Understanding the dependence of micropollutant biotransformation rates on short-term temperature shifts

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Abstract

Temperature is a key factor that influences chemical biotransformation potential and rates, on which exposure and fate models rely to predict environmental (micro)pollutant fate.

Arrhenius-based models are currently implemented in environmental exposure assessment to adapt biotransformation rates to actual temperatures, assuming validity in the 0-30°C range.

However, evidence on how temperature shifts affect the physico-chemical and microbial features in biological systems is scarce, questioning the validity of existing modelling

approaches. In this work, laboratory-scale batch assays were designed to investigate how a

mixed microbial community responds to short-term temperature shifts, and how this impacts its ability to biotransform a range of structurally diverse micropollutants. Our results revealed three distinct kinetic responses at temperatures above 20°C, mostly deviating from the classic Arrhenius-type behavior. Micropollutants with similar temperature responses appeared to undergo mostly similar initial biotransformation reactions, with substitution-type reactions maintaining Arrhenius-type behavior up to higher temperatures than oxidation-type reactions. Above 20°C, the microbial community also showed marked shifts in both composition and activity, which mostly correlated with the observed deviations from Arrhenius-type behavior, with compositional changes becoming a more relevant factor in biotransformations catalyzed by more specific enzymes (e.g. oxidation reactions). Our findings underline the need to re-examine and further develop current environmental fate models by integrating biological aspects, to improve accuracy in predicting the environmental fate of micropollutants.

Introduction

Understanding the environmental fate of manufactured chemicals, such as pharmaceuticals and pesticides, and the factors that affect their potential persistence is of crucial importance to accurately evaluate their impact on the environment and public health¹. Biotransformation is a significant removal pathway for micropollutants in the environment, and its rate is an influential parameter in exposure and fate models². Chemical biotransformation is particularly responsive to changes in environmental factors, with temperature playing an important role by directly affecting process rates and microbial populations (e.g., growth, composition and biomass). To date, scientists, engineers and regulators temperature-correct the kinetic parameters of (bio)chemical processes in environmental models using a classic Arrhenius relationship, which works under the assumption that there is a single, rate-limiting, thermally activated process, and that the activation energy is independent of temperature. Although the Arrhenius equation was originally developed for chemical degradation processes (i.e.,

hydrolysis), over time, the approach has been implemented in various settings, including models of biological systems (such as activated sludge models ASM³ or pesticide fate model in soil-plant system PEARL⁴), and it is generally accepted to be valid in the 0-30°C range^{5,6}. However, several studies have reported deviation from the Arrhenius relationship for a number of chemicals⁷⁻⁹ as well as biochemical processes^{8,10}. In these studies, deviations from the Arrhenius relationship generally occurred with the decrease of temperature, where higher or lower reactivities, and therefore a decrease or increase of the apparent activation energy, were recorded^{9,11,12}. A number of mathematical and theoretical models have been suggested in order to interpret such observations, but a universal approach has yet to be agreed upon^{10,13,14}. In particular, for biotransformation in microbial systems, there is a lack of experimental data for many chemicals and for different temperature regimes. This prevents a full understanding of how temperature variations may impact enzyme-mediated chemical reactions and microbial communities relevant for biotransformation, and whether and how these should be integrated into existing exposure modelling frameworks. Both natural (e.g., rivers, soil) and engineered systems (e.g., full-scale wastewater treatment plants WWTPs) are subject to frequent temperature fluctuations, not only due to seasonal changes, but also on a more short-term basis (e.g., diurnal)^{15,16}. For instance, weather phenomena, such as snow melt and intense precipitation events, the occurrence of which is intensified by climate change¹⁷, or the operation of batch units in upstream industrial processes¹⁸, can greatly change the temperature over short periods (hours/days). Such temperature variations can occur over a much broader range than currently assumed in environmental models. For instance, daily extremes from 5 up to 40°C have been recorded in biological treatment systems located in (sub)tropical regions¹⁹, and daily temperature fluctuations of similar magnitude have been measured in surface soils in temperate areas²⁰. Furthermore, temperature is also a critical parameter in estimating half-lives from laboratory biodegradation tests (such as in OECD chemical testing²¹), where there is a debate regarding at which temperature tests should be

performed and how to extrapolate laboratory results to other temperatures²². Such extensive ranges of temperature fluctuations question to what extent the thermal properties of a single reaction can be extrapolated to the more complex microbial community level. In fact, additional factors might start playing a role in mixed biological systems, such as microbial community growth and activity, and resource limitations or temperature acclimation^{23, 24}. This work aims to fill the knowledge gap regarding the effect of short-term temperature variation on the kinetics of micropollutant biotransformation in biological systems. Activated sludge was chosen as a model system for the experimental studies, due to its intrinsic microbial complexity and the facility to manipulate and maintain homogeneous process conditions in laboratory studies. Furthermore, biotransformation laboratory assays with activated sludge can provide valuable information on the kinetics of mixtures of chemicals not only for full-scale WWTPs, but also for alternative biological systems, such as soil²⁵. A series of laboratory-based assays were carried out to closely monitor both micropollutant biotransformation, and compositional and activity changes in the microbial community under short-term temperature manipulations, over a 4-40°C range. The experimental data were then used to validate existing Arrhenius-based models, to more robustly define their applicability domain.

