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Effects of estrone and organic carbon exposure on the transformation of estrone†

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Exposure of biomass to estrone (E1) and alternate organic substrates was studied to determine whether cometabolism or multiple substrate utilization is an operating mechanism for the transformation of E1 and if feeding intervals affect the selection of E1 degrading bacteria. Biomass generated in membrane bioreactors (MBRs) was capable of degrading E1 regardless of E1 exposure. Nevertheless, pre-exposed biomass had higher E1 transformation rates ($P = 0.05$) and un-exposed biomass showed a clear lag phase (6 h) prior to E1 transformation. These results are consistent with and strongly suggest metabolic transformation of E1 via multiple substrate utilization. In the feeding interval study, longer intervals between feeding periods selected for E1 degraders at high organic carbon loads ($100 \text{ mg COD L}^{-1} \text{ d}^{-1}$; $P = 0.018$), but had no effect at low organic carbon loads ($30 \text{ mg COD L}^{-1} \text{ d}^{-1}$; $P = 0.32$). A lag phase was observed in E1 transformation during famine periods but was absent during feast periods. This result indicates that the presence of other organic carbon substrates speeds the transformation of E1. This research is the first to demonstrate evidence for the role of multiple substrate utilization in the transformation of E1 and suggests operating conditions to improve selection for and activity of E1 degrading bacteria.

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Water impact statement

Estrone (E1) is a critical micropollutant due to its ubiquity and effects on aquatic life. The effects of microbial exposure to E1 and alternate organic substrates were studied to determine if E1-degradation by a mixed microbial community proceeds via multiple substrate utilization or cometabolism. Additional work was done examining the effects of feeding cycles in conjunction with organic carbon concentrations. Removal patterns show that E1 transformation proceeds metabolically (multiple substrate utilization), while feeding cycle duration effects were dependent on organic carbon concentration. These results suggest potential approaches for improving E1 removal in wastewater treatment systems.

1. Introduction

The ubiquitous nature¹ and adverse ecological effects^{2–4} of estrogens in surface water are well known. Municipal wastewater treatment plants (WWTPs) are one of the primary sources of estrogens and other micropollutants to the environment.⁵ Improving and optimizing micropollutant removal in municipal WWTPs is therefore critical. Estrone (E1) is of particular concern because it is a major contributor to estrogenic activity in treated wastewater^{6,7} and observed E1 removal rates across WWTPs vary widely.^{8–11} Nevertheless, simple transformations of steroidal estrogens result in a dramatic drop in estrogenicity,¹² precluding the need to stimulate complete mineralization. A more fundamental understanding of micropollutant transformation, and in particular

E1 transformation, is needed to provide the scientific underpinning for improved WWTP operation.

Because micropollutants are, by definition, present at low concentrations that are unlikely to sustain growth, it is thought that microbial transformation of these compounds takes place either fortuitously, not benefiting the microbe and induced by another substrate (cometabolism),¹³ or that these micropollutants are metabolized in conjunction with other substrates that cumulatively enable growth (multiple substrate utilization).¹⁴ As a micropollutant, E1 is unlikely to sustain microorganisms specialized in E1 degradation¹⁵ and so E1 transformation in WWTPs has been attributed to both cometabolism and multiple substrate utilization.^{16–18} While cometabolic transformation of E1 by the ammonia monooxygenase enzyme has been refuted,¹⁹ it is not clear whether the bacteria responsible for E1 transformation in wastewater treatment are capable of utilizing this compound as a carbon and/or energy source, or if transformation is cometabolic. Furthermore, identifying key microbes to study the mechanism for the initial step of E1 transformation in wastewater

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treatment is difficult because of a wide variety of possible microbial candidates,^{20–22} strain-level E1 transformation ability,^{21,23} and possible initial transformation products that do not require carbon uptake.²⁴

