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Towards characterizing the genetic basis of trace organic contaminant biotransformation in activated sludge: The role of multicopper oxidases as a case study

A. Athanasakoglou[1*] and K. Fenner[1,2,3]

[1] Department of Environmental Chemistry, Swiss Federal Institute of Aquatic Science and Technology (Eawag), 8600 Dübendorf, Switzerland
[2] Institute of Biogeochemistry and Pollutant Dynamics, ETH Zürich, 8092 Zürich, Switzerland
[3] Department of Chemistry, University of Zürich, 8057 Zürich, Switzerland

(E-mail: anastasia.athanasakoglou@eawag.ch, kathrin.fenner@eawag.ch)

*Corresponding author

Abstract

Activated sludge treatment leverages the ability of microbes to uptake and (co)-metabolize chemicals and has shown promise in eliminating trace organic contaminants (TrOCs) during wastewater treatment. However, targeted interventions to optimize the process are limited as the fundamental drivers of the observed reactions remain elusive. In this work we present a comprehensive workflow for the identification and characterization of key enzymes involved in TrOCs biotransformation pathways in complex microbial communities. To demonstrate the applicability of the workflow, we investigated the role of the enzymatic group of multicopper oxidases (MCOs) as one putatively relevant driver of TrOCs biotransformation. To this end, we analyzed activated sludge metatranscriptomic data and selected, synthesized and heterologously expressed three phylogenetically distinct MCO-encoding genes expressed in communities with different TrOCs oxidation potential. Following the purification of the encoded enzymes, we screened their activities against different substrates. We saw that MCOs exhibit significant activities against selected TrOCs in the presence of the mediator compound 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and, in some cases, also in the presence of the wastewater contaminant 4'-hydroxy-benzotriazole (4-OH-BT). In the first case, we identified oxidation products previously reported from activated sludge communities, and concluded that in presence of appropriate mediators bacterial MCOs could contribute to biological removal of TrOCs. Similar investigations of other key enzyme systems may significantly advance our understanding of TrOCs biodegradation and assist the rational design of biology-based water treatment strategies in the future.

Keywords
laccases; metatranscriptomes; micropollutants; redox mediators; sludge; wastewater

Synopsis
This research integrates bioinformatics and biochemistry with high resolution mass spectrometry to elucidate the contribution of a specific enzymatic group in trace organic contaminant biotransformation during wastewater treatment.

5632 words + 2 Figures (900 words) + 2 Tables (600 words)

Graphical Abstract

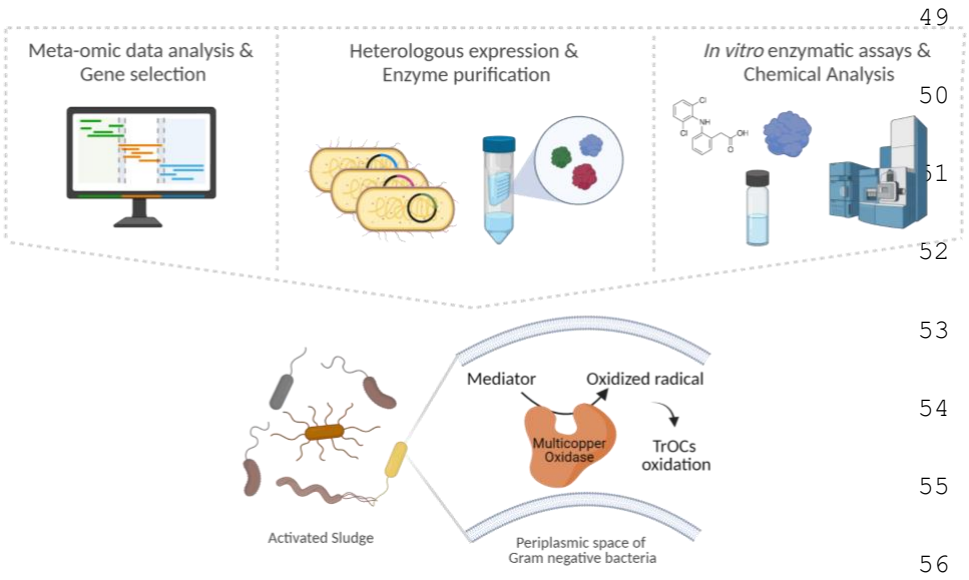

Figure created with Biorender

INTRODUCTION

Treated wastewater has been identified as a major source of trace organic contaminants (TrOCs) discharge into the aquatic environment [1, 2]. The inherent toxicity of many of these compounds and their metabolites, as well as the often unknown additive effects of chemical mixtures render these TrOCs a potential threat for aquatic biodiversity and human health[3-6].

Conventional wastewater treatment plants (WWTPs) are designed to remove solid wastes, dissolved organic matter and macronutrients, such as phosphorus and nitrogen. During the different treatment processes, TrOCs can be partially removed through sorption to particulate matter, abiotic degradation, volatilization or biological transformation[7]. While the first three processes are solely dependent on the physicochemical properties of the compounds and can be influenced by operational parameters, the latter additionally leverages the ability of microbes to uptake and (co)-metabolize chemicals through diverse enzymatic activities[8, 9]. Given the complexity of microbial communities present in wastewater[10] and the plasticity of biological

metabolism[11], opportunities to improve and optimize the observed activities are numerous[12, 13]. However, for the majority of biochemical reactions taking place during biological treatment, the enzymes and enzymatic mechanisms involved are poorly understood. Yet, for targeted interventions, it is essential to identify the underlying genetic components and experimentally validate the functionality of the encoded enzymatic machinery. So far, efforts towards this direction mostly focused on functional characterization of genes amplified from cultured strains[14, 15], assays with commercially available, purified enzymes[16-18] and gene inhibition experiments[19-21]. A more comprehensive approach to target, identify and characterize genes directly from environmental samples is lacking.

