

# Biotransformation of Chemicals in Water–Sediment Suspensions: Influencing Factors and Implications for Persistence Assessment

Carolyn Seller, Mark Honti, Heinz Singer, and Kathrin Fenner\*



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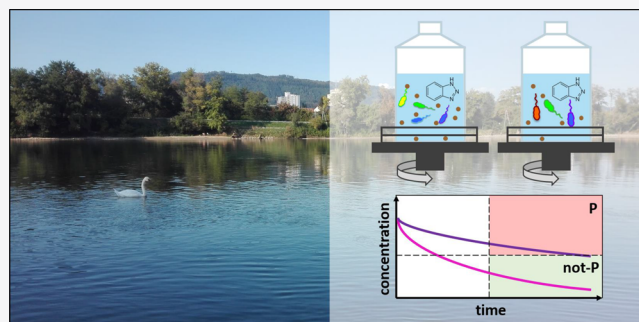


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**ABSTRACT:** Chemicals' half-lives derived from biotransformation simulation studies are central metrics for persistence assessment in international regulatory frameworks. To determine the persistence of chemicals released to the aquatic environment, paradigm shifts in recent and ongoing revisions of chemical legislation assign increasing importance to OECD 309 simulation studies. OECD 309 studies were designed to target biotransformation in natural water (pelagic test) or in water amended with sediment (suspension test). Suspension tests bear several advantages over the pelagic test, most importantly, employing higher bacterial cell densities, which promote biotransformation of various chemicals at observable rates. However, experience with suspension tests is limited. In this study, we followed the fate of 43 pharmaceuticals, pesticides, and industrial chemicals in various suspension test setups and elucidated parameters influencing biotransformation kinetics and half-lives derived thereof. Besides striking intrastudy variability between replicates, we found that differences in sediment origin and bacterial cell density resulted in chemical half-lives that were different by up to 2 orders of magnitude, making persistence classification rather uncertain. However, data suggested that test systems employing bacterial cell densities close to the upper limit of what is commonly observed in natural surface waters (i.e.,  $10^7$  cells  $\text{mL}^{-1}$ ) yielded increased and more uniform biotransformation of chemicals.



## INTRODUCTION

Chemicals can enter surface waters through various pathways and bear the potential to harm aquatic ecosystems. Actual concentration levels of chemicals in surface waters depend on their environmental persistence, defined by a chemical's recalcitrance toward biotic and abiotic transformation.<sup>1–3</sup> In recent years, chemicals' half-lives as indicators of their persistence are becoming increasingly central metrics for hazard and risk assessment in international regulatory frameworks.<sup>1,3–5</sup>

The OECD has developed a series of test guidelines used by professionals involved in chemical persistence assessment and accepted by international regulatory frameworks, e.g., by the REACH regulation of the European Union.<sup>1,6</sup> Two test guidelines are relevant for chemicals that may enter surface waters: the OECD 308 guideline, which targets biotransformation in aquatic sediments, and the OECD 309 guideline, which assesses biotransformation in the pelagic water body.<sup>7,8</sup>

In November 2018, the European Medicines Agency (EMA) released a draft revision of their environmental risk assessment guidelines for marketing authorization of pharmaceuticals, whose review and revision process is ongoing. A major paradigm shift in the new draft guideline is that OECD 309 rather than OECD 308 tests may become the cornerstone of persistence assessment for pharmaceuticals.<sup>9,10</sup> The draft guideline thus follows REACH regulations, which also

emphasize using OECD 309 tests for assessing the persistence of industrial chemicals.<sup>11</sup>

The OECD 309 guideline allows two experimental setups, i.e., testing biotransformation in natural water (pelagic test) or in water amended with sediment to a concentration of  $\leq 1$  g solids  $\text{L}^{-1}$  (suspension test).<sup>8</sup> To date, OECD 309 studies are mostly carried out as pelagic tests. Pelagic tests pass the validity criteria described in the current OECD 309 guideline when the examined water contains biomass concentrations of minimally  $10^3$ – $10^4$  cells  $\text{mL}^{-1}$ .<sup>8</sup> However, in fact, bacterial cell densities are higher in most natural surface waters, ranging from  $10^4$  to  $10^7$  cells  $\text{mL}^{-1}$ , with a global average of  $10^6$  cells  $\text{mL}^{-1}$ .<sup>12–15</sup> Low biomass concentrations in pelagic tests, which are nevertheless deemed valid, lead to the effect that very low levels of biotransformation are observed for a large majority of substances and potentially biodegradable compounds may be assessed as persistent. In case a compound is actually biotransformed in a pelagic test, despite the low levels of

