Environmental Science & Technology

Policy Analysis

pubs.acs.org/est

Simulation Studies to Explore Biodegradation in Water–Sediment Systems: From OECD 308 to OECD 309

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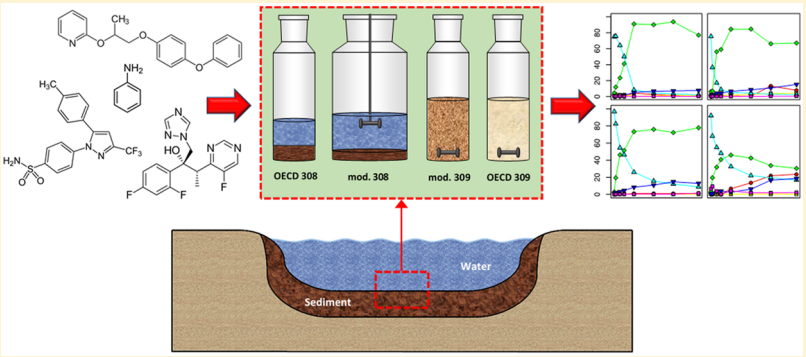
Supporting Information



ABSTRACT: Studies according to OECD 308 and OECD 309 are performed to simulate the biodegradation of chemicals in water–sediment systems in support of persistence assessment and exposure modeling. However, several shortcomings of OECD 308 have been identified that hamper data evaluation and interpretation, and its relation to OECD 309 is still unclear. The present study systematically compares OECD 308 and OECD 309 and two variants thereof to derive recommendations on how to experimentally address any shortcomings and improve data for persistence and risk assessment. To this end, four ^{14}C-labeled compounds with different biodegradation and sorption behavior were tested across standard OECD 308 and 309 test systems and two modified versions thereof. The well-degradable compounds showed slow equilibration and the least mineralization in OECD 308, whereas the modified systems provided the highest degree of mineralization. Different lines of evidence suggest that this was due to increased oxygenation of the sediment in the modified systems. Particularly for rapidly degrading compounds, non-extractable residue formation was in line with degradation and did not follow the sediment–water ratio. For the two more slowly degrading compounds, sorption in OECD 309 (standard and modified) increased with time beyond levels proposed by equilibrium partitioning, which could be attributed to the grinding of the sediment through the stirring of the sediment suspension. Overall, the large differences in degradation observed across the four test systems suggest that refined specifications in test guidelines are required to reduce variability in test outcomes. At the same time, the amount of sediment and its degree of oxygenation emerged as drivers across all test systems. This suggests that a unified description of the systems was possible and would pave the way toward a more consistent consideration of degradation in the water–sediment systems across different exposure situations and regulatory frameworks.

INTRODUCTION

The degradability of a compound in different environmental compartments is a fundamental factor determining its environmental fate and therefore plays a decisive role in regulatory chemical risk assessment. Simulation studies carried out according to guidelines OECD 308 and OECD 309 represent the most important test systems for providing kinetic biodegradation data at the water–sediment interface to be used for (i) persistence assessment and (ii) risk assessment within different European and international regulatory frameworks for chemicals,[1−5] plant protection products,[6−10] and biocides[11−13] as well as human and veterinary pharmaceuticals.[14−19]

Received: March 3, 2016
Revised: May 27, 2016
Accepted: May 27, 2016

DOI: 10.1021/acs.est.6b01095
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

In recent years, several shortcomings of the OECD guideline 308[20] on "Aerobic and Anaerobic Transformation in Aquatic Sediment Systems" have been discussed in various publications.[21−29] Even if the study is performed correctly, results are often uncertain and difficult to interpret. The main points of criticism are:

- The recommended sediment−water (S−W) ratio of 1:3 to 1:4 (v/v) does not reflect the conditions in the natural environment and shifts equilibrium mass distribution excessively toward the sediment phase.[21] Consequently, rapid dissipation from the water phase into the sediment phase is often observed, which can result in unrealistically high levels of non-extractable residues (NER). Furthermore, no clear guidance is given on how to interpret NER and how they should be considered in risk assessment.[21]
- Due to continuous exchange between sediment and water, as well as degradation at the water−sediment interface, the generation of compartment-specific degradation half-lives for comparison to persistence criteria is challenging.[26] Disappearance half-life (DT_{50}) values can be easily determined but are dependent on the test design and therefore cannot be considered robust indicators of persistence. In contrast, transformation half-life ($DegT_{50}$) values are less dependent on the test design, but their determination from the data is challenging and leads to considerable uncertainty.[24]
- Due to the stagnant and stratified test design of OECD 308 studies, anaerobic areas in the sediment cannot be avoided. According to Bowmer et al.,[28] the OECD 308 thus reflects multiphasic processes for degradation and distribution between water, a thin aerobic sediment layer, and a deeper anaerobic sediment layer.
- Finally, the test design of OECD 308 does not consider flow velocity and sediment dynamics, and it is therefore not considered suitable to represent conditions in flowing surface-water bodies.[21,29]

