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Identifying Functional Groups that Determine Rates of Micropollutant Biotransformations Performed by Wastewater Microbial Communities

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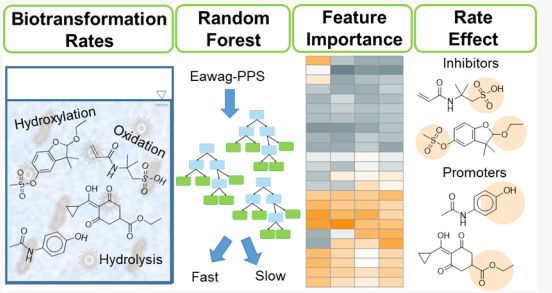
ABSTRACT: The goal of this research was to identify functional groups that determine rates of micropollutant (MP) biotransformations performed by wastewater microbial communities. To meet this goal, we performed a series of incubation experiments seeded with four independent wastewater microbial communities and spiked them with a mixture of 40 structurally diverse MPs. We collected samples over time and used high-resolution mass spectrometry to estimate biotransformation rate constants for each MP in each experiment and to propose structures of 46 biotransformation products. We then developed random forest models to classify the biotransformation rate constants based on the presence of specific functional groups or observed biotransformations. We extracted classification importance metrics from each random forest model and compared them across wastewater microbial communities. Our analysis revealed 30 functional groups that we define as either biotransformation promoters, biotransformation inhibitors, structural features that can be biotransformed based on uncharacterized features of the wastewater microbial community, or structural features that are not rate-determining. Our experimental data and analysis provide novel insights into MP biotransformations that can be used to more accurately predict MP biotransformations or to inform the design of new chemical products that may be more readily biodegradable during wastewater treatment.



KEYWORDS: *biotransformation, random forest, micropollutants, wastewater, biotransformation kinetics, biotransformation products, microbial community*

INTRODUCTION

The influent to municipal wastewater treatment plants (WWTPs) contains hundreds of organic micropollutants (MPs) including pharmaceuticals, personal care products, pesticides, and industrial chemicals.[1−4] Wastewater microbial communities residing in the biological processes of WWTPs can biotransform some MPs to variable extents, but it is difficult to predict which types of MPs will be biotransformed in any specific WWTP or the extent of biotransformation.[5−8] At the fundamental level, MP biotransformation depends on the presence of specific enzyme catalysts and a chemical structure that is amenable to biotransformation by one or more specific enzyme catalyst. However, it has been difficult to identify causal associations between specific enzyme catalysts and MP biotransformations because MPs are typically present in wastewater at very low concentrations and in the presence of relatively high concentrations of readily biodegradable organic carbon substrates; as a result, MPs are unlikely to serve as growth substrates, and instead, their biotransformation is frequently attributed to cometabolic processes.[2,9,10]

Without a known link to specific enzyme catalysts, the prediction of MP fate during wastewater treatment is generally based on links between chemical structures and specific types of biotransformations. For example, the Eawag-pathway prediction system (Eawag-PPS)[11,12] relies on a curated database of 1503 literature-reported biotransformations to derive 249 biotransformation rules (btrules) that predict specific biotransformations at certain functional groups. Similarly, the BIOWIN modules within EPISUITE[13] estimate the probability of rapid biodegradation of organic chemicals with empirical constants derived for certain functional groups based on the results of standardized biodegradation tests and regression techniques. Despite the value of these tools, the majority of data used to develop them were derived from either pure culture systems and/or in a mineral medium in which the test chemical was present as the primary carbon source at a high concentration. These conditions are selected to induce metabolic processes and are unlikely to represent MP

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biotransformations performed by wastewater microbial communities.

In recent years, a number of studies have reported on the biotransformation of certain types of MPs in self-consistent test systems that are more representative of WWTPs.[8,14−18] Data from these studies have enabled a more generalized understanding of the types of biotransformations performed by wastewater microbial communities. For example, a study of amide-containing MPs revealed differences in biotransformations driven by the extent of N-substitution,[19] and a study of amine-containing MPs revealed a set of previously unreported biotransformations involving N-acylation reactions.[20] However, few studies have considered trends in biotransformations performed by wastewater microbial communities simultaneously among multiple functional groups as a means to more broadly generalize our understanding of MP biotransformations. Machine learning algorithms have been increasingly used in the field of environmental science and engineering and provide a new approach to analyzing complex environmental systems where patterns across multiple variables are not easily recognizable.[21] We expect that coupling broad and self-consistent MP biotransformation datasets with emerging machine learning algorithms will lead to novel insights into the ways in which MPs are biotransformed during wastewater treatment.

The objectives of this study were to: (i) estimate the biotransformation rate constants for 40 MPs in bioreactors seeded with four independent wastewater microbial communities; (ii) identify the biotransformation products for the 40 MPs in each of the four independent wastewater microbial communities as a means to infer specific biotransformations; and (iii) use machine learning algorithms to discover links between the chemical structure or observed biotransformations and estimated biotransformation rates. We used random forest models to classify the estimated biotransformation rates for each of the four wastewater microbial communities as "fast" or "slow" based on the presence and absence of specific functional groups or observed biotransformations. Our experimental data and analysis allowed us to identify 30 functional groups that we define as either biotransformation promoters, biotransformation inhibitors, structural features that can be biotransformed based on uncharacterized features of the wastewater microbial community, or structural features that are not rate-determining.

