The Effect of Regional and Seasonal Variability in Wastewater Microbial Community Structure on the Biodegradability of Room Temperature Ionic Liquids

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The Effect of Seasonal and Regional Variation of the Wastewater Microbial Community Structure on the Biodegradability of Room Temperature Ionic Liquids

A thesis presentation by Steven W. Aiello for the Lee Honors College of Western Michigan University

Written under the supervision of Dr. Kathryn Docherty

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Abstract:

Biodegradability is an important consideration in the design of novel chemicals. At the end of their function, chemicals should break down into harmless degradation products to avoid toxic effects as a result of environmental persistence. To test the biodegradability potential of novel chemicals, a protocol designed by the Organisation for Economic and Cooperative Development (OECD) is used. This protocol states that in a mixture of the chemical of interest, a microbial inoculate, and mineral media, 70% of the chemical must degrade within a 10-day window of a 28-day experiment in order to be considered readily biodegradable. The protocol states that the microbial inoculate can be obtained from one of several possible locations, such as a Wastewater Treatment Plant (WWTP), at any time point. Because of this, the microbial community is taken as a constant, when instead it has shown to be variable. In multiple OECD-driven experiments using the same chemical of interest, different biodegradation results were recorded. This is problematic, as the data collected may not be useful and will not provide the correct information concerning the fate of the chemical once it enters the environment. In this experiment, we tested the biodegradability of novel green chemicals, Ionic Liquids (ILs). Varying biodegradation from previous experiments make them an ideal candidate for this experiment. We predict that the microbial community involved in the biodegradation process varies by the location and the time point during the year it is collected. In this experiment, we collected microbial inoculate from two different sampling sites at three time points in a year, for use in an experiment prepared in accordance with the OECD protocol. We quantified biodegradation by measuring the absorbance of the pyridinium or imidalizolium ring (depending on the IL used), as well as the total organic carbon concentrations (June experiment only). Additionally, we performed Terminal Restriction Fragment Length Polymorphism (TRFL-P) analysis to provide insight into the microbial
community present. Our results suggest that microbial community structure varies between locations and across different time points in a year, and that this variation affects the biodegradation of ILs.

Introduction:

The twelve Principles of Green Chemistry state that biodegradability of a novel chemical is paramount to its green design (Anastas and Warner 1998). Thus, “green” designer chemicals should break down at the end of their function and not persist in the environment. In order to create more sustainable practices, chemical engineers develop novel green chemicals with their biodegradability potential in mind. This is to ensure that the chemical will be biologically metabolized to harmless biomass and CO₂ while it is processed in a typical wastewater treatment plant (WWTP), and will not be released as a persistent waste stream into the environment. To evaluate the biodegradability of novel chemicals, many investigators use a standardized protocol designed by the Organisation for Economic and Cooperative Development (OECD). This protocol outlines a standard experimental procedure in which the novel chemical of interest is the sole carbon source added to a mixture of mineral media and a microbial inoculate collected from a typical WWTP aeration tank. The microbial community contained within the aeration tank is responsible for biodegradation of the chemical of interest, and is treated as a constant, regardless of the source. If 70% of the chemical is metabolized within a 10-day window of a 28-day test period, then the chemical is defined as “readily biodegradable” and is assumed to meet the green criteria for biodegradability.

While a useful standardized metric, the OECD protocols for biodegradability do not provide any information on the community members in the microbial inoculate. The community
is treated as a constant, but may in fact vary based on the timing of sampling and the specific WWTP sampled. For example, a study that employed 454-pyrosequencing techniques to investigate the microbial community structure of twelve WWTPs discovered the dominant phyla and trace community members to be different across the sampled WWTPs (Hu et al. 2012). Furthermore, another recent study examined the microbial community structure of a WWTP aeration tank over a period of 4 years, and showed significant differences in the relative abundances of dominant genera with time (Ju et al. 2014).

