Contaminants in Aquatic and Terrestrial Environments

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Exploring the specificity of extracellular wastewater peptidases to inform the design of sustainable peptide-based antibiotics

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Abstract

New antimicrobial peptides are emerging as promising alternatives to conventional antibiotics because of their specificity for target pathogens and their potential to be rapidly hydrolyzed (i.e., inactivated) by extracellular peptidases during biological wastewater treatment, thereby limiting the emergence and propagation of antibiotic resistance in the environment. However, little is known about the specificity of extracellular peptidases derived from wastewater microbial communities, which is a major impediment for the design of sustainable peptide-based antibiotics that can be hydrolyzed by wastewater peptidases. We used a set of natural peptides to explore the specificity of dissolved extracellular wastewater peptidases. We found that enzyme-catalyzed hydrolysis occurred at specific sites and that a subset of the these hydrolyses were conserved across enzyme pools derived from three independent wastewater microbial communities. An analysis of the amino-acid residues flanking the hydrolyzed bonds revealed a set of residue motifs that were linked to enzyme-catalyzed hydrolysis and are therefore candidates for incorporation into new and sustainable peptide-based antibiotics.

Keywords

Biological wastewater treatment, peptide-based antibiotics, antibiotic resistance, extracellular enzymes, biotransformation, green chemistry

38 **TOC graphic**

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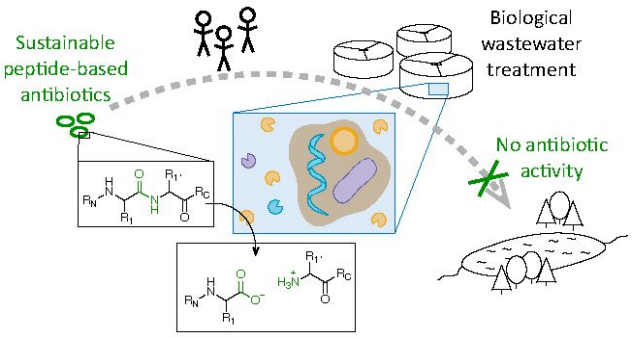

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Introduction

Due to the increasing number of infections caused by antibiotic-resistant bacteria, the development of potent and sustainable antibiotics is one of the greatest challenges of the 21st century.[1–3] Most antibiotics are only partially metabolized in the human body and, consequently, a fraction of them is excreted and conveyed to centralized wastewater treatment facilities.[4–6] As a result, wastewater microbial communities utilized in the biological processes within centralized wastewater treatment facilities are chronically exposed to antibiotics and contribute to the emergence and propagation of antibiotic resistance.[7–10] Additionally, antibiotics that persist during wastewater treatment can have profound effects on the ecosystem services provided by microbial communities in natural systems downstream from wastewater treatment facilities and lead to further emergence and propagation of antibiotic resistance in these downstream systems.[10–12]

To enable the development of sustainable antibiotics for the benefit of environmental and public health, we believe that new antibiotics should be evaluated for their fate during biological wastewater treatment, along with conventional metrics such as their efficacy, specificity, toxicity, and stability in the human body.[13] Complete inactivation of antibiotics during biological wastewater treatment would protect downstream microbial communities from the selective pressures and stresses caused by antibiotics in the environment. Further, we expect that the rapid inactivation of antibiotics by dissolved extracellular enzymes secreted by wastewater microbial communities would protect the wastewater microbial communities themselves from the selective pressures and stresses caused by antibiotics.[14] In fact, it has been recently demonstrated in controlled experiments with structured microbial communities that

extracellular processes that inactivate antibiotics reduce the selective pressures that lead to the emergence and propagation of antibiotic resistance.[15]

Antimicrobial peptides, such as the recently characterized teixobactin,[16] thanatin,[17,18] streptocidin D,[19] albomycin,[20] malacidin,[21] tachyplesin II,[22] and murepavadin[23] are promising not only because of their specificity for target pathogens,[13,16,24,25] but also for their potential to be rapidly hydrolyzed (i.e., inactivated) by extracellular peptidases during biological wastewater treatment.[14,26,27] However, previous studies on the biotransformation of antibiotics that contain peptide bonds showed that not all peptide bonds are rapidly hydrolyzed by wastewater peptidases. For example, one study reported only 50% removal of vancomycin during biological wastewater treatment,[28] and only a fraction of that removal can be attributed to enzyme-catalyzed peptide hydrolysis. Similarly, we found in our previous study that the hydrolysis of daptomycin by extracellular enzymes derived from wastewater microbial communities was slow.[14]

Besides the limited amount of available data on the hydrolysis of peptide-based antibiotics during wastewater treatment, the specificity of extracellular wastewater peptidases has never been systematically explored. This knowledge gap limits our ability to predict the biodegradability of existing peptide-based antibiotics or to design peptide-based antibiotics to be rapidly hydrolyzed by extracellular wastewater peptidases. Previous research on wastewater peptidases has solely been conducted with probe molecules that target a small number of peptidases capable of hydrolyzing the bond between a particular amino-acid residue and a fluorescent moiety.[29,30] Whereas methods that provide detailed information on peptidase specificity have also been established,[31] the potential of these methods to assess the peptidase specificity of wastewater enzymes has yet to be assessed.

In this study, we explored the specificity of dissolved, extracellular, *endo*-cleaving peptidases (i.e., enzymes that hydrolyze peptide bonds other than the terminal bonds in a peptide) derived from wastewater microbial communities by incubating a set of natural peptides with enzymes extracted from aerobic bioreactors within centralized wastewater treatment facilities. We measured the biotransformation of the parent peptides, identified product peptides for hydrolyzed parent peptides, and compared the resulting peptidase specificity patterns among extracts from three independent wastewater microbial communities. The identification of peptide bonds that are hydrolyzed by extracellular peptidases across independent wastewater microbial communities is an essential and significant step towards understanding the fate of peptide-based antibiotics during biological wastewater treatment and creating opportunities for the design of more rapidly hydrolyzed and more sustainable antibiotics.

Materials and Methods

Chemicals

A list containing all chemicals used in this study along with their suppliers is provided in the Supplementary Information (SI).

Preparation of the parent peptide library

We digested bovine serum albumin (BSA) according to a protocol that was slightly adapted from the supplier of trypsin. In brief, we dissolved 2.4 mg of BSA in 500 µL LC-MS grade water containing ammonium bicarbonate (100 mM), calcium chloride (1 mM), and sodium dodecyl sulfate (0.1% w/w). Then, we added dithiothreitol (DTT, 20 µL of a 500 mM aqueous solution) and incubated the solution at 60 °C for 1 h. After cooling the solution to room temperature, we added iodoacetamide (IAA, 20 µL of a 1 M aqueous solution), incubated the solution at 37 °C in the dark for 30 min, and added DTT (10 µL of a 500 mM aqueous solution). Subsequently, we added a freshly prepared solution of trypsin (1 µg/µL in aqueous acetic acid (50 mM)) and incubated the resulting solution for 24 h at 37 °C. To clean up peptides, we added trifluoroacetic acid to a final concentration of 0.1% v/v and used a peptide desalting column (Thermo Fisher, article number: 89852) according to the supplier's protocol. To elute the peptides from the column, we used an aqueous solution of acetonitrile (50% v/v) containing trifluoroacetic acid (0.1% v/v). Subsequently, we evaporated the solvent using a Speed Vac Concentrator (Savant, SVC-100H), resolubilized the peptides in 1.2 mL of an aqueous solution of acetonitrile (50% v/v) containing formic acid (0.1% v/v), and stored the solutions at -20 °C. In addition to the BSA:trypsin ratio suggested in the supplier's protocol (i.e., 100:1 w/w), we tested BSA:trypsin ratios of 300:1 and 1000:1 and analyzed the resulting solutions by HPLC-HRMS/MS (**Figure S1**). Because this analysis revealed no consistent trend of peptide abundance with respect to the