MATERIALS AND METHODS

MP Selection. We selected 40 MPs that contain either amide, amine, ester, or ether functional groups for this study. These four functional groups were selected because many MPs contain one or more of these functional groups, and we expect biotransformation at these locations to be an important determinant of MP fate during wastewater treatment.[19,20] A list of the selected MPs categorized by their major functional group along with their commercial use, CAS number, and chemical structure is provided in Table S1 of the Supporting Information (SI). A stock solution of each MP was prepared at 1 g L^{-1} in the respective solution solvent; a list of MP suppliers, purities, and solution solvents is provided in Table S2. A MP solution mixture was then prepared with each MP present at 90.9 mg L^{-1} in a solution of 6.6:3.3:1 MeOH:H_2O:EtOH. The MP solution mixture was used for preparing calibration standards and for spiking incubation experiments and was stored at −20 °C until use. Details of the preparation of the MP solution mixture and suppliers of all solvents and consumable reagents are provided in the Supporting Information.

Wastewater Microbial Communities. We sampled wastewater microbial communities from three WWTPs in New York State. The WWTPs were selected to represent different types of aerobic biological processes including conventional activated sludge (WWTP1), a sequencing batch reactor (WWTP2), and conventional activated sludge with an extended aeration system (WWTP3). From WWTP1, we sampled in the winter (WWTP1_win) and summer (WWTP1_sum) to assess temporal differences in the functioning of a wastewater microbial community from a single WWTP. We used a 0.5 L plastic bucket affixed to a 3 m aluminum pole to sample wastewater microbial communities from the center of each aeration basin at a depth of at least 0.3 m. Sampled wastewater microbial communities were transferred to two 1 L amber glass bottles, capped, and immediately transported to our laboratory where 0.5 L of each sample was then combined into a single 1 L amber glass bottle and prepared for incubation experiments. More details of WWTP sampling dates and operating conditions are provided in Table S3.

Incubation Experiments. All incubation experiments were conducted in 100 mL amber glass reactors (Corning) and in triplicate as previously described.[22−24] Briefly, triplicate reactors were prepared by combining 28.8 mL of a wastewater microbial community with 1.2 mL of phosphate buffer (pH 7.0, concentration: 500 mM). The reactors were then spiked with the MP solution mixture to achieve a starting concentration of 100 μg L^{-1} for each MP and placed on a rotary shaker at 20 °C; all incubation experiments were spiked within 5 h of sample collection at the respective WWTP. We note that a starting concentration of 100 μg L^{-1} is on the high end of the range of MP concentrations expected in the influent of WWTPs[25] but was essential for this study to allow for the detection of biotransformation products that are formed in relatively low abundance.[22] We further note that our experimental procedure results in trace amounts of organic solvent (<0.1%) in our bioreactors; previous studies have demonstrated that this amount of organic solvent has no effect on the biotransformation of certain tested MPs.[19,26] We collected 0.5 mL samples from each reactor after 5 min, 2 h, 6 h, 18 h, and 30 h, transferred the samples to a 1.5 mL centrifuge tube (Eppendorf), and centrifuged the samples at 13,000 rpm for 5 min at 4 °C. Then, 400 μL of the supernatant was transferred to a 2 mL amber glass vial (VWR), capped, and stored at −20 °C until analysis. We note that the incubation experiment from WWTP3 was sampled at 42 h after spiking instead of at 30 h. Control experiments were conducted with the same procedure, except control reactors were autoclaved twice (120 °C, 1.3 bar, 20 min, 4 h apart) and spiked 24 h after the start of the active incubation experiment.

Sample Analysis. We adopted a previously described analytical method for MP quantification.[3,22,23] Briefly, we used reversed-phase liquid chromatography (Ultimate 3000, Thermo Scientific) coupled to high-resolution quadrupole-orbitrap mass spectrometry (HRMS, QExactive, Thermo Scientific) with 20 μL injections of samples stored at 4 °C during the analysis. Samples were separated using a mobile phase gradient consisting of LC−MS grade water (OmniSolv, 58201, solvent A) and methanol (OmniSolv, 58215, solvent B)—both containing 0.1% (v/v) formic acid—over an XBridge C18

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column (Waters, 186003021, particle size: 3.5 μm, flow rate: 0.2 mL/min, gradient properties: 0−5 min: 5% B, 5−21 min: 5% B − 95% B (linear increase), 21−25 min: 95% B, 25−30 min: 5% B). We collected full-scan mass spectrometry (MS) data (100−800 m/z, resolution 140,000) in electrospray ionization (ESI) mode using rapid polarity switching. Data-dependent MS^2 scans were triggered using the masses (m/z) of all parent MPs and their predicted biotransformation products with dynamic exclusion set at 6 s. For absolute quantification of MPs, we analyzed a calibration series (concentration range: 2−100 μg/L) prepared in a filter-sterilized, matrix-matched extract (activated sludge centrifuged and filtered with a 0.22 μm PVDF syringe filter) that was autoclaved twice. Concentrations were then calculated using peak areas obtained with Xcalibur Quan Browser (Thermo Scientific, Version 3.1). Limits of quantification were determined by the lowest linear calibration point with five sticks and the presence of a diagnostic fragment. More details of the analytical and acquisition parameters for each of the 40 MPs are provided in Table S4 of the Supporting Information.

