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Perspective

Methodological Advances to Study Contaminant Biotransformation: New Prospects for Understanding and Reducing Environmental Persistence?

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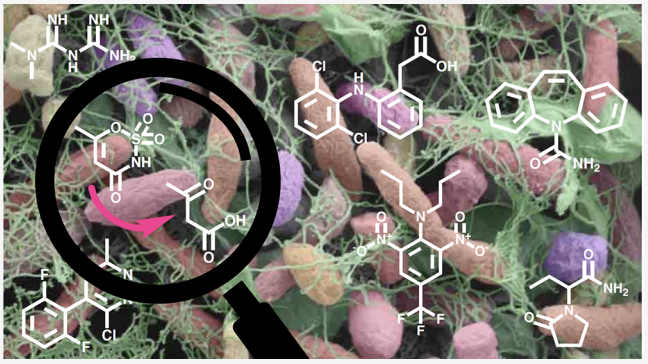
ABSTRACT: Complex microbial communities in environmental systems play a key role in the detoxification of chemical contaminants by transforming them into less active metabolites or by complete mineralization. Biotransformation, i.e., transformation by microbes, is well understood for a number of priority pollutants, but a similar level of understanding is lacking for many emerging contaminants encountered at low concentrations and in complex mixtures across natural and engineered systems. Any advanced approaches aiming to reduce environmental exposure to such contaminants (e.g., novel engineered biological water treatment systems, design of readily degradable chemicals, or improved regulatory assessment strategies to determine contaminant persistence *a priori*) will depend on understanding the causal links among contaminant removal, the key driving agents of biotransformation at low concentrations (i.e., relevant microbes and their metabolic activities), and how their presence and activity depend on environmental conditions. In this Perspective, we present the current understanding and recent methodological advances that can help to identify such links, even in complex environmental microbiomes and for contaminants present at low concentrations in complex chemical mixtures. We discuss the ensuing insights into contaminant biotransformation across varying environments and conditions and ask how much closer we have come to designing improved approaches to reducing environmental exposure to contaminants.



1. DRIVERS BEHIND BIOTRANSFORMATION: CHANGES AT LOW CONCENTRATIONS

Microbes, including bacteria, archaea, fungi, and other small eukaryotes, harbor a tremendous diversity of catabolic capacities and are nearly ubiquitous. They therefore play a key role in reducing environmental exposure to chemical contaminants by transforming them into less bioactive compounds, which can continue all the way to complete mineralization.[1,2] Compared to other types of environmental transformations such as photolysis or abiotic redox reactions, microbial transformation is generally considered to be the most important degradation process in terms of mass balance.[3] However, there is a growing body of evidence that increasing levels of chemical contamination in the environment directly impair human and environmental health[4] (see, e.g., loss of invertebrate biodiversity in pesticide-polluted streams[5] or compromised reproduction in humans due to exposure to endocrine-disrupting chemicals[6]). Given the need to manage increasingly closed local and regional water cycles[7] and the ever-increasing production and consumption of chemicals,[8] we can no longer just passively rely on microbes to reduce contaminant levels. Rather, we should aim to actively take advantage of their capabilities to decontaminate water resources by developing optimized engineered systems at critical control points of the water cycle (e.g., at wastewater and drinking water treatment facilities) or by designing new chemicals in a way that conserves their intended activity while rendering them readily transformable to innocuous substances in the environment. Designing systems and/or chemicals optimized for microbial transformation requires understanding (i) the causal links between contaminant removal and the specific agents of biotransformation (i.e., relevant microbes and catabolic pathways down to the enzyme level) and (ii) the conditions under which biotransformation can occur.

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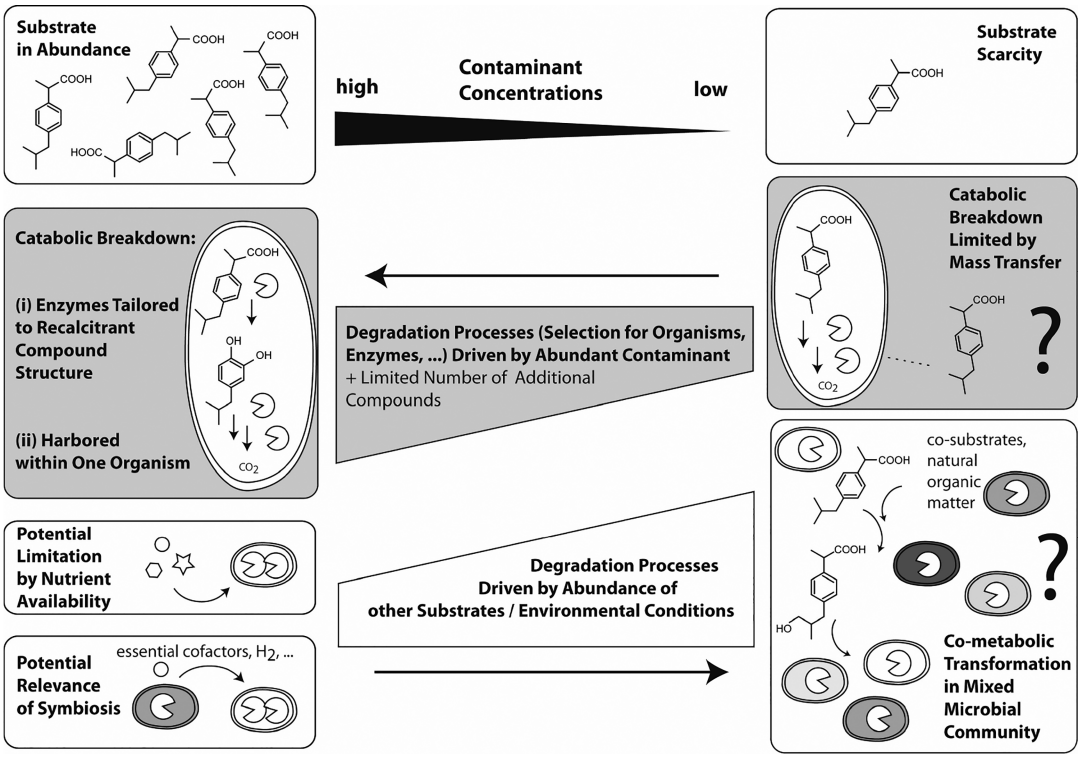

Figure 1. Biodegradation as driven by an interplay among environmental conditions, compound structure, compound concentration, and potential active agents of biotransformation. The selective pressure imposed by the substrate decreases at lower concentrations, so that environmental conditions become more important. Consequently, drivers of biodegradation may change fundamentally when moving from high (left) to low (right) concentrations.