Distinguishing between the mechanisms of cometabolism and multiple substrate utilization is not merely a theoretical exercise, because either would have important and different implications for process optimization. Biomass exposure to E1 and/or other organic carbon substrates will result in different E1 removal patterns depending on the transformation mechanism. If cometabolism is the key E1 degradation mechanism in wastewater, prior exposure to estrogens will not affect removal rates, but the presence of cometabolites will be crucial. Alternatively, if multiple substrate utilization is the key mechanism, exposure to E1 will provide a theoretical competitive advantage, as has been suggested by others,²⁵ though it may not be large enough to detect. Additionally, organic carbon exposure would affect E1 removal by multiple substrate utilizers, theorized to be slower growers,²⁶ resulting in beneficial or harmful community selection. In fact, a previous study showed supporting evidence for this with organic carbon having positive effects *via* biomass growth but eventual selection against E1 degraders after repeated doses of high organic carbon concentrations.²⁷

The purpose of this research was to determine which mechanism, cometabolism or multiple substrate utilization, controls the transformation of E1 and to explore the effects of biomass exposure to E1 or other organic carbon substrates on E1 transformation. Due to the aforementioned difficulties of identifying key E1 degrading bacteria, changes in removal rates and patterns in mixed-microbial communities were used to identify the key mechanism. To accomplish this, loss of E1 was measured to obtain the desired information regarding the initial step of E1 transformation. Two sets of studies were performed. First, biomass generated in a membrane bioreactor (MBR) with and without exposure to E1 was used in kinetic studies to determine whether cometabolism or multiple substrate utilization was the key mechanism involved in E1 transformation. Next, experiments were performed to determine if the adverse selection effects observed in a previous study conducted in our laboratory²⁷ could be mitigated by reducing organic carbon concentrations or varying the period between feeding cycles. Additionally, transformation rates during feast and famine periods during these cycles were compared. Together, these studies provide a much clearer picture of E1 removal in wastewater treatment systems.

2. Materials and methods

2.1 Chemicals and synthetic wastewater

E1 and deuterated or ¹³C-labeled E1 were obtained from Sigma and Cambridge Isotopes, respectively. The recipe for synthetic wastewater was adapted from Boeije *et al.*²⁸ and contained (per L): 75 mg urea, 11 mg ammonium chloride, 12 mg sodium uric acid, 25 mg magnesium phosphate

dibasic trihydrate, and 20 mg potassium phosphate tribasic. The synthetic wastewater also contained a carbon source made up of the following (per L, for 100 mg chemical oxygen demand (COD) per L nominal concentration): 6 mg bacteriological peptone, 51 mg sodium acetate, 6 mg dry meat extract, 17 mg glycerine, 21 mg potato starch, and 25 mg low fat milk powder. The carbon source was diluted or concentrated for carbon feeds of various strengths.

2.2 Sludge seed

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

2.3 Batch systems

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

2.4 E1 exposure experiment

Continuous flow MBRs were operated as described in a previous paper²⁷ to culture biomass with and without exposure to E1. Briefly, MBRs (150 mL) were operated with an HRT of 8 h and an SRT of 10 d and fed a synthetic wastewater with a COD of 100 mg L^{–1} for a 30 d period (3 SRTs). MBRs were either exposed or not exposed to E1 over this 30 d period (10 µg L^{–1} E1 in the influent or no E1 in the influent, respectively). Each paired treatment was run in triplicate, with each pair sharing the same sludge seed. Following the 30 d period, the reactors were sacrificed and the biomass recovered by centrifuging. The biomass from each MBR was resuspended in a 1 L batch reactor, and biomass concentration in each reactor was determined *via* a volatile suspended solids (VSS) test.²⁹ Additional biomass was collected for Illumina analysis. Previously collected effluent from the MBRs was used as reactor liquor. This effluent had pH of 7.4, ammonia concentration of 18.2 mg L^{–1}, and COD of 12 mg L^{–1}. E1 was added to each batch reactor at a concentration of 10 µg L^{–1}, and E1 removal was monitored over a 22 h period.