Biotransformation Product Analysis. We used the Eawag-PPS[11] to generate a list of predicted biotransformation products for each of the 40 MPs as previously described.[22,27,28] The Eawag-PPS uses a database of btrules to predict biotransformation products based on recognized structural features of the parent MP (e.g., bt0024 is triggered by an ester functional group and results in the prediction of ester hydrolysis products). A list of relevant btrules, triggering functional groups, product functional groups, reaction types, and aerobic likelihoods is provided in Table S5; the btrules triggered by each of the 40 MPs are provided in Table S1. We used the Eawag-PPS to predict initial biotransformations for each MP with relative reasoning turned off and including all aerobic likelihoods. For amine-containing MPs, we also manually predicted the structures of the acylation products described in the study by Gulde et al.[20] We generated SMILES for each of the predicted biotransformation products and used JChem for Excel (2019 version 19.26.0.571) to calculate the exact mass of the $[M + H]^+$ and $[M - H]^-$ ions for each predicted TP. We then used Xcalibur Qual Browser (Thermo Scientific, Version 3.1) to manually screen the HRMS acquisitions for evidence of biotransformation product formation. Evidence of biotransformation product formation includes: (i) peak areas greater than 1E5; (ii) reasonable peak shape; (iii) presence of a peak in the active reactors and absence of a peak (or a peak area less than 1E4) in control reactors; and (iv) increasing or increasing and then decreasing peak area over time.[20] The resulting list of candidate biotransformation products was further vetted by comparing MS spectra and MS^2 fragmentation data to theoretical MS spectra or *in silico* MS^2 fragments generated by Mass Frontier (ThermoScientific). We assigned confidence levels to our final list of putative biotransformation products based on conventions established in the field[29] as follows: all experimental and analytical evidence supports a single biotransformation product structure (level 2); all experimental and analytical evidence supports multiple biotransformation product isomers (level 3); experimental evidence supports the biotransformation product structure but no MS^2 fragments match in Mass Frontier (level 3a); experimental evidence supports the biotransformation product structure but noisy MS scan (level 3b); experimental evidence supports the biotransformation product structure but no MS^2 data are acquired because of the intensity of parent ions (level 3c).

Data Analysis and Random Forest Classification Models. We used data from the active and control incubation experiments to estimate pseudo-first-order biotransformation rate constants for each MP in each of the four experiments as previously described[19,26] and detailed in the Supporting Information. We then used random forest classification models to identify predicted btrules or observed biotransformations that are important in classifying the biotransformation rates of the MPs in each wastewater microbial community.[30] We opted to use a decision tree-based algorithm because of the nonlinear dependence of biotransformation rates on chemical characteristics.[30,31] All random forest classification models were created using the R computing environment[32] in RStudio[33] (R version 4.0.4, RStudio version 1.1.463) using the package *randomForest*.[34,35] We used binary matrices (1 for presence and 0 for absence) of either predicted btrules or observed biotransformations for each MP in each wastewater microbial community as the predictor variables used for model classifications. If multiple biotransformations could be assigned to one observed biotransformation product (e.g., monohydroxylations), all possible btrules for that observed biotransformation product were given a value of 1. We selected a biotransformation rate constant of 0.5 d^{-1} as a threshold to define "fast" and "slow" biotransformations. This threshold defines a rate that results in approximately 50% disappearance of a MP over the course of our incubation experiments. Model performance was evaluated using prediction accuracy and out-of-bag (OOB) error estimates resulting from bootstrap aggregation as a means of cross-validation. We assessed the sensitivity of the model accuracy and OOB error by systematically changing the number of variables to be randomly selected at each tree split (*mtry*) from 1 to 64 and the number of trees (*ntree*) produced in the model ensemble from 1 to 1000; changes in these parameters resulted in only small changes in OOB error; therefore, typical values were chosen for the final models (*mtry* = 8 and *ntree* = 1000). We also evaluated the sensitivity of the model accuracy and OOB error by systematically changing the biotransformation rate constant threshold from 0.1 to 1.0 d^{-1} and confirmed that a biotransformation rate constant threshold of 0.5 d^{-1} was appropriate for this study.

RESULTS AND DISCUSSION

Examination of Progress Curves. The raw experimental data for the biotransformation of the 40 MPs spiked in reactors seeded with the four wastewater microbial communities are plotted as progress curves (concentration versus time) in Figure S1 through Figure S4. Several important observations are worth noting. First, the biotransformation experiments were repeatable as demonstrated by nearly identical progress curves across triplicate reactors with no apparent biodegradation lag times. Second, no significant abiotic transformations were observed for the MPs in the control reactors, with the exception of bupropion, which was abiotically transformed in all of the control reactors. Third, the progress curves of phthalimide and acetylsalicyclic acid exhibit erratic concentrations in all of the bioreactors, and the progress curves of sertraline exhibit zero or negative concentrations in bioreactors seeded from WWTP3; therefore, phthalimide and acetylsalicyclic acid are not included in further analyses and sertraline is not included in further analyses of WWTP3. Finally, several other MPs either exhibit unexpectedly high concentrations in

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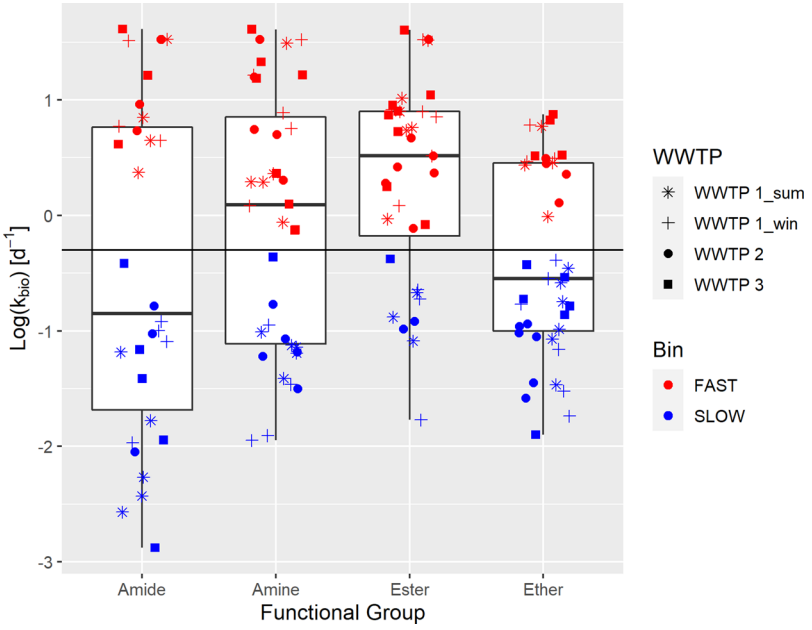