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degrader biomass provided, variability among studies with different water samples is high.<sup>16,17</sup> Previous pelagic tests revealed up to 20-fold differences in half-lives of certain substances, depending on the origin of employed waters.<sup>16,17</sup>

Increasing inoculum concentrations by adding sediment-borne biomass may therefore increase the environmental relevance of OECD 309 studies. Furthermore, previous research suggests that increased degrader biomass and pre-exposure of microbial communities to chemicals support their biotransformation, reduce lag phases, and increase the reproducibility of laboratory tests.<sup>16–23</sup> However, to the best of our knowledge, so far, only a limited number of studies assessed biotransformation of chemicals in water–sediment suspensions and the chemicals were limited to few model compounds.<sup>24–26</sup> Moreover, these studies did not investigate the influence of bacterial cell densities on biotransformation kinetics, nor did they provide general conclusions about the reproducibility and comparability of suspension tests when conducted in water and sediment sourced from different locations.

Therefore, we here explored biotransformation behavior of 43 pharmaceuticals, pesticides, and industrial chemicals that are ubiquitously present in wastewater treatment plant effluents and surface waters,<sup>27–31</sup> and hence are environmentally relevant, in various suspension tests, and gathered consistently derived persistence information. To study the effect of testing different microbial communities with varying bacterial cell densities, our experiments were conducted in suspensions sampled from an agriculture- and wastewater effluent-impacted river and a pristine pond at two sediment concentrations, one of them within and one beyond OECD 309 standards. From this, we elucidated experimental parameters that generally influence biotransformation in laboratory experiments across a wide range of chemicals. We hereby aim to support the ongoing EMA revision process with timely experimental evidence on the suspension test of the OECD 309 guideline, which will likely gain significant importance for persistence assessment across relevant regulatory frameworks for various chemicals.<sup>9,11</sup>

## MATERIALS AND METHODS

Detailed information about laboratory methods and data analysis is provided in the [Supporting Information \(SI1 and SI2\)](#). In brief, suspensions containing 1 and 10 g solids L<sup>-1</sup> were prepared with water and sediment sampled from the Rhine (R, Mumpf, Switzerland) and from Cressbrook Mill Pond (CMP, Derbyshire, U.K.). Suspensions are referenced with a code indicating sampling site, sediment concentration in grams per liter, and sampling time in the case of Rhine suspensions. The lower-biomass suspensions are R1-Fall, R1-Spring, and CMP1, and the higher-biomass suspensions are R10-Fall and CMP10. Sampling of the Rhine was done twice, i.e., in fall and in spring, to obtain environmental samples with comparable physicochemical properties ([Table SI2](#)) but different microbial communities. This investigation of seasonality was performed in 1 g solids L<sup>-1</sup> suspensions to be consistent with current OECD 309 standards.

Sediment was kept in suspension with an orbital shaker and additionally with a magnetic stirrer in the case of CMP sediment (CMP1-Stirrer/CMP10-Stirrer). Sterile hydrolysis and sorption experiments were conducted alongside the respective biotransformation experiments to distinguish

compound removal via biotransformation from abiotic transformation or phase transfer.

Test systems were spiked with a mixture of 43 test compounds to a concentration of 1 µg L<sup>-1</sup> each. Dissipation of the parent compound was monitored by analyzing up to 11 subsamples taken from the water phase of each experimental vessel over a time course of >54 days. Chemical analysis was performed on an Agilent Triple Quad MS instrument coupled to a HPLC system.