131 BSA:trypsin ratio, we pooled the purified peptides that resulted from the three
132 digestions and used the resulting solution for all incubation experiments.
133
134 *Wastewater microbial community sampling, enzyme extraction, and protease activity*
135 *measurements*
136 We sampled wastewater microbial communities from the aeration tanks of three
137 municipal wastewater treatment facilities in New York State (Ithaca, Dryden,
138 Trumansburg). Microbial community sampling and enzyme extraction were performed
139 as previously described.[14] In brief, we collected microbial communities by taking a 1 L
140 grab sample from each treatment facility, transported them to the lab in a glass bottle,
141 aliquoted them into 50 mL plastic centrifuge tubes, and extracted dissolved
142 extracellular enzymes by centrifugation (4000 x g, 5 min, Legend XTR centrifuge,
143 Thermo Scientific) and subsequent filter-sterilization (0.22 μm, PVDF syringe filters,
144 Restek). For each microbial community, we performed triplicate extractions and we
145 used the three resulting extracts for incubation experiments. This level of experimental
146 replication was chosen to capture differences with respect to enzyme activity between
147 the 50 mL aliquots, extraction efficiency, incubation experiments, and peptide analysis.
148 Prior to centrifugation, we added potassium phosphate (final concentration: 20 mM) to
149 stabilize the pH at 7.4. To determine the general protease activity of the enzyme
150 extracts, we used the EnzChek Protease Assay kit (Thermo Fisher, E6638) and a
151 microplate reader (Tecan, Infinite M200-pro; excitation wavelength: 485 nm, emission
152 wavelength: 530 nm) with black, flat bottom 96-well plates (Corning, 3991). On each
153 plate, we ran incubations with Milli-Q water in addition to wastewater enzyme extracts.
154 These control incubations were used to correct for run-to-run variations of fluorescence
155 measurements and for non-enzymatic probe hydrolysis.
156

Peptide hydrolysis experiments

Incubation experiments of parent peptides with wastewater enzymes were conducted immediately after enzyme extraction (i.e., < 3 h after sampling at the wastewater treatment facilities). Therefore, we spiked 15 µL of a solution containing the purified parent peptides to a 2 mL centrifuge tube containing 1.5 mL of either the enzyme extract or LCMS-grade water that was pH-stabilized (pH 7.4) using potassium phosphate (20 mM). We additionally performed an incubation of enzyme extracts to which we added 15 µL of a mock digest (i.e., a solution that was treated the same way as digestions, but did not contain BSA). These controls showed that none of the parent peptides was present at a substantial concentration in any of the wastewater extracts (i.e., peak area before peptide spiking < 0.4% of peak area after peptide spiking). Immediately after spiking, we mixed the solution by vortexing and incubated it at room temperature under horizontal shaking (180 rpm). At the sampling time points, we transferred 150 µL of the incubation solution into a new plastic centrifuge tube and incubated the tube at 75 °C for 10 min in a Dry Bath Incubator (Fisher Scientific, 11-718-2) to inactivate the enzymes and to stop enzymatic hydrolysis.[32] We performed a control experiment to confirm that the parent peptides persisted during this heat treatment (**Figure S2**). Immediately after the heat treatment, we incubated the tube on ice for 1 min, centrifuged the tube at 15'800 x g, for 1 min, and transferred the supernatant into a plastic HPLC vial (SUN SRI, 501-354) for storage at -20 °C until analysis.

Identification and relative quantitation of parent and product peptides

We used high-performance liquid chromatography (HPLC, Ultimate 3000, Thermo Scientific) coupled to high-resolution tandem mass spectrometry (HRMS/MS, QExactive, Thermo Scientific) for peptide identification and relative quantification.

Briefly, we injected 20 µL of peptide solution and separated the peptides with an Acclaim PepMap 100 C18 column (Thermo Scientific, catalog number: 164572, length: 150 mm, inner diameter: 1 mm, particle size: 3 µm) at a flow rate of 40 µL /min and with the following mobile phase composition (A: LC-grade water; B: LC-grade methanol – both contained 0.1% v/v formic acid): 0-2 min: 0 % B, 2-80 min: 0% B – 55% B (linear increase), 80-90 min: 55% B – 90% B (linear increase), 90-94 min: 90 % B, 94-95 min: 90% B – 0% B, 95-100 min: 0% B). For detection, we performed full-scan MS acquisitions (scan range: 160 – 1800 m/z, resolution: 70'000, AGC target: 1E6, Maximum IT: 200 ms) in positive electrospray ionization (ESI) mode and Top10 MS^2 acquisitions (resolution: 17'500, AGC target: 1e5, Maximum IT: 100 ms, isolation window: 1.0 m/z, NCE (stepped): 20, 25, 30, dynamic exclusion time: 6 s)[33]. ESI parameters were as follows: sheath gas: 15, aux gas: 5, S-lens: 70.

We used the open-source software Skyline (version 4.2.0) for data analysis.[34] For parent peptide identification, we first predicted peptides by applying the *in silico* digestion tool within Skyline on the amino-acid sequence of BSA (UniProt-ID: P02769) and screened the collected HPLC-HRMS/MS data for these peptides (Skyline settings: trypsin specificity: KR/P, missed cleavages: 1, modifications: cysteine acylation, lengths: ≥ 5amino acids, precursor charges: +2 and +3, fragment ion type: y, fragment charges +1 and +2). For product peptide identification, we first predicted all products that result from the hydrolysis of one peptide bond in the parent peptide of interest and screened the collected HPLC-HRMS/MS data for these products (Skyline settings: lengths: ≥ 3 amino acids, precursor charges: +1, +2 and +3, fragment ion type: y, fragment charges +1 and +2). We applied the following additional criteria to both parent and product peptides: precursor mass deviation < 2 ppm, at least one fragment with a mass deviation < 5 ppm, matching isotope pattern of the precursor (isotope distribution dot-product

208 > 0.9), and a reasonable chromatographic peak shape. For relative peptide quantitation,
209 we integrated peak areas using the automated algorithm included in Skyline and
210 manually verified the integration boundaries.

Results

Preparation and characterization of parent peptides

To prepare a set of natural parent peptides, we digested bovine serum albumin (BSA) with trypsin – a peptidase that specifically hydrolyzes peptide bonds C-terminal of lysine and arginine (**Figure 1A**). Using high-performance liquid chromatography coupled to high-resolution tandem mass spectrometry (HPLC-HRMS/MS), we detected 42 parent peptides that were predicted by an *in silico* digestion of BSA with trypsin (we later refer to them as T1 – T42). Amino-acid sequences and acquisition parameters of these peptides are provided in **Table S1** and in **Figure S3**. Control experiments, in which the peptides were incubated in pH-buffered Milli-Q water for 48 h, demonstrated that the peak areas of all 42 peptides remained constant during the incubation and that non-enzymatic hydrolysis can therefore likely be neglected in experiments with wastewater enzyme extracts (**Figure S4**).

Because the specificity of many peptidases is governed by specific pairs of amino-acid residues,[31] we characterized the parent peptides based on the residue pairs they contain. We found that more than 40% of all possible residue pairs (i.e., 169 out of 400) occurred in our set of parent peptides (**Figure 1B**). While many of the residue pairs occurred once, some occurred more frequently (e.g., the residue pair alanine-aspartic acid occurred seven times). We note that we excluded the residues located at the amino- and the carboxyl-terminus of each parent peptide from this analysis because the focus of this study is on *endo*-cleaving peptidases. We also note that, because the parent peptides contained only ~ 4% of all possible three-residue motifs (i.e., 318 out of 8000), we restricted the analyses of this study to focus on residue pairs.

