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Letter

Increasing the Environmental Relevance of Biodegradation Testing by Focusing on Initial Biodegradation Kinetics and Employing Low-Level Spiking

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ABSTRACT: The environmental relevance of standard biodegradation tests such as OECD 309 has been questioned. Challenges include the interpretation of changing degradation kinetics over the 60−90 incubation days and the effects of chemical spiking on the microbial community. To ameliorate these weaknesses, we evaluated a modified OECD 309 test using water and sediment from three Swedish rivers. For each river, we had three treatments (no spiking, 0.5 $\mu g\ L^{-1}$ spiking, and 5 $\mu g\ L^{-1}$ spiking). The dissipation of a mixture of 56−80 spiked chemicals was followed over 14 days. Changes in dissipation kinetics during the incubation were interpreted as a departure of the microbial community from its initial (natural) state. The biodegradation kinetics were first-order throughout the incubation in the no spiking and 0.5 $\mu g\ L^{-1}$ spiking treatments for almost all chemicals, but for the 5 $\mu g\ L^{-1}$ treatment, more chemicals showed changes in kinetics. The rate constants in the no spiking and 0.5 $\mu g\ L^{-1}$ treatments agreed within a factor of 2 for 35 of 37 cases. We conclude that the environmental relevance of OECD 309 is improved by considering only the initial biodegradation phase and that it is not compromised by spiking multiple chemicals at 0.5 $\mu g\ L^{-1}$.

KEYWORDS: *biodegradation, river water, sediment, micropollutants, OECD 309*

INTRODUCTION

The assessment of chemical degradation is a key element in many international regulatory frameworks for organic chemicals, and it is crucial to understanding the environmental fate, exposure levels, and environmental risk of contaminants.[1−5] Compared to other types of environmental degradation, biodegradation is generally recognized as the most important degradation process in terms of mass balance,[5−7] affecting the fate of almost all organic chemicals in the environment and thus an important determinant of exposure.[8] However, there are widespread concerns about the environmental relevance and robustness of existing standard procedures for assessing the biodegradation of chemicals in aquatic environments.[9−17]

Measuring biodegradation rates in the environment is possible under some conditions[18] but difficult and costly. Consequently, almost all measurements are done in the laboratory. Ready biodegradation tests are generally the first tier in persistence assessment, but they have limited environmental relevance[16] and are of little value for exposure assessment. The OECD 309 test is the most recognized method for measuring biodegradation rates in surface water and is the recommended higher-tier test for persistence assessment under the European chemicals legislation REACH.[3] It is used to measure biodegradation by spiking the test chemical (concentrations of $\leq 10\ \mu g\ L^{-1}$ are preferred) into aerobic natural waters or water−sediment systems with low suspended sediment concentrations (≤ 1 g of solids L^{-1}).[19] However, chemical spiking has been shown to influence the microbial community and chemical attenuation, which creates doubts about the environmental relevance of the biodegradation test.[10,20−22] As an alternative, a nonspiked test has been suggested as it provides a better representation of biodegradation in the environment.[10,11] However, this test is constrained by the requirement for background contamination of the test chemicals in the environment of interest at quantifiable concentrations.

A second concern with OECD 309 relates to the interpretation of the chemical attenuation data. The method prescribes that if variable biodegradation kinetics are observed then the biodegradation rate constant should be determined from the period showing the fastest degradation.[19] Changes in biodegradation kinetics are a manifestation of the departure of the test system from its initial state. Over the long (60−90 day) incubation period of the OECD 309 test, variable

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biodegradation kinetics typically occur as a period of slower degradation (commonly called the lag phase) followed by more rapid degradation, followed in turn by slow degradation. By reporting the rate constant from the rapid phase, OECD 309 describes the properties of a system that has departed from its initial state. The environmental relevance of the test result is thus questionable.