Figure 1. Distribution of pseudo-first-order biotransformation rate constants of MPs organized by their primary functional group. The solid horizontal line in the middle of each box represents the median, and box ends are the 25th and 75th percentiles. The threshold selected to define relatively fast and relatively slow biotransformation rate constants (0.5 d^{-1}) is marked on the plot with a solid line.

the bioreactors (e.g., 2,6-dimethoxyphenol and serotonin in WWTP1_win, WWTP1_sum, and WWTP2) or >50% adsorption in the control reactors (e.g., amitriptyline and haloperidol); however, these phenomena did not affect our ability to estimate pseudo-first-order biotransformation rate constants (because pseudo-first-order rate constants are not influenced by the initial concentration[19,26]) or identify putative biotransformation products.

Biotransformation Kinetics. We used the raw experimental data to estimate pseudo-first-order biotransformation rate constants as described in eq S1. The average and standard deviation of the estimated biotransformation rate constants are provided in Table S6. When we observed complete disappearance of an MP by the second sampled time point (e.g., for acetaminophen, DCD, and serotonin), the estimated biotransformation rate constants were in the range of 30 to 50 d^{-1}; because we cannot be certain that complete biotransformation did not occur more rapidly than this, we report the estimated rate constant as >30 d^{-1}. The average values of the estimated biotransformation rate constants are presented in box plots organized by their primary functional group in Figure 1.

Based on data from previous studies, we hypothesized that amide- and ester-containing MPs would be biotransformed rapidly and that amine- and ether-containing MPs would be biotransformed slowly.[19,36−38] To test this hypothesis, we selected a biotransformation rate constant of 0.5 d^{-1} as a threshold to define relatively fast and slow biotransformations. We found that, in aggregate and in agreement with our expectation, ester-containing MPs were biotransformed relatively fast and that ether-containing MPs were biotransformed relatively slowly. However, in contrast to our expectation, amide-containing MPs were biotransformed relatively slowly and amine-containing MPs were biotransformed relatively fast. We attribute these deviations from our expectations to two separate phenomena. First, the distribution of biotransformation rate constants for the amide-containing MPs is bimodal with three MPs (acetaminophen, gabapentin lactam, and propachlor) undergoing fast biotransformations across all wastewater microbial communities and the remaining MPs mostly undergoing slow biotransformations. Therefore, although we selected amide-containing MPs that had relatively simple structures, there are electronic or steric factors that limit the biotransformation rates of some of the selected amide-containing MPs.[19] Second, the distribution of biotransformation rate constants for the amine-containing MPs is skewed by data from WWTP3, which had estimated biotransformation rate constants >0.43 d^{-1} for all of the amine-containing MPs. Therefore, we conclude that catalytic activity unique to the wastewater microbial community derived from WWTP3 is driving the unexpected aggregate findings for the amine-containing MPs. We suspect that this phenomenon can be explained by the higher solid retention time in the extended aeration system in WWTP3.[14]

Despite these aggregate observations, the biotransformation rate constants among MPs in each of the primary functional group categories span three to four orders of magnitude, and there are individual MPs in each functional group category that have biotransformation rate constants that can be classified as very fast (>5 d^{-1}) or very slow (<0.05 d^{-1}). Additionally, there are MPs from all primary functional group categories that exhibit both fast and slow biotransformation rate constants across the four incubation experiments; these include DEET (amide-containing MP), albuterol, and pseudoephedrine (amine-containing MPs), gibberellic acid and warfarin (ester-containing MPs), and gemfibrozil (ether-containing MP). Together, this analysis of biotransformation rate constants demonstrates that the primary functional groups are, alone, not rate-determining and that other features of the MP structure or the microbial community are important in determining biotransformation rates across wastewater microbial communities.

MP Biotransformations. We next aimed to identify biotransformation products for each of the MPs as a means

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Table 1. List of Biotransformation Products Identified in Each Wastewater Microbial Community with Citations to Previous Reports in the Literature