Out of all the different types of TrOCs biotransformation reactions, oxidations are the most frequently observed initial attacks that render chemical compounds more susceptible to further degradation[22]. Supported by the notion that these reactions often proceed via non-specific enzymatic mechanisms[23], several members of the diverse and promiscuous enzyme class of oxidoreductases have been associated with TrOCs biotransformations[24-27]. Among them, multicopper oxidases (MCOs) (also known as laccases) show a wide distribution in several bacterial and fungal phyla and have demonstrated activities against phenolic and non-phenolic substrates[27-29]. A purified multicopper oxidase from the fungal species *Trametes versicolor*, for instance, has been shown to catalyze the transformation of bisphenol A, diclofenac, mefenamic acid and triclosan with rates strongly influenced by pH, enzyme concentration or temperature[30]. In the presence of low molecular weight compounds that could act as redox mediators, the substrate range of this enzyme was further broadened to include other environmental contaminants, including the pesticide isoproturon[31, 32] and a number of antibiotics, like ciprofloxacin and sulfamethoxazole[18]. Different types of mediators with different reaction mechanisms have been reported. In the case of the synthetic compound 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic

96 acid (ABTS), the mediated reactions proceed via electron transfer, whereas the so-called -NOH
97 type mediators (e.g. 1-hydroxybenzotriazole) promote a hydrogen atom abstraction[33]. MCOs from
98 different taxonomic groups seem to have significantly different structural and functional
99 properties[29], as well as varying redox potentials[34] and different enzyme-mediator complexes can
100 result in oxidations of different substrates[17]. Consequently, the aforementioned studies with
101 purified, model fungal enzymes did not provide any insights into whether and to what extent this
102 enzymatic group contributes to *in vivo* TrOCs degradation, e.g., during activated sludge treatment,
103 where bacterial strains dominate the communities. Yet, given that MCOs are widespread enzymes
104 and molecules of natural and synthetic origin with redox mediator potential are present in
105 wastewater[35, 36], this mechanism might indeed, among others, lead to elimination of TrOCs in
106 wastewater.
107 In this study, we propose a workflow to investigate the role of relevant enzymatic groups in the
108 (co)-metabolism of contaminants during biological wastewater treatment. We demonstrate the
109 workflow for the case of MCOs and, in doing so, shed more light onto their role in TrOCs
110 degradation during activated sludge treatment. To this end, we first analyzed metatranscriptomic
111 data from activated sludge bacterial communities with different TrOCs oxidation potential[37, 38]. We
112 identified, synthesized and heterologously expressed three phylogenetically and structurally
113 distinct multicopper oxidase genes and tested the activities of the purified enzymes in *in vitro*
114 assays with wastewater-relevant TrOCs as substrates. To further investigate the role of these
115 catalytic activities in real wastewater, we tested different reaction conditions and explored the
116 potential of the industrial chemical 4'-hydroxy-benzotriazole (4-OH-BT), present in wastewater, as
117 mediator for MCO-catalyzed transformation reactions. Taking into account the enzymes'
118 subcellular localization in the periplasmic space and the reactions' transformation products, we
119 propose a putative *in vivo* model for the role of MCOs in TrOCs biotransformation by activated

sludge microbes.

MATERIALS AND METHODS

We define a workflow that consists of 5 steps (for an overview, see SI, Fig. S1) aiming to identify and characterize genes involved in TrOCs biotransformation reactions. The workflow integrates an *in vivo* experiment with given environmental communities and quantitative or qualitative calculation of their biotransformation capacity on select chemicals. Once these data are available, metagenomic and/or metatranscriptomic sequencing and assembly, followed by comparative analysis of the resulting gene and protein sequences enables the identification of enzymatic group(s) of interest. After selection of specific candidates, gene synthesis and heterologous expression in model systems like *Escherichia coli* allows the purification of the corresponding enzymes and screenings of their activities with various substrates *in vitro*. Appropriate analytical techniques are subsequently used to identify the *in vitro* reactions and assign functions to the tested genes and enzymes. In the final step of the workflow, an attempt to link the *in vitro* to the *in vivo* results can either be done directly *(*e.g., *in vivo* assays with the strains from which the selected genes originate, quantification of gene expression in environmental samples) or indirectly (e.g., transformation products comparisons, reaction conditions agreement).

In vivo experiment with environmental communities

For our study, we used previously reported results obtained by Achermann et. al 2018[37], and Mansfeldt et. al. 2019[38]. Briefly, the experimental setup included six activated sludge bioreactors treating municipal wastewater and operating at a gradient of solids retention times (SRTs, 1 to 15 days, 2 replicates per SRT, 12 samples in total). A number of TrOCs was spiked in each bioreactor and their biotransformation reactions and corresponding rate constants were determined. Sludge

144 samples were collected from each reactor and following RNA extraction and library preparation,
145 were sequenced using the Illumina pipeline 2.4.11 (Raw data are available under the EBI accession
146 number: ERP024418, 41.2−54.3 million reads per sample).
147
148 **Metatranscriptomic data analysis**
149 The raw data from RNA-Seq of the activated sludge communities[37] were assembled.) A detailed
150 description of the bioinformatics analysis is provided in the Supporting Information (SI, S1.1
151 Bioinformatics and sequence analysis). Briefly, after quality filtering, adapter and rRNA sequences
152 removal, the paired-end reads from each sample were assembled using MEGAHIT v2.4.2[39]. Genes
153 were predicted with Prodigal v2.6.3[40] and annotated in Eggnog Mapper[41]. All genes of interest
154 (MCOs) were extracted, compared and candidates for further characterization were selected
155 (more details in results section).
156
157 **Chemicals, genes and bacterial strains**
158 In total, 19 TrOCs (SI, Fig. S2, Table S1), previously found to be oxidatively biotransformed by
159 activated sludge communities[37] were selected and divided into four groups based on the type of
160 initial biotransformation reaction. Mix 1 contained phenylureas (chlorotoluron, diuron,
161 isoproturon and metoxuron) that were found to undergo dealkylation, hydroxylation or
162 dihydroxylation in wastewater. Mix 2 included chemicals that had been transformed via *S*- and/or
163 *N*-oxidations (irgarol, ranitidine, terbutryn and amisulpride) whereas mix 3 contained chemicals
164 that underwent amine or amide dealkylation (furosemide, valsartan, bezafibrate). Mix 4 contained
165 compounds that were expected to be hydroxylated (ketoprofen, gemfibrozil, clofibric acid,
166 iprovalicarb, capecitabine and diclofenac) and, finally, mix 5 included the sulfonamide antibiotics
167 sulfamethoxazole and sulfadiazine, for which oxidation but also conjugation reactions had

previously been observed in activated sludge communities. All chemical standards were purchased (Sigma-Aldrich GmbH, Dr. Ehrenstorfer GmbH, HPC 113 Standards GmbH, Honeywell Specialty Chemicals, and Toronto Research Chemicals), and the selected genes were codon-optimized and synthesized by Thermo Fisher Scientific Inc. into the commercially available pRSETA plasmid vector that contains a T7 promoter and an N-terminal polyhistidine (6xHis) tag.