Materials and methods

Biotransformation batch assays. Detailed descriptions of the batch experiments, sampling and analytical methods are given in the Supporting Information (SI). Briefly, activated sludge (2.0 ± 0.03 g Total Suspended Solids/L) was collected from the nitrification basin of a municipal WWTP (Neugut WWTP, Dübendorf, Switzerland), and used immediately as inoculum for the batches. Sterile 100 mL amber Schott bottles (autoclaved at 121°C and 103 kPa for 20 min) constituted the reactors for five parallel laboratory scale assays. One series of batches was maintained at the original temperature of the full-scale WWTP (20°C, control

series), while the remaining batches were kept at four different temperatures (4, 10, 30, 40°C), in order to evaluate how short-term temperature changes would impact a temperate biological system. The experimental temperatures were achieved by using benchtop incubators or temperature-conditioned rooms (see SI). Each experimental set-up was composed of triplicate reactors for biotransformation experiments (50 mL of untreated activated sludge), and duplicate reactors for sorption (SCs, 50 mL of activated sludge autoclaved twice, 24 hours apart, at 121°C and 103 kPa for 20 min) and abiotic controls (ACs, 50 mL filtered, 0.2µm pore size, Sartorius Stedium, Göttingen, Germany, and autoclaved at 121°C and 103 kPa for 20 min activated sludge). SCs were used to evaluate losses due to sorption processes, and ACs to quantify abiotic transformation processes, such as hydrolysis. After allowing the batches to reach the selected experimental temperature (1 hours), each reactor was spiked with a stock solution (6mg/L) containing 87 micropollutants to obtain a final concentration of 6 µg/L of each chemical in each reactor. Prior spiking, the solvent (ethanol) of the micropollutant stock solution was evaporated under a stream of nitrogen and resuspended in nanopure water (Barnstead Nanopure, Thermo Scientific), in order to contain the presence of organic solvents (< 5% of total volume), which may interfere with the chromatographic detection. Additional chemicals (e.g., acesulfame, aspartame, cyclamate, metalaxyl, metoprolol, saccharine, sucralose) were also monitored, but were not spiked as known to be present in high concentrations in the influent of WWTPs²⁶. The micropollutants (see Table S1 in SI) were chosen based on their physico-chemical characteristics, their occurrence in sewage and natural systems, their presumed biotransformation reaction (from *in silico* predictions²⁷ or literature²⁸) and availability of reference compounds. In the biotransformation reactors, the first sample (2 mL, time = 0) was taken immediately after spiking and subsequently eight samples were regularly taken over a period of up to four days. For the ACs, samples were taken on day 1 (t=0), 2 and 3. For SCs, a single sample was taken 15 hours after spiking, and compared to ACs at t=0 to estimate the extent of sorption. The supernatant of the activated

sludge samples (centrifuged 10 min at 14,000 rpm) was spiked with isotope-labelled internal reference compounds (2 µg/L final concentration in the sample vial) and analyzed for micropollutants concentrations using reverse-phase liquid chromatography coupled to a hybrid quadrupole Orbitrap mass spectrometer (QExactive Plus, Thermo Scientific), as described in Achermann et al.²⁸. Triplicate sampling from each reactor at the second time point was used to determine the procedural and analytical accuracy. At each sampling time point, pH and temperature were recorded using aHQ30d Flexi Meter (Hach Lange, Vesenaz, Switzerland).

Kinetic evaluation. Micropollutant quantification was done using TraceFinder software 4.1 (Thermo Fisher Scientific). For each reactor, biotransformation rate constants (k_{bio}) were calculated from the logarithmic concentration-time series, assuming single first-order kinetics based on a linear regression model (“censReg” package in R v. 3.5.1²⁹, with left limit set at 3% of the initial spiked concentration), in order to account for instrumental limitations (insufficiently defined chromatographic peaks or when < LOQ). The quality of the kinetic fits was evaluated by visual assessment and by referring to the following goodness-of-fit parameters: R^2 (>0.98), residual patterns, residual standard error σ and chi-square test χ^2 (as per FOCUS guidelines³⁰). The kinetic fits that did not pass the quality checks were excluded for further analysis. For the remaining micropollutants, biotransformation rate constants were estimated for each individual experiment and their arithmetic mean calculated for the replicate reactors. The k_{bio} values were not biomass-nor-sorption-normalized, as each bioreactor was run at a similar initial biomass concentration of 2.0 ± 0.03 g/L.

Comparison of kinetic trends, for each micropollutant across the temperature range, was facilitated by using auto-scaled rate constants ($\tilde{k}_{bio,MP}$)³¹. Auto-scale rate constants were

calculated using the command “scale” in R v. 3.5.1, as $\tilde{k}_{bio,MP} = \frac{k_{bio,MP} - \bar{k}_{bio,MP}}{sd_{MP}}$,

where $k_{bio,MP}$ is the rate constant for micropollutant MP, $\bar{k}_{bio,MP}$ and sd_{MP} are the mean rate constant and the standard deviation across all temperatures for the same micropollutant MP. No analysis of biotransformation products was carried out in this particular work. Rather information on micropollutant reaction pathways were sourced from Achermann et al.²⁸.

Modelling of temperature effect on micropollutants biotransformation. The dependence of micropollutant biotransformation kinetics on temperature was evaluated using the following three prominent Arrhenius-based modelling approaches:

Classic Arrhenius describes an exponential decrease of k_{bio} with inverse temperature, where the activation energy (E_a) for the reaction is assumed to be independent of temperature (eq.1):

$$\ln k_{bio} = \ln A - \frac{E_a}{R} \left(\frac{1}{T} \right) \quad (1)$$

with $\ln A$ being the pre-exponential factor and R the universal gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$). In this case, E_a can be mathematically derived from the slope of the Arrhenius plot ($\ln k_{bio}$ vs $1/T$).

