate			x
Calcium formate			x
Lauramine oxide			x
Liquitint™ Blue			x
Dimethicone / polydimethyl silicone			x

Appendix B: Extracellular Enzyme Assay Preparation and Additional Results

Figure B-1 shows the typical microtiter plate set-up that was prepared for each extracellular enzyme assay.

Figures B-2 through B-7 present example data that results from such an assay for each enzyme.

Figures B-8 and B-9 present a time series of glycosidase and aminopeptidase activity throughout the course of the experiments described in Chapter 4. Because experimental conditions were not changed in R1 (Figure B-8) over the course of the experimental period, but the data for each of these data series appeared like they might be increasing over time, a linear regression of each enzyme activity in R1 on day of operation was performed. Thereafter hypothesis testing was performed to determine if the null hypothesis that the slope of the line was equal to zero could be rejected. For all five enzyme activities in R1, the null hypothesis could not be rejected at α=0.10.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	250 µL DI	250 µL $NaHCO_3$	200 µL Sample R1 + 50 µL $NaHCO_3$	200 µL Sample R2 + 50 µL $NaHCO_3$		50 µL 4-MUB + 200 µL $NaHCO_3$	50 µL 4-MUB + 200 µL Sample R1	50 µL 4-MUB + 200 µL Sample R2	50 µL 7-AMC + 200 µL $NaHCO_3$	50 µL 7-AMC + 200 µL Sample R1	50 µL 7-AMC + 200 µL Sample R2
B	Empty	250 µL DI	250 µL $NaHCO_3$	200 µL Sample R1 + 50 µL $NaHCO_3$	200 µL Sample R2 + 50 µL $NaHCO_3$		50 µL 4-MUB + 200 µL $NaHCO_3$	50 µL 4-MUB + 200 µL Sample R1	50 µL 4-MUB + 200 µL Sample R2	50 µL 7-AMC + 200 µL $NaHCO_3$	50 µL 7-AMC + 200 µL Sample R1	50 µL 7-AMC + 200 µL Sample R2
C	Empty	250 µL DI	250 µL $NaHCO_3$	200 µL Sample R1 + 50 µL $NaHCO_3$	200 µL Sample R2 + 50 µL $NaHCO_3$		50 µL 4-MUB + 200 µL $NaHCO_3$	50 µL 4-MUB + 200 µL Sample R1	50 µL 4-MUB + 200 µL Sample R2	50 µL 7-AMC + 200 µL $NaHCO_3$	50 µL 7-AMC + 200 µL Sample R1	50 µL 7-AMC + 200 µL Sample R2
D	50 µL 4-MUB-α-g + 200 µL $NaHCO_3$	50 µL 4-MUB-α-g + 200 µL Sample R1	50 µL 4-MUB-α-g + 200 µL Sample R2	50 µL 4-MUB-β-g + 200 µL $NaHCO_3$	50 µL 4-MUB-β-g + 200 mL Sample R1	50 µL 4-MUB-β-g + 200 mL Sample R2	50 µL 4-MUB-β-N-AG + 200 µL $NaHCO_3$	50 µL 4-MUB-β-N-AG + 200 µL Sample R1	50 µL 4-MUB-β-N-AG + 200 µL Sample R2	50 µL 4-MUB-PO_4 + 200 µL $NaHCO_3$	50 µL 4-MUB-PO_4 + 200 µL Sample R1	50 µL 4-MUB-PO_4 + 200 µL Sample R2
E	50 µL 4-MUB-α-g + 200 µL $NaHCO_3$	50 µL 4-MUB-α-g + 200 µL Sample R1	50 µL 4-MUB-α-g + 200 µL Sample R2	50 µL 4-MUB-β-g + 200 µL $NaHCO_3$	50 µL 4-MUB-β-g + 200 mL Sample R1	50 µL 4-MUB-β-g + 200 mL Sample R2	50 µL 4-MUB-β-N-AG + 200 µL $NaHCO_3$	50 µL 4-MUB-β-N-AG + 200 µL Sample R1	50 µL 4-MUB-β-N-AG + 200 µL Sample R2	50 µL 4-MUB-PO_4 + 200 µL $NaHCO_3$	50 µL 4-MUB-PO_4 + 200 µL Sample R1	50 µL 4-MUB-PO_4 + 200 µL Sample R2
F	50 µL 4-MUB-α-g + 200 µL $NaHCO_3$	50 µL 4-MUB-α-g + 200 µL Sample R1	50 µL 4-MUB-α-g + 200 µL Sample R2	50 µL 4-MUB-β-g + 200 µL $NaHCO_3$	50 µL 4-MUB-β-g + 200 mL Sample R1	50 µL 4-MUB-β-g + 200 mL Sample R2	50 µL 4-MUB-β-N-AG + 200 µL $NaHCO_3$	50 µL 4-MUB-β-N-AG + 200 µL Sample R1	50 µL 4-MUB-β-N-AG + 200 µL Sample R2	50 µL 4-MUB-PO_4 + 200 µL $NaHCO_3$	50 µL 4-MUB-PO_4 + 200 µL Sample R1	50 µL 4-MUB-PO_4 + 200 µL Sample R2
G	50 µL L-Leu-AMC + 200 µL $NaHCO_3$	50 µL L-Leu-AMC + 200 µL $NaHCO_3$	50 µL L-Leu-AMC + 200 µL $NaHCO_3$	50 mL L-Leu-AMC + 200 mL Sample R1	50 mL L-Leu-AMC + 200 mL Sample R1	50 mL L-Leu-AMC + 200 mL Sample R1	50 mL L-Leu-AMC + 200 mL Sample R2	50 mL L-Leu-AMC + 200 mL Sample R2	50 mL L-Leu-AMC + 200 mL Sample R2			
H	50 µL L-Gly-AMC + 200 µL $NaHCO_3$	50 µL L-Gly-AMC + 200 µL $NaHCO_3$	50 µL L-Gly-AMC + 200 µL $NaHCO_3$	50 µL L-Gly-AMC + 200 µL Sample R1	50 µL L-Gly-AMC + 200 µL Sample R1	50 µL L-Gly-AMC + 200 µL Sample R1	50 µL L-Gly-AMC + 200 µL Sample R2	50 µL L-Gly-AMC + 200 µL Sample R2	50 µL L-Gly-AMC + 200 µL Sample R2			

Figure B-1 – Microtiter plate set-up for extracellular enzyme assay.