Against this scientific background, this study was designed to test whether an OECD 309-like test with chemical spiking at low concentrations gives biodegradation kinetics comparable to those of the same test without spiking. To this end, we used river water and sediment from three wastewater-impacted rivers to conduct a series of OECD 309 experiments modified so that they maximally mirrored field conditions. In each experiment, we set up three treatments for comparison: no spiking, 0.5 $\mu g\ L^{-1}$ spiking, and 5 $\mu g\ L^{-1}$ spiking. The dissipation of a mixture of 56 or 80 test chemicals was followed over the incubation period for the spiked treatments, while for the no spiking treatment, the dissipation of chemicals already present in the sampled water and sediment was measured. The biodegradation rate constant (k) for each test chemical was estimated and compared between the nonspiked treatment and spiked treatments. We tested whether biodegradation was first-order from the start of the experiment, and k was determined from the initial period of first-order biodegradation kinetics. Finally, the biodegradation kinetics of some compounds not present in the spike solution were determined through nontarget analysis to assess whether spiking affects the degradation of other chemicals.

MATERIALS AND METHODS

The experimental procedure was based on the OECD 309 test protocol with five significant modifications. (1) The sediment concentration was increased to 50 g of wet solids L^{-1}, as a higher sediment concentration has been suggested to yield more reproducible results.[12,13] (2) Multiple chemicals were added to each reactor. (3) The amount of chemical spiked was set to 0.5 $\mu g\ L^{-1}$, or the chemicals were not spiked at all, with a 5 $\mu g\ L^{-1}$ treatment for comparison. (4) The incubation bottle was sealed to prevent outgassing of CO_2 from the oversaturated river water, which would change the pH and hence potentially affect degradation rates.[9] (5) The length of the test was reduced to 14 days, as long incubation periods increase the risk of causing changes in the microbial community, i.e., departure from environmental conditions.[13]

Details about the test chemicals, biodegradation experiments, and data analysis are provided in the Supporting Information (S1). In brief, sediment and water were sampled from three wastewater-impacted rivers in Sweden: Fyrisån (FYR), Enköpingsån (ENK), and Hågaån (HAG). The incubations were spiked with an aqueous solution of test chemicals [56 chemicals for FYR and ENK and 80 for HAG (Table S1)]. The test chemicals were expected to occur in surface waters, and most of them had a weak tendency to sorb to sediment [log D_{OW} < 3 (Table S1)], including pharmaceuticals, industrial chemicals, agrochemicals, food additives, and cosmetics. Three treatments were used for each river: nonspiked incubation (N); 0.5 $\mu g\ L^{-1}$ per chemical (low level, L), which is close to the concentration limit for the reliable quantification of attenuation kinetics with our analytical method; and 5 $\mu g\ L^{-1}$ (high level, H). A sterile sorption control and a water control were conducted for the H treatment. Five incubations were conducted for each treatment. All experiments were carried out in the dark at river temperature (FYR, 10 °C; ENK, 18 °C; and HAG, 20 °C). Up to 10 water samples were collected from each flask over 14 days and analyzed using high-resolution mass spectrometry.

The degradation rate constant (k) was calculated independently for each incubation using linear least-squares regression of the natural logarithm of the chemical's peak area versus time. When the difference between the k derived from the first 2 days of data and the k derived from the full data set was >20%, biphasic elimination kinetics were considered. For those chemicals showing biphasic kinetics, k was estimated separately for each phase, yet only the k derived from the initial phase was used for comparison between treatments. The criterion for the end of the initial phase was a change in k of >10% when including one more time point. If the estimated k was not significantly different from 0 (regression analysis in Excel; $p > 0.05$), the chemical was classified as persistent with an undefined k. All measurements below the limit of quantification (LOQ) or not in the linear range of the calibration curve were removed. Dissipation in the sorption control was subtracted from dissipation in test treatments prior to calculating k (S5). Regressions were rejected when there were fewer than three time points above LOQ or when R^2 was <0.65. Statistical differences among the three treatments (N, L, and H) were evaluated using a two-tailed t test.

RESULTS AND DISCUSSION

Quality Assurance. Dissolved oxygen (DO), pH, and conductivity during the incubation were stable and close to field conditions for FYR and ENK (S2, Figure S1). In HAG, DO decreased markedly after 3 days, presumably because of a high oxygen demand for this river. Therefore, only the first 3 days of data were used for HAG. The marked changes suggest that completely sealing the bottles is not appropriate for rivers with a high oxygen demand. In such cases, some exchange of the headspace is necessary to sustain oxygen levels while preventing significant outgassing of CO_2.