*Quadratic Arrhenius*¹¹ represents a deviation from the classic Arrhenius by an additional quadratic term in $1/RT$ (eq.2):

$$\ln k_{bio} = \ln A + \frac{B}{RT} + \frac{C}{(RT)^2} \quad (2)$$

where C accounts for the deviation from a classic linear Arrhenius type-temperature dependence ($C > 0$ concave, and $C < 0$ convex Arrhenius plot). According to this theory, E_a still relates to the slope of the Arrhenius plot, however it is no longer a constant but rather linearly depends on $1/T$ (eq. 3):

$$E_a = -\frac{d \ln k_{bio}}{d(1/RT)} = -B - \frac{2C}{RT} \quad (3)$$

167 *Deformed Arrhenius*¹² applies an algebraic deformation on the exponential function of the
 168 classical Arrhenius relationship (eq. 4):

$$169 \quad \ln k_{\text{bio}} = \ln A + \frac{1}{d} \ln \left(1 - d \frac{\varepsilon}{RT} \right) \quad (4)$$

170 where d describes the deviation or curvature in the Arrhenius plot, and which converges to
 171 eq.1 for $d \rightarrow 0$ and $\varepsilon \rightarrow E_a$ (a constant). In this approach, E_a is again a function of T (eq.5):

$$172 \quad E_a = - \frac{d \ln k_{\text{bio}}}{d(1/RT)} = \varepsilon \left(1 - \frac{d\varepsilon}{RT} \right)^{-1} \quad (5)$$

173 Model fits were assessed using a variation of Mallows' C_p statistics (eq.6 as in Fenner et al.
 174 ³²), which takes into account differences in the number of fit parameters, thus preventing
 175 overfitting:

$$C_p = \frac{\text{RSE}}{n} + \frac{2p\hat{\sigma}^2}{n} \quad (6)$$

176 where RSE is the sum of the residual squared errors, n is the number of data points used in the
 177 fitting, p is the number of fit parameters in the model, and $\hat{\sigma}^2$ is an estimate of residual
 178 variance, calculated as $\text{RSE}/(n-p)$. C_p values close to or smaller than p indicate stronger model
 179 fits. Modelling and statistical analysis were conducted using R v. 3.5.1.

180 **Microbial analysis.** Detailed descriptions of DNA and RNA extractions and amplicon
 181 libraries preparation are given in SI. Briefly, samples for microbial analysis (2 mL) were
 182 collected from each of the triplicate reactors, four hours after spiking the micropollutants,
 183 when it was assumed that all the micropollutants were undergoing biotransformation and the
 184 enzymatic activity of the microbial community fully expressed. Pelleted cells (centrifugation
 185 at $10000 \times g$ for 5 min) were preserved in RNeasy lysis buffer (Qiagen, Crawley, UK) and
 186 at -20°C until used. DNA and RNA were simultaneously extracted from each sample using

the AllPrep DNA/RNA Mini Kit (Qiagen), following the manufacturer's instruction. Two negative controls, in which the sludge sample was not added, were included in the extraction protocol. Total RNA samples were reverse transcribed to produce the complementary DNA (cDNA), using SuperScript III Reverse Transcriptase Kit (Invitrogen) and random primers, following the manufacturer's instruction. Individual amplicon sequencing libraries were prepared from each of the genomic (gDNA) and complementary DNA (cDNA) samples using a ligation approach, and primers 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3') containing the Illumina overhang adapters (see SI). In order to assess the presence of artefacts, errors and bias in library preparation, sequencing and bioinformatics steps, amplicon libraries (in duplicate) were also prepared for the two negative controls, obtained from the extraction step, and the ZymoBIOMICS Microbial Community Standard (Cat No. D6306, Zymo Research), used as a positive control. The final amplicon libraries were pooled equimolarly and sequenced on an Illumina MiSeq System (Illumina, USA) at the Genetic Diversity Centre (ETH Zurich), following a PE300 method. FastQC v 0.11.2 was used to quality check the raw sequencing data, followed by trimming to 253bp sequence length using PRINSEQ-lite v.0.20.4³³. USEARCH V.8.1.1756³⁴ was then used to merge the remaining reads (15bp minimum overlap, 100bp minimum merge-length, 5bp maximum error), primers trimmed using cutadapt v1.5 (error rate of 0.01 and wildcard allowed), a final quality filtering was done using PRINSEQ-lite v.0.20.4, and then sequences were clustered into operational taxonomic units (OTUs) with 97% similarity threshold. Taxonomy was assigned using usearch_global with 70% identity against the SILVA 16S database (release 128), followed by SINTAX with 70% identity cutoff. The final dataset was analyzed using phyloseq 1.24.0 and vegan packages in R v. 3.5.1 as follows.

Species richness (H_0), Shannon (H_1) and Simpson (H_2) diversities (Hill numbers) across the temperature ranges were estimated (on rarefied data) as $H_\alpha = (\sum_{i=1}^S p_i^\alpha)^{\frac{1}{1-\alpha}}$, where S is the number of "species" in the sample and p_i is the proportional abundance of each species³⁵.

Higher α values accentuate more abundant species rather than rare species. Difference in the microbial community compositions between temperatures was quantified using Bray-Curtis dissimilarity index on untransformed data, and graphically represented using a Principal Coordinate Analysis (PCoA, using the R-phyloseq function “ordinate”). The statistical significance of temperature-driven community diversity was tested by permutational multivariate analysis of variance (PERMANOVA), using the R-vegan function “adonis”. Further, the similarity percentage procedure (SIMPER) was used to identify the taxa that primarily contributed to the Bray-Curtis dissimilarity measures, using a cut-off of 70% cumulative contribution, with at least 1% significance. Comparison of trends in relative abundance, for each SIMPER-selected OTU across the temperature range, was facilitated by using auto-scaled relative abundances, as for the auto-scaled rate constants. Spearman rank-correlation or Pearson’s product moment correlation (using R-vegan package) were performed to evaluate potential correlation between changes in relative abundances of the SIMPER-selected OTUs (present and active) and changes in micropollutant kinetics across the temperature range.