2.5 Feast-famine experiment

The impact of the interval between COD addition on E1 removal was examined using batch reactors. Two experiments were performed. In each experiment a 10 L batch reactor was seeded with cryopreserved activated sludge, synthetic wastewater with 100 mg L^{–1} COD, and 10 µg L^{–1} E1. After a 5 d period, this 10 L reactor was split into 3 sets of triplicate



Table 1 Feeding and kinetic study schedules

	Low organic carbon		High organic carbon	
	Feeding schedule (in mg per L COD)	Total organic carbon added over 6 d cycle (mg COD per L)	Feeding schedule (in mg L ⁻¹ COD)	Total organic carbon added over 6 d cycle (mg COD per L)
1d feed	30 each day	180	100 each day	600
3d feed	90 on days 0, 3, 6, 9, 12	180	300 on days 0, 3, 6, 9, 12	600
6d feed	180 on days 0, 6, 12	180	600 on days 0, 6, 12	600
Kinetic studies	Days 6, 11, 12		Days 6, 12	

1.0 L reactors. The reactors received 10 $\mu\text{g L}^{-1}$ of E1 on day 0 and were operated for 12 d following one of the two feeding regimes shown in Table 1, resulting in exposure to a total of either 180 or 600 mg L⁻¹ COD over a 6 d experimental cycle.

E1 (10 $\mu\text{g L}^{-1}$) was added periodically to each reactor (Table 1) and the E1 removal rate at that time point was determined over a period of 12 h. Biomass was also collected at the time of each E1 kinetic study and quantified by VSS. The experiments were identical with the following exceptions: (1) different total COD concentrations were fed over a 6 d experimental cycle (Table 1) and (2) an E1 transformation study was performed on day 11 during the low organic carbon experiment, but was not performed during the high organic carbon experiment.

2.6 Sample processing and analysis

Detailed procedures for E1 and DNA sampling and analysis are provided in the ESI†. Briefly, E1 samples were processed *via* solid phase extraction and silica gel cleanup based on the method by Ternes *et al.*³⁰ Processed samples were analyzed *via* LC-MS as described in Tan *et al.*²⁷ or by LC-MS-MS with methods adapted from Di Carro *et al.*³¹

DNA samples were extracted using the FastDNA spin kit (MP Biomedicals, Solon, OH), the 16S region amplified using primers described by Muyzer *et al.*,³² and prepared for Illumina sequencing as described by Bartem *et al.*³³ Illumina sequencing was carried out on an Illumina MiSeq platform at the University of Minnesota Genomics Center (Saint Paul, MN, USA). Illumina sequence reads were processed using Quantitative Insights Into Microbial Ecology (QIIME)³⁴ and clustered using uclust.³⁵ Reference sequences for each OTU were compared to the Greengenes reference database.³⁶ UNIFRAC was used for principle coordinate analysis of communities.³⁷

2.7 Data analysis

E1 removal rates and confidence intervals were obtained from the linear regression function in Microsoft Excel, while the student *t*-test (paired *t*-tests of E1 degradation rates for each pair of exposed/non-exposed reactors seeded with the same sludge seed in the E1 exposure experiments, paired *t*-tests to compare reactor performance in feeding cycles over time, pooled *t*-tests to compare different reactor conditions) were performed using the relevant function in the same program.

Lag phases for E1 degradation in the first study were visually distinct, and were identified by plotting degradation curves on a log scale to determine when first-order degradation began. Lag phases for E1 degradation in the second study were determined using residual analysis of linear regressions (log scale), omitting data points until residuals no longer showed a bias.

