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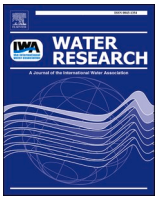

Making waves: Enhancing pollutant biodegradation via rational engineering of microbial consortia

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ABSTRACT

Biodegradation holds promise as an effective and sustainable process for the removal of synthetic chemical pollutants. Nevertheless, rational engineering of biodegradation for pollutant remediation remains an unfulfilled goal, while chemical pollution of waters and soils continues to advance. Efforts to (i) identify functional bacteria from aquatic and soil microbiomes, (ii) assemble them into biodegrading consortia, and (iii) identify maintenance and performance determinants, are challenged by large number of pollutants and the complexity in the enzymology and ecology of pollutant biodegradation. To overcome these challenges, approaches that leverage knowledge from environmental bio-chem-informatics and metabolic engineering are crucial. Here, we propose a novel *high-throughput bio-chem-informatics pipeline*, to link chemicals and their predicted biotransformation pathways with potential enzymes and bacterial strains. Our framework systematically selects the most promising candidates for the degradation of chemicals with unknown biotransformation pathways and associated enzymes from the vast array of aquatic and soil bacteria. We substantiated our perspective by validating the pipeline for two chemicals with known or predicted pathways and show that our predicted strains are consistent with strains known to biotransform those chemicals. Such pipelines can be integrated with metabolic network analysis built upon genome-scale models and ecological principles to rationally design fit-for-purpose bacterial communities for augmenting deficient biotransformation functions and study operational and design parameters that influence their structure and function. We believe that research in this direction can pave the way for achieving our long-term goal of enhancing pollutant biodegradation.

1. High-throughput identification of bacterial strains to rationally assemble bacterial communities for synthetic chemical biodegradation

Synthetic chemical pollution is a planetary boundary threat causing serious environmental, health and societal problems (Escher et al., 2020; Orive et al., 2022; Rockström et al., 2009). Pollution reduction can only be realized if we i) *reduce inputs* by using less chemicals and/or replacing them with more environmentally benign alternatives, and ii) *identify and enhance sink terms* by employing effective remediation and water treatment technologies (Fenner and Scheringer, 2021; Schwarzenbach et al., 2006). Although some chemicals are intrinsically persistent to known enzymatic reactions, many are biodegradable to differing degrees, making bacteria-mediated biodegradation among the most important potential sink mechanisms (Schwarzenbach et al., 2016). However, the extent of biodegradation can vary across locations (Wick and Chatzinotas, 2019) and over time, as biodegradation depends on both the structure of the chemical and its interactions with a complex ecosystem, such as the enzymatic capability of the resident microbial community and the local environmental conditions (Kolvenbach et al., 2014; Regnery et al., 2017). Therefore, when developing more benign chemicals, we need to assess their biodegradability in an environmentally relevant manner and envisage biodegradation solutions at the point of application (e.g., agricultural fields, households) or disposal (e.g., wastewater treatment plants) based on approaches similar to those developed for their biodegradability assessment. However, advancements in biological solutions are currently hindered by the slow pace of knowledge gain on the enzymes and bacterial strains that catalyze and/or stimulate

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chemical biotransformations and their functioning in dynamic environmental conditions (Hübner et al., 2022). Consequently, the potential and limits of chemical biodegradation remain unclear.