Results and discussion

Micropollutant biotransformations. Over the course of the biotransformation experiments, pH remained in the range of 8.0 ± 0.3 across all batches, and the temperature varied maximally by 0.7°C for each selected temperature. Among the 94 targeted micropollutants, 80 compounds were detected by high-resolution mass spectroscopy. Their analytical measurements showed overall good accuracy for the biotransformation batches, abiotic and sorption controls (overall analytical error $< 10\%$, Table S2 in SI) over all temperatures. However, 38 of those micropollutants were excluded from further analysis, as accurate rate constants could not be calculated, mostly for two reasons. Firstly, deviation from first-order kinetics (visual assessment, residuals with systematic deviations, $R^2 < 0.90$, $\chi^2 < 0.05$, σ values

above 0.5, Table S3 in SI) was observed for a number of amine-containing micropollutants (i.e., citalopram, clozapine, doxylamine, levamisol, lidocaine, mianserin, pheniramine, propranolol, venlafaxine). For these, an initial decrease in aqueous concentrations was followed by a stagnation phase. This was interpreted as being consistent with a competing ion-trapping mechanism in activated sludge, previously described by Gulde et al.³⁶. Atenolol and ticlopidine were the only amine-containing micropollutants that did not show any evidence for the ion-trapping mechanism in our batches, confirming the predominance of bacterial biotransformation in these cases, as previously reported for atenolol³⁶. However, ticlopidine was not further investigated, due to strong sorption (50-70% across temperatures), which would have introduced biases in the pseudo-first order kinetics analysis. Secondly, additional compounds were excluded because they were particularly persistent (< 30% removal, mostly micropollutants containing secondary and tertiary amides and triazine structures) or were biotransformed extremely fast (i.e., propachlor). Both cases resulted in poor model fits (e.g., $R^2 < 0.8$, $\chi^2 \gg 0.1$ and σ values above 0.6, Table S3 in SI) and consequently poor rate constant estimates (see Figure S2 in SI). The remaining 42 micropollutants showed an exponential decrease in concentration over time, and abiotic or sorption effects were negligible (<20%). These 42 included also compounds that showed good residuals distribution and goodness-of-fit statistical parameters (χ^2 and σ), even when R^2 value was < 0.98 (see Table S3 in SI). For all these 42 compounds, rate constants k_{bio} were calculated for each batch system, and the average k_{bio} was obtained from replicate batches run at the same temperature (Figure S3, SI).

Effect of temperature on micropollutant biotransformation kinetics. In the 20°C batches, all 42 micropollutants decreased by more than 40% over the experimental period of 72 hours, with amides undergoing hydrolysis or dealkylation being among the fastest degraded, and sulfonamides the most slowly transformed (Figure S2 in SI). Most micropollutants maintained

an exponential decrease in concentration across all the temperatures tested, with the exception of a few compounds, which displayed deviations from first-order kinetics at 40°C, showing stagnation at later time points (i.e., acesulfame, DEET and naproxen), or which were not degraded at all at extreme temperatures (4 and 40°C, see Figure S2 in SI). The (geometric) mean k_{bio} across all micropollutants at each temperature showed a gradual decrease in rate constants at temperature above 20°C, leading to a convex-shaped Arrhenius plot (green dotted line in Figure 1). However, the individual micropollutants showed distinct responses to temperature changes (Figure 2). While a positive (linear) relationship with temperature was observed in the 4-20°C range for all compounds, three major trends were found above 20°C, in which rate constants kept increasing or decreased with the increase of temperature, mostly leading to a convex-shape Arrhenius plot (see Table 1 and Figure 1).

Arrhenius-based modelling. Three Arrhenius-type modelling approaches (here named classic, quadratic and deformed Arrhenius) were tested on our experimental data, for chemicals for which data for three or more temperatures were available (see Figure S4 and Table S4 in SI). The observation of convex-shaped Arrhenius plots ($\ln k_{bio}$ vs $1/T$) for most of the micropollutants revealed clear limitations of the classic Arrhenius model (eq.1). This model only provided a fairly accurate fit up to 20°C, and not 30°C as currently assumed⁵, for most of the micropollutants (see Figure 1, and model residuals and $C_p < 0.6$ in Figure S4 in SI). The classic Arrhenius model gradually overestimated biotransformation rate constants as temperature increased further, except for the sulfonamides (see Figure S4 in SI model residuals and $C_p < 0.033$ in the 4-40°C range). Based on their observation in a laboratory soil system, Alidina et al.³⁷ had suggested the presence of a communal critical temperature (10°C) at which strong changes in micropollutant biotransformation rates occur, resulting in two distinct areas of linear relationships (below and above 10°C). However, our data revealed

kinetic changes occurring rather between 20°C to 30°C, and in a more unsystematic manner, i.e., depending on the micropollutant.

Table 1 – List of micropollutants selected for this study, their initial reaction pathways (as reported in Achermann et al ²⁸) and grouping according to temperature responses, e.g., k_{bio} increases up to 20°C (group 1) or up to 30°C (group 2; group 2b where k_{bio} at 40°C did not meet the selection criteria, but data suggested a decrease), or in the full 4-40°C range (group 3).