Previous studies indicate that variation in WWTP microbial communities may account for differences in biodegradability predictions. For example, Docherty et al. (2010) showed that the green ionic liquid (IL) 1-butyl-3-methylpyridinium bromide (bmpyrBr) was not readily biodegradable using an inoculum collected from the WWTP in South Bend, IN. Additionally, Docherty has shown that microbial inocula collected at three different time points from the South Bend WWTP yielded different predictions of biodegradability for bmpyrBr. Using a microbial inoculate collected in April 2005, bmpyrBr did not degrade; however, using a microbial inoculate collected in February 2006, bmpyrBr was classified as readily biodegradable (Docherty, unpublished data). In a study that used a microbial inoculum collected from a WWTP in Jeonju, Korea, this same IL was found to be readily biodegradable as well (Pham et al. 2010).

A comprehensive study examining how the timing and location of WWTP sampling affects novel green chemical biodegradability predictions has not been performed, and is necessary to determine whether standard biodegradability assays are useful metrics to inform green chemical design. ILs are considered to be a green chemical due to their low vapor pressure, high thermal stability, and ease of recovery from application (Gathergood 2004). ILs are used for a number of industrial applications, including use in hydrogenation, isomerization,
hydroformylation, dimerization, alkylation, Diels-Alder reactions and biocatalysis media (Zhao 2002). In this study, we examined biodegradability of three common ILs using microbial inocula collected from two WWTPs at three time points. Specifically, we examined the biodegradability of 1-octyl-3-methylpyridinium bromide (ompyrBr), 1-butyl-3-methylpyridinium bromide, and 1-butyl-3-methylimidazolium chloride (bmimCl) (Figure 1). We chose these three ILs because previous studies indicate that ompyrBr is usually classified as readily biodegradable (Harjani 2009, Docherty et al. 2007), bmpyrBr has been shown to have variable biodegradability (Docherty et al. 2010, Pham et al. 2010), and bmimCl is typically not biodegradable using the OECD standard assay (Harjani et al. 2009, Coleman et al. 2010,). We hypothesize that the wastewater microbial community structure varies regionally and temporally, and that this variation affects biodegradability predictions for these ILs.

Methods

OECD Protocol: We conducted all biodegradation experiments in this study according to the OECD 301A: Dissolved Organic Carbon (DOC) Die-Away Test (OECD 1992). This protocol dictates that if the chemical of interest (serving as the only carbon source), mixed with mineral media and activated sludge, biodegrades by 70% within a 10 day window of 28 days, the chemical can be considered readily biodegradable. To obtain the microbial inocula, we collected grab samples from the aeration tanks at the Kalamazoo Water Reclamation Plant (Kalamazoo, MI), and the South Bend Wastewater Treatment Plant (South Bend, IN) at three time points during the year: August 22, 2012, December 19, 2012 and May 28, 2013. We transported samples back to the laboratory, and aerated them for 7 days prior to beginning the experiment in order to remove labile carbon. We removed triplicate samples (45 mL) from the grab samples on the day of collection and immediately froze them at -80°C for microbial community analyses.
On the 6th day of aeration, we measured the total suspended solids (TSS) by vacuum filtering 20mL of activated sludge onto a 0.45µm pore size filter (Whatman, Pittsburgh, PA) and allowing it to dry overnight at 50°C before weighing. The TSS measured from the South Bend and Kalamazoo sludge was 1.42/0.647g, 1.01/1.47g and 1.72/1.09g for the August, December, and June experiments, respectively.