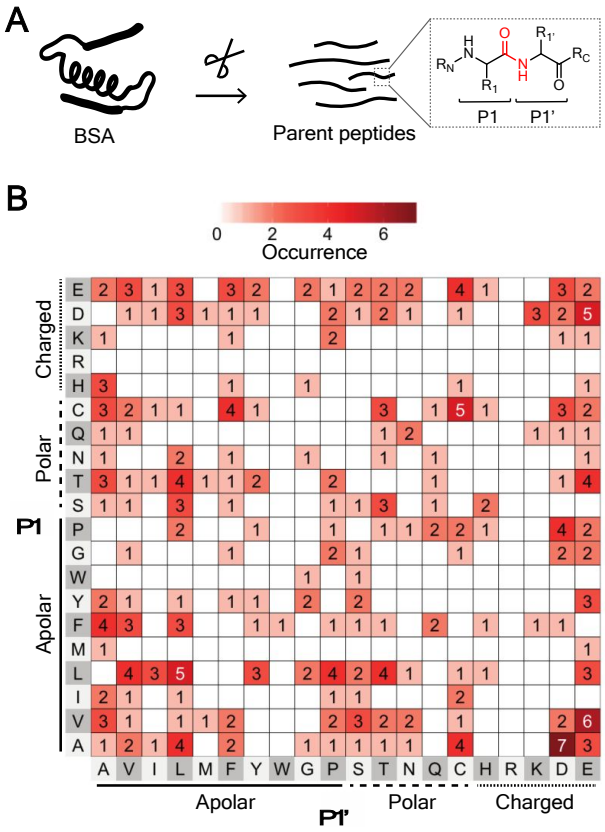

Figure 1. Preparation and assessment of parent peptides. (**A**) Parent peptides resulted from the digestion of bovine serum albumin (BSA) with trypsin. Every peptide bond links two amino-acid residues – one at the prime site (P1') and one at the nonprime site (P1). R_N: residues located towards the N-terminus, R_C: residues located towards the C-terminus. (**B**) Occurrence of non-terminal residue pairs (i.e., P1P1') in the parent peptides. Residue pairs that occurred on multiple parent peptides that shared sequences due to missed cleavages during the trypsin digestion were only counted once. Amino-acid residues are represented by their single-letter abbreviation and grouped based on the chemistry of their side chain (i.e., apolar: solid line, polar: dashed line, and charged: dotted line). Cell color and number in cell both represent the occurrence of the respective amino-acid-residue pair

Hydrolysis of parent peptides by dissolved extracellular wastewater peptidases

To assess the susceptibility of the peptide bonds in the parent peptides to hydrolysis by dissolved extracellular wastewater enzymes, we used a previously described method to extract enzymes from three wastewater microbial communities and incubated the parent peptides with the resulting enzyme extracts (**Figure 2A**).[14] We selected these wastewater microbial communities because of the different operational parameters of the wastewater treatment facilities. Key operational parameters of the

256 wastewater treatment facilities (i.e., hydraulic retention time, solids retention time, and
257 total suspended solids content) and key characteristics of the enzyme extracts (i.e.,
258 protein concentration and general protease activity measured with an assay based on
259 fluorogenic casein) are provided in **Table S2**.

260 In a preliminary experiment, we estimated the rate of enzymatic peptide
261 biotransformation by quantifying the decrease of parent-peptide peak areas during their
262 incubation with enzymes extracted from MC1 (**Figure S5**). Data from this experiment
263 show that biotransformation rates varied across parent peptides. For example, the peak
264 area of peptide T33 decreased by more than 3 orders of magnitude during an 18 h
265 incubation, while the peak areas of peptides T18 and T19 remained constant. Incubation
266 times for triplicate experiments with enzymes derived from each of the three microbial
267 communities were chosen based on the results of this preliminary experiment and the
268 protease activities provided in **Table S2**.

269

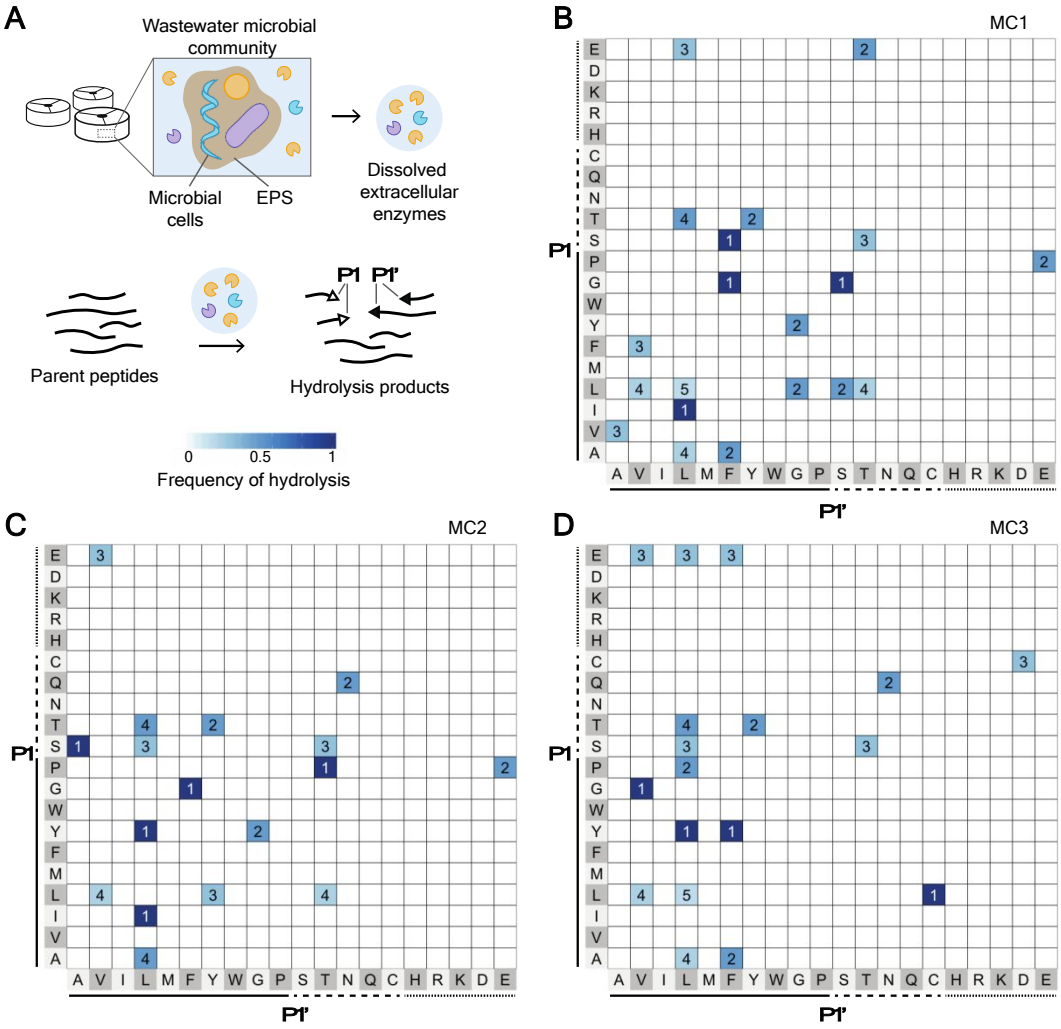

Figure 2. Identification of peptide bonds hydrolyzed by dissolved extracellular wastewater enzymes. (**A**) Enzymes were extracted from the microbial communities (MC) of three wastewater treatment facilities and incubated with the parent peptides. (**B**) – (**D**) Amino-acid-residue pairs (i.e., P1P1') flanking peptide bonds hydrolyzed by enzymes extracted from MC1 – 3, respectively. Number in cell represents the occurrence of the respective residue pair in the parent peptides; cell color represents the relative hydrolysis frequency. Residues are represented by their single-letter abbreviation and grouped based on the chemistry of their side chain (i.e., apolar: solid line, polar: dashed line, and charged: dotted line). We note that the absence of a residue pair in this depiction does not necessarily mean that the respective bond was not hydrolyzed, but that we did not find products as evidence for hydrolysis.