Deficient biodegradation functions might be due to functional bacterial strains being absent from engineered or natural systems, which raises a case for bioaugmentation or being present but showing poor performance, which could be addressed by operational or design strategies (e.g., biostimulation). So far, synthetic biology approaches and genetic engineering of functional genes in model bacteria have been proposed for enhancing biodegradation (de Lorenzo et al., 2016), but remain controversial and are hampered by concerns regarding their potential environmental impacts. Compared to genetically modified organisms, microbial communities often have the necessary functions to achieve improved biotransformations via the division of metabolic labor (i.e., where different bacterial strains specialize at performing different functions) (Hübner et al., 2022; Johnson et al., 2012; Li et al., 2021). Moreover, increasing the diversity of a bioaugmentation community increases the probability that at least some members will establish (Vila et al., 2019), resulting in higher success rates when compared to single strains. Natural communities can be enriched under selective environmental conditions; however, they remain complex with limited controllability and might not contain or maintain desired functions (Blasche et al., 2017). Defined synthetic communities (SynComs) (i.e., mixtures of selected strains), in contrast, have reduced complexity, are easier to control, and can be rationally assembled for a function of interest. Indeed, the successful establishment of an optimized and stable microalgae-bacteria consortium for glyphosate degradation highlights the potential of SynComs (Borella et al., 2023). In this rational assembly, the authors leverage a well-documented glyphosate biotransformation pathway, in conjunction with known functional gene sequences and bacterial strains associated with its degradation. This exemplifies that when such knowledge is available, SynComs can be systematically designed and fine-tuned to flourish within specific environmental settings. Yet, given the dearth of this knowledge for most of the chemicals in commerce, identifying and selecting strains from aquatic and soil microbiomes for a SynCom remains challenging. To date, when strains are not reported in the literature, SynCom assembly relies on laborious screening of strain collections (Bhatia et al., 2018; Toju et al., 2020). However, due to ever-expanding number and diversity of chemicals, relying solely on experimental approaches has become impractical. A fast *"high-throughput bio-chem-informatics pipeline"* for the identification and selection of strains that effectively catalyze a set of desired biotransformations up to pre-defined metabolites or complete mineralization would benefit both fundamental and applied microbial ecology, as well as biotechnology applications and the chemical industry. We argue that such a pipeline would also strongly benefit environmental engineering, as SynComs can be applied for bioaugmentation of deficient biodegradation functions and be used to study environmental and operational factors affecting their maintenance and performance as model communities.

To enable such advances, the available data on the biodegradation of synthetic chemicals, including biotransformation reactions and pathways, catalyzing enzymes, and resulting metabolites, have been curated in databases, including data from experiments with isolates as well as with complex soil and activated sludge communities (Eawag-BBD-PPS and its successor enviPath) (Gao et al., 2009; Wicker et al., 2016). These databases have integrated tools to predict and prioritize potential biodegradation pathways using a substructure search that applies expert curated generalized reaction rules (metabolic logic) and machine learning-based relative reasoning (Wicker et al., 2016). In comparison to databases or pathway prediction tools primarily developed for cellular metabolism, e.g., KEGG and BNICE (Hatzimanikatis et al., 2005; Ogata et al., 1999), enviPath focuses on structural elements not present in natural compounds (Wicker et al., 2016), such as aliphatic or aromatic compounds containing halo-, nitro, azo-, sulfo-, or multiple methyl groups, which improves prediction of enzyme-catalyzed reactions of synthetic chemicals. Such pathway prediction tools can help to identify functional bacterial strains, as biotransformation pathways for many chemicals are unknown. However, even if known or predicted biotransformation pathways are available, identifying bacteria that express the responsible enzymes remains challenging, as experimentally observed, or predicted biochemical reactions may lack information on the associated enzymes. Enzymes catalyzing chemical reactions are classified and catalogued by enzyme commission (EC) numbers into four levels, where the 4th level defines the exact reactants and products while the 3rd level groups reactions with shared catalytic mechanisms (Webb, 1992). Typically, amino acid sequence homology based on computational methods is applied to identify novel enzyme candidates for a function of interest (Pearson, 2013). These approaches reach their limits when attempting to link a chemical reaction to an amino acid sequence when no sequence has previously been annotated with that function.