DI – deionized water
$NaHCO_3$ – 5 mM sodium bicarbonate
4-MUB – 100 µM 4-methylumbelliferone
7-AMC – 100 µM 7-amido-4-coumarin
4-MUB-α-g – 200 µM 4-methylumbelliferyl-α-D-glucoside
4-MUB-β-g – 200 µM 4-methylumbelliferyl-β-D-glucoside
4-MUB-β-N-AG – 200 µM 4-methylumbelliferyl-β-1,4-N-glucosamide
4-MUB-PO_4 – 100 µM 4-methylumbelliferyl-phosphate
L-Leu-AMC – 200 µM L-leucine-7-amido-4-methylcoumarin
L-Gly-AMC – 200 µM L-glycine-7-amido-4-methylcoumarin

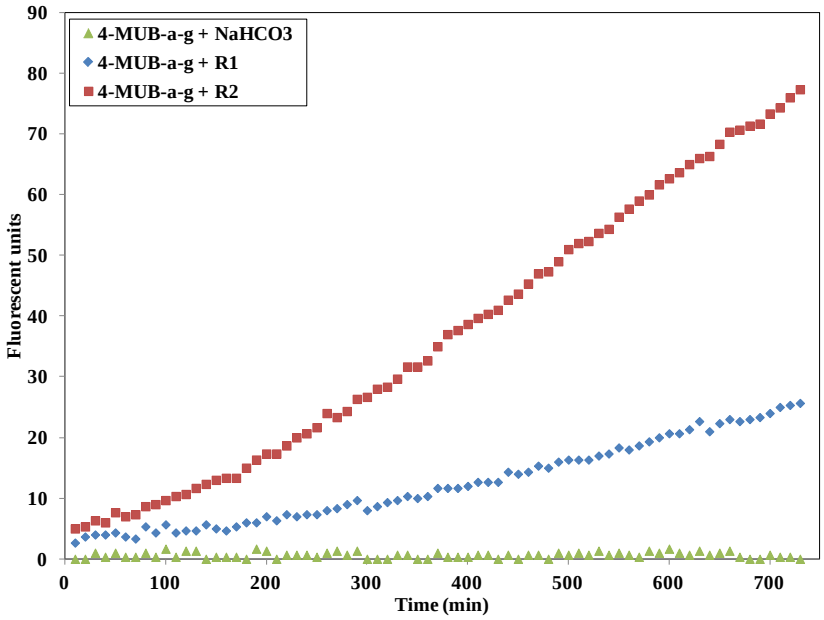


Figure B-2 – Typical response of assay for α-glucosidase activity.

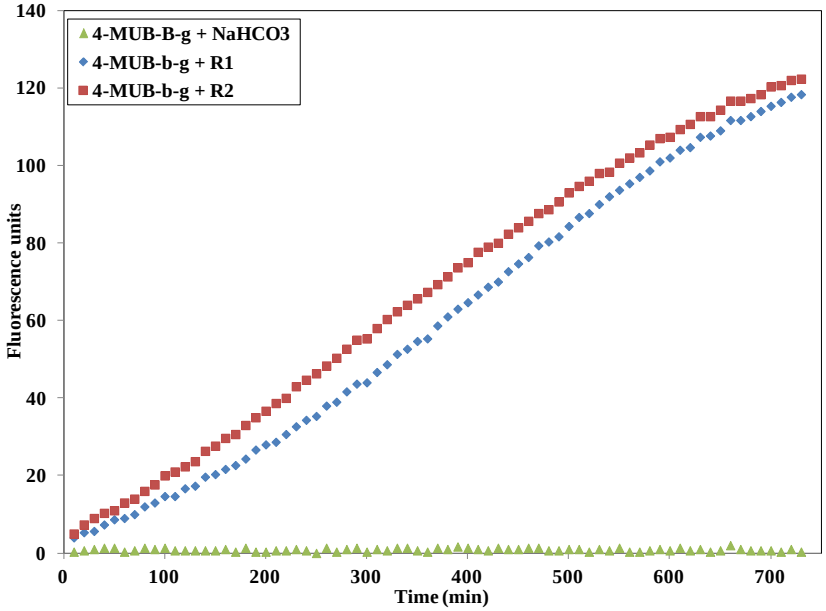


Figure B-3 – Typical response of assay for β-glucosidase activity.

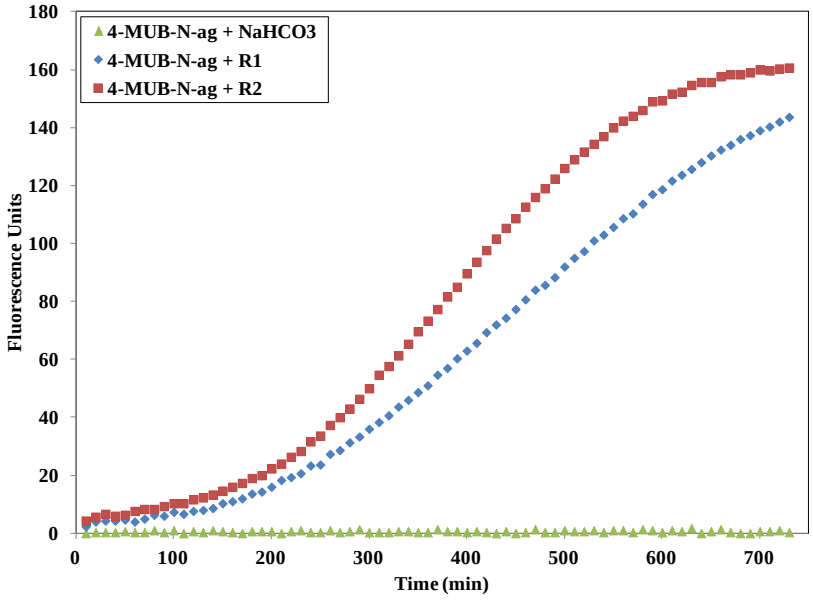


Figure B-4 – Typical response of assay for β-1,4-N-glucosamidase activity.

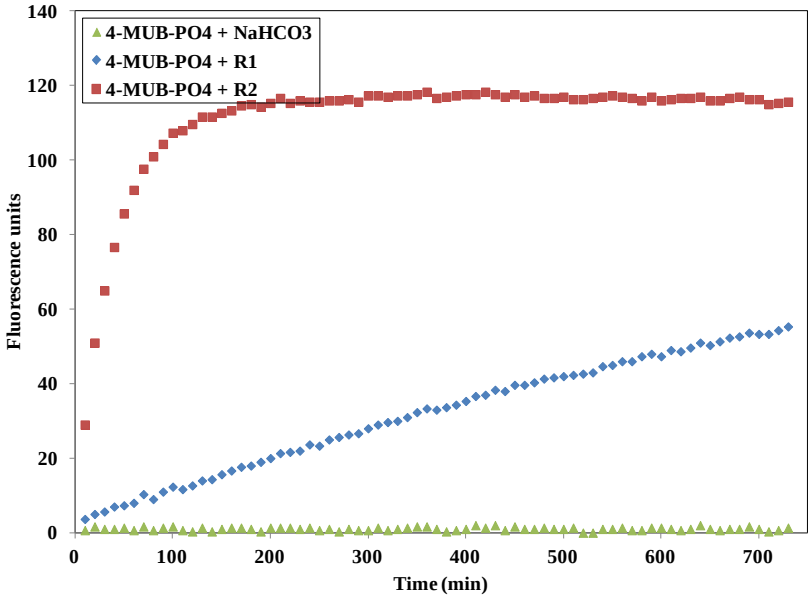


Figure B-5 – Typical response of assay for acid/alkaline phosphatase activity.