Our next step towards deciphering the specificity of wastewater peptidases was to select parent peptides that were biotransformed during their incubation with each of the enzyme extracts. Our criterion for defining biotransformation was that the mean peak area of the parent peptide at the end of the incubation was <50% and significantly smaller (t-test, $p<0.05$, $n=3$) than the mean peak area of the peptide at the beginning of the incubation (**Figure S6**). Applying this criterion resulted in the identification of nine,

seven, and twelve parent peptides that were biotransformed by extracellular peptidases derived from MC1, MC2, and MC3, respectively. Five of the parent peptides (i.e., T12, T30, T31, T33, and T36) were biotransformed by enzymes extracted from all three wastewater microbial communities. Because the above criterion is based on peak areas, we analyzed a dilution series of the parent-peptide solution to assess the relationship between peak area and peptide concentration (**Figure S7**). This analysis showed that the relationship was linear ($R^2 > 0.95$) for 35 of the 42 peptides. For the remaining 7 peptides, our results indicate that the actual extent of biotransformation during experiments with wastewater enzymes might have been less than the extent of biotransformation determined based on peak area.[35] Nevertheless, because the above selection of biotransformed parent peptides was mainly conducted to reduce the number of parent peptides for product screening (see below), we did not exclude parent peptides based on the relationship between peak area and peptide concentration.

Identification of hydrolysis products and cleaved peptide bonds

To gain insights into the sites of peptide hydrolysis, we screened the collected HPLC-HRMS/MS data for all possible product peptides that contain at least three amino acids and result from the hydrolysis of one peptide bond in the parent peptides that were selected as described above. We applied the following criteria for the identification of an enzymatic hydrolysis product: the mean peak area of a product at the end of the enzyme incubation is >100-fold and significantly larger (t-test, $p<0.05$, $n=3$) than the mean peak area of the product at the end of water control incubations and >2-fold and significantly larger (t-test, $p<0.05$, $n=3$) than the mean peak area of the product in the beginning of the enzyme incubation. Additionally, we verified that none of the products originated from the wastewater by confirming that the mean peak area

of the product at the end of the incubation was >100-fold larger than the peak area of the product in an enzyme extract to which no parent peptides were spiked. This analysis resulted in the identification of 26, 23, and 22 products for the experiments with enzymes derived from MC1, MC2, and MC3, respectively (**Tables S3-S5**). We note that we excluded products that resulted from the hydrolytic removal of one amino acid from the amino- or the carbonyl-terminus as the focus of this study is on *endo*-cleaving peptidases. However, we cannot rule out that some of the identified products resulted from multiple consecutive *exo*-type hydrolyses.

The number of identified peptide bonds that were hydrolyzed by enzymes derived from MC1, MC2, and MC3, respectively was 21, 19, and 19 (this number is lower than the number of products because we sometimes detected two products of the same hydrolysis). The residue pairs flanking the hydrolyzed peptide bonds (i.e., residue pairs that occupy sites P1 and P1') are shown in **Figure 2B-D**. Examining the results from each wastewater microbial community separately, we found that: (i) peptidases from each community hydrolyzed two different bonds surrounded by the residue pair threonine-leucine; and, (ii) peptidases from MC2 additionally hydrolyzed two different bonds surrounded by the residue pair alanine-leucine. For the remaining residue pairs flanking hydrolyzed peptide bonds, we detected only one incident of hydrolysis.

Comparison of peptide hydrolysis among wastewater microbial communities

We next examined the dataset to assess how the observed peptide hydrolyses compared among the three tested wastewater microbial communities. We found that six peptide bonds were hydrolyzed by peptidases derived from all three communities, twelve bonds were hydrolyzed by peptidases derived from two communities, and nine, six, and six bonds were only hydrolyzed by peptidases derived from MC1, MC2, and

MC3, respectively (**Figure 3A**). A closer examination of the amino-acid-residue pairs flanking the 18 peptide bonds that were hydrolyzed by peptidases from at least two microbial communities revealed that leucine is highly abundant at the P1' site of these bonds (**Figure 3B**). While leucine makes up less than 11% of the potential P1' positions in the parent peptides (**Figure 1B**), it makes up more than 44% of the P1' positions of peptide bonds that were hydrolyzed by peptidases from at least two wastewater microbial communities. In other words, 24% of the peptide bonds that have leucine at the P1' site were hydrolyzed by peptidases from at least two communities (**Figure 3B**). Additional information on the 18 peptide bonds that were hydrolyzed by peptidases from at least two communities (i.e., sequences of parent and product peptides and peak areas of product peptides) are provided in **Table S6**.