MP	btrule	Reaction	WWTP1_win	WWTP1_sum	WWTP2	WWTP3	MP	btrule	Reaction	WWTP1_win	WWTP1_sum	WWTP2	WWTP3
DEET[19]	bt0012 bt0013 bt0036 bt0334	Monohydroxylation		X	X		DCD	bt0024	Ester hydrolysis	X	X	X	X
	bt0012 bt0013 bt0036 bt0334	Monohydroxylation	X	X	X	X	Dehydroacetic Acid	bt0241	Monohydroxylation	X	X	X	
	bt0243	N-dealkylation	X	X	X	X	Diethyl Phthalate	bt0011 bt0013	Monohydroxylation	X	X	X	X
Propachlor[19]	bt0022	Hydrolytic dehalogenation	X	X	X	X		bt0024	Ester hydrolysis	X	X	X	X
	bt0029	Reductive dehalogenation	X	X	X	X	Dimethyl Phthalate	bt0024	Ester hydrolysis	X	X	X	X
Albuterol	bt0001	Primary alcohol oxidation		X	X	X	Gibberellic Acid	bt0024	Ester hydrolysis		X	X	X
	bt0002	Secondary alcohol oxidation	X	X	X			bt0002	Secondary alcohol oxidation	X	X	X	X
Amitriptyline	bt0242	Monohydroxylation	X	X	X	X	Malaoxon	bt0361	Phosphoester hydrolysis	X	X	X	X
	bt0242	Monohydroxylation	X	X	X	X		bt0024	Ester hydrolysis	X	X	X	X
	bt0242	Monohydroxylation	X	X	X	X	Trinexapac-Ethyl[14,26]	bt0071 bt0241 bt0242 bt0334	Monohydroxylation	X	X	X	
	bt0063	N-dealkylation	X	X	X	X		bt0044	Enol oxidation	X	X		
Haloperidol	bt0063	N-dealkylation	X	X	X	X		bt0024	Ester hydrolysis	X	X	X	X
	bt0063 bt0242	N-dealkylation or monohydroxylation	X	X	X	X	Warfarin	bt0024	Ester hydrolysis	X	X	X	X
	bt0063 bt0242	N-dealkylation or monohydroxylation	X	X	X	X		bt0291	Hydration of double bond	X	X	X	X
	bt0063 bt0242	N-dealkylation or monohydroxylation		X	X	X		bt0241	Monohydroxylation	X	X	X	X
Ketamine	bt0063	N-dealkylation	X	X	X	X	Ethofumesate[26]	bt0023	Ether dealkylation	X	X	X	X
	bt0071 bt0242	Oxidation of saturated ring or monohydroxylation		X	X	X	Gemfibrozil[14,42]	bt0036	Monohydroxylation	X	X	X	X
Methadone	bt0242 bt0333 bt0334	Monohydroxylation	X	X	X	X		bt0012 bt0036 bt0242 bt0332	Monohydroxylation	X	X	X	X
Pseudoephedrine	bt0063	N-dealkylation	X	X	X	X	Mecoprop	bt0005	Dihydroxylation	X	X	X	X
Ritalinic Acid	bt0063 bt0241	N-dealkylation or monohydroxylation	X	X	X	X		bt0012 bt0013 bt0036 bt0333	Monohydroxylation	X	X	X	X
								bt0029	Reductive dehalogenation	X	X	X	X
Sertraline[18]	bt0241	Monohydroxylation	X	X	X	X	Naproxen[43,44]	bt0005	Dihydroxylation	X	X	X	X
								bt0023	Ether dealkylation	X	X	X	X
							Oxybenzone	bt0353	Dihydroxylation	X	X	X	X
								bt0023	Ether dealkylation	X	X	X	X

to confirm the expected biotransformations at the primary functional group or identify other biotransformations. The Eawag-PPS predicted 227 initial biotransformations for 38 of the MPs (phthalimide and acetylsalicylic acid excluded as described in the preceding). We identified 46 biotransformation products for 23 of the MPs with a confidence of level 2 or level 3. The observed biotransformations are summarized in Table 1. Observed biotransformations included the expected amide (bt0067) and ester (bt0024) hydrolyses and amine (bt0063) and ether (bt0023) dealkylations along with at least one amide N-dealkylation (bt0243), monohydroxylation (bt0011, bt0012, bt0013, bt0036, bt0241, bt0242, bt0332, bt0333, and bt0334), dihydroxylation (bt0005 and bt0353), hydrolytic dehalogenation (bt0022), reductive dehalogenation

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(bt0029), alcohol oxidation (bt0001, bt0002, and bt0044), phosphoester hydrolysis (bt0361), and hydration of a double bond (bt0291), all of which are biotransformations that have been previously observed to be performed by wastewater microbial communities.[14,15,18,19,26,39−44] Details of the observed biotransformation products and the analytical data that support their tentative identification are provided in Table S7 through Table S10 and Figure S5 through Figure S50. We note that we were unable to identify biotransformation products for several MPs that exhibited fast biotransformation. This includes acetaminophen, coumarin, gabapentin lactam, serotonin, and 2,6-dimethoxyphenol. This could be the result of biotransformations that were not predicted by the Eawag-PPS, subsequent rapid biotransformation of the initial biotransformation products such that they escape detection in our experiments, or formation of predicted biotransformation products that were not detected by our analytical method (e.g., biotransformation products that have an exact mass (m/z) less than 100 Da are highly polar and are not retained on the analytical column, or are not efficiently ionized during ESI).[45] Finally, as noted in Table 1 and to the best of our knowledge, 37 of the 46 biotransformation products that we identified are reported to be formed in wastewater microbial communities for the first time.

Random Forest Models—Predicted Biotransformations (btrules). Because the four primary functional group categories were not comprehensively predictive of biotransformation rates, we next sought to identify important structural features of the MPs that are predictive of the estimated biotransformation rates in each of the wastewater microbial communities. We used random forest classification models to classify the biotransformation of each MP in each wastewater microbial community as "fast" or "slow" using a 0.5 d^{-1} biotransformation rate constant threshold and a binary matrix of predicted btrules as the predictor variables. The prediction accuracy of the random forest models was high (>95%) although the OOB error ranged between 24 and 42%. Nevertheless, the ensemble of decision trees developed from recursive partitioning on the data provides valuable insight into the relative importance of each btrule in determining the rate of MP biotransformation. The relative importance of each btrule was evaluated using the mean decrease in accuracy (MDA), which is calculated by recording the change in prediction error on OOB samples after permuting every predictor variable in each single tree (see the Supporting Information for more details on MDA). We extracted the MDA values for every btrule in each of the four random forest classification models, aggregated these values into new matrices, and used hierarchical clustering to determine trends among predicted btrules for rate classification across wastewater microbial communities. The results of this analysis are provided in Figure 2 in which we identify two main clusters of btrules.