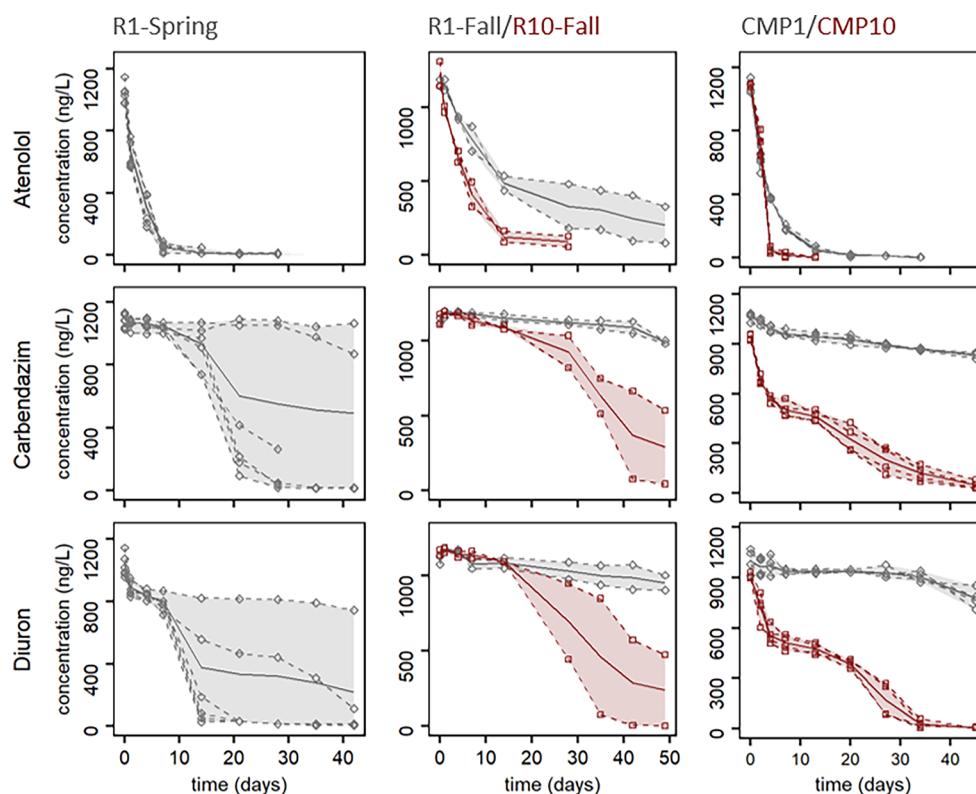
Total parent compound residues in experimental systems at a given time were calculated from measured water phase concentrations, considering a sediment–water partitioning coefficient derived from sorption experiments. Compound residues in each experimental replicate as a function of time were fitted to a first-order degradation model considering lag phases to be consistent with data evaluation recommendations in regulatory frameworks.<sup>8,32</sup> Here, we defined the total system degradation half-life (DegT<sub>50,TS</sub>) as the time interval needed to reach 50% primary degradation, once compound dissipation has started. In contrast, the total system dissipation half-life (DT<sub>50,TS</sub>) was defined as the sum of DegT<sub>50,TS</sub> and the length of the lag phase.

Bacterial cell densities were determined by sacrificing whole experimental vessels at various time points and enumerating cells, both in the water phase and in the bulk sediment, by using a BD Accuri C6 flow cytometer. Due to the necessity of sacrificial sampling, biotransformation experiments were performed with up to 18 replicates and chemical trajectories end at different time points. Still, chemicals' dissipation was monitored in at least two replicates until the end of each experiment.

## RESULTS AND DISCUSSION

**Biotransformation in Suspension Tests.** Biotransformation was distinguished from hydrolysis and sorption by comparing biotransformation experiments with their abiotic controls ([SI2.2](#)). In agreement with the results of Shrestha et al.,<sup>24</sup> results of CMP1/10-Stirrer revealed that keeping sediment in suspension with a magnetic stirrer led to grinding of particles and continuously increased sorption of chemicals, which made differentiation between transformation and sorption difficult ([SI5](#)). Therefore, data from stirrer experiments were not used further. On the basis of experiments using the orbital shaker instead, dissipation of 35 compounds could be assigned at least partially to biotransformation. Concentration–time series obtained for those compounds are shown in [Figure SI1](#).