In general, any observed contaminant biotransformation outcome (i.e., whether a given contaminant is removed, at what rate, and yielding what kind of products) is the result of a complex interplay of several factors (Figure 1). These factors are (i) the bioavailable concentration of the contaminants in question, (ii) the composition and capabilities of the microbial community (e.g., expressed metabolic pathways or single enzymes), and (iii) the presence of the proper substrates and electron donors or acceptors.[9,10] Different environmental conditions (e.g., redox conditions, temperature, humidity, nutrient status, chemical exposure, microbial residence time, etc.) determine these factors and hence exert control on contaminant biotransformation. For each individual contaminant, the extent to which it is biotransformed is in great part determined by those factors and by its chemical structure, which determines its intrinsic recalcitrance, i.e., its ability to interact with specific enzymes.

Over the past 30−40 years, a large body of biotransformation knowledge has accrued for a number of major legacy contaminants, mostly in a bioremediation context, i.e., for highly concentrated mixtures (e.g., chlorinated solvents,[11−14] BTEX,[9,15] or explosives/munitions chemicals[16]). In such a context, contaminants are typically metabolically degraded; i.e., they can be used as sources of carbon or other nutrients, and/or as electron donors or acceptors by microbes, and, therefore, drive the general biodegradation setting (i.e., selection for organisms, evolution of pathways, etc.) (Figure 1, left side). Consequently, to explore optimal conditions for bioremediation, research has focused on identifying specific degraders or consortia that become dominant under such thermodynamically favorable conditions, involving experimentation with enriched or pure cultures, characterization of metabolic pathways involving growth assays, and enzyme purification and characterization. More advanced work has focused on the intricate metabolic interactions in contaminant-degrading consortia, thus introducing a community level perspective.[17,18] This research has taught us the importance of mutualistic and symbiotic interactions within complex microbiota, where partner organisms either generate necessary electron donors (e.g., hydrogen[19]) or supply cofactors (e.g., corrinoids[20]) essential for efficient pollutant removal (Figure 1, left side).

More recently, awareness of the widespread presence of highly complex mixtures of hundreds of anthropogenic chemicals at low concentrations (i.e., in the low microgram per liter to nanogram per liter range) throughout aquatic and terrestrial environments has increased.[21] Biodegradation of these chemicals of emerging concern at low concentrations is far less understood but is key to developing and deploying strategies to reduce environmental exposure to such contaminants (Figure 1, right side). While the importance of substrate availability, i.e., the concentration of the contaminant in question, is recognized in principle,[22] bioavailability limitations have primarily been expected from diffusion through microbial biofilms,[23] or when compounds are sorbed to the solid matrix in heterogeneous environments such as soils.[24] In contrast, for suspended microbes in water, dissolved contaminant concentrations next to the cell have commonly been considered to be directly available. Yet with decreasing pollutant concentrations, it can be expected that metabolic degradation, i.e., biodegradation by abundant, specialized degraders with targeted, efficient enzymes, will become less competitive, even for fully dissolved and hence available contaminants, because energy gained from degrading the available contaminant may no longer be sufficient to fulfill the

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degraders' energy requirements. In that case, mixed substrate usage and co-metabolic ("fortuitous") transformation by promiscuous enzymes expressed for different purposes may potentially become more prominent. With this, exertion of selective pressure by the contaminant decreases, while the influence of environmental conditions, e.g., the presence of a co-substrate such as natural organic matter and the composition and activity of microbial communities (which itself is a product of the environmental conditions), on contaminant degradation increases (Figure 1, right side). The following questions specifically arise in this context. (1) Is there a concentration below which contaminant availability becomes so limiting that a niche for catabolic breakdown by degraders no longer exists? (2) If so, are there examples and circumstances under which chemicals are efficiently transformed despite low concentrations? (3) What are the underlying degradation mechanisms in those cases? Are those compounds still metabolically degraded, meaning that the complete enzymatic toolbox is hosted within one degrader or small degrader consortium that fulfill their overall energy and nutrient requirements from mixed substrate usage, or are those compounds co-metabolically ("fortuitously") transformed and hence do not serve as growth substrates for a specific degrader or degrader consortium? In the latter case, enzymes, the active agents, may no longer be harbored by a single organism only, but may be distributed over a microbial community ("metaorganism") (Figure 1, right side). An important consequence of co-metabolic degradation is that degradation might not be complete but stop at any given stage, raising questions about the fate and effect of recalcitrant transformation products thus formed.

The major challenge in acquiring mechanistic insights into biodegradation under low concentrations is that well-established direct approaches of our disciplines may no longer be adequate. Enrichment cultivations, requiring high contaminant concentrations and reducing highly diverse communities to simple, few-membered communities, are not likely to inform on complex environmental settings relevant at low concentrations. The limitations are even more pronounced if the responsible enzymes and catabolic fluxes are distributed over multiple organisms. Advanced methodological approaches are thus essential for probing and understanding contaminant biotransformation in complex mixtures with other contaminants and in complex microbial communities. In this Perspective, we therefore first survey recent methodological advances that open new windows of opportunity for gaining mechanistic insights into contaminant biotransformation at relevant environmental concentrations and at the level of complex microbial communities (section 2). We then present concrete examples of successful application of these methods to gain mechanistic insights into low-concentration contaminant transformation across varying environments and conditions (section 3). In section 4, we discuss the extent to which these emerging insights may eventually inform the design of improved chemical and engineering approaches to reduce environmental exposure to contaminants. Finally, in section 5, we put everything into context and highlight approaches that seem to have the greatest potential for future progress in contaminant biotransformation research.