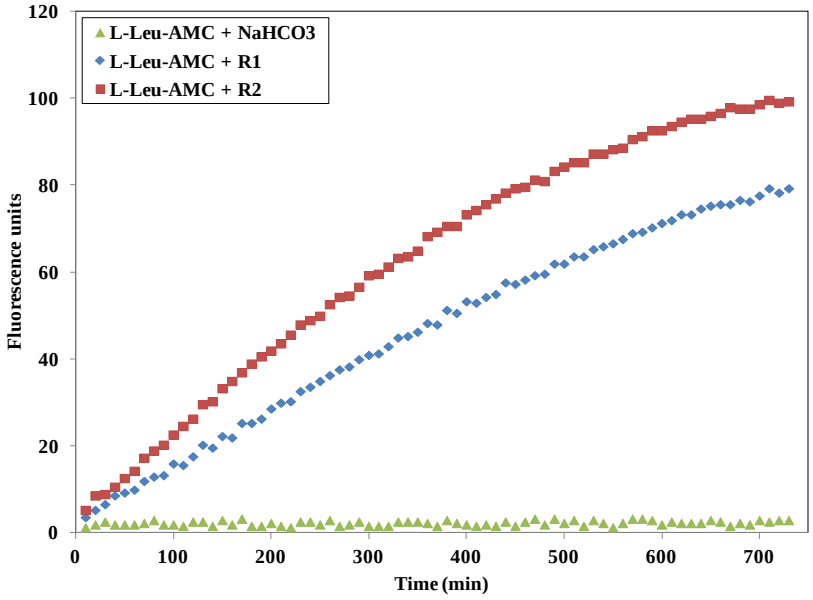


Figure B-6 – Typical response of assay for L-leucine-aminopeptidase activity.

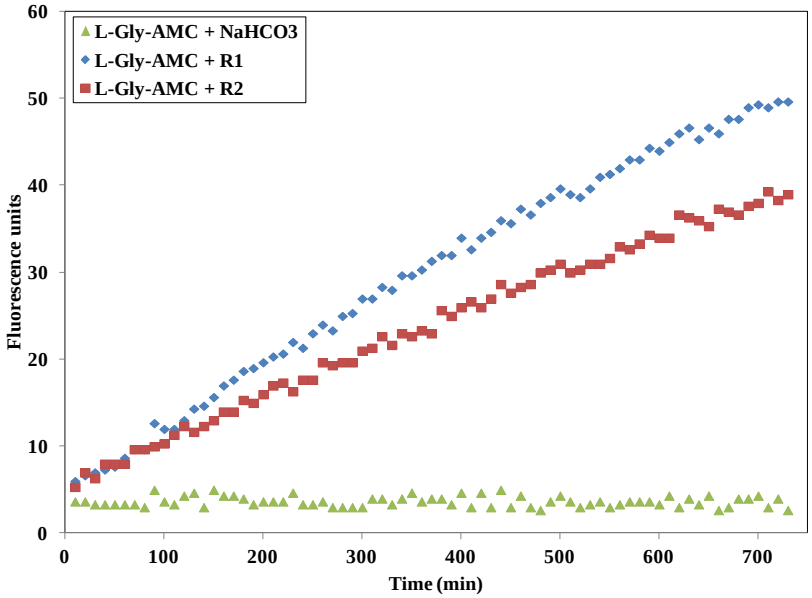
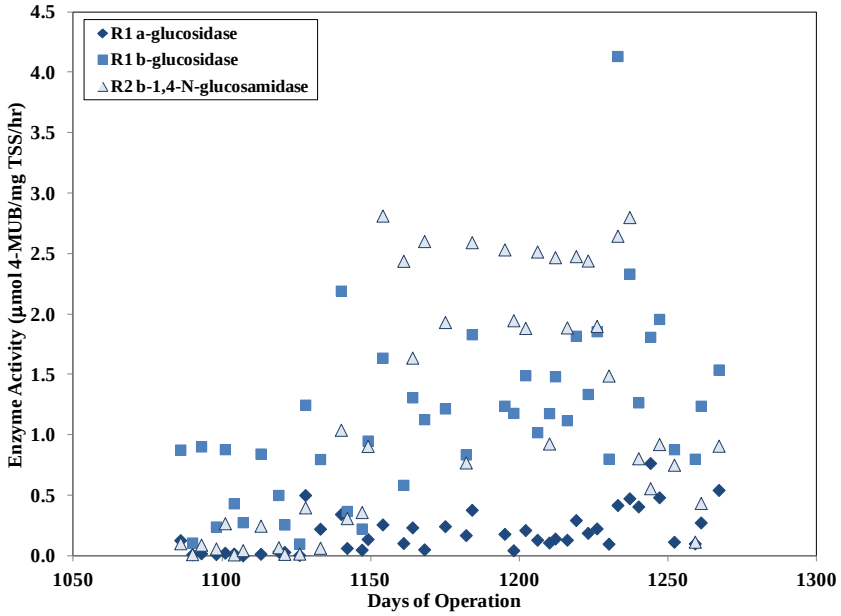


Figure B-7 – Typical response of assay for L-glycine-aminopeptidase activity.

a)



b)

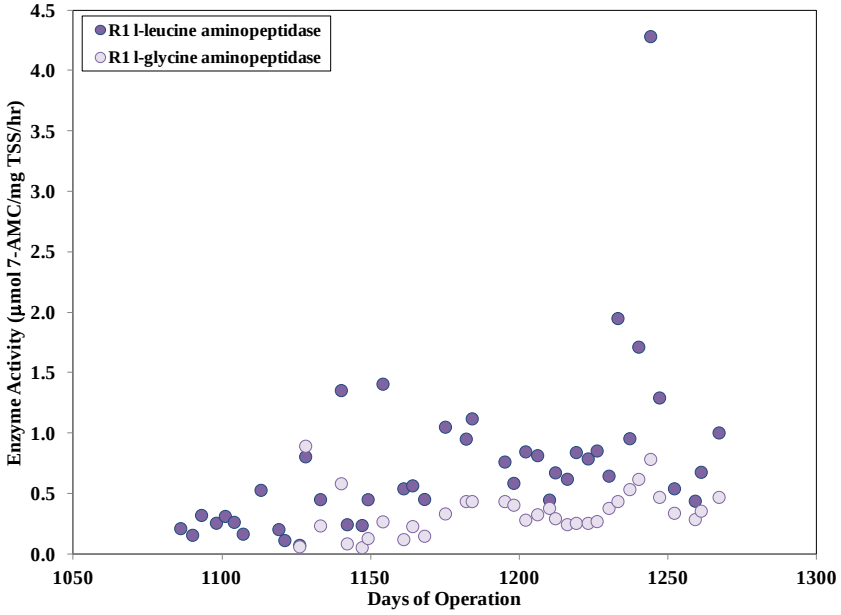
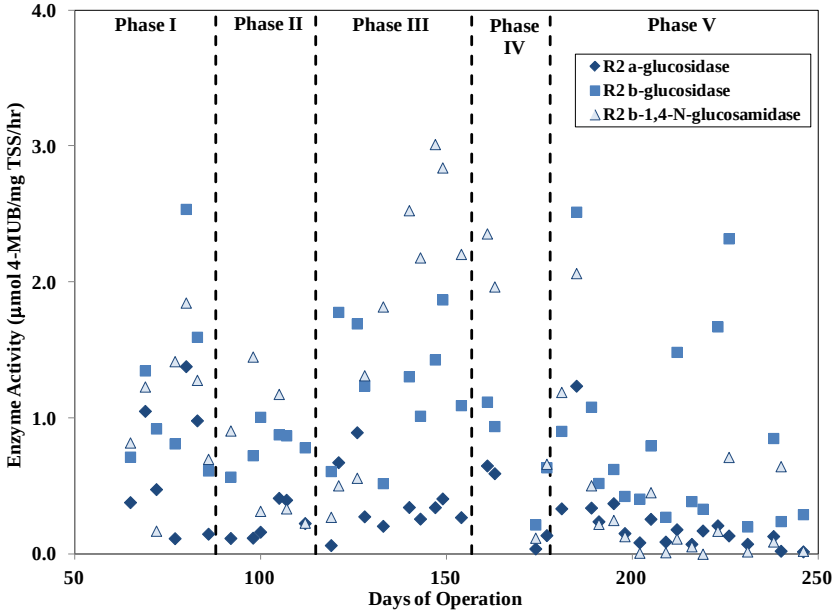