Gene expression and enzyme purification

To obtain the purified enzymes, the genes were transformed into *E. coli* DE3 cells and their expression was induced using 1 mM IPTG at 18 ^{o}C for 18 h. To obtain fully functional MCOs, 0.25 mM of $CuCl_2$ was added to the bacterial cultures, and, after 4h of shaking incubation, the flasks were left to incubate overnight at room temperature without shaking (microanaerobic conditions)[42].The cells expressing the synthetic genes were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 50% glycerol, 5 mM imidazole, 1 mM PMSF and 1 mg/mL lysozyme) before they were lysed using a french press (Avestin Emulsiflex C3). Cell debris and other impurities were removed by centrifugation and the cell extracts were incubated with Ni-NTA agarose beads (Qiagen) for 2h. The beads were collected and washed three times in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50% glycerol, 25 mM imidazole). The proteins were eluted in elution buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50% glycerol, 250 mM imidazole) and analysed in SDS-Page gel. Quantification of the proteins was done using the Bradford assay (Thermo Fisher Scientific Inc.) and the enzyme activity was calculated with the ABTS assay, as described before[43]. One unit of activity (U) was defined by the oxidation of one µmol of ABTS per min, using the extinction coefficient ε 420 nm of 36,000 M^{-1} cm^{-1}.

***In vitro* assays with trace organic contaminants and chemical analysis**

The reaction mixtures contained 100 mM ammonium acetate buffer at pH 4-6 or potassium phosphate buffer at pH 7-8, 1 mg/L substrate, 300 U/L enzyme and mediator at concentrations of 200 µM (ABTS) and 500 µM (4-hydroxybenzotriazole). At defined timepoints (for reactions with ABTS 0h, 2h, 6h, 10h, 30h and for 4-hydroxybenzotriazole 0h, 2h, 6h, 10h) 100 µl were withdrawn from each reaction vial and transferred into new glass vial inserts. An equal volume of 100% acetonitrile was added to precipitate the enzyme and stop the reactions. Centrifugation at 6000 rpm for 5 min, followed by dilution of the supernatant (1:200) in nanopure water and addition of internal standard mix (Table S1) for quantification of the substrates were done before the samples were analysed by reversed phase liquid chromatography coupled to a high-resolution tandem mass spectrometer (LC-HRMS/MS) (Q Exactive, Thermo Fisher Scientific Inc.). The analytical method used was previously reported[37].

Transformation product identification by suspect and nontarget screening

Screening for transformation products (TPs) was performed using the software Compound Discoverer 3.2 (Thermo Fisher Scientific Inc.). The workflow included peak picking, pre-filtering and comparison with a predefined suspect list. In this list, we included transformation products identified before in experiments with the activated sludge, the sequencing data of which were analysed in this study[37], as well as transformation products described in the literature. Additionally, further potential TP masses were calculated considering mass shifts of oxidative biotransformation reactions. For non-target screening, the “molecular networks” node of Compound Discoverer was used. With this feature, relationships between compounds were explored based on a pre-defined library of expected transformations and spectral similarity.

RESULTS

Multicopper oxidase distribution in activated sludge

An *in vivo* experiment assessing the TrOCs biotransformation capacity of twelve activated sludge communities cultivated at different SRTs and previously published by Achermann *et. al*[37] was the basis of the current study (SI, Fig. S1, Workflow step 1). The raw RNA-sequencing data from the twelve communities exhibiting increasing oxidative biotransformation potential with increasing SRT were retrieved and analyzed (SI, Fig. S1, Workflow step 2). Each data set was independently assembled and the resulting genes and corresponding amino acid sequences were annotated. All sequences annotated as MCOs or showing sequence similarity above 70% to characterized MCOs were extracted, creating a catalogue of expressed genes in the twelve communities (SI, S2.5 Protein Sequences). Phylogenetically widespread MCOs could be retrieved from all communities suggesting that this enzymatic group is ubiquitously present in activated sludge communities. However, the number of identified sequences was significantly higher in communities of higher SRTs, which had also been shown to more efficiently oxidize TrOCs[37] (SI, Table S2 and Table S3). To identify possible structural and functional correlations in the retrieved sequences and get more insight into their distribution across samples, we inferred their phylogenetic relationships using the Maximum-Likelihood approach at the amino acid level (Fig. 1A). The majority of sequences retrieved from the communities of lower SRTs (SRT 1 and 3 days) grouped together and separately from those of higher SRTs (SRT 5, 7, 10 and 15 days), suggesting that the two groups could have distinct origin and/or structural or functional properties.

Gene selection for functional characterization

Out of all MCOs identified (SI, Table S2) candidates were selected for further functional characterization. This selection was based on three different criteria. The final selected sequences should (1) be phylogenetically distinct and originate both from communities with high and low

oxidation potential towards TrOCs so that we can investigate putative differences in activities, (2) be full length, corresponding to one open reading frame, (3) contain conserved motifs corresponding to copper ligand motifs that are identified in functional homologues. Based on these criteria, we selected three nucleotide sequences for downstream characterization (Table 1). The first, from now on referred to as MCO1, is a sequence expressed in communities of SRT 7 days with close homologues in communities of SRT 5, 10 and 15 days. The nucleotide sequence shares 72% similarity with the *Nitrosomonas sp*. JL21 MCO, while the exact same sequence has been reported in an activated sludge metagenome deposited on JGI database (Gene ID: Ga0099877_100528 Genome ID: 3300007407 Klosterneuburg C21_HANv2). The second selected sequence, herein called MCO2, is expressed in a community with SRT 15 days and a close homologue is also present in a community with SRT 7 days. MCO2 is 91% similar to the *Nitrosomonas sp.* Nm84 MCO and close (82% similarity) to a gene found in activated sludge from Taiwan, Wenshan plant, as deposited on JGI (Gene ID: Ga0131077_127990261 Genome ID: 3300009873). Both MCO1 and MCO2 originate from a phylogenetic clade that does not include any homologue from the communities with low oxidative potential (Fig. 1A) and even though they show high sequence similarity (72%), their homology with enzymes from strains with different geographical distribution (Table 1), renders them interesting candidates for functional characterization. The third selected nucleotide sequence, from now on referred to as MCO3, is expressed in the community with SRT 1 day and is 100% similar to the *Flavobacteria bacterium* GWA2 laccase and 97% similar to a gene reported in JGI (Gene ID: Ga0070407_100213 Genome ID: 3300007470 KlosterneuburgC25_HAv2). All selected candidates contained conserved motifs, including the copper ligand histidine motifs HXHG, HXH, HXXHXH and HCHXXXHXXXXM, also found in *E. coli* (NCBI Accession Number AAC73234.1) and *Bacillus subtilis* (NCBI Accession Number NP_388511.1) characterized homologues (SI, S2.3 Protein Alignment)[44].