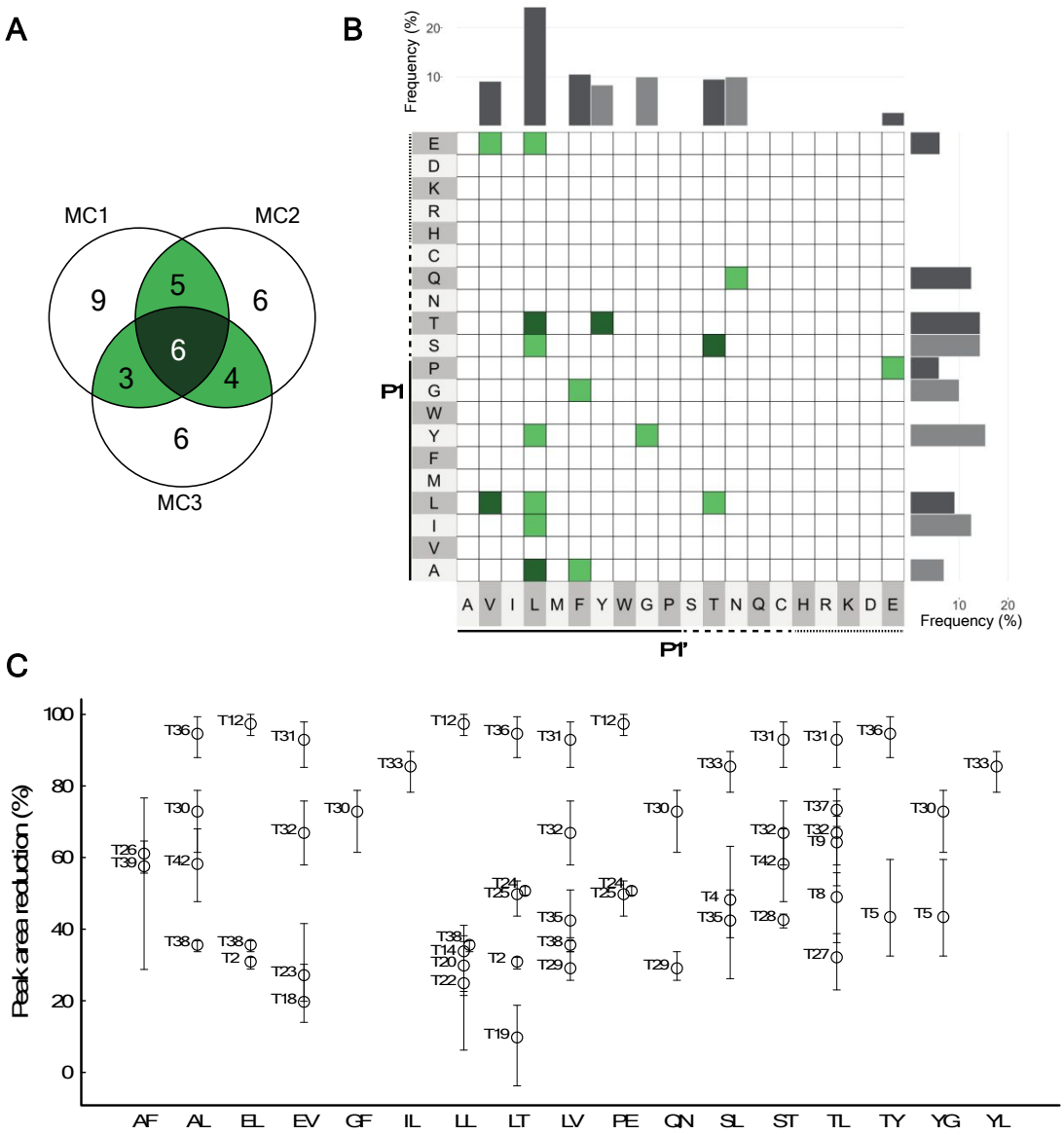

Figure 3. Overlap in hydrolyzed peptide bonds across microbial communities (MCs). (**A**) Venn diagram representing the overlap of hydrolyzed peptide bonds across the three wastewater microbial communities. (**B**) Amino-acid-residue pairs (i.e., P1P1') flanking peptide bonds that were hydrolyzed by enzymes extracted from two and three microbial communities are represented by light green cells and dark green cells, respectively. Bar plots at top and right represent the fraction of peptide bonds with the respective residue at the P1' and P1 site, respectively, that was hydrolyzed by enzymes derived from at least two microbial communities. Residues are represented by their single-letter abbreviations and grouped based on the chemistry of their side chain (i.e., apolar: solid line, polar: dashed line, and charged: dotted line). (**C**) Peak area reduction of parent peptides that contain residue pairs flanking peptide bonds that were hydrolyzed by enzymes extracted from at least two microbial communities. Points and error bars represent means and ranges, respectively, of the peak area reductions measured for the three microbial communities. Parent peptide IDs are provided next to the respective points. Black points represent parent peptides for which we detected the hydrolysis of the respective peptide bond by enzymes extracted from at least two microbial communities.

To test if the residue pairs flanking these 18 peptide bonds are generally linked to enzyme-catalyzed hydrolysis, we compared the peak area reduction of the parent peptides that contain at least one of these residue pairs (**Figure 3C**). While the mean peak area reduction across the enzyme pools derived from three wastewater communities was > 50% for some of the selected parent peptides, others showed moderate to low peak area reduction. For example, the residue pairs leucine-leucine, and leucine-threonine occurred in parent peptides for which we observed almost complete peak area reduction by enzymes from all wastewater microbial communities (i.e., T12 and T36), as well as in parent peptides with peak area reductions <10% in some enzyme extracts (i.e., T22 and T19). The observation that these three residue pairs have a leucine at the P1 site and an uncharged amino acid at the P1' site suggests that they might be substrates for similar peptidases. Therefore, we examined the residues adjacent to P1 and P1' and found that the parent peptides that were biotransformed contained uncharged residues at P2' (i.e., tyrosine C-terminal to leucine-leucine on T12 and proline C-terminal to leucine-threonine on T36), while the parent peptides that were more stable contained a charged residue at P2' (i.e., glutamic acid C-terminal to leucine-leucine on T22 and lysine C-terminal to leucine-threonine on T19).

As these findings suggest that amino-acid residues adjacent to P1 and P1' also affect hydrolysis by wastewater peptidases, we examined the residue pairs at the P2P1 and at the P1'P2' sites around the 18 bonds that were hydrolyzed by peptidases from at least two wastewater microbial communities and compared the peak area removal of the parent peptides that contain these residue pairs (**Figures S8** and **S9**). Similar to the results for P1P1' described above, the results of this analysis suggested varying degrees of biotransformation. However, we identified a set of residue pairs for which all of the parent peptides showed a mean peak area reduction >50% across the three communities.

By screening the acquired HPLC-HRMS/MS data for product peptides of the respective hydrolyses, we found that the two peptide bonds that feature the residue pair valine-serine at the P2P1 site and the four peptide bonds that feature either phenylalanine-glutamine or threonine-proline at the P1'P2' site were hydrolyzed by peptidases derived from at least two microbial communities (**Figures S8** and **S9**). Examining the residues adjacent to these three residue pairs showed that the residues C-terminal of valine-serine and N-terminal of phenylalanine-glutamine and threonine-proline were all uncharged.

401 **Discussion**

402 The suitability of the set of parent peptides for this first study on wastewater
403 peptidase specificity was demonstrated by the good coverage of amino-acid-residue
404 pairs (i.e., 40% of all possible residue pairs occurred at least once) and by the variation
405 in the estimated extent of biotransformation among the parent peptides. For some
406 peptides, we found a peak area reduction >80% during incubations with dissolved
407 extracellular wastewater peptidases. The duration of these incubations (i.e., 6 h, 3.8 h,
408 and 3.5 h for enzymes derived from MC 1, MC 2, and MC 3, respectively) was in the
409 range of typical hydraulic retention times (HRTs) of conventional activated sludge-
410 based wastewater treatment processes and shorter than HRTs of other types of
411 biological wastewater treatment processes such as sequencing batch reactors. This
412 finding is promising with respect to the inactivation of peptide-based antibiotics during
413 biological wastewater treatment. However, we note that future research will need to
414 validate the activity and specificity of extracellular peptidases from other types of
415 biological wastewater treatment processes and to assess a potential temporal variability.
416 Furthermore, biotransformation rates need to be obtained by absolute quantitation using
417 synthetic peptides. The slow biotransformation of some parent peptides is in agreement
418 with the observed recalcitrance of existing antibiotics containing peptide bonds during
419 biological wastewater treatment (i.e., daptomycin and vancomycin).[14,28]

420 The variation in the extent of biotransformation among parent peptides, together
421 with the relatively small number of observed hydrolyses, suggests that the investigated
422 peptidases exhibited substrate specificity and that the pool of dissolved extracellular
423 wastewater peptidases is of a relatively narrow complexity. We expect that both
424 peptidases with a broad specificity, as well as a highly complex pool of specific
425 peptidases, would have resulted in a larger number of hydrolyzed peptide bonds and in

more similar extents of biotransformation across different peptides. Defining the specificity of dissolved extracellular wastewater peptidases is an important prerequisite for the identification of peptide bonds that can be rapidly hydrolyzed during biological wastewater treatment. Future work is needed to assess the hydrolyzability of amino-acid-residue pairs not covered in this work, motifs consisting of more than two amino-acid residues, and motifs containing D-amino acids or non-native amino acids.[31,36]

The finding that a substantial fraction of the hydrolysis sites overlapped across the peptidases derived from three independent wastewater microbial communities suggests that some peptidase specificity is conserved across wastewater microbial communities. This overlap in specificity might be linked to the core wastewater microbial community that was recently characterized from 269 wastewater treatment facilities on six continents.[37] It could also be expected, however, that taxonomically unique communities exhibit conserved functional redundancy in the target substrates of their peptidases, assuming the microorganisms are growing on similar substrates in a similar environment. Irrespective of the underlying reason, the overlap in peptidase specificity among wastewater microbial communities is a promising result for the design of peptide-based antibiotics that are rapidly biodegraded during biological wastewater treatment.