To address this challenge, we propose to leverage knowledge from the field of metabolic engineering, where enzyme and sequence prediction tools such as BridgIT and BridgIT+ have recently been developed which associate chemical structures with enzymes for the effective biosynthesis of chemicals, among other applications (Hadadi et al., 2019; MohammadiPeyhani et al., 2023). To tailor the predictions to biodegradation of synthetic chemicals, the reaction mechanism predictions from BridgIT can be enriched with enviLink. enviLink is a new data module in enviPath that links generalized biotransformation rules used for synthetic chemical biotransformation predictions in enviPath to 3rd or 4th level EC classes (Schmid and Fenner, 2021). We hypothesize that these tools can help to identify and rationally select SynCom strains for target synthetic chemicals based on the known or predicted biotransformation pathways without requiring mechanistic pathway-gene sequence information for those exact chemicals. More specifically, we propose a concrete bio-chem-informatics pipeline (Fig. 1) composed of three steps: (i) extract/predict biodegradation pathways for a synthetic chemical of interest (enviPath), (ii) rank exact/enriched 4th level EC numbers with linked amino acid sequences for each reaction step (enviLink & BridgIT), and (iii) generate an amino acid sequence profile to search sequence databases for bacterial strains harboring the targeted enzymatic function (BridgIT+). As a proof of concept, we applied the pipeline to identify potential biodegrading bacterial strains for two chemicals and evaluated whether our predicted strains are consistent with strains known to biotransform those chemicals. Our goal is to illustrate how such bio-chem-informatics pipelines can inform rational design and assembly of fit-for-purpose SynComs for advancing pollutant biodegradation.

2. The bio-chem-informatics pipeline in action

We selected atrazine and diuron as proof-of-concept chemicals as previous studies identified bacterial strains and communities capable of mineralizing these chemicals and their transformation products (Bers et al., 2013; Bhardwaj et al., 2015; Billet et al., 2021; Breugelmans et al., 2007; Cai et al., 2003; De Souza et al., 1998; Dejonghe et al., 2003; Devers-Lamrani et al., 2014; Esquirol et al., 2018; Jiang et al., 2011; Qingyan et al., 2009; Sørensen et al., 2008; Sorensen et al., 2013; Strong et al., 2002; Vaishampayan et al., 2007; Yao et al., 2011; Zhang et al., 2018). This allowed us to assess how well our pipeline can recapitulate experimentally validated knowledge. For assessing reliability, we chose atrazine, which has a well-characterized biotransformation pathway, and used the hydrolytic pathway from the enviPath database. To evaluate the feasibility of using predicted pathways, we selected diuron, which has a partially characterized biotransformation pathway, and utilized only the predicted pathway from enviPath and Eawag-BBD/PPS.

In our pipeline, BridgIT identifies similarities in reactive sites of an orphan (or predicted) reaction with well-characterized KEGG reactions (i.e., 4th level EC numbers) and their amino acid sequences. The calculated similarity metric helps identify 4th level EC numbers that closely resemble the orphan reaction mechanism (Hadadi et al., 2019).