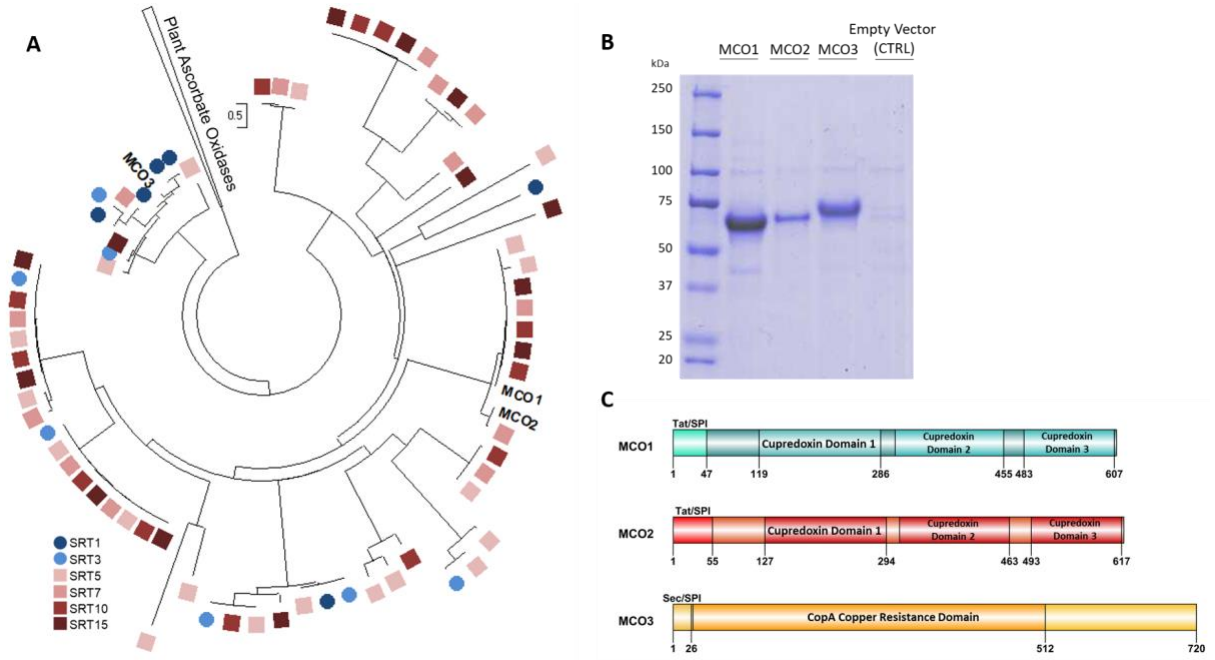

Figure 1. A. Phylogenetic tree of all multicopper oxidase proteins identified in the assembled metatranscriptomes of communities with low (SRT1, 3) and high (SRT 5, 7, 10, 15) TrOCs oxidation activities. Selected candidates for characterization are indicated (MCO1, MCO2, MCO3). The tree was rooted with an outgroup of plant ascorbate oxidases. All of the protein sequences are available in the SI. **B.** SDS-PAGE gel with the recombinant purified enzymes. Protein markers with known size (in kDa; kiloDalton) is shown in lane 1, MCO1 in lane 2, MCO2 in lane 3, MCO3 in lane 4 and the empty vector control (CTRL) in lane 5. **C.** Structural domains and signal peptides identified in the selected candidates. Tat/SPI: Twin arginine translocation signal peptide; Sec/SPI: general secretory signal peptide; CopA: copper resistance A domain

The selected sequences also contain different conserved domains (Fig. 1C). MCO3, alongside with all the sequences from its clade on the phylogenetic tree, is an enzyme that contains a CopA domain, which corresponds to copper resistance proteins. These proteins are responsible for copper detoxification in bacteria[45]. Unlike CopA proteins, the other two candidates, MCO1 and MCO2, have a different domain organization. They contain three cupredoxin domains[46] with the type (T1) copper center located in the C-terminal domain and the type 2/type 3 (T2/T3) trinuclear cluster at the interface between domains 1 and 3. During the catalytic cycle, electrons are transferred from T1 to T2/T3 clusters to enable substrate oxidation and reduction of dioxygen to water [47-49]. Overall, MCO1 and MCO2 share 72% sequence similarity at the amino acid level and share a common domain structure that differs from MCO3.

Table 1. Genes selected for characterization, along with their closest homologues in single strains (Blast hits) and in metagenomic datasets (JGI/IMG hits). For each sequence, the presence and amino acid position (pos.) of signal peptides (Tat/SPI: Twin arginine translocation signal peptide; Sec/SPI: general secretory signal peptide) and the conserved domains are indicated.