2. NOVEL METHODS FOR ELUCIDATING BIOTRANSFORMATION AT TRACE LEVELS

In the following, we focus on recent methodological advances that are, or have the potential to be, instrumental in providing mechanistic insights into contaminant biotransformation at low, environmentally relevant concentrations and at the level of complex microbial communities. These methods can support us in answering two major questions in this context. (a) Is the contaminant transformed or even fully degraded? (b) Can the observed degradation activity be linked to specific microbial community functions (i.e., responsible organisms or enzymes)? Figure 2 summarizes the extent to which the different methods presented in the following contribute to answering these questions. One specific feature of this type of research is its need for being highly interdisciplinary, bringing together methods from analytical chemistry, molecular biology, high-throughput experimentation, and data sciences.

2a. High-Resolution and High-Sensitivity Chemical Analytics for Detecting Chemical Contaminant Transformation. The detection and characterization of low-level contaminant transformation in the environment has been revolutionized by two major technological advancements in analytical chemistry: high-resolution mass spectrometry (HRMS) and compound-specific isotope analysis (CSIA). These are complemented by different labeling-based approaches that can be applied in the case of metabolic degradation and will be presented separately further below.

Toward the turn of the century, the advent of softer ionization techniques, readily coupled to both gas and liquid chromatography, allowed for the detection of intact molecular ions. Robust and sensitive HRMS instruments now make the detection and quantification of low concentrations (i.e., down to a few nanograms per liter) of individual contaminants possible, also in complex contaminant mixtures and without the need for spiked reference standards.[25] For transformation research, this has opened two unprecedented possibilities. First, transformation products can be sought[26,27] and identified with reasonable certainty and accuracy[28] using suspect and nontarget workflows both in batch experiments and in full-scale treatment systems.[29] This allows characterization of contaminant degradation with respect to not only parent compound half-lives but also the actual enzymatic transformation reaction(s) that has taken place. Second, the possibility of resolving individual compound signals now allows us to study transformation processes in complex substance mixtures, generating information about biotransformation outcomes (e.g., rates and products) for many, structurally diverse chemicals under fully consistent conditions (see section 3a for an example). The sensitivity of these methods even allows the observation of biotransformation at ambient contaminant concentrations present in the environments of interest (e.g., impacted rivers). This allowed the demonstration, for instance, that biotransformation kinetics for some compounds differed between experiments that were run with the chemical residue levels present in the environment (down to50 ng/L) compared to those spiked to a specific, higher starting concentration of, e.g., 50 μg/L. This study highlighted the importance of understanding how community pre-exposure to contaminants and relative spike levels influence observed biotransformation kinetics.[30]

CSIA, the second analytical innovation, accesses a previously untapped source of information, naturally occurring stable

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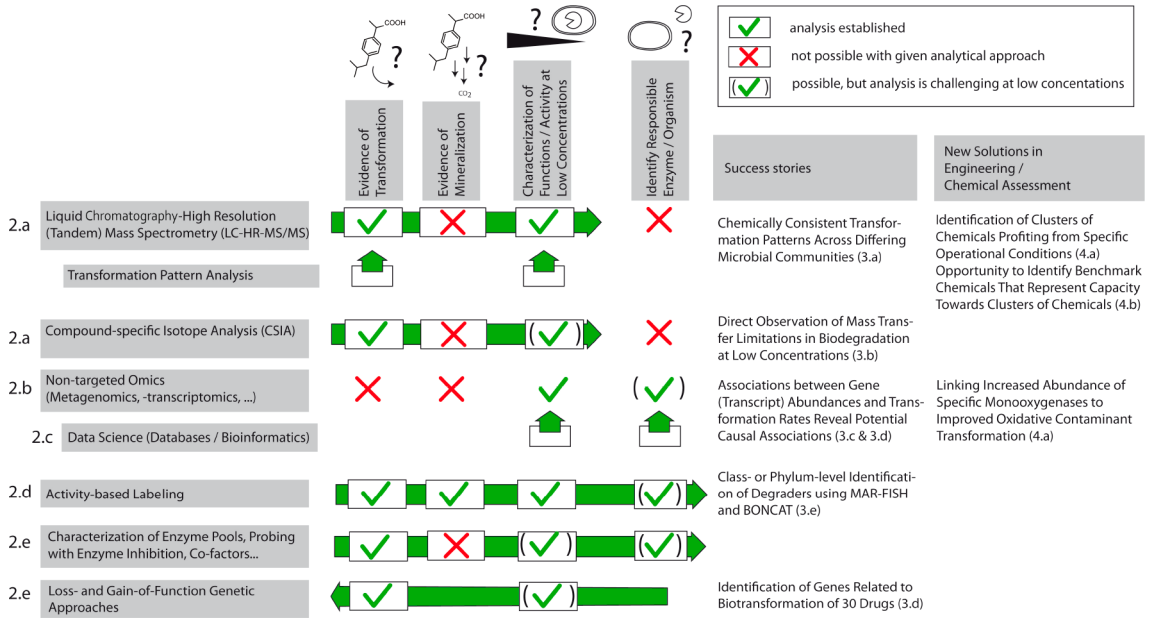

Figure 2. Realized and potential contributions of novel analytical and experimental methods for elucidating the mechanisms and agents of biotransformation at trace levels and for conceiving new solutions for environmental engineering and chemical assessment.

isotopes in organic contaminants. Analysis by gas or liquid chromatography, coupled to online conversion to CO_2, N_2, or H_2, and subsequent isotope ratio mass spectrometry (IRMS) (ref 31 and references cited therein) can measure contaminant isotopic signatures ($^{13}C/^{12}C$, $^{15}N/^{14}N$, etc.) and deliver two different types of information. First, isotopic fingerprinting can elucidate different origins of the same chemical compound. Second, gradual changes in contaminant isotope ratios (over time or with transport distance) can detect natural contaminant degradation, because enzyme reactions usually prefer molecules with light isotopes (kinetic isotope effect). This leaves molecules with heavy isotopes behind, resulting in characteristic changes in contaminant isotope ratios that allow the detection of biodegradation. CSIA of multiple elements (C, Cl, or N) within the same contaminant can even reveal reaction-specific characteristic isotope effect patterns. This made it possible to discriminate underlying transformation mechanisms, such as different atrazine degradation pathways and diverging reaction mechanisms in reductive dehalogenases.[32,33] In section 3b, we give an example of how CSIA can further be used to discriminate between diffusion and enzymatic biotransformation as rate-limiting steps in contaminant degradation. For studies at low contaminant concentrations, CSIA poses the challenge that rare isotopes (^{13}C and ^{15}N) need to be analyzed with the same precision as their more abundant counterparts. To this end, optimized and validated extraction methods need to be developed that push respective quantification limits into the submicrogram per liter range.[34]