3. Results and discussion

3.1 Effect of E1 exposure on E1 transformation: evidence for multiple substrate utilizers

E1 transformation took place in both reactors with and without prior exposure to E1 (Fig. 1). Prior E1 exposure, however, correlated with higher removal rates and the lack of a lag phase. The transformation of E1 in all reactors clearly shows that E1 degraders do not require the presence of E1 to grow. A lag phase of 6 h was observed in cultures without prior E1 exposure, suggesting induction of E1 transformation enzymes. Indeed, growth of minor communities is unlikely to explain the end of the lag phase due to the low amount of E1

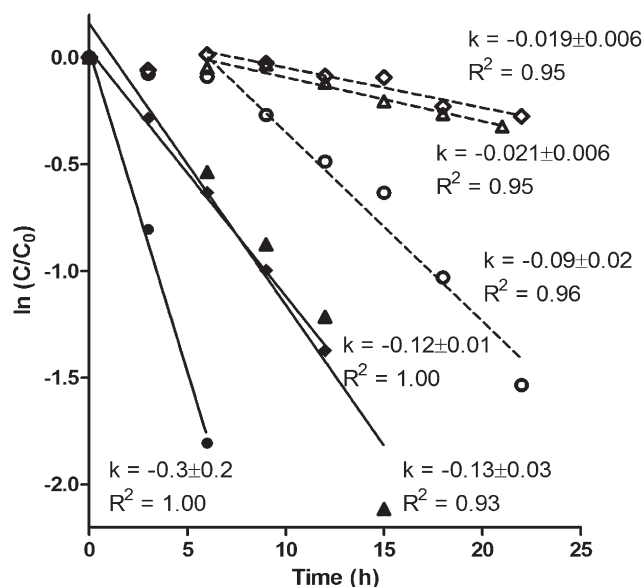


Fig. 1 Transformation of E1 by biomass with (closed symbols) and without (open symbols) prior exposure to E1. Symbol shapes (diamonds, circles, and triangles) represent paired reactor sets started from the same sludge seed. First-order removal rate coefficients are shown as k values. Error values on the k values represent 95% confidence intervals.



present, and in particular, the short time frame over which the lag period ended. This stands in contrast to typical cometabolic patterns where lag phases are not observed apart from inhibition or enzyme induction by cometabolites.^{38–40} Neither would explain the presence or absence of lag phase in our study as reactor liquor was identical across reactors. The higher E1 removal rates, shown through paired *t*-tests, in biomass with prior exposure to E1 ($P = 0.05$) suggests that either (1) prolonged exposure to E1 resulted in higher up-regulation of E1 transformation enzymes than the short-term exposure achieved, and/or (2) the ability to degrade E1 provided a competitive advantage within some niche, resulting in higher growth than would be expected based on the energy available in E1 alone. Both explanations point toward metabolic transformation of E1.

Principal coordinate analysis of Illumina sequences from the mature MBRs, shown in Fig. 2, showed no broad community effects from E1 exposure. Instead, the initial sludge seed appeared to drive variation in communities (Fig. S1†). Analysis of operational taxonomic units at the genus level and at the species level for abundant genus (data not shown), did not show enrichment resulting from E1 exposure, suggesting that (1) exposure to E1 resulted in up-regulation of transformation enzymes rather than a larger population of E1 degraders, (2) E1 degraders are a very small fraction of the population, and/or (3) multiple genus/species may degrade E1. The inability to detect enrichment of rare OTUs is unsurprising, due to the large number of reactor replicates that would have been required.⁴¹

The E1 degradation patterns observed for communities with and without prior E1 exposure demonstrate that multiple substrate utilization is involved in the transformation of E1. This hypothesis has been suggested by others.^{25,26,42} Prior evidence for the role of multiple substrate utilization in E1 transformation includes: higher degradation rates associated with municipal wastewater biomass compared to industrial wastewater biomass;⁴³ improved E1 transformation with

extended exposure to E1;²⁵ possible metabolic transformation of E1;⁴⁴ and higher rates of estrogen degradation in the presence of diverse food sources, which may favor multiple substrate utilization.⁴² The results shown herein, however, are the first that we are aware of that directly compare the effect of E1 exposure on similarly cultured biomass, providing strong evidence for the role of multiple substrate utilization in the biodegradation of E1.