Generally, compound dissipation via biotransformation was faster in suspensions with increased sediment content. Least compound losses were observed in CMP1 and R1-Fall; only six substances showed up to 50% removal by the end of those experiments, while dissipation of most substances could be observed in R10-Fall and CMP10. Correspondingly, bacterial cell densities were lowest in CMP1 and R1-Fall with averages of  $5 \times 10^6$  and  $1.6 \times 10^7$  cells mL<sup>-1</sup>, respectively. As increasing sediment content led to increased sediment-borne biomass, higher average cell densities of  $4 \times 10^7$  and  $3.2 \times 10^7$  cells mL<sup>-1</sup> were measured in R10-Fall and CMP10, respectively. In R1-Spring, we measured an average of  $2.2 \times 10^7$  cells mL<sup>-1</sup>, meaning that cell densities did not differ strongly between R1-Fall and R1-Spring. Strikingly though, most compounds dissipated much faster from various replicates of R1-Spring compared to R1-Fall. This suggests that the extent of



**Figure 1.** Concentrations of atenolol, carbendazim, and diuron measured in the water phase of R1-Spring, R1-Fall, R10-Fall, CMP1, and CMP10. Data for suspensions containing 1 g solids L<sup>-1</sup> are colored gray with measured data represented as diamonds, and data for suspensions containing 10 g solids L<sup>-1</sup> are colored red with measured data represented as squares. Measurement points belonging to the same experimental replicate are connected with dashed lines. The solid lines show the average concentration calculated from the plotted experimental replicates, and shaded areas indicate the spread of the concentrations measured at the same time point.

compound removal via biotransformation is influenced by not only bacterial cell densities but also by the composition or activity of microbial test communities. The latter two parameters have been demonstrated to undergo seasonal variations in surface waters,<sup>33–35</sup> which agrees with our findings of varying biotransformation capacities in environmental samples sourced during different seasons.

Besides interstudy variations, we observed drastic differences between replicates of the same study once biotransformation of compounds reached a detectable range. We expressed those intrastudy variations as the spread between maximum and minimum concentrations of one compound in different replicates at the same time point. Especially during R1-Spring and R10-Fall, intrastudy variations increased over time; in R1-Spring, the average spread between trajectories increased from 270 ng L<sup>-1</sup> after 13 days to 430 ng L<sup>-1</sup> after 28 days (Figure S12). Intrastudy variations in biotransformation kinetics were lowest in CMP10 with a spread of <90 ng L<sup>-1</sup> regardless of the time point. Described inter- and intrastudy variations are exemplarily illustrated for atenolol, carbendazim, and diuron in Figure 1.

Interestingly, the extents of variation in biotransformation kinetics coincide with the extents of variation in bacterial cell densities. These variations, expressed as the coefficient of variation (CV) calculated from flow cytometry measurements in sacrificed replicates, were greatest in R1-Spring and R10-Fall, with CVs of 74% and 84%, respectively, and lowest in CMP10 with a CV of 44% (Figure S14). If we assume that different densities in different replicates at different time points could be considered an approximate indicator of the extent of

community dynamics during the experiments, our data suggest that larger community dynamics coincide with increased intrastudy variabilities. However, we acknowledge that this hypothesized relationship would certainly profit from actual time series obtained from individual experimental vessels, and additional analysis of not only cell densities but also microbial community composition and activity.

Generally, little variation was observed for rapidly degrading compounds, i.e., atenolol, bezafibrate, and fenoxycarb. Literature suggests that those compounds are biotransformed by enzymes widespread among bacteria,<sup>36–39</sup> supporting our observation that biotransformation readily occurred in different suspension tests. However, inter- and intrastudy variations in chemical removal indicate that most of our test compounds seem to have been transformed by enzymes less widespread or only expressed under specific conditions. Rare enzymes have a lower probability of occurrence at lower inoculum concentrations, and their emergence in different test systems strongly depends on how the microbial community evolves over time.<sup>19,39–41</sup> Coherently, a previous study in activated sludge showed that biotransformation of acesulfame, phenylureas (i.e., diuron and isoproturon in our study), and carbendazim strongly depends on the solids retention time and hence community composition, suggesting a need for enzyme activities not always present in activated sludge.<sup>36</sup> In our study, inoculum concentrations of around 10<sup>7</sup> cells mL<sup>-1</sup> were required to increase the likelihood of providing sufficient specific degraders/enzymes to yield observable dissipation of most compounds from water–sediment suspensions. Qualitatively aligned with our findings, a recently conducted



international ring test showed that increasing cell densities in biodegradation tests with marine waters (OECD 306<sup>42</sup>) from  $10^5$  to  $10^7$  cells  $\text{mL}^{-1}$ <sup>143</sup> allowed for less variable characterization of biotransformation of five test compounds. With our much broader set of compounds, we showed that increasing inoculum concentrations to the upper limit of what is commonly observed in natural surface waters<sup>15</sup> increased the probability to trigger biotransformation reactions; however, significant inter- and intrastudy variations could still occur.