We set up the experiment using triplicate biotic and abiotic controls and IL treatments, with 33 acid washed and autoclaved 1L Pyrex bottles. Abiotic controls received ILs and mineral media, but no microbial inoculate, to test for abiotic degradation of the IL. Biotic controls received mineral media and microbial inoculate, but no ILs, to control for autotrophic growth. We prepared mineral media by combining 800 µL Ultrapure autoclaved sterile water and analytical grade reagents into 1L acid washed, sterile pyrex bottles. We added 0.22 µm (Millex, Billerica, MA) filter-sterilized stock concentrations of media to all bottles to achieve final concentrations of: 0.085g L⁻¹ KH₂PO₄ (VWR, Radnor, PA), 0.2175g L⁻¹ K₂HPO₄ (Sigma-Aldrich, St. Louis, MO), 0.344g L⁻¹ Na₂HPO₄ (Sigma-Aldrich), 5mg L⁻¹ NH₄Cl (Acros Organics, West Chester, PA), 36.4 mg L⁻¹, 36.4 mg L⁻¹ CaCl₂·2H₂O (Sigma-Aldrich), 22.5mg L⁻¹ MgSO₄·7H₂O (Acros Organics), and 0.25mg L⁻¹ FeCl₃·6H₂O (Sigma-Aldrich).

We prepared stock solutions containing 10 g C L⁻¹ of each IL in acid-washed volumetric flasks. We made all stock solution by dissolving 1g of IL in ultrapure water, and diluted to make 100 mL of stock solution in a volumetric flask. ILs were either purchased from Iolitec, Inc (Tuscaloosa, AL; bmpyrBr and ompyrBr) or provided by Dr. Stuart Jones (University of Notre Dame, Notre Dame, IN, bmimCl). We added 13, 9.6 or 9.5 mL of 10 g C L⁻¹ bmimCl, bmpyrBr or ompyrBr, respectively, to the experimental treatment and abiotic controls to achieve a final concentration of 50 mg C L⁻¹ for each IL.
Following aeration, we added 5 g TSS of microbial inoculate to each of the experimental treatment and biotic control bottles. We diluted all bottles to 1L with sterile Ultrapure water and loosely capped all bottles. The bottles were then shaken aerobically, in the dark, at room temperature, at a speed of 150rpm for an incubation period of 28 days.

**Absorbance vs. TOC:** To quantify biodegradation throughout the course of the experiments, we measured absorbance every 2 days during the August and June experiments, and weekly during the December experiment, over a 28 day period. In the June experiment only, we also measured dissolved organic carbon (DOC) concentrations. The OECD protocol specifies DOC measurements, but similar trends in data indicate that absorbance is an equally effective method of determining biodegradability of these ILs (Docherty et al. 2010). To measure absorbance, we vacuum filtered 300 µL of each sample using a GF/F Filter (Whatman, Pittsburgh, PA), placed into a UV 96-well plate (Costar, Vernon Hills, IL). We read absorbances of pyridinium ILs at 265 nm and absorbance of imidazolium at 210 nm using a 96-well Microplate Spectrophotometer (Epoch, Biotek, Winooski, VT) in order to detect breakdown of the ring structure. We collected 20mL DOC samples from the filtrate, re-filtered them through a 0.22 µm syringe filter (Millex, Billerica, MA), and acidified them with 100uL of 2N HCl. We analyzed DOC samples at the University of Notre Dame using a Shimadzu 5000 Total Organic Carbon analyzer with autosampler to detect metabolism of carbon by the microbial community.