For the three treatments, N, L, and H, biodegradation rate constants (k) of 17, 36, and 42 chemicals in FYR, 9, 31, and 42 chemicals in ENK, and 29, 52, and 58 chemicals in HAG, respectively (Figure S6 and Table S5), were estimated, including chemicals with and without apparent degradation. For the chemicals with k values significantly different from 0, k was obtained for at least four of the five replicates in 83% of the cases. The coefficient of variation of k across replicate incubations was <25% for 57−67% of the chemicals in the N treatment, 63−77% in L, and 73%−93% in H (S3, Figure S3). The results demonstrate that the modified OECD 309 test with spiking can reproducibly generate k values while maintaining test conditions close to those in the environment.

The "Lag Phase" Contains Relevant Kinetic Information. In the N treatment, 12, 7, and 18 chemicals with k values significantly different from 0 were determined for FYR, ENK, and HAG, respectively (Figure S6 and Table S5). Biodegradation of all of these chemicals began immediately, with >95% of the replicates showing consistent dissipation from the start of the incubation. Two chemicals in FYR and ENK and one chemical in HAG showed a concentration increase during the first 2 days, which was attributed to a change in distribution between sediment and water arising from shaking. This phenomenon has been observed in nonspiked tests before.[9] Otherwise, dissipation kinetics were first-order with a constant k until the end of the experiment or until the signal

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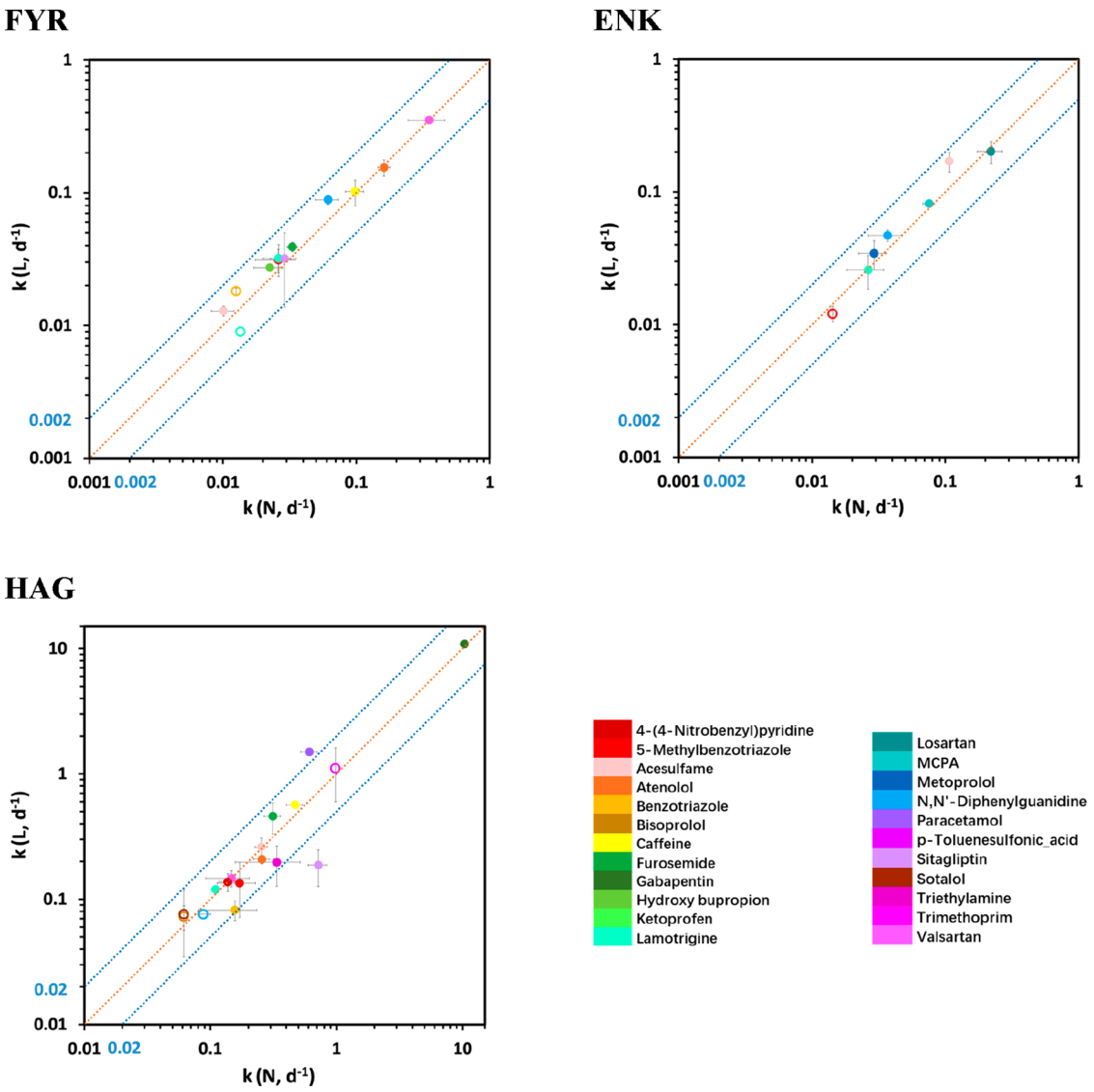