Figure B-8 – Glycosidase activity (a) and aminopeptidase activity (b) measured in R1. No assay for l-glycine aminopeptidase activity was performed until Day 1126.

a)



b)

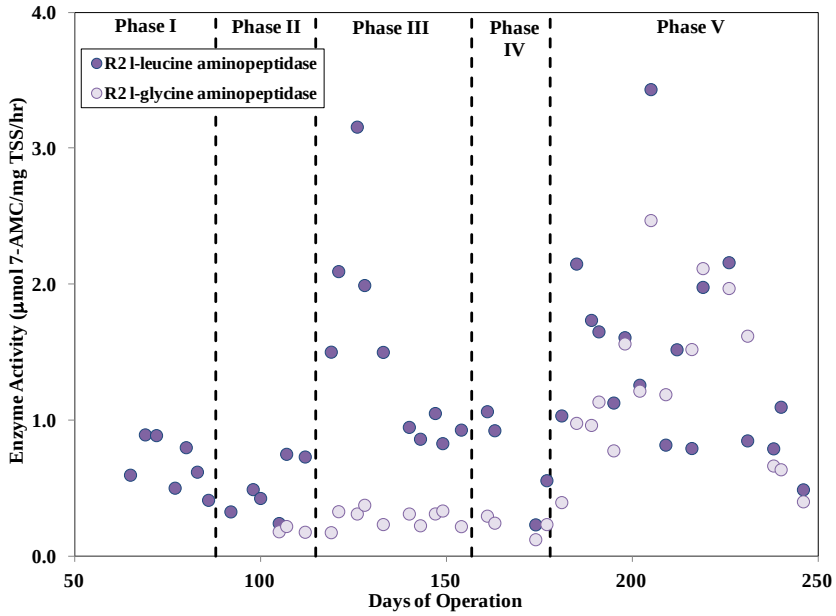


Figure B-9 – Glycosidase activity (a) and aminopeptidase activity (b) measured in R2. No assay for l-glycine aminopeptidase activity was performed until Day 105.

Appendix C: MBR and Chemostat Pyrosequencing Results

Microbial genomic DNA was extracted using a MoBio PowerSoil Extraction Kit (MoBio Laboratories, Inc.; Carlsbad, CA). DNA samples were sent to Research and Testing Laboratory (Lubbock, TX) for pyrosequencing targeting the 16S rRNA gene for bacteria. Percentage results by genus are presented below.

	MBRs		Chemostats	
Genus	R1	R2	ISS High P	SGW High P
Pseudomonas	47	2.3	1.8	37
Cupriavidus	19.1	2.1	0.011	0.016
Chitinophaga	12.3	12.6	0	0
Herbaspirillum	7.1	1.6	0	0
Burkholderia	4.0	0.69	0.084	0.17
Ralstonia	2.9	2.8	0.38	0.064
Azospira	1.9	0.031	2.3	0.19
Variovorax	0.99	0	0	0
Afipia	0.78	0.061	0.032	0.064
Pandoraea	0.73	0.041	0.21	0.080
Escherichia	0.46	0.17	0.042	0.016
Dyella	0.36	0.22	0.011	0.032
Mitsuaria	0.32	1.2	0	0
Rhodocyclus	0.28	1.4	2.4	0.37
Curvibacter	0.28	0.010	0	0
Acinetobacter	0.15	0	0.26	0.064
Delftia	0.14	0.24	45	7.7
Rudaea	0.14	0.13	0.59	0.21
Lactobacillus	0.11	0	0	0
Veillonella	0.098	0.4885	0	0
Frateuria	0.084	0.0204	0	0
Serratia	0.084	0.010	0.021	0.048
Enterobacter	0.070	0.33	0	0.032
Achromobacter	0.056	63	0.11	0.72
Niastella	0.056	0.051	0	0

	MBRs		Chemostats	
Genus	R1	R2	ISS High P	SGW High P
Clostridium	0.056	0.020	0	0
Shigella	0.056	0.010	0.011	0
Finegoldia	0.056	0	0	0
Acidovorax	0.042	0.092	0.47	1.4
Enterococcus	0.042	0.031	0	0
Streptococcus	0.042	0.010	0	0
Klebsiella	0.028	2.1	0	0
Bradyrhizobium	0.028	0.47	0.59	0.14
Sphingobacterium	0.028	0.36	0	0
Roseateles	0.028	0.17	0	0
Sediminibacterium	0.028	0.031	0.063	0.048
Terrimonas	0.028	0.020	0	0
Chryseobacterium	0.028	0	0.011	2.3
Rhodanobacter	0.028	0	0	0
Staphylococcus	0.014	3.8	0	0
Stenotrophomonas	0.014	0.46	0.19	13
Alcaligenes	0.014	0.16	0.43	0.032
Beta	0.014	0.11	0.12	0
Flavobacterium	0.014	0.092	0	0
Bacteroides	0.014	0.051	0.021	0
Pantoea	0.014	0.041	0	0
Dechloromonas	0.014	0.020	0.19	0.016
Agrobacterium	0.014	0.010	1.4	11
Vibrio	0.014	0.010	0.032	1.7
Massilia	0.014	0	0	0
Thiolamprovum	0.014	0	0	0
Aeromonas	0.014	0	0	0
Caulobacter	0	1.2	1.6	0.59
Zoogloea	0	0.19	26	4.2
Telmatospirillum	0	0.18	0	0
Citrobacter	0	0.092	0	0
Azonexus	0	0.081	10	0.95
Sphingomonas	0	0.081	0	0.032
Proteus	0	0.051	0	0
Microbacterium	0	0.041	0	0