Guideline OECD 309[30] on "Aerobic Mineralization in Surface Water" is intended to determine biodegradation in aerobic natural waters. It is run at low substance concentrations under fully aerobic conditions. A pair of variants are suggested: the "pelagic" test with surface water only and the "suspended sediment test". The latter variant is strongly related to OECD 308 but might potentially exhibit several advantages over OECD 308 for determining biodegradation through sediment-associated microorganisms. In particular, it can be expected to show less irreversible sorption and, hence, potentially also less NER formation due to the finely distributed sediment particles and the low S−W ratio. However, only limited experiences are available with OECD 309 so far, and we are not aware of any systematic comparison of the results of OECD 308 and OECD 309, nor of the different options to perform OECD 309 (e.g., with or without sediment, different sediment concentrations, and stirring or shaking).

Against this background, the objective of the study was to systematically compare OECD 308 and OECD 309 with respect to the above-mentioned shortcomings and to derive recommendations on how to experimentally address some of them. For this purpose, two modified versions, i.e., OECD 308 with a thinner sediment layer and OECD 309 with higher sediment content, were applied besides the standard tests. These experimental systems were intended to bridge the gap between OECD 308 and 309 in terms of amount of sediment and redox conditions. The four test systems were then compared in terms of their degradation and sorption behavior by testing on four different compounds. In this paper, the experimental systems as well as results for the four test compounds are presented and discussed. Special emphasis is placed on the comparison between standard and modified systems with respect to the points of criticism mentioned above.

MATERIALS AND METHODS

Test Compounds. Test compounds were chosen to comprise a range of sorption and biodegradability. Thus, two rather weakly sorbing compounds with high and low biodegradability, as well as two strongly sorbing compounds with high and low biodegradability, were chosen. For compound selection, the following aspects were additionally considered: (i) compounds generating volatile major metabolites other than CO_2 were preferably avoided, (ii) ^{14}C-labeled standards had to be in stock or available commercially at a reasonable price, and (iii) compounds should not readily degrade under anaerobic conditions to facilitate cross-comparison between standard OECD 308 and the other test systems. On the basis of these criteria, the following test compounds were selected: aniline hydrochloride ([U-ring-^{14}C], CAS no. 142-04-1, readily biodegradable, weak sorption, Hartmann Analytics), pyriproxifen ([Pyridyl-2(6)-^{14}C], 95737-68-1, high degradability, strong sorption, Selcia), voriconazole ([Triazol ring-^{14}C], 137234-62-9, slow degradability, weak sorption, Quotient Bioresearch), and celecoxib ([Pyrazol ring-^{14}C], 169590-42-5, intermediate degradability, strong sorption, Quotient Bioresearch).

Sediments and Water Used. A pair of sediments were collected with low organic carbon (OC) (sand, 77.5%; silt, 17.3%; clay, 5.2%; and OC, 0.8%) and high OC (sand, 22.9%; silt, 58.4%; clay, 18.6%; and OC, 7.2%) from locations Wenne and Wingeshausen, Germany. The sediment parameters were determined according to ISO 11277 (2009)[31] (see Table S1). The background concentrations of the selected test compounds in the sediment were not analyzed because we used ^{14}C-labeled test compounds for the tests.

Both the bioactivity and the physicochemical properties of the sediment are crucial parameters influencing the fate of the test compounds in the different experimental systems. However, because not all 32 simulation tests could be run in parallel, it was decided (deviating from OECD 308) to collect the sediment for all individual tests at once and to freeze it at −20 °C in individual portions until use. This should ensure the same physicochemical sediment characteristics at the start of each test. To check the influence of freezing on the microbial activity and composition of the sediment, we performed aniline mineralization experiments, RNA analysis, and fumigation analysis with the sediment before freezing and after thawing for each test (see Figures S2−S4 for method details and results). Before the start of each experiment, surface water was collected from the two locations from which sediment had been sampled previously.