Among the peptide bonds that were hydrolyzed across more than one wastewater microbial community, we identified amino-acid-residue motifs that were linked to hydrolysis. The identification of these motifs will enable predictions of the biotransformation of peptide-based compounds during biological wastewater treatment.[38–40] For example, the frequent occurrence of leucine at the P1' site of hydrolyzed peptide bonds is particularly noteworthy. We searched the Merops database for peptidases that preferentially hydrolyze peptide bonds with leucine at the P1' site

and found that a substantial number of the deposited peptidases belong to the M4 family of bacterial metalloproteases, which are secreted to extracellularly hydrolyze proteins and peptides for bacterial nutrition (e.g., griselysin, vimelysin, bacillolysin, and pseudolysin).[36] The fact that gene transcripts that encode for two of these peptidases (i.e., bacillolysin (EC 3.4.23.28) and pseudolysin (EC 3.4.24.26)) were detected in a previous study on wastewater metatranscriptomes suggests that these peptidases might indeed be key players in the pool of extracellular wastewater enzymes.[41]

Complementary experimental work will need to assess the pharmacokinetics and *in vivo* stability of wastewater-labile peptide-based antibiotic drug candidates. For example, an intravenously-administered antimicrobial peptide would not be an effective drug if it were susceptible to rapid hydrolysis by human blood peptidases. Previous research showed that human blood peptidases preferentially hydrolyze peptide bonds flanked by arginine/lysine (P1) and alanine/serine (P1'),[42] which suggests that some of the peptide bonds we found to be hydrolyzed by wastewater peptidases might be stable in human blood.

The identification of motifs associated with rapid hydrolysis by dissolved extracellular wastewater peptidases will create opportunities for the (re-)design of promising antimicrobial peptides such as streptocidin D, malacidin A, thanatin, and teixobactin (**Figure S10**).[13,43] On the way towards sustainable peptide-based antibiotics, we see great potential in future studies that apply omics-based approaches to seek links between the peptidase specificities described herein and the identity of extracellular wastewater peptidases.[44–47] Ideally, such studies will identify peptidases that are abundant and active across wastewater microbial communities, but absent from the specific part of the human body to which the respective antibiotics would be applied. The motifs targeted by such wastewater peptidases could then be incorporated into

476 peptide-based antibiotics to render the latter rapidly biodegradable in wastewater and
477 therefore benign with respect to the emergence and propagation of antibiotic resistance
478 in wastewater.
479

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Supporting Information. Additional information on parent peptides, product peptides, and wastewater treatment processes. Supplementary visualizations of experimental data. Supplementary Materials and Methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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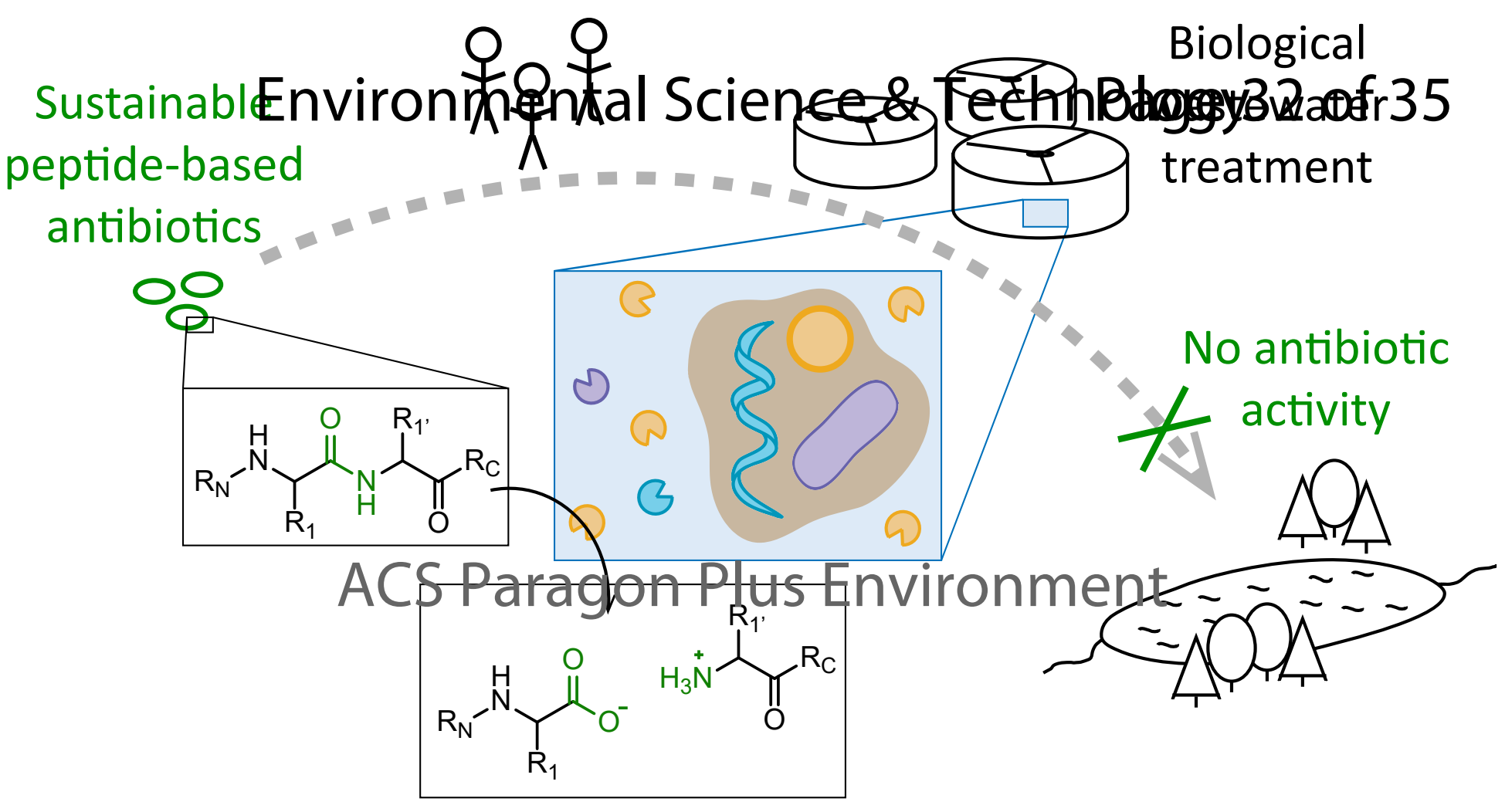
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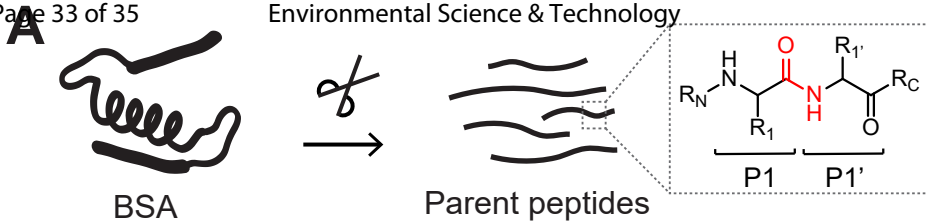