Figure 1. Biodegradation rate constant k in the L treatment vs the N treatment, with one plot for each river. Each data point is an average of measurements from one to five replicates. Error bars represent the standard deviation. The area between the two blue dashed lines represents agreement within a factor of 2. A hollow symbol indicates that k was obtained from only one replicate.

fell below the LOQ. Because no exogenous chemicals were introduced, the microbial population in N was adapted to the available substrates. We, therefore, expected a stable bacterial community and thus stable biodegradation kinetics because there was no need for the microbial population to respond to new stimuli. The results indicate that it is possible to study environmental biodegradation in a laboratory test without disturbing the natural system to the extent that biodegradation is notably affected.

Similar results were obtained for the L treatment, for which k was significantly different from 0 for 23, 24, and 32 chemicals in FYR, ENK, and HAG, respectively (Figure S6 and Table S5). Biodegradation of almost all compounds started right from the beginning, with >95% of the replicates showing consistent first-order dissipation for the whole period. Only four chemicals showed biphasic kinetics with a slight change in k (Figure S7 and Table S6).

In the H treatment, there were 27, 33, and 33 chemicals with k significantly different from 0 in FYR, ENK, and HAG, respectively (Figure S6 and Table S5). More chemicals (10 of the 33 chemicals) showed biphasic biodegradation kinetics with an increase in k during the incubation, and this biphasic behavior was consistent between replicates. For a few chemicals (bezafibrate, metformin, and MCPA in FYR-H, *N,N′*-diphenylguanidine in ENK-H, and triethylamine in HAG-H), degradation was negligible during the first few days or k changed so quickly that there were insufficient data to estimate k for the initial period. For all other chemicals with biphasic kinetics, an initial phase with slow first-order degradation followed by at least a doubling in k was observed (Figure S7). For six of these chemicals (5-methylbenzotriazole, atenolol, caffeine, ketoprofen, MCPA, and trimethoprim), this increase in k was observed in both FYR and ENK. It was only observed for one chemical in HAG, which can be attributed to the shorter incubation period (3 days). The significant increase in k after a period of 1−8 days suggests that the high-level spiking caused changes in the microbial community that increased the biodegradation rate. One explanation could be that these chemicals experienced a background elimination by co-metabolism (reflected in the initial period of first-order