	MBRs		Chemostats	
Genus	R1	R2	ISS High P	SGW High P
Janthinobacterium	0	0.031	0	0
Dokdonella	0	0.020	0.79	0.86
Methylobacterium	0	0.020	0.011	0.91
Geothrix	0	0.020	0	0
Acidobacterium	0	0.020	0	0
Solibium	0	0.020	0	0
Streptomyces	0	0.020	0	0
Sporotalea	0	0.020	0	0
Cronobacter	0	0.020	0	0
Pelomonas	0	0.020	0	0
Pigmentiphaga	0	0.010	0.35	0.064
Mesorhizobium	0	0.010	0.063	0.14
Thauera	0	0.010	0.053	0
Diaphorobacter	0	0.010	0	0
Nitrobacteria	0	0.010	0	0
Sporomusa	0	0.010	0	0
Filimonas	0	0.010	0	0
Gloeobacter	0	0.010	0	0
Xanthobacter	0	0.010	0	0
Sarcina	0	0.010	0	0
Peptoniphilus	0	0.010	0	0
Propionivibrio	0	0	2.2	0.11
Rhizobium	0	0	0.83	6.9
Ochrobactrum	0	0	0.44	0.51
Bosea	0	0	0.24	0.048
Devosia	0	0	0.22	0.032
Thiobacillus	0	0	0.21	0
Bordetella	0	0	0.13	0.064
Filomicrobium	0	0	0.11	0
Phaeospirillum	0	0	0.042	0
Comamonas	0	0	0.032	0.16
Azoarcus	0	0	0.032	0
Meganema	0	0	0.032	0
Pseudoxanthomonas	0	0	0.021	0
Shinella	0	0	0.011	5.6

	MBRs		Chemostats	
Genus	R1	R2	ISS High P	SGW High P
Defluvibacter	0	0	0.011	1.2
Methylocystis	0	0	0.011	0
Naxibacter	0	0	0.011	0
Pedomicrobium	0	0	0.011	0
Rhodomicrobium	0	0	0.011	0
Stenoxybacter	0	0	0.011	0
Thermomonas	0	0	0.011	0
Rhodobium	0	0	0.011	0
Gulbenkiania	0	0	0.011	0
Prosthecomicrobium	0	0	0.011	0
Mycoplana	0	0	0	1.1
Xanthomonas	0	0	0	0.29
Brevundimonas	0	0	0	0.19
Sinorhizobium	0	0	0	0.14
Paenibacillus	0	0	0	0.064
Sphingobium	0	0	0	0.048
Pseudorhodoferax	0	0	0	0.032
Albidiferax	0	0	0	0.032
Faecalibacterium	0	0	0	0.016
Aminobacter	0	0	0	0.016
Phyllobacterium	0	0	0	0.016
Paracoccus	0	0	0	0.016
Kingella	0	0	0	0.016

References

Allen, L.; Bryan, S.; Woelfle-Erskine, C. Residential Greywater Irrigation Systems in California: An Evaluation of Soil and Water Quality, User Satisfaction, and Installation Costs; Greywater Action: 2012.

Almqvist, H.; Hanaeus, J., Organic Hazardous Substances in Graywater from Swedish Households. Journal of Environmental Engineering **2006,** 132 (8), 901-908.

American Public Health Association (AHPA); American Water Works Assocation; Water Environment Federation, Standard Methods for the Examination of Water and Wastewater. 20th ed.; 1999.

American Water Works Association (AWWA), Residential End Uses of Water; AWWA: November, 1999.

Andersen, H. R.; Lundsbye, M.; Wedel, H. V.; Eriksson, E.; Ledin, A., Estrogenic personal care products in a greywater reuse system. Water Science and Technology **2007,** 56 (12), 45-49.

Atasoy, E.; Murat, S.; Baban, A.; Tiris, M., Membrane Bioreactor (MBR) Treatment of Segregated Household Wastewater for Reuse. CLEAN – Soil, Air, Water **2007,** 35 (5), 465-472.

Austin Water Utility, City of Austin Water Quality Summary 1st Quarter Averages (January 1, 2008 to March 31, 2008). 2008a.

Austin Water Utility, City of Austin Water Quality Summary 2nd Quarter Averages (April 1, 2008 to June 30, 2008). 2008b.

Austin Water Utility, City of Austin Water Quality Summary 3rd Quarter Averages (July 1, 2008 to September 30, 2008). 2008c.

Austin Water Utility, City of Austin Water Quality Summary 4th Quarter Averages (October 1, 2008 to December 31, 2008). 2009.

Autian, J., Toxicity and health threats of phthalate esters: review of the literature. Environ Health Perspect **1973,** 4, 3-26.

Barlass, T., Long-running soapie ends. Sydney Morning-Herald June 13, 2011.

Berger, H., Environmentally compatible surfactants for the cosmetic industry. International Journal of Cosmetic Science **1997,** 19 (5), 227-237.

Birks, R.; Hills, S., Characterisation of indicator organisms and pathogens in domestic greywater for recycling. Environ. Monit. Assess. **2007,** 129 (1-3), 61-69.

Boczar, B. A.; Begley, W. M.; Larson, R. J., Characterization of Enzyme Activity in Activated Sludge Using Rapid Analyses for Specific Hydrolases. Water Environ. Res. **1992,** 64 (6), 792-797.

Breedveld, G. D.; Sparrevik, M., Nutrient-limited biodegradation of PAH in various soil strata at a creosote contaminated site. Biodegradation **2000,** 11 (6), 391-399.

Cases, V.; Alonso, V.; Argandoña, V.; Rodriguez, M.; Prats, D., Endocrine disrupting compounds: A comparison of removal between conventional activated sludge and membrane bioreactors. Desalination **2011,** 272 (1-3), 240-245.

Chaillou, K.; Gérente, C.; Andrès, Y.; Wolbert, D., Bathroom Greywater Characterization and Potential Treatments for Reuse. Water, Air, & Soil Pollution **2011,** 215 (1-4), 31-42.

Chappell, K. R.; Goulder, R., Enzymes as river pollutants and the response of native epilithic extracellular-enzyme activity. Environmental Pollution **1994,** 86 (2), 161-169.

Chen, G.-H.; Mo, H.-K.; Liu, Y., Utilization of a metabolic uncoupler, 3,3′,4′,5-tetrachlorosalicylanilide (TCS) to reduce sludge growth in activated sludge culture. Water Research **2002,** 36 (8), 2077-2083.

Chew, L. C. K.; Tacon, W. C. A.; Cole, J. A., Effect of growth conditions on the rate of loss of the plasmid pAT153 from continuous cultures of Escherichia coli HB101. FEMS Microbiology Letters **1988,** 56 (1), 101-104.

Christopher, H. J.; Boardman, G. D.; Freedman, D. L., Aerobic biological treatment of 2,4-dinitrotoluene in munitions plant wastewater. Water Research **2000,** 34 (5), 1595-1603.

Çiçek, N.; Franco, J. P.; Suidan, M. T.; Urbain, V.; Manem, J., Characterization and Comparison of a Membrane Bioreactor and a Conventional Activated-Sludge System in the Treatment of Wastewater Containing High-Molecular-Weight Compounds. Water Environ. Res. **1999,** 71 (1), 64-70.

Clara, M.; Strenn, B.; Gans, O.; Martinez, E.; Kreuzinger, N.; Kroiss, H., Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Research **2005,** 39 (19), 4797-4807.

Clara, M.; Windhofer, G.; Hartl, W.; Braun, K.; Simon, M.; Gans, O.; Scheffknecht, C.; Chovanec, A., Occurrence of phthalates in surface runoff, untreated and treated wastewater and fate during wastewater treatment. Chemosphere **2010,** 78 (9), 1078-1084.

Commission of the European Communities, Report from the Commission to the Council and the European Parliament: Pursuant to Article 16 of Regulation (EC) No 648/2004 of the European Parliament and of the Council of 31 March 2004 on detergents, concerning the use of phosphates. Brussels, Belgium, 2007.