Cluster 1 contains 16 btrules that were generally important for classifying biotransformation rates across the WWTPs (average MDA = 4.9 ± 2.7), indicating that functional groups triggering these btrules improve the accuracy of random forest model rate classifications. The distributions of rate constants measured for MPs that trigger these btrules are provided in Figure S51. From these distributions, we can see that MPs that trigger these btrules tend to have either mostly fast or mostly slow biotransformation rates. Therefore, the functional groups that trigger these btrules can be categorized as rate-determining biotransformation promotors or rate-determining biotransformation inhibitors, respectively. The clearest examples of this phenomenon are demonstrated by the four btrules in Cluster 1A: bt0064 (triggered by a 1-hydroxy-4-unsubstituted benzenoid), bt0286 (alkylsulfonic acid derivative), bt0029 (organohalide), and bt0242 (secondary aliphatic). All MPs that contain a 1-hydroxy-4-unsubstituted benzenoid moiety and most MPs that contain a secondary aliphatic moiety are biotransformed rapidly (>0.5 d^{-1}) by all of the wastewater microbial communities. We therefore categorize the functional groups linked to bt0064 and bt0242 as rate-determining biotransformation promotors. Conversely, all MPs that contain an alkylsulfonic acid derivative and most MPs that contain an organohalide moiety are biotransformed slowly (<0.5 d^{-1}) by all of the wastewater microbial communities. We therefore categorize the functional groups that trigger bt0286 and bt0029 as rate-determining biotransformation inhibitors. These observations agree with the literature,[31,46,47] and it is validating to identify these four structural features as very important in determining biotransformation rates among the selected MPs performed by wastewater microbial communities. It is important to note that MPs containing functional groups that are categorized as rate-determining biotransformation promotors or inhibitors may actually exhibit relatively slow or fast biotransformations, respectively, as demonstrated in Figure S51 for bt0242 and bt0029. This can be due to the presence of other functional groups in the same MP that could likewise be rate-determining. For example, certain functional groups adjacent to a rate-determining biotransformation promotor may limit MP biotransformations because of steric hindrance, whereas labile functional groups simultaneously present in an

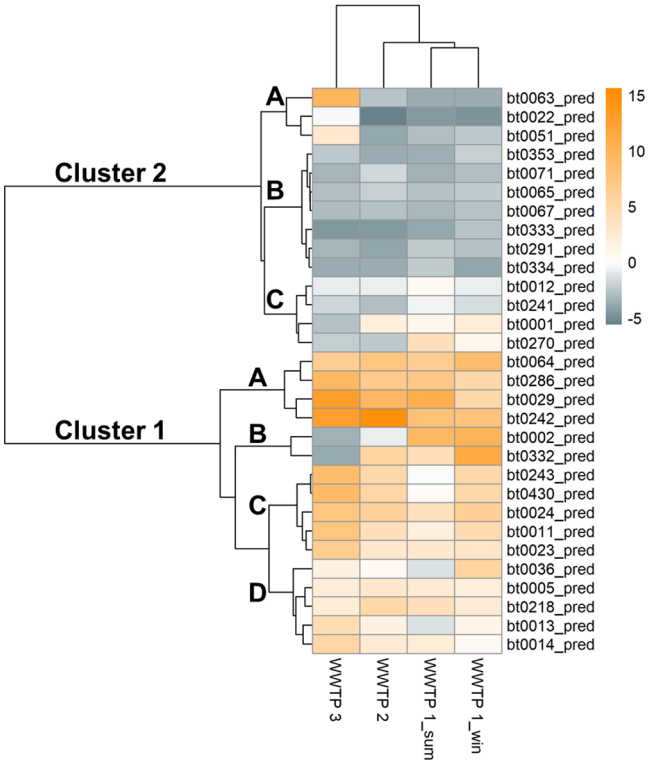

Figure 2. Heatmap of MDA for 30 predicted biotransformation rules with nonzero MDA values. Cells colored on a scale from orange to gray indicate a scale of rule importance from high to low based on accuracies of rate predictions in a specific WWTP model when including each rule.

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MP with a rate-determining biotransformation inhibitor may result in fast biotransformations of that MP. This is precisely why the ensemble of decision trees generated by the random forest models and analysis of MDA is essential to identify rate-determining biotransformation promotors and inhibitors while considering the whole structure of the MP and why the box plots in Figure S51 cannot be interpreted in isolation.

Clusters 1C and 1D likewise contain 10 btrules categorized as rate-determining biotransformation promoters or inhibitors, though their importance is not as high as the btrules in Cluster 1A. The functional groups that trigger bt0024 (ester), bt0011 (unsubstituted benzenoid at the ortho position), bt0005 (vic-unsubstituted aromatic), and bt0014 (1-hydroxy-2-unsubstituted aromatic) are categorized as rate-determining biotransformation promotors, whereas functional groups that trigger bt0243 (N-substituted amide), bt0430 (tertiary amide), bt0023 (ether), bt0036 (aromatic methyl), bt0218 (2-hydroxyethylamine), and bt0013 (unsubstituted benzenoid at the para position) are categorized as rate-determining biotransformation inhibitors. Although some btrules in Clusters 1C and 1D have an almost equal distribution between fast and slow biotransformation rates associated with each rule (e.g., bt0023, bt0013), the information gained from including these rules in the random forest model nevertheless improves MP rate classifications. This analysis confirms that ester groups are important for determining relatively fast biotransformations and that ether groups are important for determining relatively slow biotransformations performed by wastewater microbial communities. Furthermore, this analysis suggests that N-substituents on amide groups can be rate-inhibiting, which helps to explain why the amide-containing MPs selected for this study have a bimodal distribution of biotransformation rate constants. Finally, it is interesting to note that bt0011 (unsubstituted benzenoid at the ortho position) is categorized as a rate-determining biotransformation promotor and bt0013 (unsubstituted benzenoid at the para position) is categorized as a rate-determining biotransformation inhibitor. These rules are triggered by the same structural feature (unsubstituted benzenoid) but result in a monohydroxylation at the ortho or para positions, respectively. Our findings suggest that MPs with a benzenoid that is unsubstituted at the ortho position are likely to be biotransformed at a faster rate than MPs with a benzenoid that is unsubstituted at the para position.