Temperature Response	Micropollutants	Reactions ²⁸		Classic Arrhenius R^2	
Groups	Name	Pathways	Type	4-40°C	4-20°C
1	BEcIB	Amide dealkylation Hydroxylation	Oxidation	0.591	0.985
	CHLOROTOLURON	Hydroxylation Dihydroxylation Dealkylation		0.048	0.917
	CLOFIBRIC ACID	Hydroxylation		0.201	
	FUROSEMIDE	Amide dealkylation		0.363	0.907
	IPROVALICARB	Hydroxylation		0.122	0.896
	IRGAROL	S-oxidation		0.661	0.940
	ISOPROTURON	Hydroxylation Dealkylation		0.671	0.982
	RANITIDINE	S-oxidation N-oxidation		0.003	0.972
	TERBUTRYN	S-oxidation		0.696	0.946
	VALSARTAN	Amide dealkylation		0.530	0.831
	CAPECITABINE	Hydroxylation Hydrolysis	Oxidation/ Substitution	0.071	0.874
	ACESULFAME	Unknown	Unknown	0.656	0.836
	TEBUFENOZIDE	Unknown		0.848	0.994
	TRIMETHOPRIM	Unknown		0.854	0.999
2	PARGYLINE	N-oxidation	Oxidation	0.133	0.998
	BEZAFIBRATE	Amide dealkylation Hydrolysis	Oxidation/ Substitution	0.000	0.953
	ALACHLOR	Substitution Reductive dehalogenation Amide hydrolysis	Substitution	0.599	0.984
	DIMETHENAMID			0.665	0.988
	FLUFENACET			0.413	0.985
	LEVETIRACETAM			0.020	0.995
	METOLACHLOR	Substitution Reductive dehalogenation Dealkylation		0.655	0.974
	RUFINAMIDE	Amide hydrolysis		0.597	1.000
	SULFATHIAZOLE	Pterine conjugation		0.404	0.965
	TRINEXAPAC-ETHYL	Ester hydrolysis		0.577	0.814
	DEPRENYL	Unknown		0.788	0.896
	AZOXYSTROBIN	Ester hydrolysis	Substitution	0.952	0.965
	BROMOXYNIL	Nitrile hydration		0.961	0.915
2b	DEET	Unknown	Unknown	0.746	0.865
	DIMEFURON			0.837	0.951
	ETHOFUMESATE			0.814	1.000
	NAPROXEN			0.811	0.934
	THIACLOPRID			0.832	1.000
	ZONISAMIDE			0.891	0.705
3	KETOPROFEN	Hydroxylation Biphenyl pathway	Oxidation	0.904	0.912

	ACETAMIPRID	Nitrile hydration	Substitution	0.963	0.999
	ATENOLOL	Amide hydrolysis		0.913	1.000
	SULFADIAZINE	Pterine conjugation		0.968	0.999
	SULFAMETHAZINE			0.950	0.905
	SULFAMETHOXAZOLE			0.930	0.973
	SULFAPYRIDINE			0.960	0.987
	FENHEXAMID	Unknown	Unknown	0.877	0.978
	INDOMETHACIN			0.864	0.952

Figure 1 On the left, Arrhenius plots for the three micropollutants representing the three main temperature-response trends are given, together with the average kinetics across all micropollutants (geometric mean depicted with a green dotted line), and the fit to the experimental data using the classic, quadratic and deformed Arrhenius models. On the right, the corresponding activation energy predictions for each model scenario are reported.

We therefore explored the evident deviation from Arrhenius-linearity of our experimental data by implementing two alternative phenomenological Arrhenius-based formulations (quadratic and deformed Arrhenius). These approaches seemed more suitable to describe our results and

more in line with previously reported convex-Arrhenius behavior in enzyme kinetics^{10,38,39}. In our case, the deformed Arrhenius model succeeded in predicting only smaller curvatures in the Arrhenius plot (e.g., as observed for atenolol and sulfonamides), suggesting that the limited number of parameters used in eq. 4 and the restricted flexibility of the d-parameter were not sufficient to describe all the experimental data. Alternatively, the additional quadratic term in eq. 2 seemed to better capture the observed curvatures (see Figure 1, and Figure S4 in SI model residuals and $C_p < 0.45$), conferring higher flexibility and allowing extrapolation to wider ranges of temperatures.

Convexity in the Arrhenius plots for biochemical systems has previously been associated with multiple factors including: interconverting protein conformations with the appearance of catalytically inactive regions, change in enzymatic flexibility, or irreversible loss of enzymatic activity⁴⁰⁻⁴². However, previous studies³⁹ have also shown that temperature-dependent protein unfolding or oligomerization would generate highly inflated Arrhenius prefactor (A) estimations ($A > 10^{25} \text{ s}^{-1}$ [or 10^{20} d^{-1}], corresponding to $\ln A = 57 \text{ s}^{-1}$ or 46 d^{-1}), which is not the case for either the quadratic or deformed models here ($\ln A \ll 46 \text{ d}^{-1}$ see Table S4 in SI).

Hence, reasons for convexity might more likely be attributed to the presence of competing enzymatic forms or multiple coexisting enzymes, each dominating at a specific temperature range⁴³, and/or displaying different kinetics under different temperatures. In addition, possible temperature-induced variations in the composition of a system microbial community cannot be underestimated, as this could also influence the presence and/or abundance of certain enzymes in the system.