2b. Nontargeted Omics Approaches for Resolving Microbial Community Functions in Relation to Trace Contaminant Degradation. To explore the role of microorganisms in complex communities as active agents in low-level contaminant transformation (Figure 1, right side), modern high-throughput DNA sequencing technologies (Illumina, Nanopore, MinION, etc.) are becoming an essential tool. Analysis of specific marker genes, such as that of rRNA, provides information about the phylogenetic placement of populations and on community composition, today with a resolution at the genus or even species level. Sequencing of the entire DNA (metagenome) or mRNA (metatranscriptome) pool of a complex microbial community, termed metagenomic or metatranscriptomic sequencing, respectively, additionally allows us to access the metabolic and biochemical capacities of individual populations and entire communities and can thus point toward potentially active biotransformation functions in the analyzed microbiome. These technologies have enabled the generation of ample knowledge about microbiota from diverse habitats, such as oceans,[35] soil,[36] wastewater,[37] and human and animal hosts.[38] Wu et al.,[37] for instance, analyzed 16S rRNA gene sequences from more than 1200 wastewater treatment plants (WWTPs) on all six continents and used the data to elucidate which operational and environmental factors most strongly influence community composition and functioning in terms of BOD removal.

However, such sequencing approaches come with two major limitations in elucidating the main drivers of contaminant transformation. First, the identification of genes of interest in such large data sets may be all but trivial if the relevant catabolic pathways and reactions for a certain contaminant transformation have not been resolved *a priori*. Second, even if pathways are known and thus can be tracked, metagenomic or metatranscriptomic detection does not provide direct evidence that the respective pathway is actively involved in a given contaminant degradation process. In that context, it has been argued that (meta)proteomic data, i.e., the nontarget enumeration of enzymes in a given microbial community, describe the pool of enzymes potentially involved in contaminant transformation most directly[39] and hence, particularly in combination with separation of the enzyme pools by compartment (intra- and extracellular), have the greatest potential of the different omics approaches to provide new mechanistic insights. As a prerequisite, however, protein identification and annotation require the availability of sample-specific metagenome data, which need to be interpreted and annotated with due caution.[40] Also, wherever a certain

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pollutant induces the expression of a specific catabolic pathway, a variety of omics approaches (metagenomics, -transcriptomics, or -proteomics) may be successfully applied to identify functionally relevant microbiome components.[41] Yet at low contaminant levels, the expected potential lack of metabolic pathway induction, particularly for co-metabolic transformation, questions the applicability of this approach for identifying agents of transformation.

Alternatively, omics-based profiling of different microbial communities may be combined with data-driven analysis pipelines to generate correlation-based hypotheses about the link between functions of interest, e.g., contaminant biotransformation and specific microbes or genes (see section 3c for an example).[42] However, despite the first success stories, three major challenges are associated with correlational approaches. First, they often suffer from poor statistical power due to the high number of taxonomic and genetic features in a (meta)transcriptome/proteome compared to the typically limited number of observations and biological replicates. Second, the lack of standardized procedures for sample preparation, data treatment, and analysis may hamper quantitative analyses and cross-comparisons between studies. In particular, the normalization of transcript abundances across diverse communities and sequencing libraries is the subject of ongoing debate[43,44] yet may strongly affect the outcome of quantitative or correlative interpretations. Third, the reliance on gene annotation and available databases to interpret microbial functions is often compromised by the fact that new annotations build on existing annotations in databases. Error rates in functional assignment for large, uncurated sequence databases have been documented to be as high as 80%, a compounding problem that has been termed "annotation rot".[45] In the following, we therefore discuss how pertinent databases and bioinformatics tools currently being developed may aid in alleviating these annotation challenges.

2c. Databases and Bioinformatics Approaches to Link Observed Transformations to Enzyme Functions and Underlying Gene Sequences. Protein and genome databases are essential for any workflow trying to link observed biotransformation reactions to genes and enzymes potentially catalyzing them. While there are a large number of freely available resources covering enzymatic reactions and pathway maps (e.g., KEGG, BRENDA, Rhea, and MetaCyc), protein sequences including functional annotations (e.g., SwissProt, UniProt, Pfam, and EggNOG), and genomes of individual bacterial strains (e.g., proGenomes2, RefSeq, Ensembl, and PATRIC), these are mostly focused on central and secondary metabolism and contain scarce information about contaminant biotransformation. For our purpose, databases that specifically collate relevant and well-curated information about contaminant biotransformation (i.e., pathways, enzymes, metabolites, and kinetics) therefore play a crucial role. Prominent examples include the Eawag-BBD/PPS (http://eawag-bbd.ethz.ch),[46] originally developed at the University of Minnesota, and its successor, enviPath (http://envipath.org),[47] or the plastics microbial biodegradation database PMBD (http://pmbd.genome-mining.cn/home/).[48] Relative to Eawag-BBD/PPS, enviPath provides opportunities to store information about biotransformation kinetics besides pathway information and to supply both pathways and kinetics with metadata on environmental and operational conditions. These should allow the exploration of the dependence of contaminant removal on environmental conditions to learn about the causes underlying the observed variability in microbial biotransformation and to learn how to shape conditions to foster effective biotransformation (see section 3a for further discussion).