Besides the need for the emergence of specific enzymes to remove the majority of our test compounds from aquatic systems, co-metabolic processes might also have promoted compound dissipation during our suspension tests.<sup>17,39,44</sup> When comparing suspension tests employing bacterial cell densities sufficient to trigger compound removal via biotransformation (i.e., R1-Spring, R10-Fall, and CMP10), we found shortest lag phases and most uniform dissipation in CMP10. Because average cell densities, at least between R10-Fall and CMP10, did not differ much, we speculate that the comparably elevated carbon and nutrient levels in CMP10 suspensions (Table S15) might have led to a generally more active microbial community and increased co-metabolism of our test compounds.

**Suspension Tests in a Regulatory Context.** To further reflect how the observed varying biotransformation capacity of microbial test communities influences metrics for regulatory persistence assessment of chemicals derived from biotransformation kinetics in suspension tests (i.e.,  $\text{DegT}_{50,\text{TS}}$  and  $\text{DT}_{50,\text{TS}}$ ), we fitted a kinetic model (SI2) to trajectories derived from those studies in which we observed significant compound removal, i.e., R1-Spring, R10-Fall, and CMP10. A complete listing of fit parameters and evaluation statistics is provided in Table SI6. Model fits were generally good for most evaluated trajectories ( $R^2 \geq 0.8$ ). Figure 2 presents lag phases,  $\text{DegT}_{50,\text{TS}}$ , and  $\text{DT}_{50,\text{TS}}$  for substances biotransformed in R1-Spring, R10-Fall, and CMP10.

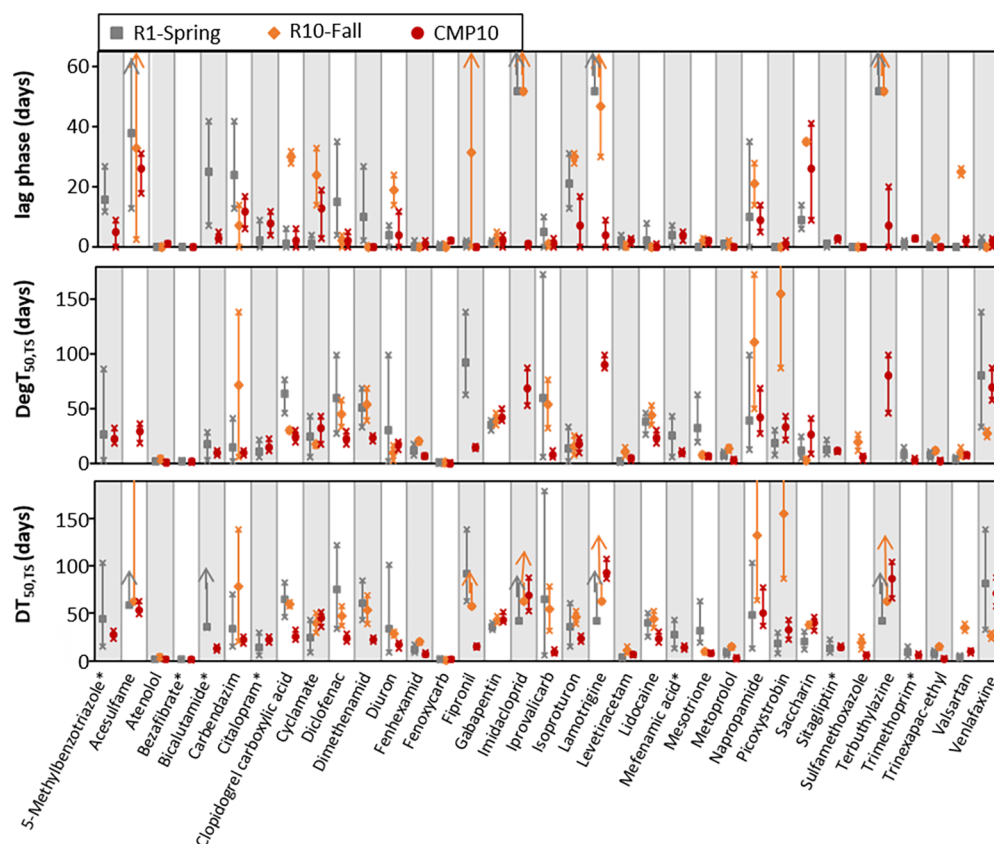
Lag phases are commonly observed in laboratory biotransformation tests;<sup>16,20,45</sup> here, lag phases occurred to various extents for all compounds, except for bezafibrate, and ranged from  $\sim 1$  to  $>63$  days, depending on the compound and experiment. Lag phases were generally shorter in tests carried out with  $10 \text{ g solids L}^{-1}$  suspensions, especially in CMP10. In addition, we observed intrastudy variations of lag phases, which were most significant in R1-Spring; differences between replicates were  $>20$  days for five compounds (i.e., acesulfame, bicalutamide, carbendazim, dimethenamid, and isoproturon). As discussed previously, biotransformation of at least three of those has been hypothesized to depend on the emergence of specific enzymes.