3.2 Effect of carbon exposure on E1 transformation

Fig. 3 shows that longer intervals between feeding cycles were beneficial for reactors receiving higher COD loads, suggesting that feeding cycles can be important for selection of E1 degraders. Among the reactors receiving higher COD loads, those fed every 6 d were the only reactors to have a statistically significant increase in overall E1 removal rates between days 6 and 12 ($P = 0.018$). Biomass normalized rates for this set of reactors also appeared to increase ($P = 0.073$). This suggests that the change in performance is not a result of only biomass growth, but also was a result of community development. At day 12, these reactors had significantly higher removal rates than those fed every day ($P = 0.002$) and rates also appeared higher than those fed every 3 d ($P = 0.057$).

Conversely, among the reactors receiving lower COD loads, the longer interval between feeding did not confer any advantage, suggesting that the importance of feeding cycles for selection of E1 degraders depends on the abundance of food. Overall and biomass-normalized removal rates were similar across these reactors on day 12 ($P = 0.32$ and 0.75 respectively). For this group of reactors, only those fed daily showed an increase in overall E1 removal rates ($P = 0.017$). This change appears to be attributable to the increase in biomass, because biomass-normalized removal rates did not change ($P = 0.85$). Additionally, biomass-normalized removal rates dropped between days 6 and 12 for the 6 d feed reactors ($P = 0.020$). It is not clear why this occurred, but overall removal rates did not change.

Famine conditions appeared to induce a lag phase prior to E1 removal, suggesting that exposure to some minimal level of organic carbon was beneficial to E1 transformation. A clear 4 h lag phase was observed in E1 transformation experiments for the 6 d feed reactors on day 11; 2–4 h lag phases seemed to occur for the 3 d feed reactors as well (Fig. S4†). No comparable lag phases were observed for any reactors during feast periods (Fig. S2–S3†). In contrast to lag phase effects, the presence of other carbon sources did not appear to affect the rate of E1 transformation, because the removal rate for a given reactor was similar on days 11 (famine) and 12 (feast). While the presence of a certain low level of organic carbon is beneficial for inducing the transformation of E1, there were no additional benefits, particularly in terms of removal rates, provided by higher organic carbon concentrations.

The difference in E1 transformation behavior in the presence and absence of exposure to other carbon sources is

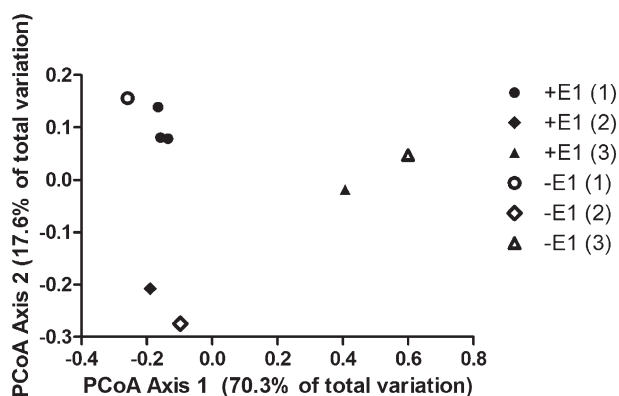


Fig. 2 Principal coordinate analysis of Illumina sequences of microbial communities with and without prior exposure to E1. Closed symbols represent biomass with E1 exposure and open symbols represent biomass without E1 exposure. One sample (+E1 (1)) was analyzed in triplicate. Reactors were run in pairs,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