Potential enzymatic-associated changes, such as changes in the structure or abundance of enzymes, would lead to temperature-driven shifts in the biotransformation capability of the system, which are not considered under the assumptions of the classic Arrhenius theory. The representation of multiple simultaneous changes in the biochemistry of the system is represented in the activation energy (E_a), which is derived from the slope of the Arrhenius

plot. According to equations 3 and 5, the overall E_a is therefore dependent on the temperature, decreasing with increasing temperature, moving from positive to negative values (see Figure 1). An explanation for the transitioning from positive to negative E_a values could be provided by Tolman's interpretation⁴⁴, in which the apparent activation energy ($E_a = E_r - E_s$) is the result of the combination of the average energy of all reacting systems (E_r) and the average energy of all reacting and non-reacting systems (E_s). Therefore, the overall E_a changes would imply that, with an increase of temperature, E_r decreases or increases less rapidly than E_s , resulting in an apparent reduced micropollutant biotransformation capability (or activity) in our batches, and justifying a passage to negative values in the overall E_a . However, these phenomenological models are unable to provide a deeper insight into the kinetic processes, and only describe the overall kinetics of the system. Further work is required to decouple the temperature-dependent kinetic processes, in order to produce a more realistic theoretical model that captures all the system dynamics.

Figure 2 Rate constant variation (auto-scaled with standard deviation as scaling factor) across batches kept at different temperatures (4 to 40°C), with darker shades indicating higher values. Grey squares indicate missing rate constant values (due to not pseudo-first order behaviour). On the left, hierarchical clustering underlines the temperature responses according to initial biotransformation reaction type (see Table 1).

Reaction-specific temperature effects. Closer investigation of the temperature responses of the biotransformation rate constants was conducted to reveal the presence of similar trends amongst chemicals. Previous studies have reported that temperature responses in chemical biodegradation are mainly driven by compound characteristics^{5,37} and/or alternative parameters, such as substrate availability⁴⁵.

Here, hierarchical clustering of chemicals as a function of the temperature-dependence of their rate constants revealed that micropollutants undergoing a similar type of

356 biotransformation reaction would display similar temperature responses, with only few
357 exceptions (see Figure 2). In particular, for chemicals undergoing oxidation-type reactions
358 (according to Achermann et al.²⁸), rate constants started to decrease at temperatures above
359 20°C (group 1, see Table 1). For instance, the thioethers irgarol, terbutryn, and ranitidine
360 clustered closely together, suggesting that their biotransformation would involve similar
361 enzymes or microbes. Indeed, similarity in their initial biotransformation reaction (i.e., S-
362 oxidation) and similarity in their response to differing conditions (i.e., different solids
363 retention times) have previously been reported^{28,46}, corroborating our finding. Another strong
364 cluster (still in group 1) included iprovalicarb and isoproturon, both undergoing initial
365 hydroxylation reactions. In contrast, substitution-type reactions seemed to endure higher
366 temperatures, suggesting that their major transformation pathways are regulated by
367 microorganisms or enzymes with higher thermostability, or that those types of enzymes are
368 more redundant and hence present across microorganisms with different temperature optima.
369 For instance, strong deviation from classical Arrhenius-type behavior only occurred above
370 30°C for the acetanilides, i.e., dimethenamide, metolachlor, alachlor and flufenacet, which
371 strongly clustered together (group 2, see Table 1). Previous studies^{28,47} have shown that their
372 biotransformation mostly occurs through initial conjugation mediated by glutathione-S-
373 transferase, a known thermostable enzyme with reported optimal activity at 30°C^{48,49}. A
374 similar temperature response was found for chemicals harbouring a nitrile group, i.e.,
375 bromoxynil and acetamiprid, which also strongly clustered with the ester azoxystrobin.
376 Interestingly, these last three chemicals completely failed to biotransform at temperatures
377 above 30°C (group 2b). Previous studies have suggested that bromoxynil and acetamiprid
378 transformation to the corresponding amide is catalysed by nitrile hydratases²⁸, whose activity
379 is known to start decreasing significantly at temperatures above 20°C due to enzyme structure
380 destabilisation⁵⁰. Azoxystrobin, in contrast, has been shown to be primarily transformed
381 (hydrolysis) by the action of protozoa³⁶, which are unable to survive for more than few hours

above 37°C⁵¹. Only a small group of micropollutants (i.e., most sulfonamides and three amides) showed a temperature response that was more in line with a classic Arrhenius relationship, i.e., increasing reaction kinetics with increasing temperatures across the entire temperature range (group 3 in Table 1). It has previously been suggested that the four sulfonamide antibiotics sulfadiazine, sulfamethazine, sulfamethazole, and sulfapyridine mainly undergo pterin-conjugation in activated sludge systems (batch and full scale plants⁵²). This pathway is believed to be initiated by the dihydropteroate synthase enzyme, which is a central enzyme in the folate synthesis pathway, and hence essential for all bacterial cell growth⁵². It is worth noticing the distinct behavior of sulfathiazole, which shows an evident deviation from the Arrhenius prediction above 30°C (group 2, Table 1), in contrast to the rest of the sulfonamides studied. This singular behavior might be due to the presence of a secondary oxidative transformation of the thiazole group, which has been previously hypothesized to explain deviating behavior of sulfathiazole⁴⁶. For atenolol⁵³ and indomethacin⁵⁴, it is likely that their initial hydrolysis is catalysed by amidohydrolase enzymes, which are known to be rather thermostable, i.e., to positively respond to temperature increases of up to 70°C⁵⁵.

These evident reaction-specific trends in temperature responses confirmed the hypothesized involvement of a complex array of differing enzymes and microbial species in micropollutant biotransformation reactions. These findings thus also highlight the key role played by the microbial community, and the need to understand how the temperature impacts its composition and (co)metabolism.