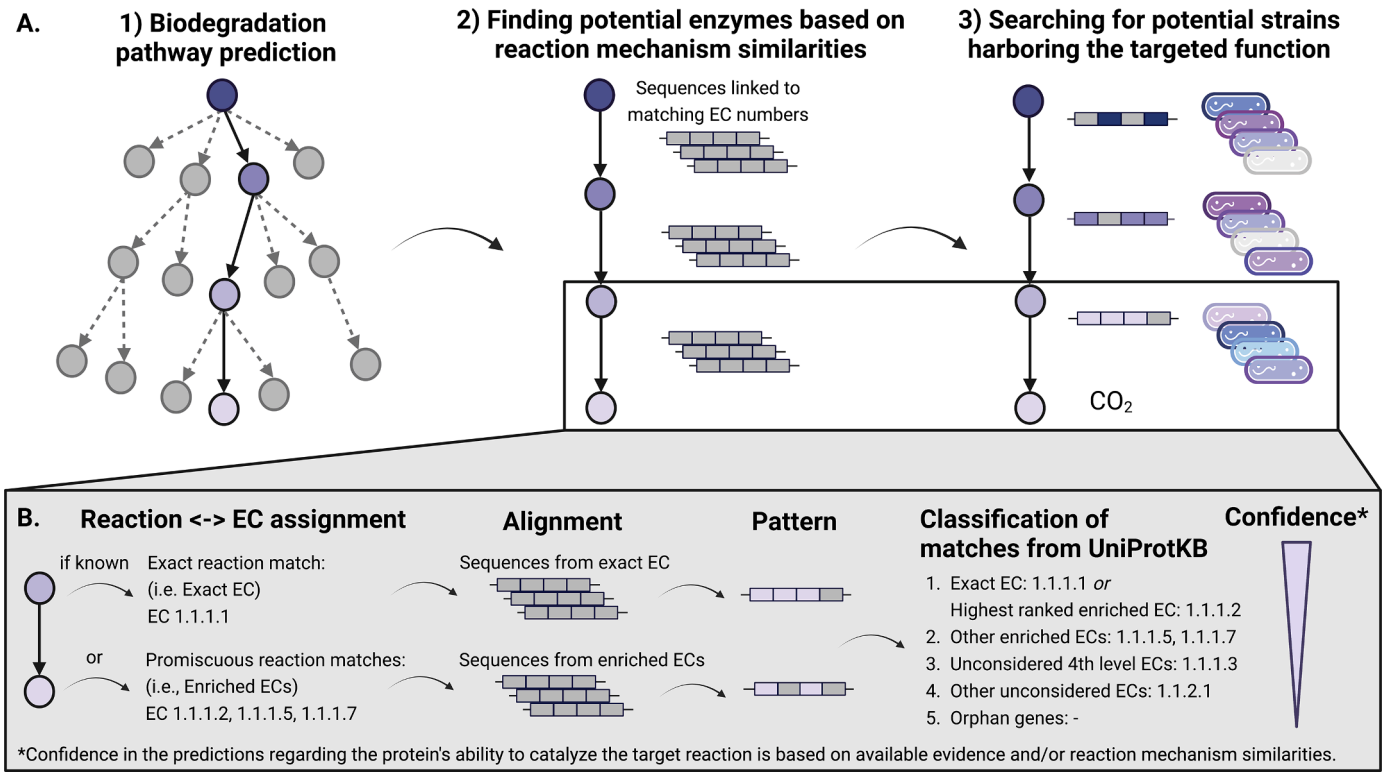

Fig. 1. Proposed pipeline for the identification of functional bacteria for the biodegradation of synthetic chemicals. Panel A: 1) The transformation products (TPs) of a chemical (purple) are illustrated with different shades of purple (likely/prioritized TPs) or gray (unlikely TPs). Biotransformation reactions are shown by dashed or solid arrows, where the later indicates the likely biotransformation pathway. 2) The potential amino acid sequences, which are identified by reactive site similarities between predicted and well-characterized reactions (EC numbers) are illustrated for the biotransformation pathway of a chemical (purple). 3) Predicted bacterial strains potentially harboring the targeted enzyme profiles for the biotransformation pathway of interest. Panel B: A more detailed overview of the 2nd and 3rd steps, i. e., the assignment of EC numbers to the reactions, alignment of the sequences, pattern generation and the classification of the predicted gene sequences based on the EC numbers. Components of the figure were created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Combining BridgIT with enviLink, we identified exact and potential 4th level EC numbers that describe the reaction mechanisms for each biotransformation reaction (Fig. 1(B)). Subsequently, we aligned the amino acid sequences linked to these EC numbers using BridgIT+ and generated a promiscuous amino acid sequence profile (i.e., a position specific scoring matrix). To identify bacterial strains likely to express the targeted enzymatic function, we used BridgIT+ to perform PSI-BLAST against the UniProtKB (Swiss-Prot & TrEMBL) database, focusing on bacteria only and setting a conservative homology significance threshold (e-value) below 10^{-20} (for more details on BridgIT and BridgIT+, see Hadadi et al., 2019 and MohammadiPeyhani et al., 2023).