Test System and Sample Preparation. S−W ratios (w/w) of 1:3, 1:10, 1:100, and 1:1000 were used for the preparation of OECD 308, modified OECD 308, modified OECD 309, and OECD 309 test systems, respectively. For OECD 308 and modified OECD 308, stratified water−sediment systems were prepared using an amount of 50 g of sediment (dry weight basis) per incubation vessel. A suspended sediment test was performed for OECD 309 and modified OECD 309 tests. Although OECD 309 allows stirring or shaking to maintain the water−sediment suspension, stirring with a magnetic stirrer was chosen for almost

DOI: 10.1021/acs.est.6b01095
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 1. Comparison of Test System Characteristics

test system	OECD 308 (standard)		OECD 308 (modified)		OECD 309 (modified)	OECD 309 (standard)
compartments	stratified	stratified	stratified, H_2O stirred	stratified, H_2O stirred	mixed, stirred	mixed, stirred
sediment type	low OC	high OC	low OC	high OC	high and low OC	high and low OC
water [mL]	150	150	500	500	300	300
height [mm]	60	43	42	39	–	–
sediment [g dw][a]	50	50	50	50	3	0.3
height [mm]	20	38	5	9	–	–
interfacial area [cm^2]	22	22	90	90	–	–
S–W ratio (w/w)	1:3	1:3	1:10	1:10	1:100	1:1000

[a]g dw = sediment dry weight in grams.

all experiments because the coarse sediment settled rapidly on an orbital shaker. After the analysis of results for the standard and modified OECD 309 studies, it was decided to exemplarily repeat the standard OECD 309 experiments for celecoxib and maintain the suspension by shaking (orbital shaker at 120 rpm) rather than stirring. For all experimental setups, the samples were preincubated for 3–4 weeks at 20 °C in the dark before compound application to allow the sediment microflora to adapt to test conditions after thawing.

The test design of the modified OECD 308 was consistent with the standard OECD 308 except for two main modifications. A lower S–W ratio of 1:10 (w/w) and larger incubation vessels (1.5 L) were used, resulting in a thinner sediment layer and a larger interfacial area. Furthermore, the water body was stirred from above using an overhead magnetic stirrer without disturbing the sediment layer and avoiding as much as possible sediment resuspension. OECD 309 and modified OECD 309 only differed in the amount of sediment used. A comparison of test system characteristics is shown in Table 1.

Test Conditions and Sampling. After sample preparation and preincubation, the ^{14}C-radiolabeled test compounds were applied as recommended by OECD 308 and OECD 309 guidelines. In determining the starting concentrations of the different compounds in the different test setups, the main goal was to keep the compound-to-sediment ratio as constant as possible. However, we had to compromise on this goal due to limits of detection of the analytical method as well as limited water solubility, especially for pyriproxifen and celecoxib. The starting concentrations for aniline and voriconazole were 1 mg/L for OECD 308 (standard and modified) and 0.1 mg/L for OECD 309 (standard and modified). For pyriproxifen and celecoxib, starting concentrations of 0.1 and 0.05 mg/L were used in OECD 308 (standard and modified) and OECD 309 (standard and modified), respectively.

Flow-through systems were used to investigate the degradation of the test compounds within a period of 60 days at 20 ± 2 °C in the dark. The incubation vessels were arranged in parallel and connected to traps for collecting evolved carbon dioxide (1*N* NaOH) and other volatile intermediates (ethylene glycol). The traps were replaced at intervals of 7 days during the test. Whole incubation vessels (duplicates) were sacrificed for analysis at eight sampling time points (0, 1, 2, 4, 7, 14, 21, 28, and 60 days after application for aniline and 0, 1, 4, 7, 14, 28, 42, and 60 days after application for the other test compounds). A positive control (aniline) and a sterile control (sediment and water autoclaved 3-fold for 30 min at 121 °C) were run in parallel with all test systems to confirm that the test systems contained an active microbial population and to check if any abiotic degradation took place, respectively.