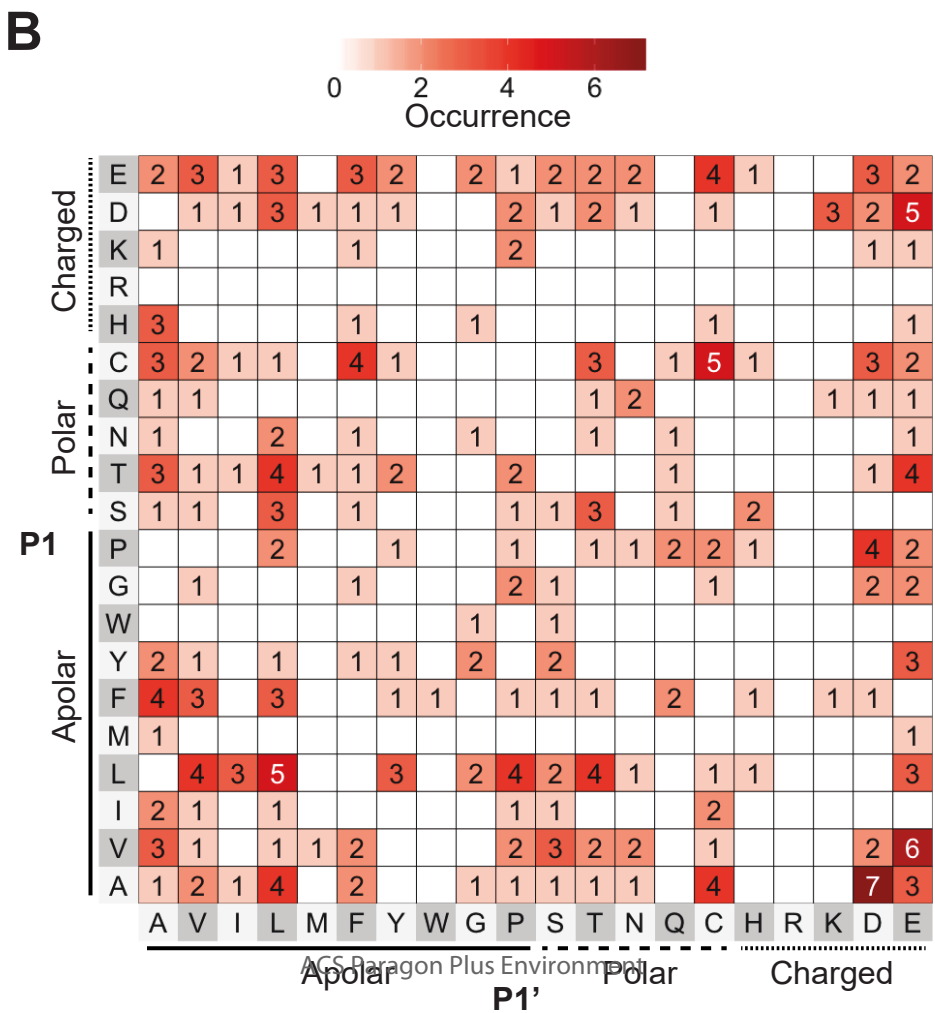

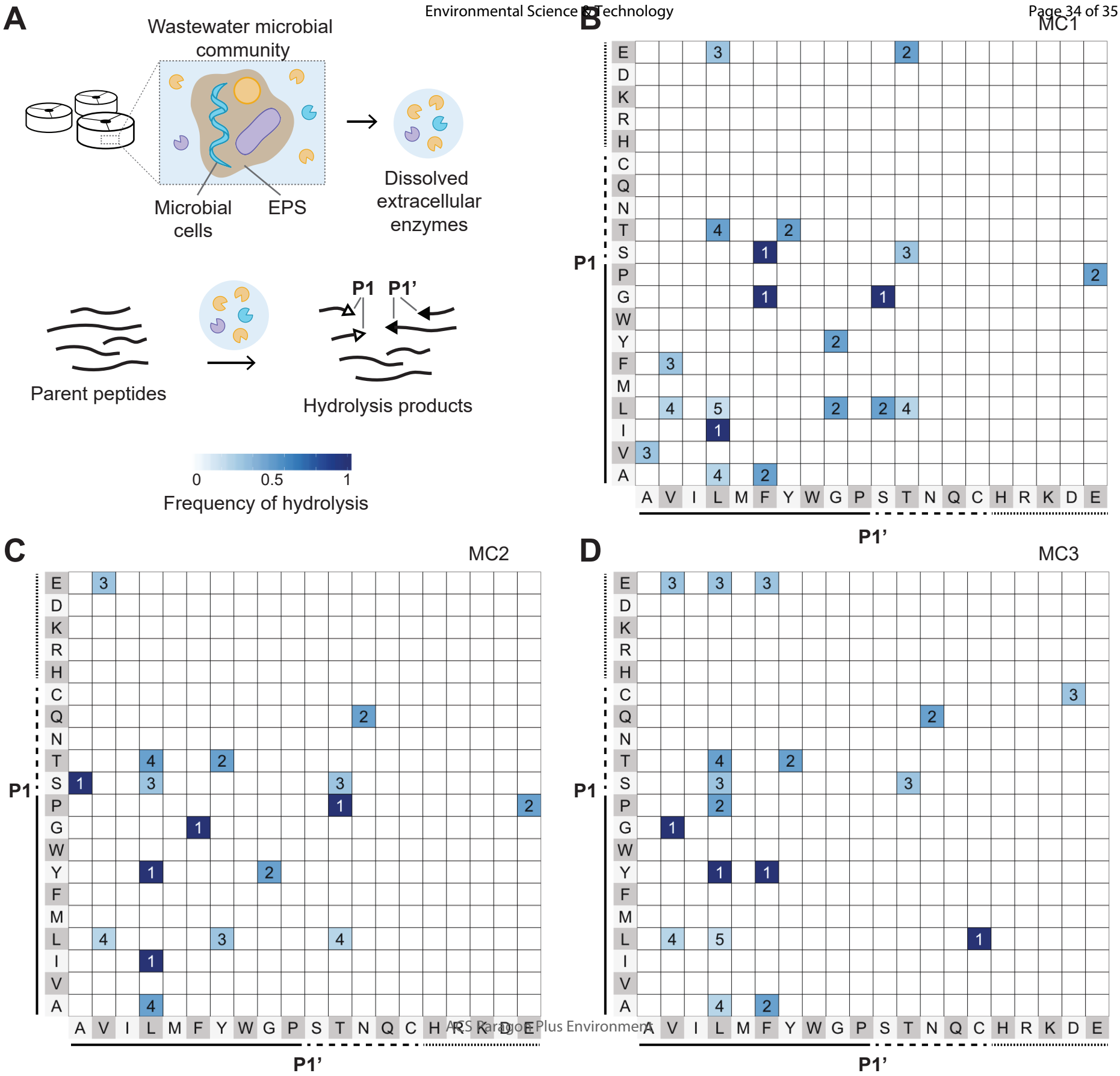

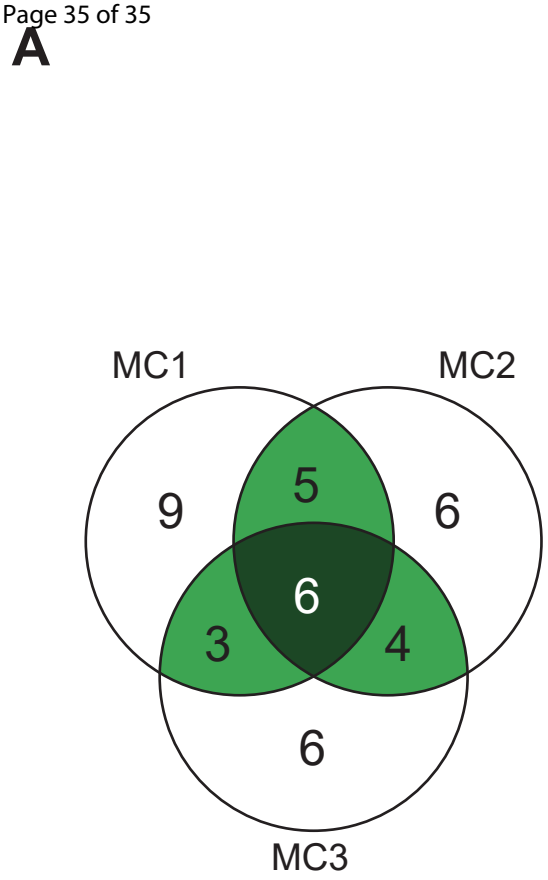
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