We found that our predicted strains included known strains reported in the literature (Fig. 2) as well as new uncharacterized strains (the complete list of predicted strains, top 1000 matches, can be accessed via the link https://doi.org/10.25678/0008YV). For atrazine, which has well-characterized biotransformation mechanisms and exact EC matches (enviLink), our pipeline correctly identified known bacterial strains among the top 10 bacterial strains ranked by the e-values of corresponding amino acid sequences and those are listed in Fig. 2(A). Analogously, reactions with exact EC matches, representing distinct enzymatic activities, are expected to have low rates of false positives among the top ranked enzymes and corresponding bacterial strains (Fig. 1(B)).

In cases where reaction steps lack an exact EC match, as observed for the entire diuron pathway (Fig. 2(A) and (C)), our pipeline ranked enriched EC numbers based on similarities in reaction mechanisms using BridgIT and based on expert judgement using enviLink. Our approach enabled the successful identification of many functional bacteria involved in diuron degradation at the strain or species level among the top 1000 matches, except for Reaction 1, as listed in Fig. 2(A). The limitation in associating known bacterial strains with Reaction 1 was due to a knowledge gap, as no hydrolases for substituted phenylurea structural elements are so far linked to an EC number, and no closely related EC numbers could be identified other than ureases or aryl amidases, EC 3.5.1.-. Nonetheless, bacterial strains predicted for Reaction 1 might still possess enzymatic potential due to shared catalytic mechanisms, requiring experimental validation. If enzymes (e.g., phenyl urea hydrolases puhA, puhB and phh) are already reported in the literature but not yet linked to any EC numbers, the bacterial strains harboring these genes can be considered alongside the predicted strains (see Reaction 1, Literature – gap filing, Fig. 2(A) and (C)). In conclusion, such pipelines can effectively identify both known and potential enzymes, as well as bacterial strains suitable for SynCom assemblies, provided that relevant associations based on reaction mechanisms can be established. In other cases, high-throughput bio-chem-informatics pipelines can facilitate targeted mechanistic studies on enzyme characterization by narrowing down the pool of potential candidates.

We can rank or prioritize the amino acid sequences predicted by our pipeline by three criteria: (i) e-values, (ii) assigned reaction mechanisms, and (iii) the type of evidence supporting the existence of the predicted amino acid sequences (i.e., protein existence identifier in UniProt). We can then further filter the highest ranked sequences, along with their corresponding bacterial strains, based on metadata related to their preferred environmental conditions. To further narrow down the number of predicted strains, and inform and evaluate the SynCom assembly, we see a great potential in integrating metabolic network analysis that builds on genome-scale models (Colarusso et al., 2021; Zaramela et al., 2021). This approach has the potential to infer observed