However, current regulatory guidelines do not specify how to consider lag phases when assessing a substance's persistence, i.e., whether  $\text{DegT}_{50}$  or  $\text{DT}_{50}$  is to be used as a decisive persistence measure.<sup>9,11,32</sup> Because lag phases are a sign of microbial adaptation and reduced lag phases could be speculated for compounds continuously or repeatedly released to the aquatic environment (e.g., pharmaceuticals or pesticides), it has been argued that  $\text{DegT}_{50}$  should be used as a persistence metric.<sup>17,21–23</sup> However, our experiments do not directly support this hypothesis as we determined shortest and least variable lag phases in suspensions employing a microbial community sourced from a pristine environment (CMP10). Therefore, and in light of the current lack of understanding of the observed variability in lag phases, the use

of  $\text{DT}_{50}$  values to assess persistence would seem to be the more cautious and environmentally protective approach. It needs to be noted though that  $\text{DT}_{50,\text{TS}}$  of a given compound can range from a few days to  $>100$  days due to the combined effect of varying lag phases and  $\text{DegT}_{50,\text{TS}}$ ; see, e.g., 5-methylbenzotriazole, carbendazim, diuron, or iprovalicarb in Figure 2 and Table SI6. Similarly, variations in half-lives of 1 or 2 orders of magnitude have previously been observed in OECD 308 or OECD 309 studies for several of our test compounds, including acesulfame, diclofenac, trimethoprim, and venlafaxine.<sup>16,26,46</sup>

To evaluate the effect of variable half-lives in a regulatory context, we performed an exemplary persistence assessment according to EMA and REACH criteria.<sup>9,11</sup> Detailed assessment outcomes are provided in SI9. As expected from the significant differences between trajectories observed in various experiments and their replicates, persistence assessment outcomes were ambiguous for approximately half of the test compounds. Due to differences of up to 2 orders of magnitude in  $\text{DegT}_{50,\text{TS}}$  and  $\text{DT}_{50,\text{TS}}$ , persistence assessment outcomes were ambiguous relative to not only the rather strict persistence criteria used in regulatory frameworks of the EU<sup>9,11</sup> but also relative to higher persistence cutoff criteria used in other legislations, e.g., in the United States.<sup>1</sup>

**Environmental Significance.** The underlying assumption of persistence assessment based on laboratory simulation studies is that the outcomes reflect compound behavior in the environment.<sup>2,7,8</sup> To explore this assumption relative to our results, we compared them to the results of two studies reporting concentration measurements of 12 of our test compounds along the Rhine (i.e., carbamazepine, cyclamate, diclofenac, isoproturon, lamotrigine, saccharin, sitagliptin, sulfamethoxazole, trimethoprim, and three confidential compounds).<sup>27,47</sup> In large streams, only compounds with half-lives in the range of the average travel time of all wastewater treatment plant inputs can be expected to show a clear dissipation signature. In case of the Rhine, where wastewater treatment plants are distributed all along the river, this results in a low average travel time of approximately 7.7 days.<sup>47</sup> As a consequence, only substances with half-lives of  $<10$ – $20$  days are significantly removed from the Rhine. For sulfamethoxazole, sitagliptin, isoproturon, carbamazepine, cyclamate, diclofenac, lamotrigine, and one confidential compound, continuously increasing concentrations along the Rhine have been reported. While persistence classification based on our data was rather uncertain for the first three compounds, the latter five were indeed consistently classified as persistent (Table SI7). Trimethoprim was reported to be removed  $>80\%$  along the Rhine;<sup>47</sup> accordingly, it was classified as non-persistent in most of our tests. Saccharin stood out in that it showed strong concentration fluctuations along certain parts of the Rhine.<sup>27</sup> This may result from industrial sources with effluent concentrations above typical municipal wastewater concentrations<sup>48</sup> but could also mean that biotransformation of saccharin depends on certain, spatially varying conditions. Indeed, considerable variations in lag phases and  $\text{DegT}_{50,\text{TS}}$  for saccharin indicate that its removal is determined by specific microbiological conditions. The latter would suggest that variable outcomes from different suspension tests may be indicative of strong spatial and temporal fluctuations in biotransformation potential of a given compound in the aquatic environment where the composition and activity of