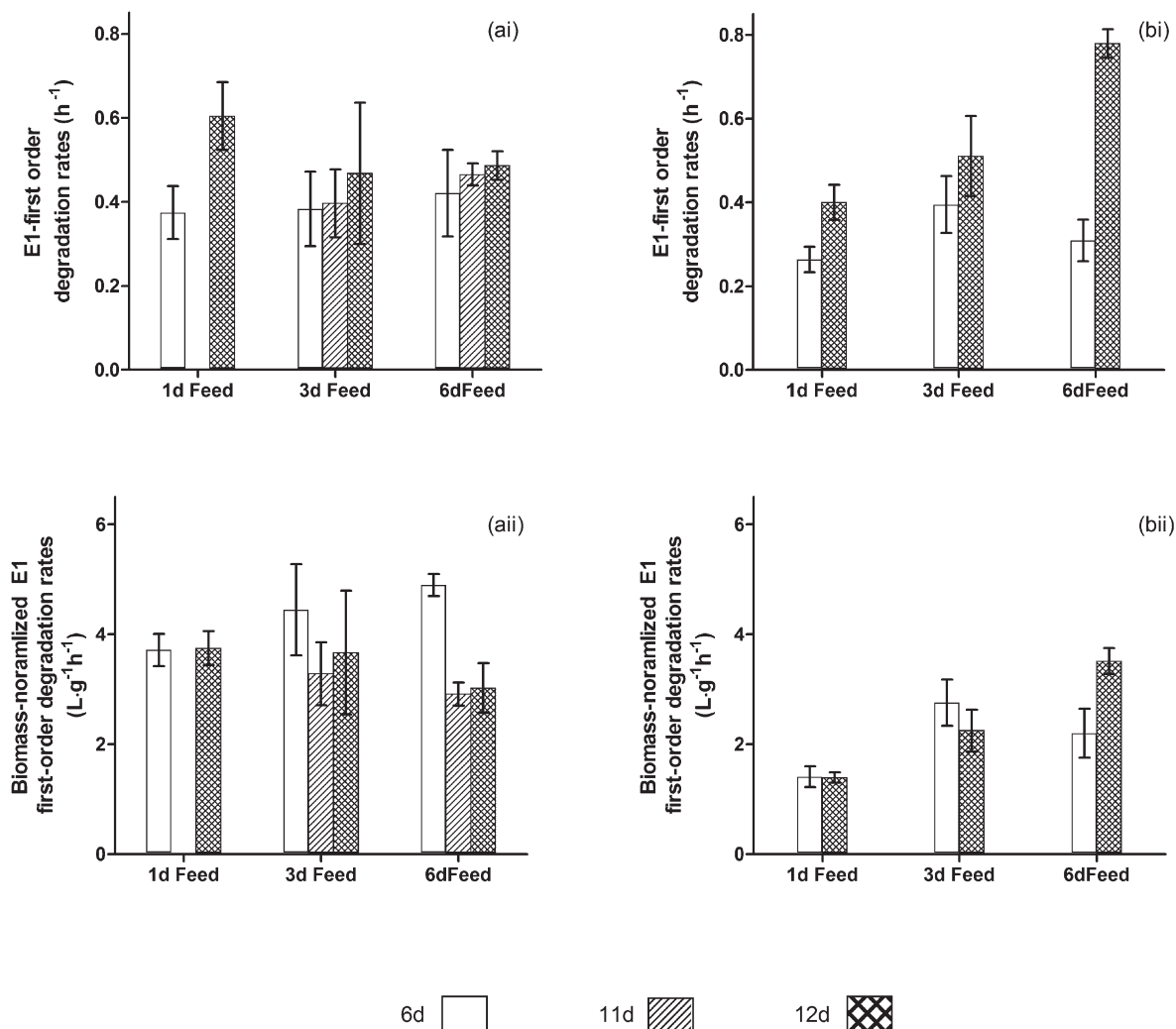


Fig. 3 E1 removal rates in feeding cycle experiments. Figures (ai) and (bi) show overall removal rates for reactors receiving low and high organic carbon loads, respectively, while figures (aii) and (bii) show removal rates normalized to biomass concentrations (as measured by VSS) for reactors receiving low and high organic carbon loads, respectively. Error bars represent the standard deviation for triplicate reactors.