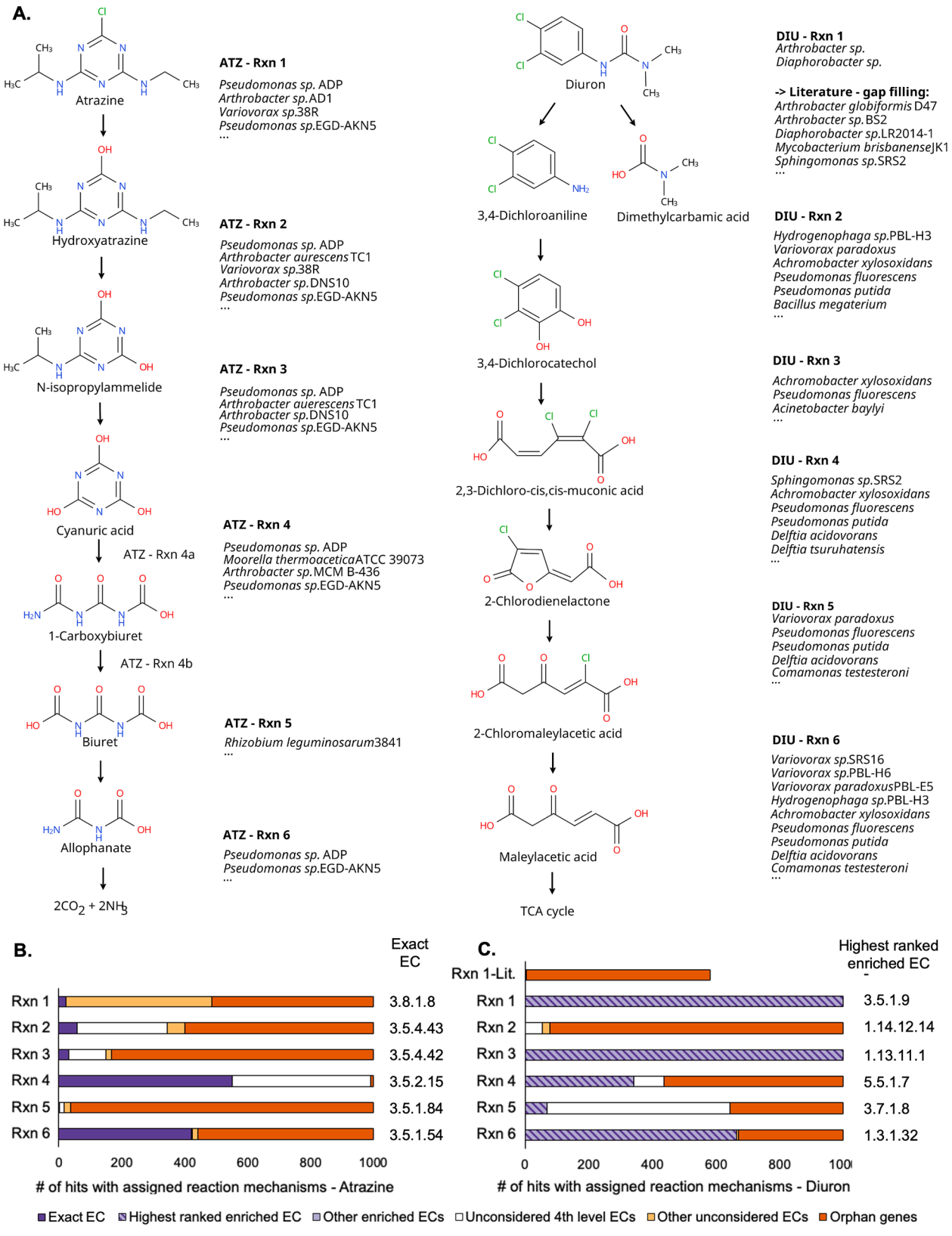

Fig. 2. Predicted bacterial strains/species matching to known degraders reported in the literature for the atrazine and diuron pathways (A). The 1000 highest-ranked predicted homologous sequences by *e*-values and their assigned reaction mechanisms for atrazine (B) and diuron (C) pathways. The predicted sequences are classified by their corresponding EC numbers in the following manner: exact EC (purple), highest ranked enriched EC (purple-lilac), other enriched 4th level EC numbers (lilac), that are considered in multiple sequence alignment, other unconsidered 4th level (white) or other unconsidered EC numbers (peach) and orphan genes (coral) which are not assigned to any EC number. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phenotypes or the feasibility of required phenotypes among the candidate members of a SynCom (Macchi et al., 2021) as well as to test consortia configurations with an optimized goal, as demonstrated by FLYCOP (García-Jiménez et al., 2018). Metabolic network analysis has the capability to recapitulate the interactions determined by carbon-source availability even in a more complex environment than a reduced SynCom, as shown for leaf microbiota ecology (Schäfer et al., 2023). Moreover, genome-scale models can provide insight into the design of complex culture media or key co-substrates to support the growth of identified degraders and ensure the maintenance of SynComs after augmentation (Ofaim et al., 2020). However, to ensure the biological compatibility of predicted strains or their ability to acquire the predicted function, experimental validation is essential before proceeding with a SynCom assembly. Thus, high-throughput or automated wet-lab methods are crucial for validating even the highest ranked bacterial strains as well as for studying interactions of bacterial strains in the process of SynCom assembly.