On the basis of these and other databases containing pathway and enzyme information, reaction mining and machine learning methods have been applied to develop a number of derivative, online tools that predict likely microbial contaminant biotransformation reactions and pathways based on chemical structure, such as Eawag-PPS,[49] enviPath,[47] BNICE,[50] and Catalogic.[51] Information contained therein can also be used to link observed or predicted transformation reactions to enzyme classes that can likely catalyze the respective transformation reaction[52−55] and hence support a more targeted mining of omics data for associations between genes or enzymes and specific biotransformation reactions.[42]

One general limitation when trying to associate genes with observed biotransformation reactions is that only a limited number of biodegradation genes fall into families with distinct and limited enzymatic activities such as *s*-triazine ring opening[56] or fumarate addition.[57] Instead, most biodegradation reactions are catalyzed by enzymes in large protein families that are multifunctional. This requires more sophisticated sequence analysis to find signatures of specific reaction subclasses. In a biosynthesis context, for instance, AdenylPred was developed as an online tool (http://z.umn.edu/adenylpred) that can predict substrate type for adenylate-forming enzymes on the basis of their sequence.[58] More toward biodegradation, a similar machine learning approach has been developed that predicts substrates of bacterial nitrilases on the basis of sequence.[59] These and similar approaches rely on classifying multifunctional families into subfamilies based on their sequence similarity. By choosing appropriate similarity cutoff criteria, the user can observe clusters of highly related sequences that generally correlate with specific functions. These tools have delineated numerous biodegradation pathways and their enzymes and as such are contributing to combating "annotation rot".[60,61] A parallel approach is to directly predict the binding and reactivity of specific pollutants by known enzymes with X-ray structures via docking and molecular dynamics simulations.[62,63] This is particularly useful with pervasive microbial biodegradative enzymes, for example, with aromatic hydrocarbon oxygenases, for which many X-ray structures of divergent members of the class are available. Once enzymes with predicted reactivity toward specific substrates are identified, sequence signatures derived from BLAST-type alignments can be used to identify closely related genes and enzymes in other microorganisms.

2d. Labeling-Based, Targeted Molecular or Cellular Approaches for Tracing Transformation Pathways and Responsible Organisms. Labeling represents a further paramount approach for linking process-relevant microbial populations to specific pollutant-degrading functions within complex microbiota.[64] Process-based labeling typically involves addition of a labeled substrate, along with the detection of label incorporation (mostly stable isotopes or fluorogenic substrates) into degradation products, cellular biomarkers, enzymes, or entire cells. While labeled degradation products enable us to distinguish between co-metabolic transformation and catabolic breakdown to CO_2, incorporation in biomolecules provides a unique angle for linking a process to responsible microorganisms. However, while some of the respective labeling tools have already found their way into

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bioremediation practice,[65] their application to pollutant degradation at low concentrations or even to co-metabolic biotransformation remains a major challenge. The most important limitation is the substantial amount of label (e.g., ^{13}C) that needs to be assimilated from the pollutant into biomass to allow for canonical process-based labeling strategies, such as stable isotope probing (SIP) of nucleic acids.[66]

Therefore, a major research need lies with the invention and application of labeling strategies capable of tracing low-level or co-metabolic activities. Here, recently emerging indirect and combinatorial labeling strategies can possibly lead the way. First, if hypotheses about the "main" metabolism of co-metabolic degraders are available, parallel labeling strategies can be designed to substantiate co-metabolic degradation. For instance, the monooxygenase systems of methanotrophs are known to be capable of co-metabolic micropollutant degradation[67] and methylotrophs have been reported as labeled in experiments with ^{13}C-labeled micropollutants.[68] Experimental settings with switched labeled methylotrophic substrates and micropollutants could directly link such metabolic activities for the same complex inocula.[68] Next, SIP studies with labeled contaminants have been conducted in parallel across wide ranges of contaminant concentrations (e.g., for endocrine disruptors[69]), thus attempting to extrapolate the involvement of identified populations at low concentrations *in situ*.

Recent advances in the detection of labeled gene products (transcriptome-SIP and protein-SIP[70,71]), albeit accomplished only for catabolic degradation to date, suggest that such methods may also be useful for dissecting functional adaptations during the co-metabolic degradation of contaminants within complex microbiota, if assumptions about the main catabolic activities of degraders are at hand. However, technical thresholds of labeling and detection thresholds may again limit actual applications at low concentrations. Here, advanced mass spectrometric approaches (e.g., NanoSIMS[72]) or high-sensitivity approaches for detecting cellular labeling such as Raman spectroscopy-based cell sorting, which can recognize and sort out labeled cells at the single-cell level,[73] may offer important advantages. These approaches may be particularly promising when combined with general activity-based labeling strategies using ^{18}O- or deuterium-labeled water.[73,74] In that case, rigorous comparative experiments with and without added contaminants can potentially identify cells specifically involved in contaminant degradation and thus provide access for downstream single-cell genomics or even strain cultivation.[75] Indirect labeling can also alleviate notorious limitations in acquiring ^{13}C-labeled substrates for organic contaminants, which either can be very costly or are not commercially available. Such "next-generation physiology" approaches[75] using cellular labeling with isotopic or fluorogenic substrates will be indispensable to truly link specific microbial populations to contaminant degradation in the environment.

2e. From Targeted to High-Throughput Screening Approaches for Uncovering Enzyme Activity and Specificity in Contaminant Transformation. A number of techniques have been developed and used regularly in different contexts to confirm enzymatic activity on specific substrates. These should also be highly instrumental as a second confirmatory tier to the workflows described above in cases in which there are strong hypotheses about the activity of a limited range of enzymes on a number of potential substrates. Such techniques include enzyme inhibition or addition of cofactors (e.g., refs 76 and 77) and heterologous cloning and expression of target genes in host cells to confirm activity on specific substrates in whole cell assays or with purified enzymes. More recently, the latter approach has been developed further into more of a screening approach by using prior bioinformatics analysis to identify clusters of enzyme homologues that are suspected to catalyze certain types of transformation reactions, followed by cloning and experimental testing of the gene products on a range of potential substrates. These screening approaches typically rely on the availability or synthesis of substrates that produce fast readouts upon enzymatic conversion (e.g., fluorogenic probes[78] or products with specific absorbances such as *p*-nitrophenolate[79,80]).