Enzyme name	Blast best hit (% Similarity)	IMG best hit (% Similarity)	Signal Peptide Prediction	Conserved domains
MCO1	*Nitrosomonas* sp. (99%)	Protein in Genome ID:3300007407 Klosterneuburg C21_HANv2 (100%)	Tat/SPI pos. 1-47	3 cupredoxin domains
MCO2	*Nitrosomonas* sp. Nm84 (91%)	Protein in Gene ID: Ga0131077_127990261 Genome ID: 3300009873 Activated sludge WWTP from Taiwan - Wenshan plant (82%)	Tat/SPI pos. 1-55	3 cupredoxin domains
MCO3	*Flavobacteria* bacterium GWA2_35_26 (100%)	Gene ID: Ga0070407_100213 Genome ID: 3300007470 KlosterneuburgC25_HAv2 (97%)	Sec/SPI pos. 1-26	CopA

As a final step before synthesis of the genes, we investigated the presence of signal peptides in the selected sequences. Presence of these pre-sequences would inhibit the expression of soluble proteins in *E. coli*, preventing the purification and consequently the further characterization of the enzyme. Using a signal peptide prediction tool (SI, S1.1 Bioinformatics and sequence analysis), we identified a Tat/SPI (Twin arginine translocation signal peptide) signal peptide in MCO1 (amino acids 1-47) and MCO2 (amino acids 1-55). A Sec/SPI (general secretory signal peptide) pre-sequence was identified in MCO3 (amino acids 1-26). This type of pre-sequence is responsible for for enzyme translocation across the bacterial cytoplasmic membrane and into the periplasmic space[50]. In the cloning design, we truncated the signal peptide sequences, to achieve optimum expression of the recombinant proteins. Additionally, the selected genes were also codon optimized for *E. coli* expression.

Functional characterization of MCOs with and without mediators

Plasmids (pRSETA, Thermo Fisher Scientific Inc.) carrying the three different genes were expressed

in *E. coli* BL21 DE3 strains (SI, Fig. S1, Workflow step 3). After purification, analysis in SDS-PAGE gel confirmed the size and purity of the three encoded enzymes (Fig. 1B). To confirm the functionality of the purified enzymes, we calculated the enzymatic activity (U/L) after incubation in buffers of different pHs (4-8) and over 24h (SI, Fig. S3). The results of this screening showed that all enzymes retained activity for 24 h in all buffers tested, with the best activities obtained at pH 8, where the enzymes retained more than 20% of their initial activity. These results allowed to rule out the possibility of enzyme instability leading to early reaction termination and, thus, allowed us to determine the maximum time (24 h) that we would monitor the *in vitro* assays with the different substrates.

To investigate the activities of the three enzymes against TrOCs (SI, Fig. S1, Workflow step 4), the purified enzymes were initially tested in reactions with five different mixtures including a total of 19 chemical substrates (Methods Section and SI, Table S2, Fig. S2). *E. coli* cells transformed with an empty vector and subjected to the same purification steps were always used in parallel reactions as controls. In the control samples, proteins from the *E. coli* cell extract that bind non-specifically to the Ni-NTA agarose beads are eluted to ensure that the observed activities stem from the recombinant proteins and not from background enzymes. Out of the 19 chemicals tested in the first screening, only the pharmaceutical diclofenac was found to be oxidized by all three MCOs at pH 5 (SI, Fig. S4). To further examine the role of pH in the oxidation of diclofenac, we then assayed diclofenac as individual compound with all three enzymes in buffers with pH ranging from 4 to 8. In this second screening, only MCO1 was found to be able to oxidize diclofenac and the best activity was obtained at pH 6, where diclofenac was degraded by up to 30%. Since our pH tests showed that the enzymes retained activity for 24h at all pH values (SI, Fig. S3), we assume that the enzyme stability did not affect the reactions and that the activity of the other two enzymes towards diclofenac is weaker and, thus, not consistent across experimental repetitions (SI, Fig S5).

The possibility that some of the other compounds acted as mediators in the reactions with substrate mixes is ruled out, as none of the other substrates was degraded.

In the next steps, we investigated whether the addition of redox mediators could lead to expansion of the MCO substrate range. Addition of the model substrate ABTS confirmed the activity of our enzymes and resulted in degradation of isoproturon, metoxuron, amisulpride, ranitidine, furosemide, diclofenac and the sulfonamides sulfamethoxazole and sulfadiazine (Fig. 2A), while the rest of the screened compounds remained unaffected (SI, Fig. S6). At least one compound per mix was degraded, leading to the conclusion that enzyme activity in the assay mixture was not inhibited by the chemicals present (substrates or transformation products).

As ABTS is a synthetic compound that is not expected to be found in wastewater and given that we are interested in describing putative mechanisms *in vivo* under real wastewater conditions, we chose to test a more relevant compound as mediator. One of transformation products of the globally used corrosion inhibitor benzotriazole[51], namely 4-hydroxy-benzotriazole (4-OH-BT), can be found in WWTPs around Switzerland at concentrations of up to 4100 ng/L[52]. Based on structural similarity to another known laccase mediator, 1-hydroxy-benzotriazole (1-OH-BT), and its relatively high concentrations in wastewater[53, 54], we selected 4-OH-BT as a putative mediator to test. One of the enzymes, MCO1, showed the best activities with 4-OH-BT, with the system transforming ranitidine to 100% and diclofenac to more than 50% at pH 8. The conversion of 4-OH-BT was confirmed by the decreased 4-OH-BT peak area over time (SI Fig. S7). Similarly, the pharmaceuticals diclofenac, amisulpride and the sulfonamide antibiotics were also degraded, though to a lesser extent (compared to control samples). The MCO3-4-OH-BT system was active against metoxuron (Fig. 2B), but no other compound seemed to be degraded with 4-OH-BT as mediator (Fig. 2B and SI, Fig S8).