**Figure 2.** Lag phases,  $\text{DegT}_{50,TS}$ , and  $\text{DT}_{50,TS}$  of biotransformed substances in R1-Spring, R10-Fall, and CMP10. Data for R1-Spring, R10-Fall, and CMP10 are colored gray, orange, and red, respectively. Squares, diamonds, and circles show an average value calculated from the experimental replicates, and the error bars extend to the smallest and largest value found for the respective parameter, displayed as crosses. When lag phases exceeded the duration of the experiment,  $\text{DegT}_{50,TS}$  could not be determined for the respective experimental replicate. When the lag phase exceeded the duration of the experiment in only one replicate, an average  $\text{DegT}_{50,TS}$  was still calculated with the values from the remaining three replicates of R1-Spring or CMP10. If lag phases exceeded the duration of the experiment in half of the replicates, no averaged  $\text{DegT}_{50,TS}$  values are shown in this graph. Upward-pointing arrows in the lag phase and  $\text{DT}_{50,TS}$  data indicate that lag phases of at least one replicate exceeded the duration of the experiments and that the actual average and the actual maximum are higher than indicated in the graph. Compounds marked with asterisks were not included in the R10-Fall study. Note that the y-axis for the lag phases covers a different scale.

microbial communities also vary spatially and temporally.<sup>23,28,46,49–51</sup>

Still, significant variations make the interpretation of biotransformation study outcomes challenging. It is therefore worth noting that we achieved most robust persistence classifications with a test system containing a 10-fold higher sediment content and at least 3 orders of magnitude higher bacterial cell densities than minimally required in the current OECD 309 guideline.<sup>8</sup> Our experimental results thus suggest that one approach to yield increased and more uniform biotransformation would be to increase bacterial cell densities and to provide sufficient nutrients in a test system. The fact that an increased sediment content increased the robustness of suspension test outcomes is also in line with low intrastudy variations commonly observed in OECD 308 experiments,<sup>24,46,52</sup> which employ water:sediment ratios of 3:1 or 4:1 (v/v).<sup>7</sup> Alternatively, rather than trying to decrease variability in biotransformation tests, different data evaluation tools may allow the interpretation of test outcomes despite significant variations; in benchmarking, for example, test outcomes for a given compound would be assessed relative to a set of reference compounds with well-established environmental behavior.<sup>2</sup> We hope that further exploration

of these emerging concepts will be facilitated with the rich set of biotransformation data that we present here.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.estlett.0c00725>.

Details about experimental methods (including chemical and biological analysis), data evaluation, persistence assessment, and additional graphs and tables (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

**Kathrin Fenner** – Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland; Department of Chemistry, University of Zürich, 8057 Zürich, Switzerland; [orcid.org/0000-0001-6068-8220](https://orcid.org/0000-0001-6068-8220); Email: [kathrin.fenner@eawag.ch](mailto:kathrin.fenner@eawag.ch)

### Authors

**Carolyn Seller** – Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland; Department of Chemistry, University of Zürich, 8057 Zürich, Switzerland

Mark Honti – MTA-BME Water Research Group, 1111 Budapest, Hungary

Heinz Singer – Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland; [orcid.org/0000-0002-8542-3699](https://orcid.org/0000-0002-8542-3699)

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.estlett.0c00725>

## Notes

The authors declare no competing financial interest.

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