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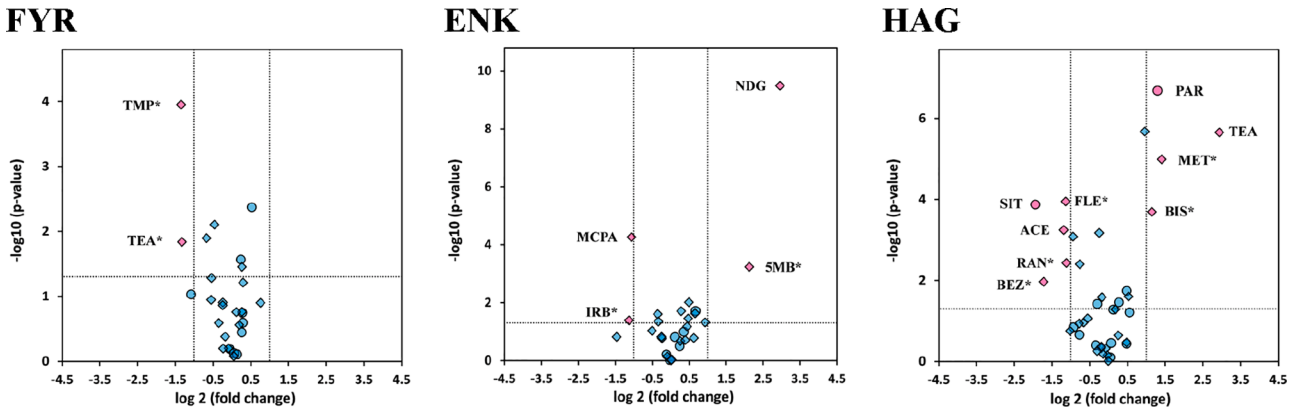

Figure 2. Plot of the quotient of the k values from different treatments (L/N, circles; H/L, diamonds). The X coordinate is log 2 of the quotient (which corresponds to the fold difference), and the Y coordinate is −log 10 of the p value of the t test comparing the two treatments. Each dot represents a chemical. The horizontal line indicates a p value of 0.05; for data points above this line, the difference between the two treatments is statistically significant. The two vertical lines indicate a 2-fold difference; for data points between these lines, the difference between the treatments was <2-fold. The red-colored data points have a >2-fold difference that is statistically significant. The abbreviations of the chemicals are listed in Table S1. An asterisk beside the abbreviation indicates that k could not be determined in N for this chemical.

degradation) accompanied by metabolic degradation by specialized organisms that grow when the chemical concentration is high enough until they are sufficiently numerous to degrade a significant portion of the substrate (reflected in the increase in k after several days).

Comparison of Biodegradation Kinetics in L and N. The k values for each chemical from N and L were compared (Figure 1). There was a good agreement (<2-fold difference) for all chemicals in FYR and ENK. In HAG, only two chemicals, sitagliptin and paracetamol, showed a >2-fold difference. The dissipation of sitagliptin in HAG-L was difficult to distinguish from that in the sorption control (Figure S5); hence, k for sitagliptin in HAG-L is uncertain. The degradation of paracetamol was rapid, which agrees with previous findings,[10] and k was positively correlated with the initial paracetamol concentration in HAG.

Not all of the chemicals for which dissipation could be measured yielded k values significantly different from 0. In total, 5, 2, and 20 chemicals in the N treatment were classified as persistent in FYR, ENK, and HAG, respectively (Figure S8), including some chemicals that have been reported to be persistent elsewhere, such as carbamazepine, tramadol, and venlafaxine.[10,11,23,24] The larger number of persistent compounds in HAG can be attributed to the shorter incubation period (3 days). All compounds that displayed persistence in N were also persistent in L for all three rivers.

Comparison of Biodegradation Kinetics in H and L. The k values from the H, L, and N treatments agreed well (Figure 2 and Figures S10 and S11). In FYR, ENK, and HAG, 91%, 83%, and 76% of chemicals, respectively, displayed a fold difference between L and H that was <2 or not statistically significant (Figure 2). Most of the chemicals that showed a >2-fold difference in k between L and H that was statistically significant also showed biphasic kinetics in the H treatment (compare Figure 2 and Figure S7). For these chemicals, errors arising from the selection of the time points to include in the calculation of the initial k could contribute to the larger differences. Thus, while spiking at higher levels can reduce the demand for sensitive analytical methods, there can be a cost in the form of fewer time points that can be used for determining k and, consequently, increased uncertainty in k.

We note that there would have been a much poorer agreement between H and L on one hand and N on the other for the chemicals displaying biphasic kinetics if we had taken the k from the second phase (i.e., in accordance with the OECD 309 protocol) instead of the initial phase (Figure S7 and Table S6).