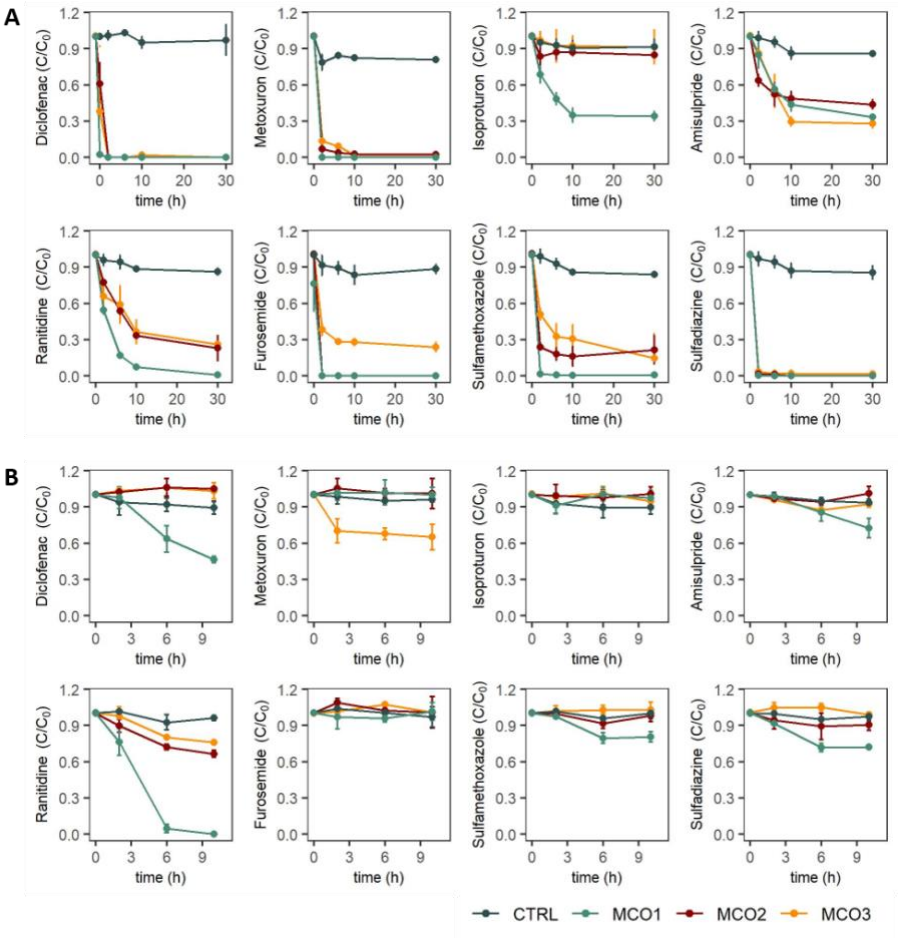

Figure 2. Concentration time series (C/C_0) for 8 TrOCs in *in vitro* reactions with 3 recombinant enzymes (MCO1, MCO2 and MCO3) and an empty vector control (CTRL) in presence of mediator ABTS at pH 5 (**A**) and 4-OH-BT at pH 8 (**B**).

Taken together, the results from the assays with mediators suggest that the type of mediator is critical for the substrate range of MCOs and determines the degree to which each substrate is transformed.

Link to activated sludge reactions: characterization of the transformation products

In order to investigate whether the reactions we observed *in vitro* could indeed be taking place in activated sludge (SI, Fig. S1, Workflow step 5), we investigated whether the transformation products (TPs) formed agree with those that have been reported from biological wastewater treatment or activated sludge batch experiments. This is possible for cases where compounds that are initially transformed by oxidation are not further degraded by other enzymes during activated

363 sludge treatment. For the eight compounds that were found to be transformed by the MCO –
364 ABTS system, we were able to identify TPs that are generated via different types of oxidation
365 reactions (Table 2). All the transformation products and more details, including the MS2 spectra
366 and structural proposals and the respective confidence levels according to Schymanski *et. al*.
367 (2014)[55], are provided in the Supporting Information (SI, S2.4 Transformation Product Analysis).
368 For diclofenac, we identified the hydroxylated derivatives 4'-OH-diclofenac and 5'-OH-diclofenac,
369 the corresponding quinone imines 4'-OH-diclofenac quinone imine or 5'-OH-diclofenac quinone
370 imine, diclofenac benzoic acid and another TP with molecular mass of 223 (TP223). The
371 hydroxylated diclofenac derivatives, as well as the benzoic acid have been identified as major TPs
372 in activated sludge, nitrifying sludge, heterotrophic sludge[56] and in a hybrid moving bed biofilm-
373 activated sludge reactor, where the quinones were also present[57]. 4'-OH-diclofenac was also
374 identified in an aerobic membrane bioreactor[58]. For metoxuron, we identified transformation via
375 *O*-demethylation at the methoxy group. Interestingly, Achermann et. al. (2018)[37] also observed a
376 demethylation reaction for metoxuron in batch activated sludge experiments, though at the urea
377 N-methyl group. Isoproturon and amisulpride TPs that we identified have been also identified in
378 activated sludge batch experiments and one of ranitidine's TPs has been previously found in
379 nitrifying bacterial cultures that are likely present in activated sludge[59]. For furosemide and the
380 sulfonamide antibiotics, we did not identify any oxidation products and it is likely that those
381 substrates form coupling products with the intermediate mediator radical. While we did look for
382 larger masses that could be related to such products in our HR-MS spectra, we could not detect
383 any non-target features that would qualify as coupling products in terms of intensity and expected
384 time courses. In the reactions with 4-OH-BT it was not possible to identify any TPs, possibly due to
385 the lower transformation rates or due to the formation of coupling products.
386

Table 2. Transformation products identified in reactions with multicopper oxidases and ABTS as mediator

Parent Compound	Transformation Product	Formula	Calculated Mol. Weight	Literature reports
Diclofenac	4' or 5 -Hydroxydiclofenac	C14H11Cl2NO3	311.0115	56-58
	4' or 5' hydroxydiclofenac quinone imine	C14H9Cl2NO3	308.99584	
	Diclofenac benzoic acid	C13H9Cl2NO2	281.00099	
	TP323	C15H11Cl2NO3	323.01144	
Metoxuron	TP215	C9H11ClN2O2	214.05074	
Isoproturon	TP223	C12H18N2O2	222.1367	37
Amisulpride	amisulpride desethyl	C15H23N3O4S	341.1409	37
	TP259	C10H14N2O4S	258.0674	
Ranitidine	TP285	C11H15N3O4S	285.0783	59
	TP300	C12H20N4O3S	300.1255	