There are also a number of techniques emerging that allow the discovery of enzymatic activity on contaminants of interest in a more untargeted manner. They typically work with cell-free lysates[81] and often involve some splitting of the enzyme pool into smaller pools, which facilitates downstream (meta)-proteomic analysis if they show activity on specific contaminants of interest. Splitting might be achieved by different techniques such as gel electrophoresis and activity testing on gel slices,[82] or a number of physical and physicochemical treatment steps, e.g., separating the activated sludge enzyme pool into extracellular, EPS-bound, and intracellular fractions.[83] Another proteomics-based approach to identifying contaminant-degrading enzymes employs contaminant-specific chemical probes designed to pull down enzymes that convey a biotransformation of interest, as elegantly applied for the identification of β-glucuronidases.[84] The currently probably most untargeted and high-throughput tools for uncovering microbiome-encoded genes involved in contaminant biotransformation take advantage of loss-of-function and gain-of-function genetic approaches, which combine genetic screening of whole genomes with phenotypic (e.g., antibiotic resistance) or high-throughput mass spectrometry-based readouts (see section 3d for an example).[85,86]

3. EMERGING INSIGHTS FROM APPLICATION OF NOVEL METHODS

Using a combination of existing and emerging technologies as introduced above, a number of novel insights into low-concentration contaminant transformation in complex microbial communities were recently achieved.

3a. HRMS-Based Analysis Reveals Transformation-Specific Patterns. Emerging sets of consistent information about both biotransformation kinetics and reactions across large numbers of diverse chemicals, now accessible through HRMS/MS analysis, allow us to study patterns of contaminant biotransformation as a function of environmental and operational conditions and across different microbial communities. Kinetic analysis of biotransformation experiments with differently conditioned activated sludge and riverine biofilm communities revealed characteristic trends for groups of substances undergoing similar types of initial transformation reactions,[87−89] suggesting that shared enzymes or enzyme systems that are conjointly regulated catalyze biotransformation reactions within such groups. In a number of recent studies exploring the influence of environmental or operational conditions on contaminant biotransformation across large sets of literature-retrieved data, global models embracing all

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compounds were therefore compared to class-specific models for groups of contaminants hypothesized to undergo similar initial biotransformation reactions. The latter generally showed improved performance in explaining observed variability in biotransformation kinetics.[90,91] However, given that substrate specificities of enzymes are strong and difficult to predict,[59] more such analyses across wider collections of compounds and different microbial systems are warranted to better understand the robustness of such reaction-specific groupings. Further explorations should also provide additional insights into whether the observation of reaction-specific grouping is exclusive to contaminants being transformed co-metabolically by the same unspecific enzyme system(s) or whether such patterns also emerge in the case of metabolic turnover.

3b. Mass Transfer Limitations to Metabolic Degradation as Uncovered by CSIA. Recently, compound-specific isotope fractionation unequivocally demonstrated a largely overlooked role of mass transfer limitation in contaminant degradation at low concentrations. Isotope fractionation is fully expressed if enzymatic turnover is limiting but becomes masked if mass transfer into bacterial cells becomes rate-determining. With bacteria cultivated on atrazine in a chemostat, a drastic decrease in the level of isotope fractionation was observed at a threshold concentration of ~50 μg/L atrazine,[73,74] and a similar decrease in isotope fractionation at lower concentrations was observed for the herbicide metabolite 2,6-dichlorobenzamide (BAM) in an inoculated sediment tank.[92] First, this direct observation of mass transfer limitation demonstrated that diffusion into the cells through the cell membrane can become rate-limiting when concentrations decrease to levels close to the Monod/Michaelis–Menten constant of enzymatic breakdown[93−95] (Figure 1, right side). Second, it revealed that the onset of mass transfer limitation occurs at residual concentrations that are orders of magnitude higher than the nanogram per liter concentrations at which products of biotransformation are observed by LC-HRMS in the environment. Third, mass transfer limitation appeared to trigger adaptation at the cellular level so that growth and turnover rates of catabolic degradation became significantly smaller once this threshold was reached.[94,96] Because substrate uptake is a general prerequisite for metabolic degradation, mass transfer through the cell membrane is expected to become limiting at low concentrations eventually in all bacteria. Further studies may help us to understand how such mass transfer thresholds vary among organisms, in the presence of other substrates and as a function of molecular properties and enzymatic transformation rates.

3c. Association Mining Links Gene Transcripts to Biotransformation Reactions. The observation of reaction-specific variability in contaminant biotransformation across different microbial communities suggests the possibility of identifying genes for certain prevalent biotransformation reactions that would allow characterization of a given microbial community for its capacity to perform the respective reaction on a range of structurally related compounds. Under the assumption of co-metabolic transformation, Achermann et al.[97] have therefore searched for correlations between biotransformation rate constants and gene transcript abundances across a series of activated sludge communities grown at different retention times. They limited the risk of identifying noncausal relationships by restricting the gene search space to those that encode enzymes potentially catalyzing the observed biotransformation reactions. In doing so, they were able to demonstrate linear and proportional relationships between biotransformation rate constants and relevant gene transcripts both for nitrification as a major community function and, more importantly, for biotransformation of two nitrile-containing contaminants (i.e., bromoxynil and acetamiprid) present at trace concentrations. While conversion of bromoxynil by nitrile hydratase had been known before, evidence of activity of nitrile hydratase-type enzymes on acetamiprid was only demonstrated later.[97,98] Similar correlational approaches between kinetic and gene (transcript) abundance data have also been used by others to highlight enzyme classes potentially involved in pharmaceutical biotransformation during wastewater treatment[99] and in contaminant biotransformation in soil columns representing managed aquifer systems.[100,101]

3d. Gain-of-Function Genetic Libraries Highlight Relevant Enzyme Functions in Gut Microbiota. A recent study illustrated the use of high-throughput mass spectrometry screening and genetic gain-of-function screens to identify microbial agents (i.e., strains and enzymes) that are responsible for the microbial metabolism of medical drugs in gut microbiota.[85] Using a combinatorial pooling strategy, 271 drugs of maximal structural diversity were tested for potential biotransformation by 76 sequenced bacterial isolates from the human gut. Almost two-thirds of the tested drugs were found to be biotransformed by at least one gut isolate, and each tested bacterial strain transformed between 12 and 76 different pharmaceuticals. Next, a gain-of-function approach was employed to experimentally test all gene-encoded enzymes of the bacterial strains to be found most versatile in their ability to catalyze contaminant transformations. Concretely, the genomic DNA of a given strain was sheared into 2−8 kb fragments, which were cloned in an *Escherichia coli* expression vector, resulting in tens of thousands of *E. coli* clones. The biotransformation capacity of these clones was then tested against the original mixture of drugs to identify actively biotransforming clones and enzymes. With this approach, four of the most competent previously tested bacterial strains were profiled, and 30 genes that collectively biotransformed 20 different drugs were identified. Finally, the identified microbial agents were assessed as potential bioindicators for the biotransforming capacity of complex microbial communities for specific drugs. For this purpose, metagenomic sequencing of fecal communities was combined with the communities' *in vitro* biotransformation rates, which demonstrated significant association between the abundance of the biotransforming enzymes/bacterial strains and the experimentally determined biotransformation rates. Altogether, this study illustrated how the combination of high-throughput mass spectrometry, bacterial culturing, and genetics can provide mechanistic insight into biotransformation processes at the molecular level and how this information could be exploited to identify bioindicators that explain and potentially predict the behavior of complex microbial communities.