further evidence of multiple substrate utilization of E1 and gives further insight into this mechanism. As in the previous experiment, if cometabolism were the mechanism of transformation, no lag phase would be expected during the famine period. Additionally, during this period, slower removal rates or the absence of E1 transformation might be expected as a result of a lack of cometabolites. As such, the similarities in removal rates between day 11 (famine) and 12 (feast) are consistent with multiple substrate utilization. We had not predicted, however, a lag phase during famine conditions based on this mechanism. At least one other study has observed a similar reduced lag phase for micropollutant transformation not attributable to microbial growth and following exposure to supplementary organic carbon.⁴⁵ It is known that oligotrophic stress can cause de-repression of the catabolome,⁴⁶ facilitating multiple substrate utilization. The prevailing view of this behavior is that of multiple substrate utilizing bacteria producing a large variety of enzymes at all times to scavenge food.^{15,46} We hypothesize that in addition to de-repression

and constant production of a variety of enzymes, exposure to carbon sources may up-regulate the production of this suite of enzymes. Further work needs to be performed to explore and confirm this idea, which would lead to a better understanding of the metabolic activity of bacteria under low organic carbon conditions.

Recovery time from exposure to high concentrations of organic carbon may be important for the selection of E1 degraders. In a previous paper,²⁶ we observed that repeated exposure to high levels of organic carbon selected against E1 degraders. We had also observed that cultures typically took between 3–5 d to develop E1 degrading capabilities. Consequently, these microbes might be outcompeted by faster growing bacteria when subjected to daily feeds. The current study tested the hypothesis that these effects could be mitigated by longer intervals between feeding, and the improved performance of the reactors receiving the higher COD load at 6 d intervals is consistent with this idea. A possible alternative is that the longer interval in feeding cycles may have



promoted cell death and generated microbially derived organic carbon that may also enhance the activity of E1 degraders.⁴⁷ The reactors exposed to lower levels of organic carbon did not seem to be subject to the same selective pressures. Interestingly, the reactors receiving lower levels of organic carbon had higher biomass to substrate yields than their counterparts (Table S1†). This phenomenon, observed previously by others as well,⁴⁸ may be associated with lower growth rates.^{48,49} If so, this would be consistent with the idea of E1 degraders requiring conditions that favor slower growing bacteria.

3.3 E1 and carbon exposure: strategies for optimizing E1 transformation

Optimizing E1 transformation requires generating a sufficient quantity of biomass while maintaining a selection pressure for E1 degraders. Understanding that these bacteria are multiple substrate utilizers rather than specialist degraders or cometabolizers points us in certain directions for achieving optimization. This study illustrates that carbon and E1 exposure are critical for selecting for E1 degrading bacteria. While it is not practical to control E1 exposure in wastewater treatment systems, organic carbon concentrations are a key operating parameter. In this study, we observed that reactors receiving lower COD loads tended to have higher biomass-normalized removal rates. Conversely, the highest overall E1 removal rates (not biomass normalized) were observed in the 6d feed reactors receiving higher COD loads, in which more biomass was present. This is consistent with our previous paper,²⁷ that showed that high COD concentrations, rather than high COD loads, select against E1 degraders. Conventional continuous stirred tank reactors can achieve high COD loads concomitant with low COD concentrations. While CSTRs are more typically used for industrial wastewater treatment to mitigate against toxicity, they could also have a role in improving E1 transformation in municipal wastewater. An alternate strategy of recovery time between feeding periods was explored in this study, and also appears to be a feasible option. This approach, however, may cost more in terms of sludge storage requirements. In addition to E1 and organic carbon exposure, there are further factors that may influence selection of E1 degraders that need to be further explored, including the diversity of food sources, as theorized by Racz *et al.*,⁴² and the quality of organic carbon available,⁴⁷ which could have selection effects on these slower growing, multiple substrate utilizing bacteria and could also suggest alternative design approaches such as membrane bioreactors.

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