DISCUSSION

The role of multicopper oxidases in TrOCs biotransformation

A number of studies have investigated the activities of purified MCOs against TrOCs [18, 31, 32, 60] and proposed their application for post-treatment, enzymatic removal of persistent contaminants[16, 29, 61]. However, their role during biological wastewater treatment remains unexplored. In this study, we therefore aimed to obtain a more comprehensive understanding of their potential contribution to TrOCs elimination during activated sludge treatment, which could also point towards opportunities to devise novel biocatalysts and guide the design of treatment systems that promote their expression (e.g., physico-chemical conditions, presence of appropriate mediator

compounds etc.) . A number of factors play a role in whether or not a substrate will be oxidized by specific MCOs, namely i) the structure and redox potential of the enzyme, ii) the structure and redox potential of the substrate[62], iii) the ambient pH and the concentrations of the enzyme and reactants[31]. In our first screening of 19 chemical compounds against the three MCOs, only the pharmaceutical diclofenac was directly oxidized by the enzymes (SI, Fig.S4), an activity that was more stable and could be robustly reproduced only with MCO1 when the enzymes were incubated with diclofenac as a sole substrate (SI, Fig. S5). Those differences, possibly, reflect the varying redox potential differences between the substrate and the T1 copper center of the three MCOs, which ultimately determine the thermodynamic feasibility of the reaction[63]. Indeed, diclofenac was previously found to be hydroxylated to different extent by both fungal[31, 60] and bacterial[16] MCOs. For the other compounds screened, it is possible that the overall low redox potential of the selected bacterial MCOs does not allow for efficient oxidations. It is also possible that steric hindrance to substrate docking of specific substrates prevents their oxidation, as it was previously found that different phenols and anilines were not oxidized by MCOs with redox potential high enough to allow one-electron abstraction[64]. Another significant parameter to take into account is the ambient pH that may affect both the enzymatic stability and the redox potential of the substrates. The three selected enzymes in our study retained their activities at pH 5-8 for a period of 24 h, but under the conditions tested, only MCO1 oxidized diclofenac, with an optimum at pH 6. As we set out to elucidate putative mechanisms taking place *in vivo*, and more concretely during wastewater treatment, we should take into account the feasibility of MCO-catalyzed reactions in the source-organisms and at relevant conditions. According to studies in *E. coli*, the pH of the periplasmic space equilibrates with the external pH[65]. Thus, we hypothesize that the physiological pH for the TrOCs reactions would be between 7.5-8.5, which is the pH typically observed during activated sludge treatment[37]. The activity of MCO1 against diclofenac at this pH range was still

422 detectable, supporting the possibility that the periplasmic MCOs of gram-negative bacteria could
423 contribute to the direct oxidation of diclofenac. This is further corroborated by the fact that the
424 periplasmic space is accessible to molecules smaller than 600 Daltons through transporters
425 (porins) that enable non-specific diffusion. As shown for clinically isolated strains, porins together
426 with antibiotic degrading enzymes play a central role in antibiotic resistance[66].
427 The involvement of MCOs in the oxidation of not only diclofenac but other TrOcs is more evident
428 in the presence of redox mediators that act as electron shuttles between enzyme and substrate.
429 This phenomenon was first described in laccases from white-rot basidiomycetes during lignin
430 biodegradation and, since then, many natural and synthetic mediators have been identified for
431 improved lignin and other recalcitrant chemicals' degradation [17, 32, 33, 67, 68]. Reactions with
432 mediators proceed via two distinct steps. In the first step, the MCO oxidizes the mediator to
433 produce a free radical species. In the second step, the radical diffuses away from the enzyme's
434 active site and, in turn, oxidizes other substrates[69]. The first reaction requires the mediator binding
435 to the enzyme's active site. Studies previously investigated structural properties that affect the
436 affinity of ABTS binding into the active site of different *T. versicolor* laccase isoenzymes revealed a
437 key role of an Asp residue (position 206 in isoenzyme α, accession number: AAW29420.1[70]). This
438 residue is conserved in MCO1 and MCO2, but is replaced by a Lys in MCO3 (SI, Fig. S9). That could
439 explain the overall lower activities observed in our assays with this enzyme (Fig. 2A). The second
440 reaction is non-enzymatic and influenced only by structural parameters of the two reacting
441 substrates. In our system, addition of the mediator ABTS indeed led to observable transformation
442 for eight of the TrOCs tested (Fig. 2A). According to Margot *et. al*. [30, 32], ABTS is oxidized by the *T.*
443 *versicolor* MCO to its radical cation ($ABTS^{\bullet+}$) within a few minutes, and subsequently, at a slower
444 rate, to the corresponding di-cation ($ABTS^{2+}$). Based on their experimental evidence, they have
445 suggested that $ABTS^{2+}$ is the intermediate radical that oxidizes substrates according to their one-

446 electron oxidation potential. We retrieved calculated one-electron oxidation potentials[71] for the
447 four phenylurea compounds that we studied. The reported values indicated that metoxuron has
448 the highest potential, followed by isoproturon, chlortoluron and diuron. Indeed, our results
449 showed that metoxuron is well oxidized in all three MCO-ABTS systems run at the pH of maximal
450 ABTS activity (pH 5) (Fig. 2A). In contrast, isoproturon was removed to a lesser extent and in only
451 one of the MCO-ABTS systems, whereas the two phenylureas with the lowest one-electron
452 oxidation potential were not removed in any of the systems (SI, Fig. S6). In agreement with Margot
453 *et al.* [30], these findings suggest that the chemical oxidation of the substrates by the oxidized
454 mediator might indeed represent the rate-limiting step in MCO-ABTS systems at acidic to neutral
455 conditions, and that the observed rate is related to the substrates' respective one-electron
456 oxidation potentials.
457 Taken together with the fact that the transformation products found in the reactions with ABTS
458 radicals show good agreement with transformation products identified in wastewater and in
459 activated sludge experiments (Table 2), our findings point toward a potential contribution of MCO-
460 mediator systems to TrOCs oxidation reactions during wastewater treatment. This would require
461 the presence of mediators other than ABTS, which are abundant in wastewater, diffuse into the
462 periplasmic space and potentially also have optimal activity at the neutral to alkaline conditions
463 typical of wastewater. Arguably, dissolved organic matter contains a variety of low molecular
464 weight compounds with functional groups with electron-accepting or donating properties (e.g.,
465 phenolic moieties, quinones, thiols)[35, 36]. Yet, those functional groups are also found in several
466 known contaminants present in wastewater. One of those, bisphenol A, has been tested as a
467 mediator compound for MCOs and was shown to enhance removal of diclofenac and flufenamic
468 acid[30, 60]. Our test using 4-OH-HBT as a potential mediator indeed led to increased transformation
469 for five substrates (Fig. 2B) at pH 8. Although all three tested enzymes led to the elimination of 4-