3e. Activity-Based Labeling Pinpoints Bacterial Classes Involved in Contaminant Degradation. As mentioned above, a process-based, specific labeling of degrader biomarkers or cells with (stable) isotopes or fluorogenic substrates is not yet routinely applied to low-level or co-metabolic pollutant breakdown. Still, a few promising demonstrations exist, such as a recent phylum level assignment of distinct pharmaceutical degradation capacities at microgram per liter concentrations in wastewater biofilms via micro-autoradiography FISH (MAR-FISH).[102] Although no further

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ACS EST Water 2021, 1, 1541−1554

taxonomic or catabolic detail was provided, labeled populations all belonged to minor phyla within the sludge microbiota, and the study is an important proof of principle that degraders of low-concentration contaminants can be successfully labeled via substrate queries. In a commendably comprehensive study, the desulfurization of perfluorooctanesulfonate (PFOS) at low to high nanogram per liter concentrations was recently linked to distinct microbial clades within Antarctic coastal waters.[103] Here, indirect cellular labeling with bio-orthogonal non-canonical amino acid tagging (BONCAT)[75] revealed that distinct members of the Gammaproteobacteria, *Roseobacter*, and SAR11 groups were most responsive to PFOS amendment. Flanked by clearly apparent community level transcriptome adaptations within the microbial sulfur metabolism, this study shows how even the incomplete degradation of perfluorinated compounds at low concentrations can be traced by cellular labeling approaches.

4. APPROACHING NEW SOLUTIONS IN ENGINEERING AND CHEMICAL ASSESSMENT BY TAKING ADVANTAGE OF ADVANCES IN CONTAMINANT BIOTRANSFORMATION RESEARCH

4a. Design of Novel Engineered Treatment Systems. Processes based on biodegradation principles are an intriguing alternative for removal of chemicals from water due to lower energy requirements as compared to those of physiochemical processes, no generation of residuals, little or no need for auxiliary chemicals, and the possibility of tailoring them to transform specific chemicals or structures. The advances in analytical chemistry and biomolecular microbiology can now provide insights into the presence and nature of contaminants before and after treatment, and we can use all of the metadata to understand what it takes in terms of physical, chemical, and biological environments to bring about effective biotransformation.

These advantages have already been well recognized in the fields of chemical engineering and industrial biotechnology where contemporary concepts of modifying complex microbial communities for targeted biotransformation of well-defined chemicals are established, known as synthetic biology (SynBio) or metabolic engineering.[104,105] However, while these concepts hold promise, they have not yet delivered beyond the laboratory scale (with some notable exceptions, e.g., PETase[106]) and applying these concepts to engineered applications for water treatment is still challenging. While scalability could likely be resolved, efficiency is a determining factor for adoption of a new treatment process. For the design of a compact, efficient, and competitive process, short hydraulic retention times are desired, requiring concentrated amounts of active biomass with enhanced biotransformation rates. Given the mostly low contaminant concentrations, neither of these conditions is likely to occur naturally, thus requiring a number of engineering interventions to provide a robust and efficient process. These might include a combination of enhanced biofilm processes, physical partitioning, and tailored chemical reactions in bulk solution and on surfaces. For instance, competent degrader strains may be introduced into the biological treatment system, which, however, need to be reliably sustained over extended time periods to minimize restart periods and process down times.[107,108] Other operational elements that may be considered in the design of novel biological processes include targeting, enriching, and delivering proper degraders or consortia, maintaining suitable and stable redox conditions, preconditioning (i.e., preacclimation in side streams; bioaugmentation), and maximizing exposure (i.e., by improving bioavailability and attachment of biofilms).

Addition of suitable degraders has been successfully used in drinking water treatment settings under ambient conditions, for instance, for the pesticide metabolite 2,6-dichlorobenzamide (BAM), which is very persistent and one of the most frequently detected contaminants in groundwater. Instead of application of advanced post-treatment, augmenting conventional sand filters with the previously identified degrader strain *Aminobacter* sp. MSH1 resulted in efficient biodegradation of BAM at environmentally relevant concentrations.[109] While performance was limited by a loss of degraders, it was later improved by immobilization of the *Aminobacter* using carriers.[110]

Extending solid retention times (SRTs) and applying higher dissolved oxygen levels during conventional activated sludge (CAS) processes can increase microbial diversity and the number of opportunities for co-metabolic biotransformation of chemicals.[91,111] In particular, employing biofilm-based systems after conventional secondary treatment can offer a high degree of biodiversity within the biofilm structure and operational conditions that are easier to adjust.[112] A tight control of more favorable operational conditions creates niches for establishment of consortia that are capable of degrading chemicals that persist under normal conditions. This was demonstrated recently at lab and full scale by establishing sequential biofilters characterized by carbon-starving and oxic conditions resulting in significantly improved removal of mixtures of chemicals at ambient concentrations occurring in impaired surface water and wastewater effluents.[113,114] Functional gene presence and expression profiles derived from metagenomic and metatranscriptomic analyses of these systems suggest higher ratios of genes associated with the biodegradation and metabolism of diverse contaminants, including vanillate monooxygenase (EC 1.14.13.82), 2-hydroxyhexa-2,4-dienoate hydratase (EC 4.2.1.132), and 2-oxopent-4-enoate hydratase (EC 4.2.1.80).[101]

4b. Development and Authorization of New Chemical Substances. New capabilities brought about by modern sensitive analytical techniques for generating biotransformation data for multiple chemicals in different environments under realistic conditions also create new opportunities to strengthen chemical regulation. Environmental persistence, a keystone parameter in chemical regulation, is assessed with laboratory tests that provide quantitative outcomes that are difficult to reproduce and have an unknown relationship to biodegradation rates in the environment. The emerging data will make it possible to explore the relationship between laboratory test outcome and environmental biodegradation rates and how this relationship depends on chemical structure and environmental conditions.