**Polymerase Chain Reaction (PCR) amplification and Terminal Restriction Fragment Length Polymorphism (T-RFLP):** Samples originally collected from the aeration tanks were thawed and filtered through a 0.22 µM filter (Millipore, Billerica, MA). The entire filter-plus-filtrate was placed into the bead-beating tube of a Powersoil DNA isolation kit (Mobio Laboratories Inc., Carlsbad, CA) for microbial community DNA extraction. We checked DNA
extraction quality by running 10 µL of extract on a 2% agarose gel for 2 hours. Following extraction, we performed PCR to amplify a region of the 16S rRNA gene in Bacteria. We performed 3 replicate PCRs on each of the 3 replicates of extracted DNA from each time point, with a positive control containing *E. coli* DNA, and with negative controls containing sterile water. PCR mastermix was added to each sample and control. The mastermix was composed of 100µL PCR Premix E (Epicentre, Madison, WI), 2.5µL Alpha-casein (Sigma-Aldrich), 6.5µL sterile water, 0.5µL Taq Polymerase (New England Biolabs Inc.), and 0.5µL 8F-FAM (5’-GAGTTTGATCCTGGCTCA-3’) and 926R (5’-CCGTCAATTCCTTTRAGTTT-3’) primers. Triplicate reactions for each DNA extraction were performed in an Eppendorf Mastercycler Pro using the following conditions: 1 min at 72°C, 45 sec at 94°C, 1 min at 53°C, 1 min at 72°C, 15 min at 72°C for 34 cycles, idling at 10°C when finished. Following PCR, all products were run on a 2% agarose gel at 130 V for 2 h. Individual bands were excised using a sterile scalpel blade and placed into sterile microcentrifuge tubes. Excised bands were purified using an UltraClean GelSpin DNA Extraction Kit (MoBio Labs, Inc, Carlsbad, CA). Following clean-up, triplicates were pooled and restricted using HhaI restriction enzyme (New England Biolabs, Ipswich, MA) at 37°C for 2 hours. This enzyme cuts double-stranded DNA at the sequence 5’-…GCG▼C-3’ and 3’…C▲GCG-5’. Following restriction, samples were cleaned using a DNA Clean and Concentrator-5 kit (Zymo, Irvine, CA) and eluted in 6 mL of final elution buffer. DNA concentrations were measured using a Qubit 2.0 fluorometer (Invitrogen, Grand Island, NY); all were within the range of 10^3 ng/mL. The samples were sent to the University of Illinois Urbana-Champaign Core Sequencing Facility for fragment analysis using an Applied Biosystems 3730x1 automated sequencer with 50cm capillary arrays.
**Fragment and Statistical Analysis:** We analyzed fragments using Applied BioSystems Peak Scanner Software v1.0. We used a No Peak Primer (NPP) analysis method and the GeneScan 600LIZ sizing standard. With the data collected from PeakScanner, we organized the TRFs into groups with similar base pair sizes (within 4 base pairs, above or below), and summed the peak heights. We performed statistical analyses for absorbance measurements using SYSTAT v 10.0. We performed Repeated Measures ANOVA for all time-series experiments using a general linear model, One Way ANOVA, at each time point. We assessed all statistics using a 95% significance level (α= 0.05).

**Results/Discussion:**

**Wastewater Treatment Plant Characterization:** In order to characterize the microbial communities at each WWTP, we synthesized information about the wastewater from each plant at the time of sampling and from each day of a week prior (table 1). On average, TSS were twice as high in the Kalamazoo aeration tank in comparison to the South Bend WWTP across all time points. There was a large phosphoric acid accidental release upstream to the Kalamazoo WWTP in June 2012, which called for neutralization by ferric chloride. It is possible that this upset somehow affected the community structure of the Kalamazoo WWTP. The amount of dissolved oxygen was similar at both plants during the December 2012 and June 2013 time points, but was 50% higher at the Kalamazoo plant during thein August 2012. It was consistently slightly warmer at the Kalamazoo plant (an average of 2.3°C) than at the South Bend plant during the time of sampling, which may be responsible for the measured shifts in community structure. Previous studies (Ju et al. 2014) have shown temperature shifts to be a driving factor in community composition.
**Microbial Community Characterization:** We performed T-RFLP analysis on the activated sludge collected at each time point to gain insight into the microbial community structure.

Proportions of TRFs in each of the six communities are shown in Figure 2. In August 2012, the Kalamazoo sample had 20 (+/- 1.7) unique TRFs and the South Bend samples had 15 (+/- 1.3) unique TRFs. In December 2012, the Kalamazoo sample had 17 (+/- 3.7) unique TRFs and the South Bend samples had 22 (+/- 5.6) unique TRFs. In June 2013, the Kalamazoo sample had 17 (+/-3.3) unique TRFs and the South Bend samples had 18 (+/- 4.6) unique TRFs. Each WWTP sample contained a few of the same TRFs with a consistent peak height in each community.