Cluster 1B contains two btrules that are important for determining biotransformation rates in WWTP1_win, WWTP1_sum, and WWTP2 but not in WWTP3. The functional groups that trigger bt0002 (secondary alcohol) and bt0332 (aliphatic methyl [H0]) can generally be categorized as rate-determining biotransformation inhibitors, but MPs that trigger these btrules are biotransformed rapidly in WWTP3. This interesting observation supports our conclusion that the wastewater microbial community derived from WWTP3 has unique catalytic activity that promotes biotransformations associated with specific types of structural features. Therefore, we conclude that the functional groups triggering bt0002 and bt0332 are sometimes rate-determining biotransformation inhibitors, but some wastewater microbial communities can have catalytic enzymes to transform MPs that contain these functional groups under certain conditions.

Cluster 2 contains 14 btrules that are generally not important for classifying biotransformation rates across the WWTPs (average MDA = −2.0 ± 1.5), indicating that the functional groups triggering these btrules are not rate-determining. The distributions of rate constants measured for MPs triggering these btrules are provided in Figure S52. These distributions again demonstrate that MPs that trigger these btrules can have fast or slow biotransformation rates. However, the functional groups triggering the btrules in Cluster 2 (and Cluster 2B in particular) are not important for determining these distributions. Nevertheless, there are interesting nuances that can be discovered in these distributions, particularly for btrules contained in Cluster 2A and Cluster 2C. Cluster 2A contains three btrules that are important for determining biotransformation rates in WWTP3. The MPs that trigger bt0063 (amine), bt0022 (halomethyl or dihalomethyl derivative), and bt0051 (2- or 3-substituted carboxylate) tend to have biotransformation rates that are both fast and slow across WWTP1_win, WWTP1_sum, and WWTP2 but are biotransformed rapidly in WWTP3. Cluster 2C contains four btrules that are important for determining biotransformation rates in WWTP1_win and WWTP1_sum. The MPs that trigger bt0012 (unsubstituted benzenoid at the meta position), bt0241 (tertiary aliphatic), bt0001 (primary alcohol), and bt0270 (aromatic methyl derivative) tend to have biotransformation rates that are both fast and slow across WWTP2 and WWTP3 but are biotransformed rapidly with more consistency in WWTP1_win and WWTP1_sum. Therefore, we conclude that these btrules that show variable levels of importance across the four wastewater microbial communities represent structural features that are amenable to variable degradation based on the catalytic activity of the microbial community. Finally, we note that bt0067 (amide hydrolysis) is generally unimportant for predicting biotransformation rates within the four wastewater microbial communities. This is notable because it challenges our initial hypothesis but is in agreement with recent literature that demonstrates the importance of structural features around the amide bond in determining the extent of amide hydrolysis.[22,48]

Random Forest Models – Observed Biotransformations. We were also interested in determining whether the biotransformations that we observed would be more predictive of biotransformation rates than predicted btrules. With a complete set of biotransformation data, one would expect the actual biotransformations to be more predictive of biotransformation rates, but it is likely that our analysis of biotransformation products was incomplete and some important and rate-determining biotransformations that were predicted may not have been observed (e.g., for acetaminophen or gabapentin lactam). Nevertheless, we used a binary matrix of the observed biotransformations to construct random forest models for each of the four wastewater microbial communities. Unlike the random forest models developed with predicted btrules, the binary matrix for observed biotransformations was different for each wastewater microbial community depending on the biotransformation products that were observed in each experiment. The prediction accuracy of the four random forest models was lower (>78%) than the models developed with predicted btrules, and the OOB error ranged between 37 and 68%. We extracted the MDA values for each observed btrule and present the resulting heat map in Figure 3 in which we identify three main clusters of btrules. Rate constant distributions among the three clusters are provided in Figures S53 and S54.

Cluster 1 and Cluster 2B consist of observed biotransformations represented by rule bt0024 (ester hydrolysis) and bt0242 (monohydroxylation of a secondary aliphatic) and are

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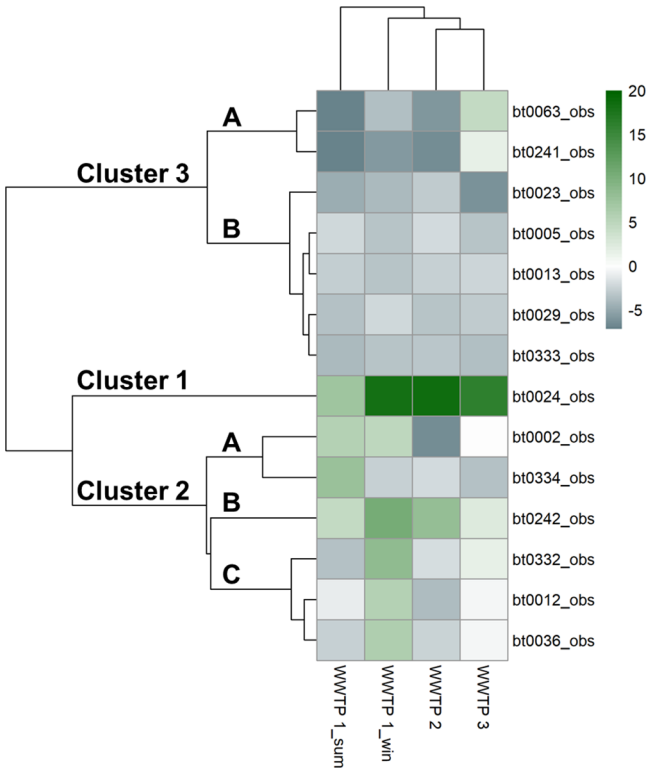