In the future, advancements in the quality of protein structure predictions and the speed of protein structure alignments for homology analysis will open additional avenues for identifying functional bacteria (van Kempen et al., 2023). As protein structures are functionally better conserved than the amino acid sequences, a structure similarity-based assessment can eliminate a potential limitation of reaction mechanism associations based on EC numbers, in particular for large and multifunctional protein families. Incorporating protein structure predictions and structure-based homology analysis into the last step can enhance the accuracy of identifying functional bacteria, further increasing the reliability and efficiency of such pipelines.

3. Perspective — Integrating ecological principles for fit-for-purpose SynCom assembly

The strength of such pipelines is most pronounced when designing SynComs to target multiple compounds within a mixture rather than focusing solely on individual chemicals. This is particularly pertinent because numerous pollutants with uncharacterized pathways and associated enzymes are often present in the environment as complex mixtures. It is also crucial to account for the concentrations of these pollutants and other substrates, as these concentrations play a vital role in shaping the physiological mechanisms involved in their degradation within an ecological framework. Namely, synthetic chemicals can be substrates for bacteria via different physiological processes; (i) diauxic metabolism of chemicals for sequential substrate growth at high concentrations, (ii) co-utilization of chemicals for mixed simultaneous substrate growth at low concentrations, and (iii) co-metabolism through fortuitous biotransformation by promiscuous enzymes regardless of the concentrations of synthetic chemicals. These different mechanisms can take place in continuum in the environment under varying concentrations of synthetic chemicals and bulk substrates (Hübner et al., 2022) and can inform the selection of strains from the list of predictions.

While there is a consensus on these physiological processes, the questions remain "*How and under which substrate conditions and governing physiologies can we rationally assemble SynComs for engineering biodegradation?*". In the presence of structurally similar natural substrates, co-metabolic biotransformation could occur by multiple bacteria, leading to extensive biodegradation (Richter et al., 2008). However, because this is not energetically productive and occurs fortuitously, the rational assembly of SynComs for co-metabolism will remain challenging. Therefore, for engineered and natural ecosystems, we believe that either *high synthetic chemical concentrations regardless of bulk substrate availability* or *low synthetic chemical concentrations and low bulk substrate availability* are the worthwhile scenarios, for which this bio-chem-informatics pipeline combined with the kinetic theory of optimal pathway length can well inform the rational assembly of SynComs. The kinetic theory of optimal pathway length argues that partial catabolism can be advantageous over complete catabolism when the concentration of the parent compound is high, as limited protein resources are optimally allocated among fewer catabolic reactions (Heinrich and Schuster, 1996), which can lead to cross-feeding relationships among bacteria (Pfeiffer and Bonhoeffer, 2004). Conversely, shorter metabolic pathways become less beneficial as the concentration of the parent compound decreases.