These fragments were 54, 78, 84, 90, 191, and 200 base pairs (bps) in size. Most of the communities also contained fragment sizes corresponding to 60, 74, 94, 197, 210 and 507 bps. The only TRF specific to the Kalamazoo plant across all three time points was 217 bp, while TRFs specific to the South Bend plant across the three time points included fragments that were 530, 537 and 543 bps. Additionally, there are several rare TRFs that are only present in some or one of the microbial communities examined. Fragments corresponding to 131, 343 and 353 bp were present only at the Kalamazoo plant during August. Fragments that were 136 and 537 bp were only present at the South Bend plant during August. A 376 bp TRF was present only at the Kalamazoo plant during December, and 263 bp, 391 bp, and 454 bp TRFs were present at the South Bend plant during December only. The only Kalamazoo-specific TRF present during the June experiment was 572 bp in size; the only South Bend-specific peak present during the June experiment was 607 bp in size. Noteworthy peaks are 530bp and 543bp, because these peaks are both present during the December and June experiments, in which ompyrBr was mineralized using sludge taken from the South Bend Plant, as described below.
Multivariate analyses of these communities indicate that the three replicates for each WWTP-date are highly similar, but that both sampling date and WWTP location significantly influenced microbial community composition (S. Jones, personal communication). Other studies have shown that microbial communities vary temporally and geographically. For example, a study by Ju et al. (2014), which analyzed microbial community structure of a WWTP over the course of 4 years, demonstrated a seasonal oscillatory pattern in microbial community structure. They concluded that the shifts in community structure do not lead to a change in WWTP function, based on the results from an experiment in which they tagged established functional sequences of proteins, and monitored their relative abundance over time. Similarly, a study by Kim et al. (2013) evaluated the seasonal variation of general and rare taxa of a WWTP, and found that the general taxa richness remained consistent, while the rare taxa became more rich with each sample point. They concluded that despite the small shifts in community structure function with respect to protein degradation did not change with time. However, the conclusion that microbial community structure does not impact WWTP function does not necessarily apply to our study, which examines specific chemical biodegradation processes instead of general WWTP functions, such as carbohydrate, protein and nitrogen metabolism, sludge retention time, or the amount of mixed liquor solids. Our study analyzes the ability of the community to degrade a single pollutant in a lab scale environment, which is unlike the processing of multiple pollutants on an industrial scale.

**Standard Biodegradability Tests: August.** In the August biodegradability experiments, none of the ILs tested were completely biodegraded (Figure 3 A-F). However, in the test pairing the microbial inoculate from Kalamazoo with ompyrBr, we observed a significant reduction in absorbance at 265nm in the experimental treatments as compared to the abiotic control (p=0.03,
significant decreases in absorption in the experimental treatment began on day 17 (p=0.002, df=4) and continued until the final day of the experiment (day 28, Figure 2B). Our results indicate that ompyrBr was partially metabolized by the Kalamazoo microbial inoculate in the August test, but that it was not completely metabolized during the 28-day period. There was no significant reduction in absorbance as compared to the abiotic control in any of the other IL-microbial inoculate combinations, indicating that no other ILs were metabolized (Figure 3A-F).

We did observe a significant difference between the experimental treatments and abiotic controls in both bmimCl (p<0.0001, df=4) tests using both WWTP inoculates. However, this difference results from a higher absorbance in the bmimCl experimental treatments than in the abiotic controls, not a decrease in absorbance that would indicate biodegradation (Figure 3E-F). This increased absorbance at 210 nm in the bmimCl experimental treatments is likely due to the interfering absorbance of organic matter in the organism, such as peptide bonds, which also absorb at 210nm.