Constable, G.; Somerville, B., A Century of Innovation: The Engineering That Transformed Our Lives. Joseph Henry Press: 2003.

Côté, P.; Buisson, H.; Pound, C.; Arakaki, G., Immersed membrane activated sludge for the reuse of municipal wastewater. Desalination **1997,** 113 (2–3), 189-196.

Dargnat, C.; Teil, M.-J.; Chevreuil, M.; Blanchard, M., Phthalate removal throughout wastewater treatment plant: Case study of Marne Aval station (France). Science of The Total Environment **2009,** 407 (4), 1235-1244.

Donner, E.; Eriksson, E.; Revitt, D. M.; Scholes, L.; Lützhøft, H. C. H.; Ledin, A., Presence and fate of priority substances in domestic greywater treatment and reuse systems. Science of The Total Environment **2010,** 408 (12), 2444-2451.

Elonen, C., Standard Operating Procedure (SOP): "Assay Methods for Determination of Sediment Extracellular Enzyme Activity Determination by Fluorescence". U.S. EPA, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division - Duluth, MN, Ed. 2005.

Elonen, C., Re: Question about Extracellular Enzyme Activity Analyses. Van Epps, A., Ed. 2012.

Eriksson, E.; Andersen, H. R.; Madsen, T. S.; Ledin, A., Greywater pollution variability and loadings. Ecological Engineering **2009,** 35 (5), 661-669.

Eriksson, E.; Auffarth, K.; Eilersen, A.-M.; Henze, M.; Ledin, A., Household chemicals and personal care products as sources for xenobiotic organic compounds in grey wastewater. Water SA **2003,** 29 (2), 135-146.

Eriksson, E.; Auffarth, K.; Henze, M.; Ledin, A., Characteristics of grey wastewater. Urban Water **2002,** 4 (1), 85-104.

Eriksson, E.; Donner, E.; Ledin, A., Presence of selected priority and personal care substances in an onsite bathroom greywater treatment facility. Water Science & Technology **2010,** 62 (12), 2889-2898.

European Commission Scientific Committee on Cosmetic Products and Non-food Products Intended for Consumers (ECSCCPNFPIC), Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Concerning Diethyl Phthalate. 2002.

Fangyue, L.; Wichmann, K.; Otterpohl, R., Evaluation of appropriate technologies for grey water treatments and reuses. Water Science & Technology **2009,** 59 (2), 249-260.

Fendinger, N. J.; Begley, W. M.; McAvoy, D. C.; Eckhoff, W. S., Determination of alkyl sulfate surfactants in natural waters. Environmental Science & Technology **1992,** 26 (12), 2493-2498.

Finley, S.; Barrington, S.; Lyew, D., Reuse of Domestic Greywater for the Irrigation of Food Crops. Water Air Soil Pollut **2009,** 199 (1-4), 235-245.

Foreman, C. M.; Franchini, P.; Sinsabaugh, R. L., The Trophic Dynamics of Riverine Bacterioplankton: Relationships Among Substrate Availability, Ectoenzyme Kinetics, and Growth. Limnology and Oceanography **1998,** 43 (6), 1344-1352.

Freedman, D. L.; Payauys, A. M.; Karanfil, T., The Effect of Nutrient Deficiency on Removal of Organic Solvents from Textile Manufacturing Wastewater During Activated Sludge Treatment. Environ. Technol. **2005,** 26 (2), 179 - 188.

Friedler, E.; Gilboa, Y., Performance of UV disinfection and the microbial quality of greywater effluent along a reuse system for toilet flushing. Science of The Total Environment **2010,** 408 (9), 2109-2117.

Friedler, E.; Katz, I.; Dosoretz, C. G., Chlorination and coagulation as pretreatments for greywater desalination. Desalination **2008,** 222 (1–3), 38-49.

Friedler, E.; Kovalio, R.; Galil, N. I., On-site greywater treatment and reuse in multi-storey buildings. Water Science & Technology **2005,** 51 (10), 187-194.

Fuls, J. L.; Rodgers, N. D.; Fischler, G. E.; Howard, J. M.; Patel, M.; Weidner, P. L.; Duran, M. H., Alternative Hand Contamination Technique To Compare the Activities of Antimicrobial and Nonantimicrobial Soaps under Different Test Conditions. Appl. Environ. Microbiol. **2008,** 74 (12), 3739-3744.

Gage, M. A.; Gorham, E., Alkaline phosphatase activity and cellular phosphorus as an index of the phosphorus status of phytoplankton in Minnesota lakes. Freshwater Biology **1985,** 15 (2), 227-233.

Gaulke, L. S., On-site wastewater treatment and reuses in Japan. Proceedings of the Institution of Civil Engineers-Water Management **2006,** 159 (2), 103-109.

Ghyoot, W.; Verstraete, W., Reduced sludge production in a two-stage membrane-assisted bioreactor. Water Research **2000,** 34 (1), 205-215.

Glenn, R. T. Regulatory Issues Associated with Greywater Reuse. Colorado State University, Fort Collins, Colorado, 2012.

Gogra, A. B.; Yao, J.; Sandy, E. H.; Zheng, S.; Zaray, G.; Koroma, B. M.; Hui, Z., Cell surface hydrophobicity (CSH) of Escherichia coli, Staphylococcus aureus and Aspergillus niger and the biodegradation of Diethyl Phthalate (DEP) via Microcalorimetry. Journal of American Science **2010,** 6 (7), 78-88.

González-Mariño, I.; Quintana, J. B.; Rodríguez, I.; Cela, R., Simultaneous determination of parabens, triclosan and triclocarban in water by liquid chromatography/electrospray ionisation tandem mass spectrometry. Rapid Communications in Mass Spectrometry **2009,** 23 (12), 1756-1766.

Granger, D. A.; Butler, B. J.; Barker, J. F., A Case of Phosphorus Limiting Monoaromatic Hydrocarbon Biodegradation in Groundwater. Bioremediation Journal **1999,** 3 (3), 213 - 221.

Gross, A.; Azulai, N.; Oron, G.; Ronen, Z.; Arnold, M.; Nejidat, A., Environmental impact and health risks associated with greywater irrigation: a case study. Water Science & Technology **2005,** 52 (8), 161-169.

Environmental Working Group, Skin Deep Cosmetic Safety Database. http://www.ewg.org/skindeep/ (accessed March 3, 2013).

Gunther, F., Wastewater treatment by greywater separation: Outline for a biologically based greywater purification plant in Sweden. Ecological Engineering **2000,** 15 (1), 139-146.

Hankin, L.; Sands, D. C., Bacterial Production of Enzymes in Activated Sludge Systems. Journal (Water Pollution Control Federation) **1974,** 46 (8), 2015-2025.

Harrow, D. I.; Felker, J. M.; Baker, K. H., Impacts of Triclosan in Greywater on Soil Microorganisms. Applied and Environmental Soil Science **2011,** 2011.

Hernández-Leal, L.; Temmink, H.; Zeeman, G.; Buisman, C. J. N., Removal of micropollutants from aerobically treated grey water via ozone and activated carbon. Water Research **2011,** 45 (9), 2887-2896.

Hernández Leal, L. a.; Vieno, N.; Temmink, H.; Zeeman, G.; Buisman, C. J. N., Occurrence of Xenobiotics in Gray Water and Removal in Three Biological Treatment Systems. Environmental Science & Technology **2010,** 44 (17), 6835-6842.