OH-HBT (SI, Fig. S7), most likely by oxidizing it into an intermediate reactive radical, with MCO1 this transformation seemed to proceed fastest and led to subsequent degradation of diclofenac, amisulpride and to a lesser extent the sulfonamides (Fig. 2B), whereas co-incubation of 4-OH-HBT with MCO3, conversely, resulted in selective removal of metoxuron (Fig. 2B). These differences probably reflect different degrees of enzymatic stability and resistance to enzymatic inactivation by free radical attack on the catalytic site of the enzyme[72]. Due to the fact that reactions were not as efficient or due to coupling reactions between the intermediate radical and the TrOCs, we did not observe the corresponding oxidized transformation products in these reactions. Similar results have been also reported by Margot et. al[30] in the co-incubation of diclofenac with bisphenol A. Overall, the concentrations and efficiency of different mediators modulate activity of the MCO-mediator systems and, therefore, different mediator compounds specifically relevant to wastewater treatment conditions should be further investigated. Other co-factors or co-substrates (like for example chloride[73]) that could enhance enzymatic activities and/or increase substrate affinities under physiological conditions are also worth investigating. Finally, intracellular metabolites with higher concentrations comparing to that of external contaminants could also play a role in mediating these reactions. This has been demonstrated before with the ammonia monooxygenase (AMO) of ammonia oxidizing bacteria (AOB) that transforms ammonia into hydroxylamine, with the latter abiotically reacting with some TrOCs[19].

It is noteworthy that the majority of the MCOs, including MCO1 and MCO2, identified in our study are encoded by AOB strains (SI, Table S3). Even though we did not directly quantify the expression levels of the selected genes in the present study, community composition analysis of the same samples in previous work from our group[38], showed that AOB are increasingly abundant in communities of longer SRTs. This supports our hypothesis that increased abundance of MCOs contributes to TrOCs degradation. Yet, the fact that MCO expression is also observed in

communities of shorter SRTs (i.e., MCO3), points to the possibility that the availabilities of mediators promote or limit the oxidation reactions. AOB was found to mediate TrOCs biotransformations in the past. Particularly, among other compounds, furosemide, ranitidine and amisulpride were found to be transformed by nitrifying activated sludge and inhibition experiments with allylthiourea (ATU) suggested that their transformation was mediated by enzymes other than AMO, which is commonly suspected to catalyze TrOCs biotransformations[21]. The fact that those enzymes seem to be distributed in both AOB and non-AOB strains, as well as their inhibition by ATU that depletes copper ions[74] (essential for MCO activity[75]) renders MCOs good candidates for this role. This observation highlights the long-debated discussion on the role of AOB bacteria in TrOCs elimination and the need to investigate enzymes other than AMO in our efforts to clarify the extent of their contribution[24, 76].

Elucidating the genetic basis of TrOCs biotransformations

The need to improve our understanding of the genetic and biochemical basis of pollutant degradation reactions has been highlighted repeatedly before[27, 77, 78]. For contaminants with high environmental concentrations, such as naphthalene[79] and polychlorinated biphenyls (PCBs)[80, 81], both sequence- and activity-based metagenomics have successfully been applied to identify the genetic mechanisms of their degradation. The low concentrations of TrOCs and the often co-metabolic nature of their degradation has so far prevented similar attempts[82]. Even though candidate genes potentially involved in TrOC biotransformation have been proposed, their functional characterization is usually lacking[83, 84]. The workflow presented in this study aims to exemplify the study of groups of enzymes particularly relevant to TrOCs biotransformation by leveraging two technological advances: meta-omics and gene synthesis.

Along the advantages the different ‘omic’ technologies offer, metatranscriptomics reveal only the expressed portion of the total genetic information, leaving out inactive members and

unexpressed, silent pathways that could potentially mislead the gene selection. At the same time, a major drawback associated with RNA-seq data is their oftentimes low quality (associated with the difficulty in obtaining high-quality RNA), which causes problems in gene assembly and accurate annotation. We overcame those challenges by choosing to investigate a group of ubiquitous and highly abundant genes (that can be assembled into full-length sequences) and focused on their absence/presence in the different communities rather than quantifying their expression levels and provide a description of the transcriptomic responses to prolonged SRTs. Future studies that will aim investigate such responses and other, less abundant gene families, could leverage the use of metagenomic or a combination of metagenomic and metatranscriptomic data for a more complete analysis at the genomic and transcriptomic level. To functionally characterize our gene candidates and avoid laborious and often mistake-prone PCR-based amplifications of genes, we used gene synthesis. Apart from the fast and easier access to the sequences of interest, gene synthesis also allowed us to codon-optimize the candidates for compatible expression in heterologous hosts. Our recombinant enzymes were fully functional after purification and we were able to assess their activities *in vitro* without any background activity from native enzymes and without any bottlenecks in substrate availability that is often the case for whole-cell assays.

The most challenging part of the proposed workflow comes in the final step of validating the *in vitro* results and linking them to *in vivo* observations. The indirect way of comparison of transformation products allowed us to hypothesize that in the presence of appropriate mediators, the *in vitro* characterized reactions could indeed be happening under real wastewater conditions. However, other oxidative enzymes could possibly form the same or similar transformation products. Therefore, more data on the activities of the strains where the genes originate from and measurement of their expression levels and/or protein levels will provide more robust conclusions and direct correlations in the future.

Finally, we used the proposed workflow to investigate the role of one group of oxidoreductases in TrOCs elimination reactions. However, the role of many other relevant enzymatic groups awaits elucidation. A well-known example is that of cytochrome P450 (CYP450) monooxygenases which are ubiquitous, promiscuous enzymes that catalyze reactions such as aliphatic hydroxylations, epoxidations and dealkylations and are responsible for xenobiotic metabolism in many organisms. Bacterial CYP450s have been successfully expressed in *E. coli* and the presence of endogenous, compatible redox partners has enabled easy and fast characterization using whole cell assays[85]. Beyond oxidoreductases, hydrolases have a prominent role in TrOCs biotransformations[27]. Efforts to characterize candidates from this group have been published[86, 87], but their role in activated sludge communities is yet to be determined. Our proposed methodology, adjusted to specific enzymatic groups, could provide useful insights into those and other oxidoreductases, ultimately supporting the rational design of biology-based water treatment strategies in the future.

Supporting Information

Details on bioinformatics analysis and supporting figure and tables of the proposed workflow and compounds used in the study. Additional result figures and tables. Transformation product analysis, protein sequences and alignments.

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