**Standard Biodegradability Tests: December.** In the December biodegradability experiments, our test data suggest that ompyrBr is readily biodegradable by the South Bend WWTP (figure 4A). There was no significant degradation of any other IL, but there was a slight drop in the 265nm absorbance at the end of the ompyrBr test using the Kalamazoo WWTP community. It is possible that if the test were allowed to continue, there would have been significant mineralization of the compound. The treatment of the bmimCl test using South Bend inoculate exhibited a significant difference from the abiotic control on days 14 and 28 (p=0.002, df=4 and p=0.001, df=4 for days 14 and 28 respectively). Additionally, the bmimCl treatment using Kalamazoo sludge showed a significant difference from the abiotic control on the first day of the experiment (p<0.0001, df=3). However, as with the August experiment, this difference is likely
due to a higher concentration of IL in the treatment and because of interference with other matter that absorbs around 210nm. There was no reduction in the 265nm absorbance during the bmpyrBr test for either the South Bend or Kalamazoo Plant during the December time point (Figure 4C-D).

**Standard Biodegradability Tests: June.** Similarly, ompyrBr degraded readily in the June experiment by the South Bend WWTP (Figure 5A). Starting on day 7 of this experiment, there was a significant decrease in the experimental treatment from the abiotic control (p<0.0001, df=4), and this trend continued to the end of the experiment. As with other biodegradation experiments using the bmimCl IL, there was no significant biodegradation at any time point (Figure 5E-F). The TOC data reflect a similar trend in data (Figure 6). Biodegradation of ompyrBr proceeds rapidly once it has begun, which suggests the cleaving of the ring structure. Abiotic control demonstrated a significant different from the experiment treatment for the assay performed on bmimCl using Kalamazoo inoculate (p=0.009, df=4). It is likely that this significant difference is due to reasons already mentioned- a higher concentration of IL in the treatment, and/or interference of other matter that absorbs at the 210nm wavelength.

**Summary of Standard Biodegradability Test Results:** In our study, only ompyrBr was biodegraded using the OECD DOC-Die Away standard biodegradability assay. None of our test results indicated that bmpyrBr or bmimCl was metabolized by the microbial communities from the two WWTPs tested, indicating that these ILs could pose a potential threat as an aquatic pollutant that cannot be removed by wastewater processing (Table 2). Our results were dissimilar to the biodegradation experiments reported by Pham *et al.* (2010). In that study, bmpyrBr was biodegraded using the same protocol, but it did not biodegrade during our at any time point during our experiment. In contrast, our results are similar to other published studies
(Docherty et al. 2007; 2010) in that we witnessed ready biodegradability of the ompyrBr at some
time points. Due to its longer side chain and pyridinium head group, ompyrBr is typically easier
to biodegrade than other ILs. Previous studies have shown complete mineralization of the
pyridinium ring structure (Grabinska-Sota and Kalka et al. 2004). In contrast, because of its
relatively short alkyl chain and imidazolium head group, bmimCl is often resistant to
biodegradation (Romero et al. 2007; Docherty et al. 2007).

**Connecting Biodegradability to Microbial Community Composition:** Our results indicate
that each WWTP-date combination results in a different microbial community. These
communities are treated as a constant in the OECD standard biodegradability assays, and
previous studies have shown that aeration tank microbial community structure does not impact
overall WWTP function (Kim et al. 2013; Ju et al. 2014). These studies indicate that rare species
act only as a seed bank, and that shifts in rare taxa do not influence WWTP function. However,
the community dynamics of single pollutant degradation in a controlled setting appear to be
largely different, and the rare TRFs may play a bigger role than suggested by previous
experiments.

The results of our ompyrBr biodegradation assays indicate that microbial community
composition can significantly impact biodegradability predictions of a specific chemical
compound. It is possible that the presence and relative abundance of certain TRFs affect
biodegradation efficacy and ability. During the December and June experiments, two peaks were
observed in the South Bend inoculum, at 530 and 543, in a higher proportion than during the
August sampling. The 530 peak comprised an average of 0.6% of the total peak height during the
December experiment, and an average of 4.3% during the June experiment. The 530 peak was
not present during the August experiment. The 543 peak comprised an average of 21% of the
total peak height from the June experiment, an average of 8% of the total peak height during the December experiment, and an average of 7.5% of the total peak height during the August experiment. Neither of these TRFs were present in the Kalamazoo inoculum, which suggests that they could represent the organisms that are responsible for biodegrading ompyrBr from the South Bend plant during the December and June experiments. It is also possible that these minor members in the community have an influence over other TRFs if several members of the community need to be present and act as a consortium in order for biodegradation to proceed.