Hill, B.; Elonen, C.; Jicha, T.; Bolgrien, D.; Moffett, M., Sediment microbial enzyme activity as an indicator of nutrient limitation in the great rivers of the Upper Mississippi River basin. Biogeochemistry **2010a,** 97 (2), 195-209.

Hill, B. H.; Elonen, C. M.; Jicha, T. M.; Cotter, A. M.; Trebitz, A. S.; Danz, N. P., Sediment microbial enzyme activity as an indicator of nutrient limitation in Great Lakes coastal wetlands. Freshwater Biology **2006,** 51 (9), 1670-1683.

Hill, B. H.; McCormick, F. H.; Harvey, B. C.; Johnson, S. L.; Warren, M. L.; Elonen, C. M., Microbial enzyme activity, nutrient uptake and nutrient limitation in forested streams. Freshwater Biology **2010b,** 55 (5), 1005-1019.

Huelgas, A.; Funamizu, N., Flat-plate submerged membrane bioreactor for the treatment of higher-load graywater. Desalination **2010,** 250 (1), 162-166.

Jashinski, K. B. Comparison of MBR Treatment of Real Graywater Effluent to MBR Treatment of Synthetic Graywater. The University of Texas at Austin, 2010.

Jefferson, B.; Burgess, J. E.; Pichon, A.; Harkness, J.; Judd, S. J., Nutrient addition to enhance biological treatment of greywater. Water Research **2001,** 35 (11), 2702-2710.

Jenkins, R. O.; Heald, S. C., Stability of toluene oxidation by Pseudomonas putida under nutrient deprivation. Applied Microbiology and Biotechnology **1996,** 46 (4), 388-392.

Jones, I. M.; Primrose, S. B.; Robinson, A.; Ellwood, D. C., Maintenance of some ColE1-type plasmids in chemostat culture. Molecular and General Genetics MGG **1980,** 180 (3), 579-584.

Joss, A.; Keller, E.; Alder, A. C.; Göbel, A.; McArdell, C. S.; Ternes, T.; Siegrist, H., Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Research **2005,** 39 (14), 3139-3152.

Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T., Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. Environmental Science & Technology **2002,** 36 (6), 1202-1211.

Krishnan, V.; Ahmad, D.; Jeru, J. B., Influence of COD : N : P ratio on dark greywater treatment using a sequencing batch reactor. J. Chem. Technol. Biotechnol. **2008,** 83 (5), 756-762.

Laine, A. T. Technologies for Greywater Recycling in Buildings. Cranfield University, 2001.

Lamine, M.; Samaali, D.; Ghrabi, A., Greywater treatment in a submerged membrane bioreactor with gravitational filtration. Desalination and Water Treatment **2012,** 46 (1-3), 182-187.

LaPara, T. M.; Zakharova, T.; Nakatsu, C. H.; Konopka, A., Functional and Structural Adaptations of Bacterial Communities Growing on Particulate Substrates under Stringent Nutrient Limitation. Microbial Ecology **2002,** 44 (4), 317-326.

Lesjean, B.; Gnirss, R., Grey water treatment with a membrane bioreactor operated at low SRT and low HRT. Desalination **2006,** 199 (1-3), 432-434.

Li, F.; Wichmann, K.; Otterpohl, R., Review of the technological approaches for grey water treatment and reuses. Science of The Total Environment **2009,** 407 (11), 3439-3449.

Litke, D. W., Review of Phosphorus Control Measures in the United States and Their Effects on Water Quality. PROGRAM, N. W.-Q. A., Ed. Denver, CO, 1999.

Liu, R.; Huang, X.; Chen, L.; Wen, X.; Qian, Y., Operational performance of a submerged membrane bioreactor for reclamation of bath wastewater. Process Biochemistry **2005,** 40 (1), 125-130.

Liu, Y., Chemically reduced excess sludge production in the activated sludge process. Chemosphere **2003,** 50 (1), 1-7.

Loretz, L.; Api, A. M.; Barraj, L.; Burdick, J.; Davis, D. A.; Dressler, W.; Gilberti, E.; Jarrett, G.; Mann, S.; Laurie Pan, Y. H.; Re, T.; Renskers, K.; Scrafford, C.; Vater, S., Exposure data for personal care products: Hairspray, spray perfume, liquid foundation, shampoo, body wash, and solid antiperspirant. Food and Chemical Toxicology **2006,** 44 (12), 2008-2018.

Love, N. G. The Impact of Growth in a Dual Substrate Limited Environment on the Stability and Expression of a TOL Plasmid. Clemson University, 1994.

Lu, Y.; Tang, F.; Wang, Y.; Zhao, J.; Zeng, X.; Luo, Q.; Wang, L., Biodegradation of dimethyl phthalate, diethyl phthalate and di-n-butyl phthalate by Rhodococcus sp. L4 isolated from activated sludge. J Hazard Mater **2009,** 168 (2-3), 938-43.

McCoy, M., Goodbye, Phosphates. Chemical & Engineering News January 24, 2011, pp 12-17.

Merz, C.; Scheumann, R.; El Hamouri, B.; Kraume, M., Membrane bioreactor technology for the treatment of greywater from a sports and leisure club. Desalination **2007,** 215 (1-3), 37-43.

Metcalf & Eddy, Wastewater Engineering: Treatment and Reuse. Tata McGraw-Hill: New Delhi, 2003.

Metcalf & Eddy, Water Reuse: Issues, Technologies, and Applications. McGraw Hill: New York, 2007.

Modi, R. I.; Wilke, C. M.; Rosenzweig, R. F.; Adams, J., Plasmid macro-evolution: selection of deletions during adaptation in a nutrient-limited environment. Genetica **1991,** 84 (3), 195-202.

Molina-Muñoz, M.; Poyatos, J. M.; Vílchez, R.; Hontoria, E.; Rodelas, B.; González-López, J., Effect of the concentration of suspended solids on the enzymatic activities and biodiversity of a submerged membrane bioreactor for aerobic treatment of domestic wastewater. Applied Microbiology and Biotechnology **2007,** 73 (6), 1441-1451.

Montuelle, B.; Volat, B., Impact of Wastewater Treatment Plant Discharge on Enzyme Activity in Freshwater Sediments. Ecotoxicology and Environmental Safety **1998,** 40 (1-2), 154-159.

Noack, D.; Roth, M.; Geuther, R.; Müller, G.; Undisz, K.; Hoffmeier, C.; Gáspár, S., Maintenance and genetic stability of vector plasmids pBR322 and pBR325 in Escherichia coli K12 strains grown in a chemostat. Molecular and General Genetics MGG **1981,** 184 (1), 121-124.

Nolde, E., Greywater reuse systems for toilet flushing in multi-storey buildings - over ten years experience in Berlin. Urban Water **2000,** 1 (4), 275-284.

Nolde, E., Greywater recycling systems in Germany — results, experiences and guidelines. Water Science & Technology **2005,** 51 (10), 203-210.

Oppenheimer, J. S., Roger; Burbano, Arturo; Liu, Li, Characterizing the Passage of Personal Care Products Through Wastewater Treatment Processes. Water Environ. Res. **2007,** 79, 2564-2577.