The new analytical capabilities are also strengthening the basis for the application of benchmarking techniques that build on measuring the relative behavior of the test chemical with respect to benchmark chemicals rather than quantifying absolute biodegradation rates. Wisely chosen benchmark chemicals can provide information about test system properties and correct for sources of variability in biodegradation rates.[115] Benchmarking can now be applied on large sets of experimental biotransformation data to explore the potential

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for read-across of the biotransformation rate of a given chemical between different environments. For a data set of 27 chemicals with widely ranging properties and biodegradation rates from 35 different laboratory reactors, normalization against the biodegradation rate of one of the chemicals reduced the mean between-reactor variability from 0.65 to 0.35 log unit (unpublished data). For a number of groups of chemicals that underwent similar initial biotransformation reactions, variability was even further reduced when the benchmark was chosen from that group.

In addition, after correction for bioavailability, good correlations were recently reported between biotransformation half-lives in soil obtained through regulatory simulation studies and short-term experiments with activated sludge for a set of ∼40 chemicals. This not only provides evidence that the relative biotransformation behavior of chemicals might even be conserved to some extent between environments but also highlights the potential to read-across biodegradation rates between environmental systems.[116] Using efficient, short-term tests (e.g., with activated sludge and mixtures of test chemicals) for estimating outcomes of regulatory tests might pave the way toward considering biotransformation potential at an early stage of compound development and hence support the development of environmentally benign new chemicals.[116]

In line with the new EU Green Deal strategy toward a toxic-free environment, these developments promise more precise regulatory test protocols with greater environmental relevance and eventually also experimental strategies or accurate predictive tools to include consideration of degradability in chemical development. However, in a regulatory context, uptake of new testing strategies or data evaluation protocols is slow because of the need to agree on legally defensible instruments that afford long-term planning safety to chemical companies. Here, low-key innovations on the data evaluation side, such as benchmarking, might support weight-of-evidence evaluations without the need to modify regulations.

5. LESSONS LEARNED

Despite all of the advances in gaining deeper mechanistic insights into contaminant biotransformation at relevant environmental concentrations (Figure 2), we conclude that we are just at the beginning of a system's understanding that could ultimately result in improved approaches to reduce environmental exposure to contaminants. In our opinion, the following aspects are key to reaching this goal.

First, while there is evidence suggesting that contaminant transformation at trace level concentrations and in complex mixtures by environmental microbial communities involves both metabolic and co-metabolic degradation, experimental and analytical methods for discerning the two mechanisms under relevant conditions need to be further improved. This seems essential to further understand controls of one or the other and, in a next step, being able to apply these controls to promote contaminant biotransformation in treatment systems. Specific questions of interest include the following.

- There are threshold concentrations for metabolic degradation, but why are they, at least for some compounds, lower than previously assumed? What role does mixed substrate usage play in those cases? Or may low-level degradation be partly driven by other selection criteria, e.g., that some contaminants are not primarily used as an energy or carbon source, but as a source of other essential elements (N, S, P etc.)?
- For co-metabolic degradation, are there a number of low-specificity enzymes that are behind those transformation-specific patterns seen across different microbial communities? If so, what are they and what conditions lead to their expression and/or high abundance?

Second, approaches for linking the presence of enzymes or microbes to activity and thus for helping to identify the key players of biotransformation need to be further developed and tested for different contaminants and environmental microbial communities. From Figure 2, we can identify approaches that have the greatest potential to fully identify responsible enzymes and organisms as highlighted by the green arrows. Overall, we see the most promise in techniques that are untargeted and allow for discovery, yet also believe that there is potential in improving more data-driven approaches. More specifically, these include the following.

- Next-generation, activity-based labeling approaches such as MAR-FISH, BONCAT, or Raman-based sorting of labeled cells can potentially pick out degraders in complex, realistic settings. However, this approach will most likely remain limited to primarily elucidating "metabolic degradation" (Figure 2, 2d).
- High-throughput screening libraries ("loss- and gain-of-function genetic approaches") for bacterial strains of specific interest (i.e., because of their known broad contaminant biotransformation potential) are an untargeted way of discovering abundant functions, e.g., unspecific enzymes that can co-metabolically transform a wide range of contaminants (Figure 2, 2e). While they are currently still experimentally and analytically very expensive, they do not rely on any predictions of reactions, transforming enzymes, etc., and hence help to circumvent limitations of databases such as annotation issues.
- Open repository databases that are specifically focused on contaminant biotransformation will be essential to further our understanding of specific conditions that foster biotransformation and to expand our knowledge of enzymes and strains involved in contaminant degradation. The latter should eventually also help data-driven approaches (i.e., correlation analysis between chemical and omics data) to be used to their full potential (Figure 2, 2c). In this context, it might also be of interest to include information about metabolism in gut microbiota, because gut microbes not only are exposed to many of the same chemicals we also encounter in wastewater but also might be released to WWTPs and receiving environments.[117]

Third, considering the potential highlighted by some of the success stories featured in sections 3 and 4, new solutions in engineering and chemical assessments that take full advantage of novel biological approaches are conceivable, including the introduction of key degraders or co-substrates, creating and controlling operational conditions that foster co-metabolic degradation, or induction of factors that result in upregulation of key functional genes. However, the success of such endeavors will critically rely on a better integration of disciplinary knowledge, particularly among wastewater engineers, microbiologists, and (bio)chemists. In that sense and in

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light of the recent strong push toward green and sustainable chemistry in Europe and worldwide,[118,119] we invite all peers to take advantage of the ideas and techniques presented here to work together toward using the full potential of microbes to provide for toxic-free environments.

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Notes

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