In summary, biodegradation patterns were not consistent between the two locations, or across the three time points of sampling. From these results, we can conclude that biodegradation of ILs varies geographically and regionally. Changes in microbial community structure between both locations and across three time points were observed with TRFL-P analysis, which suggests that the community structure is temporally and regionally variable.

**Future Directions:** The OECD protocol would be more accurate if it were made to include specific activated sludge communities taken from controlled sites during particular time points during the year, in order to better predict on-site biodegradation. The screening of biodegradability potential of novel chemicals could be made more stringent by a higher level of control within the protocol using a predetermined community structure consisting of the general taxa normally found at a WWTP. Biodegradability is among the highest concerns in the development of novel chemicals, but it is difficult to predict the fate of these chemicals with something as variable as the wastewater microbial community responsible for biodegradation. If a high-throughput sequence can be used to describe a consortium of microbes capable of biodegrading particular chemicals using a bioinformatics pipeline, the microbial community directly responsible for the breakdown of novel chemicals during the OECD assay can be
identified and used to implement proactive treatment methods in the place of costly cleanup and avoiding environmental harm. The Docherty lab has already begun a follow-up experiment enriching the bmimCl culture to allow biodegradation of an IL that is usual resistant to biodegradation. By extending the incubation period, some biodegradation was observed. This suggests that bmimCl can be degraded, but not readily within the parameters of the OECD protocol. This could potentially mean that certain ILs, although not readily biodegradable by OECD standards, may not persist in the environment when treated at a WWTP.

**Acknowledgments:**

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**Table 2.** A comprehensive table of the biodegradability potential of each IL from all sites and time points during the experiment. The only IL to experience any significant drop in absorbance was ompyrBr. It was partially mineralized by the Kalamazoo inoculum during August and December, and completely mineralized by the South Bend inoculum during December and June.

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Fig 1. Chemical structures of the three Ionic Liquids. 1-butyl-3-methylimidazoleum Chloride (top left), 1-butyl-3-methylpyridinium Bromide (top right), and 1-octyl-3-methylpyridinium Bromide (bottom)
Fig 2. A stacked bar graph of the relative abundance of each TRF in proportion to the total peak height of all TRFs taken from the Kalamazoo (left) and South Bend (right) plants at each time point. Each bar represents 1 of 3 replicates taken from an experimental time point. Each bar corresponds to a color assigned to each distinct TRF. (Figure is in color).
Fig 3. Absorbance measured at 265nm (pyridinium ILs) and 210nm (imidazolium IL) during the 28 days of the August experiment. The square markers indicate the abiotic control, and the circle, square, and diamond markers indicate the ompyrBr, bmpyrBr, and bmimCl experimental treatments, respectively. A star denotes a significant difference between the abiotic control and the experimental treatment.
Fig 4. Absorbance measured at 265nm (pyridinium ILs) and 210nm (imidazolium IL) during the 28 days of the December experiment. The square markers indicate the abiotic control, and the circle, square, and diamond markers indicate the ompyrBr, bmpyrBr, and bmimCl experimental treatments, respectively.
Fig 5. Absorbance measured at 265nm (pyridinium ILs) and 210nm (imidazolium IL) during the 28 days of the June experiment. The square markers indicate the abiotic control, and the circle, square, and diamond markers indicate the ompyrBr, bmyrBr, and bmimCl experimental treatments, respectively.
Fig 6. Total Organic Carbon, measured in mg C·L⁻¹, over time during the June experiment. The square markers indicate the abiotic control, and the circle, square, and diamond markers indicate the ompyrBr, bmpyrBr, and bmimCl experimental treatments, respectively.
June SB BmimCl

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June KZ BmimCl