The benefit of longer metabolic pathways and a wider niche breadth at low substrate concentrations is also consistent with the theory of mixed substrate growth (Harder et al., 1982). Evidence for growth in mixtures with individual chemical concentrations lower than 1 μg/L provides some validity for mixed substrate growth (Egli, 2010), not only for isolates growing with sugars but also for complex communities with synthetic chemicals (Baumgarten et al., 2011; Desiante et al., 2022; Hellauer et al., 2018; Richter et al., 2008). Because high concentrations can enable a synthetic chemical to serve as a growth substrate, cross-feeding SynComs can be assembled with *specialist* bacterial strains that catalyze different steps of a series of enzymatic reactions (e.g., diuron) for a structurally similar group of chemicals (e.g., phenyl-urea herbicides). In contrast, SynComs assembled to degrade a mix of synthetic chemicals at low concentrations can be composed of *complementary generalists* (i.e., strains that perform entire catabolic pathways but nevertheless specialize in different pathways) with a broader niche breath while maintaining co-existence potential. In this case, the outcome of the proposed bio-chem-informatics pipeline can be streamlined by searching for bacterial strains potentially catalyzing large numbers of enzymatic reactions for a broad range of synthetic chemicals with minimal metabolic overlap to avoid competition. Phylogenetically distinct bacterial strains can be selected to increase the co-existence potential and fitness in a SynCom composed of *complementary generalists*. Such *SynComs with complementary generalists* may have broad application in environmental engineering given the presence of many chemicals at rather low chemical concentrations.

4. Conclusions and the way forward for future applications

- Our proposed bio-chem-informatics pipeline (Fig. 1) can effectively associate potential biodegrading bacterial strains with chemical structures (Fig. 2) and can, in principle, be expanded to identify SynCom members for the collective removal of synthetic chemicals at environmentally relevant concentrations, which has been a major challenge in the field of water treatment and pollution reduction. Nevertheless, although the proposed approach is promising, such pipelines will also reach their limits for biochemically poorly characterized structural elements (e.g., fluorinated organochemicals) that are distinct from natural compounds and for which no similar biotransformation mechanisms are known. Hence, mechanistic studies will continue to be needed to expand our knowledge on enzymes catalyzing reactions with novel structural elements foreign to natural compounds or well-characterized synthetic chemicals.
- Equally important is that only a subset of the amino acid sequences encoded in a genome are expressed under a set of environmental conditions (e.g., redox, pH, temperature, humidity, availability of nutrients and micronutrients such as iron, cobalt, and zinc) (Kolvenbach et al., 2014; Regnery et al., 2017; Schwarzenbach et al., 2016). Accordingly, the environmental conditions of an engineered or natural ecosystem can be used to filter predicted bacterial strains harboring potential enzymatic functions prior to validation of expression and bioaugmentation. The bacterial strains predicted by our bio-chem-informatics pipeline can also inform on the identification or design of natural and engineered ecosystems serving as sinks by answering the question "*What are the cofactors and redox conditions required for enzymatic activity to effectively catalyze most occurring biotransformation reactions in the predicted pathways?*". Looking beyond, the pipeline could also be used to search for potential enzymes in specific metagenome assembled genomes instead

of from protein databases, and thus enable the identification of potential functional strains in native microbiota.

- From the perspective of application, microbial ecological principles predict challenges for successful bioaugmentation, such as fitness advantages of indigenous organisms over SynComs, continuous washout of the SynComs and input of indigenous organisms to the engineered system, difficulties in establishing sufficiently high abundances of SynComs to achieve robust activity, and antagonism or thriving of predators by indigenous microorganisms in case of successful inoculation (Castro-Gutierrez et al., 2022; Samuelsen et al., 2017). Therefore, collaborative efforts should be directed toward identifying deterministic factors affecting microbial community assembly, which can shuffle the cards and provide new niches for SynComs. In this regard, using a SynCom will improve our causal understanding while testing different design and operational strategies in engineered systems, since the loss or gain of a function can be linked to the members of SynComs and their characteristics.

CRediT authorship contribution statement

Sema Karakurt-Fischer: Conceptualization, Methodology, Formal analysis, Writing – original draft, Resources. **David R. Johnson:** Writing – review & editing, Resources. **Kathrin Fenner:** Writing – review & editing, Resources. **Jasmin Hafner:** Methodology, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data are deposited in the publically available Eawag Research Data Institutional Collection - Eric Open and can be accessed via the DOI link, https://doi.org/10.25678/0008YV.

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