Palmquist, H.; Hanæus, J., Hazardous substances in separately collected grey- and blackwater from ordinary Swedish households. Science of The Total Environment **2005,** 348 (1-3), 151-163.

Pidou, M.; Avery, L.; Stephenson, T.; Jeffrey, P.; Parsons, S. A.; Liu, S.; Memon, F. A.; Jefferson, B., Chemical solutions for greywater recycling. Chemosphere **2008,** 71 (1), 147-155.

Pidou, M.; Memon, F. A. S., T.; Jefferson, B.; Jeffrey, P., Greywater recycling: treatment options and applications. Engineering Sustainability **2007,** 160 (ES3), 119-131.

Prathapar, S. A.; Jamrah, A.; Ahmed, M.; Al Adawi, S.; Al Sidairi, S.; Al Harassi, A., Overcoming constraints in treated greywater reuse in Oman. Desalination **2005,** 186 (1–3), 177-186.

Price, A., Austin seeks to rewrite gray water rules as conservation measure. Austin American-Statesman July 2, 2012.

Qasim, S. R., Wastewater Treatment Plants: Planning, Design, and Operation. CRC Press: 1999.

Roesner, L.; Qian, Y.; Criswell, M.; Stromberger, M.; Klein, S. Long-term Effects of Landscape Irrigation Using Household Graywater – Literature Review and Synthesis; Water Environment Research Foundation / Soap and Detergent Association: 2006.

Sayler, G. S.; Hooper, S. W.; Layton, A. C.; King, J. M. H., Mini Review: Catabolic Plasmids of Environmental and Ecological Significance. Microbial Ecology **1990,** 19 (1), 1-20.

Shafran, A. W.; Gross, A.; Ronen, Z.; Weisbrod, N.; Adar, E., Effects of surfactants originating from reuse of greywater on capillary rise in the soil. Water Sci Technol **2005,** 52 (10-11), 157-66.

Sharvelle, S.; Roesner, L. A.; Qian, Y.; Stromberger, M.; Azar, M. N. Long-Term Study on Landscape Irrigation Using Household Graywater: Experimental Study; The Urban Water Center, Colorado State University: 2012.

Sheikh, B. White Paper on Graywater; 2010.

Shiklomanov, I. A. World Water Resources: A New Appraisal and Assessment for the 21st Century; United Nations Educational, Scientific and Cultural Organization: Paris, 1998.

Sinsabaugh, R.L.; Foreman, C.M., Activity profiles of bacterioplankton in a eutrophic river. Freshwater Biology **2001,** 46 (9), 1239-1249.

Sinsabaugh, R. L.; Antibus, R. K.; Linkins, A. E.; McClaugherty, C. A.; Rayburn, L.; Repert, D.; Weiland, T., Wood Decomposition: Nitrogen and Phosphorus Dynamics in Relation to Extracellular Enzyme Activity. Ecology **1993,** 74 (5), 1586-1593.

Sinsabaugh, R. L.; Findlay, S.; Franchini, P.; Fischer, D., Enzymatic Analysis of Riverine Bacterioplankton Production. Limnology and Oceanography **1997,** 42 (1), 29-38.

Sinsabaugh, R. L.; Hill, B. H.; Follstad Shah, J. J., Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature **2009,** 462 (7274), 795-798.

Sinsabaugh, R. S., Enzymic analysis of microbial pattern and process. Biology and Fertility of Soils **1994,** 17 (1), 69-74.

Stephenson, T.; Laine, A. L.; Jefferson, B.; Judd, S. J., Membrane bioreactors and their role in wastewater reuse. Water Science & Technology **2000,** 41 (1), 197.

Suen, W. C.; Spain, J. C., Cloning and characterization of Pseudomonas sp. strain DNT genes for 2,4-dinitrotoluene degradation. J Bacteriol **1993,** 175 (6), 1831-7.

Thermo Scientific, Product Manual for Acclaim Surfactant Plus Columns. 2012.

Top, E. M.; Springael, D.; Boon, N., Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. FEMS Microbiol Ecol **2002,** 42 (2), 199-208.

University of Toledo Center for Dead Plant Studies, Hydrolase Assays with Coumarin Substrates. 2000.

U.S. Department of Health and Human Services, Household Products Database. http://householdproducts.nlm.nih.gov/index.htm (accessed March 3, 2013).

U.S. Environmental Protection Agency (EPA), OSWER Draft Guidance for Evaluating the Vapor Intrusion to Indoor Air Pathway from Groundwater and Soils (Subsurface Vapor Intrusion Guidance). 2002.

U.S. EPA, 2012 Guidelines for Water Reuse. 2012a.

U.S. EPA, EPI Suite, 4.11; 2012b.

U.S. National Library of Medicine, Toxicological Data Network. http://toxnet.nlm.nih.gov/index.html (accessed March 10, 2013).

Vamsee-Krishna, C.; Phale, P., Bacterial degradation of phthalate isomers and their esters. Indian Journal of Microbiology **2008,** 48 (1), 19-34.

Voet, D., Biochemistry. J. Wiley: New York, 1995.

Vrba, J.; ÁLek, V. V.; Hejzlar, J.; Nedoma, J., Comparison of phosphorus deficiency indices during a spring phytoplankton bloom in a eutrophic reservoir. Freshwater Biology **1995,** 33 (1), 73-81.

Wei, Y.; Van Houten, R. T.; Borger, A. R.; Eikelboom, D. H.; Fan, Y., Minimization of excess sludge production for biological wastewater treatment. Water Research **2003,** 37 (18), 4453-4467.

Winward, G. P.; Avery, L. M.; Frazer-Williams, R.; Pidou, M.; Jeffrey, P.; Stephenson, T.; Jefferson, B., A study of the microbial quality of grey water and an evaluation of treatment technologies for reuse. Ecological Engineering **2008a,** 32 (2), 187-197.

Winward, G. P.; Avery, L. M.; Stephenson, T.; Jefferson, B., Chlorine disinfection of grey water for reuse: Effect of organics and particles. Water Research **2008b,** 42 (1–2), 483-491.

Wong, P. C.-Y., Minimizing Excess Sludge Production Through Membrane Bioreactors and Integrated Processes. In Biological Sludge Minimization and Biomaterials/Bioenergy Recovery Technologies, John Wiley & Sons, Inc.: 2012; pp 261-290.

Wouters, J. T. M.; Driehuis, F. L.; Polaczek, P. J.; Oppenraay, M.-L. H. A.; Andel, J. G., Persistence of the pBR 322 plasmid in Escherichia coli K 12 grown in chemostat cultures. Antonie van Leeuwenhoek **1980,** 46 (4), 353-362.

Yeldho, D.; Rebello, S.; Jisha, M., Plasmid-Mediated Biodegradation of the Anionic Surfactant Sodium Dodecyl Sulphate, by Pseudomonas aeruginosa S7. Bulletin of Environmental Contamination and Toxicology **2011,** 86 (1), 110-113.

Zafra-Gómez, A.; Ballesteros, O.; Navalón, A.; Vílchez, J. L., Determination of some endocrine disrupter chemicals in urban wastewater samples using liquid chromatography-mass spectrometry. Microchemical Journal **2008,** 88 (1), 87-94.

Vita

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