Biodegradation Kinetics of Chemicals Not Present in the Spike Solution. In addition to the spiked compounds, we determined the biodegradation kinetics of chemicals that were not present in the spike solution. This was done for one chemical in FYR and six in HAG (S10). The k values of all of the nontarget chemicals derived from spiking treatments (L and H) agreed well with those derived from the nonspiked treatment (Figure S12). The difference was at most 1.4-fold with no statistically significant differences between treatments (Table S7). Therefore, the effect of spiking multiple chemicals at low concentrations on the degradation kinetics of the nontarget chemicals was negligible. Hammershøj et al. found the number of spiked mixture components had a limited effect on the biodegradation half-lives when tested at environmentally relevant concentrations,[15] which agrees with our observation. The absence of mixture effects at low spiking levels makes it possible to investigate the biodegradation kinetics of a large number of target and nontarget chemicals simultaneously.

Relevance and Implications for Persistence Assessment. The OECD 309-type test has been criticized for its poor environmental relevance and high intrareplicate variability.[5,12] There has been a quest by academia, regulatory bodies, and industry to find a robust and environmentally relevant standard test for biodegradation in aquatic environments.[5] One of the problematic aspects of OECD 309 is its treatment of the lag period that is frequently observed when a chemical is spiked and incubation periods are long.[10] An initial period of slow attenuation is considered a "lag phase" in the OECD 309 protocol and neglected in evaluating biodegradation kinetics.[19] Our work shows that it is this "lag phase" that contains the relevant information for biodegradation in the sampled environment. Kinetic information gathered after the "lag phase" reflects the properties of a system that has been modified in the laboratory and for which there is no evidence that it will ever exist in the environment.

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Here, we considered the N treatment as the point of reference most representative of biodegradation in the environment.[10] It is not subject to lag phases because no chemical is spiked, and hence, it was valuable for showing that biotransformation starts immediately (or rather, presumably continues) when sediment and water are transferred from the field to the laboratory, and that the biodegradation kinetics remain first-order for several days thereafter. However, no spiking places several limitations on the test, including making it inapplicable for the evaluation of new chemicals. Our results indicate that when the modified OECD 309 is conducted using low spiking concentrations, it produces degradation rate constants comparable to those derived from the nonspiked test. We therefore conclude that low-level spiking (0.5 $\mu g\ L^{-1}$) of multiple chemicals can be used without compromising the environmental relevance of the nonspiked test, thereby greatly expanding its applicability domain. While spiking at higher concentrations risks not being environmentally relevant, conducting a series of treatments with different spiking levels starting from 0.5 $\mu g\ L^{-1}$ could provide insight into the nature of the biodegradation processes occurring in the environment, such as whether there are microorganisms present that can grow on the chemical and what concentration is required to support growth.

Although our conclusions are based on experiments with just three rivers, the rivers covered a range of temperatures and a range of levels of contamination, which makes the conclusions more robust. While the methodological questions addressed in our study are not expected to be sensitive to between-river variability, the biodegradation rates of specific chemicals are. Our work shows that by quantifying the initial biodegradation kinetics and avoiding artifacts caused by spiking, the possibilities for studying the spatial and temporal variability of biodegradation rates in the environment are markedly improved. Further virtues of our modified OECD 309 test include the following. (1) Changes in water chemistry parameters relative to field conditions are minimized. (2) The reproducibility of the test is good, successfully ameliorating the significant intrareplicate variations that have plagued OECD 309-type studies. (3) Multiple chemicals can be studied simultaneously.

McLachlan et al. suggested that applying benchmarking techniques in which chemicals are grouped on the basis of the consistency of their relative biodegradation rates in space and time has the potential for improving the assessment of chemical persistence.[25] The modified OECD 309 test enables the determination of degradation kinetics of a large number of chemicals. Hence, it can provide an experimental basis for exploring this hypothesis.

Although the modified OECD 309 test mirrors biodegradation under environmental conditions in many respects, a half-life determined in the test is not necessarily equal to the half-life in the sampled water body. This is because the half-life in the test is dependent on the sediment:water ratio, a parameter that is arbitrarily fixed (here to 50 $g\ L^{-1}$). For application in chemical regulation, there is a need to translate the laboratory results into environmental half-lives. Further work is required to quantitatively understand the relationship between the half-life in the test and the environment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.2c00811.

Details about the materials and methods and data evaluation procedures and graphs and tables containing complementary results (PDF)

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Notes

The authors declare no competing financial interest.

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