Sample Processing and Analysis. Upon the sampling of standard and modified OECD 308 systems, the supernatant water was separated from the sediment phase with a pipet. The remaining wet sediment was considered as the sediment phase and treated as bulk-phase throughout all extraction steps. For standard OECD 309, phase separation was achieved by filtration (glass-fiber filter, 0.45 μm pore size GF92 Whatman). Because sorption to the filter could be expected for celecoxib on the basis of a pretest, centrifugation (31920*g*, 20 min) was applied to separate phases for OECD 309 (standard and modified) in the case of celecoxib. For modified OECD 309, filtration was used during experiments with aniline and voriconazole. Centrifugation was used for phase separation when testing pyriproxifen in the modified OECD 309 setups (10000*g*, 30 min).

A subsample from the separated water phase was taken for the measurement of dissolved $^{14}CO_2$, which was calculated based on the difference in the radioactivity between acidified and nonacidified water samples (method details in p. S3 in the Supporting Information). An aliquot of the separated water phase was taken for liquid scintillation counting (LSC), and another subsample of the water phase was taken for repeated extraction with either solid-phase extraction (SPE) or liquid–liquid extraction (see Table S2). Likewise, the separated sediment phase was homogenized and was extracted using appropriate solvents (see Table S3). Duplicate subsamples from both the water and sediment extracts were taken for determination of radioactivity by LSC. Extracts from water and sediment were then concentrated stepwise under a gentle nitrogen stream. To get rid of possible solid residues after the concentration steps, we performed centrifugation (12300*g*, 3 min) of the concentrated extracts, and the supernatant was separated. This clear supernatant was subjected to specific chemical analysis by radio thin-layer chromatography (radio-TLC) and radio high-performance liquid chromatography (radio-HPLC; confirmatory method).

The extracted solid sediments were allowed to dry at room temperature. The dried sediment samples were homogenized using a mortar mill (RM 200 Retsch), after which an aliquot of it was taken for combustion (Packard model 307) to determine the amount of non-extractable residues. Because standard OECD 309 contained only little sediment, the complete sediment was combusted instead of an aliquot. A total ^{14}C mass balance (in percent of applied radioactivity (aR)) was set up for each individual incubation vessel at each sampling time point by summing up the radioactivity measured in the water phase, extractable sediment residues, NER, $^{14}CO_2$ trap, and the ethylene glycol trap for other volatile intermediates.

Specific Chemical Analysis. The concentrated extracts were analyzed by radio-TLC and radio-HPLC. However, for some compounds, the radio-HPLC analysis was not robust.

DOI: 10.1021/acs.est.6b01095
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Therefore, only radio-TLC analysis was used for the evaluation of the results, and the radio-HPLC served as confirmatory method for the radio-TLC analysis. Further identification of detected metabolites was not performed because the detailed elucidation of the compounds biotransformation pathways was not the objective of this study.

Radio-TLC. The TLC methods used for the respective test compounds are listed in Table S4. Quantification was based on the radioactivity that was determined by means of a FUJI BAS 1000 BioImager. The data recorded were evaluated with instrument specific software (AIDA Image Analyzer V.3.44), which converts each line into a chromatogram including peak integration.

Radio-HPLC. The test compound and its metabolites were analyzed by reversed-phase radio-HPLC. The HPLC parameters used for the parent compound analysis are listed on p. S6 in the Supporting Information.

Oxygen Measurement in the Sediment Phase (OECD 308 and Modified OECD 308). Oxygen measurements were performed with an oxygen micro sensor (Pre Sens). The micro sensor was connected to a control unit (see pp S17−18 in the Supporting Information) for an ultrafine vertical movement across the sediment layer. The oxygen saturation was measured across the top 2 mm of the sediment phase in 50 μm steps.

RESULTS AND DISCUSSION

Results for Individual Test Systems and Compounds. A compilation of test results for all compounds, experimental systems, and sediments is given in Figures S5−S8. On the basis of the ^{14}C mass balance data, the four test systems could be compared in terms of recoveries (percent of applied radioactivity; % aR) to see whether any one of them provided superior recoveries or reduced variability in test outcomes. Generally, all test systems provided high recoveries and low variation. However, a statistically significant lower overall mean recovery of all replicates in the tests (Kruskal−Wallis ANOVA; $p = 0.0005$; $n = 128$ per test system) was determined for standard OECD 309 compared to the other three test systems. Standard OECD 309 also provided the most recoveries outside 100 ± 10% aR. Mean recoveries were 88.2% aR (standard OECD 309), 90.2% aR (modified OECD 308), 92.3% aR (modified OECD 309), and 93.4% aR (standard OECD 308). The coefficient of variation (CV) [%] was between 10.2% and 14.8%, indicating good reproducibility. The mean difference between the duplicate samples at all sampling time points was in the range between 6.2% aR and 6.9% aR for all test systems. A table with all recovery data is given in Table S5.