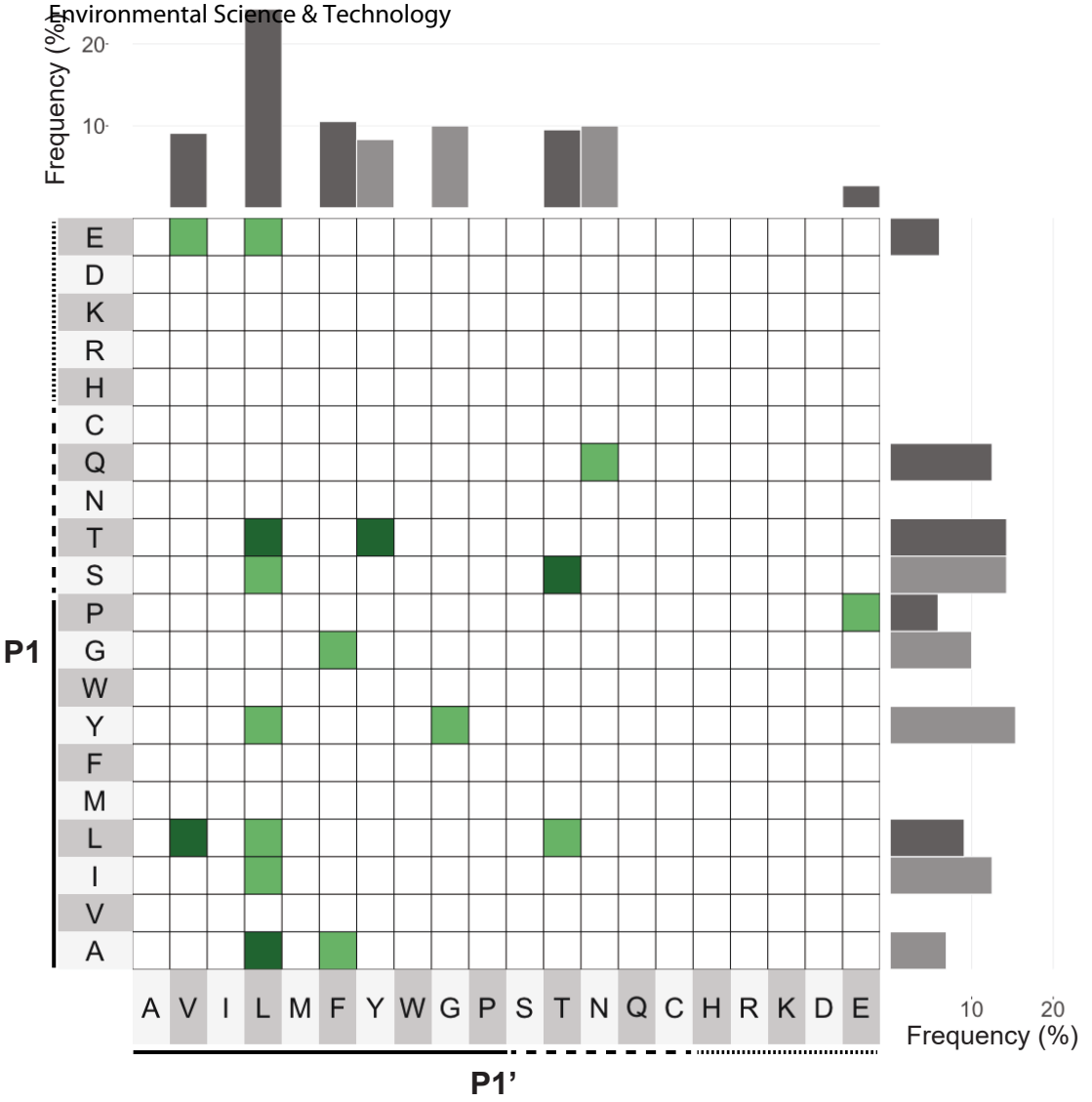
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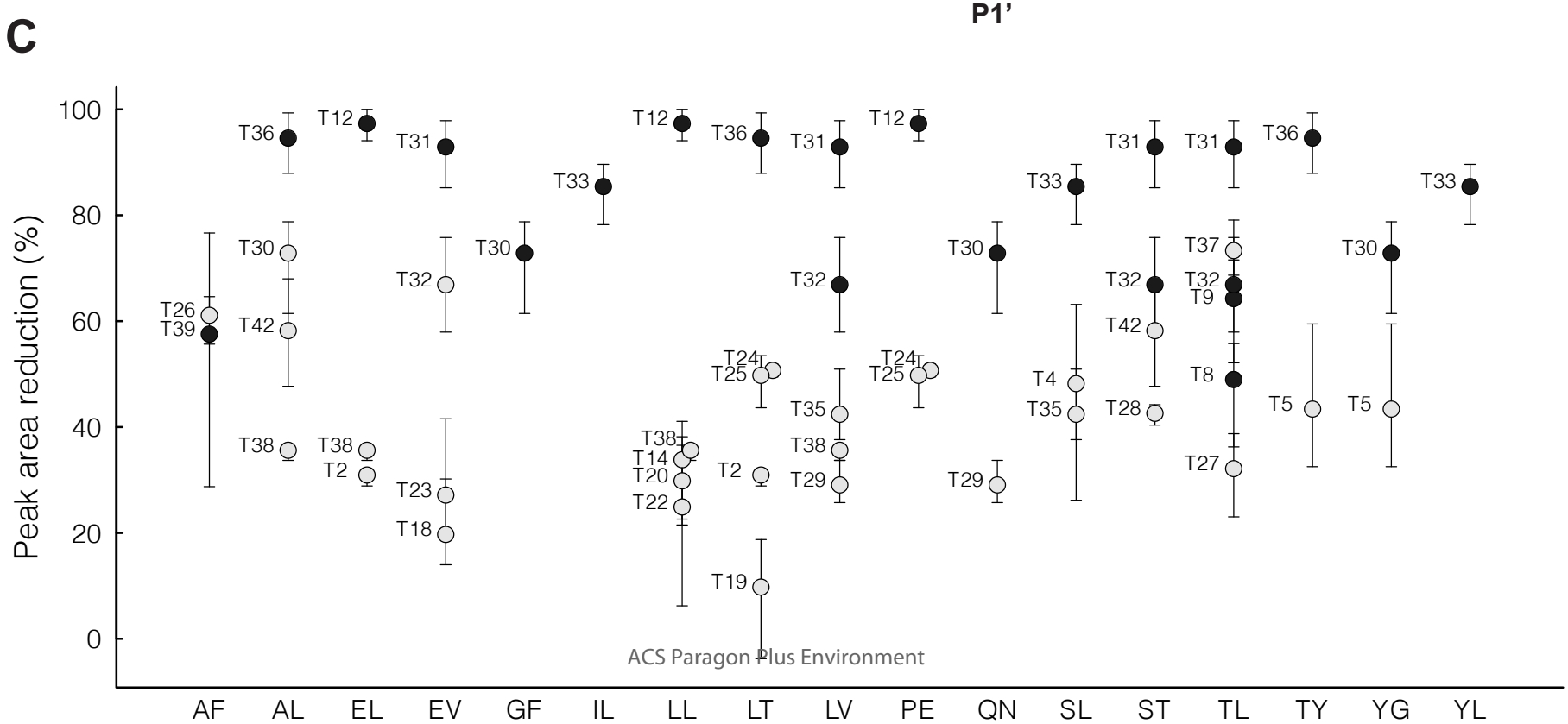
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