Figure 3. Heatmap of MDA values for observed biotransformation rules across the four WWTPs. The color scale from dark gray to dark green, where dark gray represents the most negative MDA values, and dark green shows the most positive.

categorized as rate-determining and relatively fast biotransformations in the analysis of observed biotransformations. This agrees with the analysis of predicted btrules and is corroborated by our frequent observation of the expected biotransformation products for most of the MPs triggering these btrules in each of the four wastewater microbial communities. Cluster 2A, Cluster 2C, and Cluster 3A contain seven observed biotransformations that exhibit variable degrees of importance across the four wastewater microbial communities. These include bt0063 (amine dealkylation), bt0241 (monohydroxylation of tertiary aliphatic), bt0002 (oxidation of secondary alcohol), bt0332 (monohydroxylation of an aliphatic methyl [H0]), bt0012 (monohydroxylation of an unsubstituted benzenoid at the meta position), and bt0036 (monohydroxylation of an aromatic methyl). These biotransformations all exhibited similar patterns in the analysis of predicted btrules. The only biotransformation that exhibits different behavior in the analysis of observed biotransformations is bt0334 (hydroxylation of aliphatic methyl [H2]). Rule bt0334 was not important for determining rates when we considered predicted btrules, but it is important for predicting rates in WWTP1_sum when we consider observed biotransformations. However, none of the observed biotransformation products assigned to bt0334 were unequivocal; all were level 3 annotations as monohydroxylation products. This highlights another challenge of using observed biotransformations as a means to understand biotransformation rates across wastewater microbial communities. The analysis of observed biotransformations demonstrates that observed biotransformations can be used to identify important rate-determining biotransformations; however, limitations in the size and completeness of the dataset lead us to conclude that more robust or comprehensive analyses can still be conducted with predicted btrules.

Data Compilation and Context. From our analyses, we have identified 30 structural features of MPs that can be classified as: (1) biotransformation promoters (bt0005, bt0011, bt0014, bt0024, bt0064, and bt0242); (2) biotransformation inhibitors (bt0013, bt0023, bt0029, bt0036, bt0218, bt0243, bt0430, and bt0286); (3) structural features that can be biotransformed based on uncharacterized features of the wastewater microbial community (bt0001, bt0002, bt0012, bt0022, bt0051, bt0063, bt0241, bt0270, and bt0332); and (4) not rate-determining (bt0065, bt0067, bt0071, bt0291, bt0333, bt0334, and bt0353). These findings improve our fundamental understanding of the determinants of biotransformation rates in wastewater microbial communities and provide insight into the distribution of generalist and specialist functions within wastewater microbial communities.[49,50]

We next aimed to compare our method of functional group categorization with existing methods that use structural features to predict MP biodegradability. We used BIOWIN models 1, 2, 5, and 6 within the EPISUITE[13] software from the U.S. EPA to predict the biodegradability of each of the MPs included in our study and compare the results to our observed biotransformation rates. It is important to note that the BIOWIN models were developed with data from experiments conducted under conditions that are unlikely to represent MP biotransformations performed by wastewater microbial communities and that represent different biodegradation endpoints (e.g., ultimate biodegradation or oxygen demand of the test chemical). Nevertheless, the BIOWIN models return predictions of "readily" or "not readily" biodegradable, which we directly compared to our classification of "fast" and "slow" biotransformations based on a biotransformation constant threshold of 0.5 d^{-1}. We found that the BIOWIN models could only accurately bin between 43 and 66% of our MPs across the four models and the four experiments. We attribute errors in these predictions to differences between the types of biotransformations generated in experiments conducted to train the BIOWIN models and the types of biotransformations performed by wastewater microbial communities. For example, all BIOWIN models predict that methadone will not be readily biodegradable because of the tertiary amine group. However, the biotransformation rate of methadone was fast in all of our experiments, which can be explained by the presence of a secondary aliphatic functional group (bt0242) that was identified as a rate-determining biotransformation promoter in our study. The BIOWIN models were best at predicting the biodegradability of ester-containing MPs (75% of ester-containing MP predictions from BIOWIN were correct) and the worst for amine-containing MPs (40% of amine-containing MP predictions from BIOWIN were correct). We also examined the likelihoods provided in the Eawag-PPS for each of the btrules we classified as biotransformation promoters and inhibitors. We found that the six biotransformation promotors were assigned likelihoods of likely (4) or neutral (2), whereas the eight biotransformation inhibitors were assigned likelihoods of very unlikely (1), unlikely (3), or neutral (4). These likelihood assignments likewise add support to the categorizations proposed in this study.

Environmental Implications. Despite decades of research on the removal or biotransformation of MPs during biological wastewater treatment, it remains difficult to manage MP

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removal during wastewater treatment or to predict MP biotransformations and associated biotransformation rates. In this study, we identified functional groups that promote or inhibit MP biotransformations across four independent wastewater microbial communities. We contend that the biotransformations associated with rate-determining biotransformation promoters represent generalist microbial community functions that can be readily performed by wastewater microbial communities. It is important to note that this contention warrants further validation with other MPs and other wastewater microbial communities, but this insight can be used to make predictions about promoter-containing MP biotransformations during wastewater treatment and to inform about the design of new chemical products that may be more readily biodegradable during biological wastewater treatment. We also identified functional groups that can be biotransformed based on uncharacterized features of the wastewater microbial community. We argue that the biotransformations associated with these functional groups represent specialist microbial community functions that can be performed by certain types of wastewater microbial communities. Future research should be directed to identify the taxa or metagenomic content that is causally associated with these functions as a means to more rapidly characterize the metabolic potential of a wastewater microbial community or to optimize the performance of biological wastewater treatment processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c06429.

Additional text, 54 figures, and 10 tables with details of experimental methods, confirmed biotransformation products, and random forest analysis (PDF)

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Notes

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