Effect of temperature on microbial community composition and activity. The

assumptions generated from the biotransformation data were consolidated by insights into temperature-driven microbial community changes characterized through sequencing of the

406 16S rRNA gene (gDNA) and its transcripts (cDNA), which provided information on the
407 overall present and active community members, respectively, during the experimental period.
408 After quality filtering and overlapping of forward and reverse fragments, 2,681,445 high-
409 quality sequences were retrieved with amplicons 254-255 base pairs long (99% of the total
410 reads). Positive and negative control samples demonstrated that the sequencing protocols and
411 the analysis pipeline guaranteed no coverage bias based on GC content or cell wall type,
412 indicating good taxonomic assignment independent from organism characteristics (detailed
413 data analysis in SI). The original microbiome (batch at 20°C) was dominated by the presence
414 of members of the *Comamonadaceae* (16.8%), *Rhodocyclaceae* (11.3%),
415 *Verrucomicrobiaceae* (3.7%) and *Moraxellaceae* (2.1%) families, which also appeared to be
416 among the most active member of the community (*Comamonadaceae* 13.7%,
417 *Rhodocyclaceae* 9.7%, *Verrucomicrobiaceae* 3.7%, *Moraxellaceae* 4.3%). However, not
418 necessarily these taxa exhibited the most relevant temperature-driven changes across the
419 different batches.

420 When a biological system is subject to short-term temperature changes (hours), as
421 investigated in our experimental setup, it is generally expected that the microbial metabolism
422 (enzymatic activity) would be primarily affected, while adaptation of the microorganisms
423 (compositional changes) would require longer time periods. This scenario would agree with
424 the fundamental assumption of the classic Arrhenius model, by which all the rate-limiting
425 enzyme(s) involved in a biochemical reaction (e.g., micropollutant biotransformation) would
426 have a 100% probability of being present and of maintaining its/their active conformation
427 across the temperature range. Nevertheless, there is still a lack of consensus on the minimum
428 timescale required to initiate temperature-induced microbial community compositional
429 changes. For instance, previous studies (primarily on soil and natural waters) have reported
430 that biomass acclimation to a 5°C change would occur after two weeks, if in the 15°C range,
431 and even after months, in the 30°C range⁵⁶. Our microbial analysis of short-term experiments

after 4 hours from micropollutant spiking, revealed that both microbial compositional and activity changes occurred in our batches, particularly above 20°C (Figure 3, and Adonis across 4-40°C: $R^2 = 0.641$ and $p=0.01$ for gDNA, and $R^2=0.644$ and $p=0.001$ for cDNA). These biological changes seemed to coincide with deviation from Arrhenius-type behavior in the biotransformation rates of the micropollutants here investigated at temperatures above 20°C.

Figure 3 Bray-Curtis based Principal Coordinates Analysis (PCoA) plots describing differences in composition of A) the total (gDNA) and B) active taxa (cDNA) of microbial communities across different temperatures.

Since richness and diversity have previously been related to contaminant biotransformation⁵³, we first assessed changes in these summary parameters with temperature. Reduction in diversity indices values with increasing Hill number at all temperatures shows that the overall

and active community structure was uneven and dominated by abundant taxa (see Figure S9

in SI). Temperature did not strongly impact the overall richness (H_0) of the OTUs present (gDNA) in the batches, though the number of active OTUs decreased above 20°C (a moderately strong negative Spearman's rank correlation of H_0 and H_1 with increasing temperature for cDNA, see Figure S9 in SI). At 40°C, there was a relative increase in the diversity of abundant OTUs (Shannon H_1 and Simpson H_2) of taxa present (gDNA) or conversely a relative decrease in rare taxa, while both H_1 and H_2 markedly decreased for the active (cDNA) OTUs (Figure S9 in SI), particularly between 30°C and 40°C. This seems to indicate that more rare taxa showed higher activity relative to the abundant taxa at higher temperatures.

Similarity percentage analysis (SIMPER) allowed the identification of the taxa that were strongly associated with those microbial changes (see Table S5 in SI and Figure 4).

Figure 4 Variation of relative abundances for present (gDNA, left) and active (cDNA, right) SIMPER-identified OTUs (auto-scaled values), across batches kept at different temperatures (4 to 40°C). Darker shades indicate higher values.

463

464 A small number of these SIMPER-selected OTUs corresponded to taxa commonly found in a
465 large variety of environments worldwide (from Arctic to temperate), suggesting their ability
466 to survive over a broad range of temperatures. In fact, although these OTUs were not
467 particularly abundant (averagely constituting 1.5%) in the batch microbial community at
468 20°C, their presence was maintained in all other batches, with an increase in their relative
469 abundance across the temperature gradient (Pearson's product moment correlation gDNA
470 relative abundances vs temperature: $r = 0.83, 0.93, 0.93$ and 0.97 for OTU1, OTU4, OTU23
471 and OTU29, respectively). This was the case for members of the *Verrucomicrobiaceae*
472 (OTU1)⁵⁷ and *Leptospiraceae* (OTU4) families, but also of the *Cyanobacteria* (OTU23)⁵⁸ and
473 *Planctomycetes* OM190 (OTU29)⁵⁹⁻⁶¹ phyla. Their increased presence with temperature
474 positively correlated with the Arrhenius-like increase of biotransformation rate constants for
475 the majority of the investigated micropollutants (Pearson's product moment correlation
476 gDNA relative abundances vs temperature (4-20°C), $r > 0.7$), particularly with those
477 undergoing substitution-type biotransformation that had shown to endure higher temperatures
478 (see Spearman's rank correlation in Figure S10 in SI). Previous studies have reported (or
479 speculated) the ability of these taxa to degrade a wide range of organics even under extreme
480 temperatures⁶²⁻⁶⁴, and their presence has also been directly linked to the biodegradative
481 capability in activated sludge systems^{65,66}. It is therefore plausible to assume that this group of
482 taxa played a central role in maintaining the ability to biotransform micropollutants in the
483 batches up to higher temperatures. This was corroborated by an overall increase in the activity
484 of these OTUs across the temperature gradient (see Figure 4, and Pearson's product moment
485 correlation cDNA relative abundances vs temperature: $r = 0.81, 0.75$, and 0.96 for OTU1,
486 OTU4, and OTU29, respectively), except for members of *Cyanobacteria* (OTU23), whose
487 activity showed an apparent decrease above 30°C (Pearson's product moment correlation
488 cDNA relative abundances vs temperature: $r = -0.49$ (0.85 in 4-30°C range) for OTU23).

489 However, above 30°C, OTU23 showed a higher increase in gDNA abundance than the rest of
490 the taxa in this cluster, and this most likely compensated the slight decrease in activity.
491 The remaining SIMPER-selected community members, responsible for the temperature-
492 induced microbial community shifts in the batches, were dominated by members of the
493 *Comamodadaceae* (OTU5, OTU15, OTU20 and OTU77), *Rhodocyclaceae* (OTU6 OTU12,
494 OTU13), *Caldilineaceae* (OTU95), *Roseiflexaceae* (OTU46), and *Moraxellaceae* (OTU2)
495 families. The majority of these taxa were present in high abundance (gDNA) in the 20°C
496 batches, accounting for almost 20% of the total microbial community. Previous studies have
497 reported their presence in the core community of activated sludge systems during winter⁶⁷,
498 suggesting their preference for colder habitats. This might explain their decreasing presence at
499 temperatures above 20°C, or even above 10°C for OTU2 and OTU77 (see gDNA relative
500 abundance in Figure 4). For the majority of these OTUs, also the activity appeared to drop
501 (decrease in cDNA relative abundances in Figure 4) at higher temperatures, mostly above
502 20°C but also from 10°C (OTU2) or as late as 30°C (OTU23). There was an exception for a
503 few of these taxa (OTU2, OTU15, OTU46 and OTU95), for which the increase in temperature
504 seemed to destabilize their activity, which oscillated across the temperature gradient without
505 any temperature-defined patterns. The reduced presence and activity of this group of OTUs at
506 higher temperatures mirrors the trends observed for the micropollutants biotransformation rate
507 constants (k_{bio}) that deviated from Arrhenius estimations (convex Arrhenius plot), with k_{bio}
508 gradually decreasing above 20/30°C, which, in our chemical analysis, seemed to be typical of
509 micropollutants undergoing initial oxidation-type biotransformation (see Figure S10 in SI).
510 The strong correlation of these taxa with few specific micropollutants suggested that they
511 might have employed more specific enzymes, less common amid different species, as also
512 previously reported as being characteristic of oxidation-mechanisms⁵³. For instance,
513 temperature-induced structural and activity changes in *Dechloromonas* sp (OTU6),
514 *Roseiflexus* sp. (OTU46), and *Caldilineaceae* family (OTU95) seemed to strongly correlate

with temperature-induced shifts of k_{bio} for chlorotoluron, capecitabine and iprovalicarb (overall Spearman rank correlations $r_s > 0.8$, Figure S10 in SI), in line with previous studies claiming the potential involvement of such taxa in the biotransformation of such organics through hydroxylation reactions⁶⁸.

However, the identification of specific reaction pathways of micropollutant biotransformation goes beyond the scope of the current study, and would require alternative scientific approaches, focused on formation of specific transformation products and expression of distinct enzymes involved in micropollutant biotransformation under different temperatures. Still, our 16S rRNA-based assays have revealed that short term-temperature variations indeed impact the enzymatic activity as described by Arrhenius, but also greatly affect the composition of the microbial community, which, in turn, directly determines the presence (or not) of the enzymes involved in micropollutants biotransformation. In particular, these temperature-induced compositional changes result in different sets of enzymes, and therefore specific reaction pathways, which predominate under different temperature conditions. More specifically, pathways catalyzed by “common enzymes”, such as in the majority of substitution-type reactions, will be less affected by microbial structural changes, while, when the biotransformations rely on more specific (“rare”) enzymes such as in oxidation-type reactions, the presence of specific bacterial species become a more relevant factor, by determining the probability of certain enzymes to be present and active under different temperature conditions. However, it has to be noted that our current study has investigated the impact of short-term temperature changes on a single inoculum (e.g., nitrifying activated sludge), sampled from a particular spatial location and time. It is well known that microbial communities of WWTP and other biological systems vary spatially. Therefore it is uncertain how our current results would have differed if we sampled at another location or time, though mechanisms of microbial transformation and associated rate constants for selected chemicals have been shown to be similar across different environmental compartments^{25, 69}.

Nevertheless, our findings challenge the applicability of the simplistic Arrhenius model, which does not consider potential variation in the composition of available enzymes in the microbial community, to describe temperature-dependence of microbial biotransformation. Still, further work will have to be performed to comprehend the ramifications of our findings on long-term temperature effects, prior to implementation in environmental fate models. Thus, our present work indicates the need for developing a new generation of temperature-kinetic-models that incorporates ecological phenomena, including also (co)metabolic activity changes induced by microbial compositional changes under temperature stresses. This is bound to impact the way engineered and natural biological systems are characterized and monitored, with the effective implementation of microbiological tools into routine assessments. We believe that this is essential in order to improve temperature-dependent fate models predictability and accuracy, and ultimately influence the current approaches in engineering, and chemical risk management.

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