In terms of compound behavior, the expected differences in the extent of sorption and degradation of the selected test compounds were largely confirmed by the experimental results. The experiments with aniline and pyriproxifen showed rapid (pyriproxifen) to very rapid (aniline) degradation and high mineralization, whereas voriconazole and celecoxib degraded relatively slowly, and almost no mineralization was observed. Also, the compounds exhibited a gradient of sorption affinity (pyriproxifen > celecoxib > voriconazole), which was in agreement with organic carbon−water partition coefficients (K_{oc}) measured in separate sorption experiments (see Table S7).

For aniline, we observed rapid mineralization and NER formation to an extent that the extractable amount from the sediment was less than 10% in most samples. Therefore, no observation of equilibrium partitioning was possible. Aniline further showed a highly variable pool of polar metabolites and dissolved $^{14}CO_2$ in the water phase. In some cases, unexpected volatilization of parent aniline was observed, which, however, was limited to standard OECD 308 tests (detection of parent aniline in ethylene glycol traps). In addition, aniline also rapidly formed large amounts of NER in the sterile samples (up to 91% of the applied radioactivity), indicating that NER formation for aniline to a large extent originates from abiotic (chemical) processes.[32] Overall, due to different rapid processes taking place, the contribution of the individual processes to aniline dissipation was difficult to evaluate. In the OECD 309 guideline, the use of aniline as reference compound to check the biological activity of the test system is recommended. However, on the basis of our results, we question the reliability of aniline degradation as an indicator for biomass activity.

Nevertheless, we found that the set of test compounds with varying degrees of degradability and sorption provided an ideal basis to compare the four test systems in terms of their "degradation power" and how this relates to the physicochemical conditions in these systems. Because voriconazole turned out to be quite stable, its data could be used to look at differences in sorption processes in the four test systems. Aniline and pyriproxifen could be mainly used to explore degradation in the four systems, whereas celecoxib, as a moderately degradable compound, finally allowed the observation of the interaction between the degradation and sorption processes.

Degradation across Test Systems. For both aniline and pyriproxifen, mineralization at the test end (60 day) in modified systems (OECD 308 and OECD 309) and in standard OECD 309 was higher than in the standard OECD 308 system (Figure 1). Actually, across all experiments, mineralization in modified test systems was statistically significantly higher compared to the standard OECD systems (F-Test, $p < 0.01$), and there was only

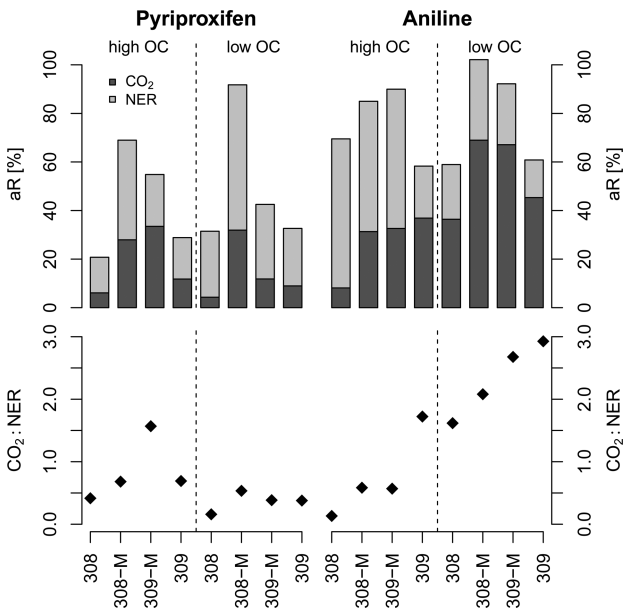

Figure 1. Comparison of mineralization and NER pool at 60 days between different test systems for two well-degradable test compounds (aniline and pyriproxifen). The lower graph shows the CO_2−NER ratio, which hints about the relation between both the processes among the test systems. The high OC and low OC refers to fine-textured sediment with high organic carbon content and coarse sediment texture with low organic carbon content, respectively. aR[%]: percent total applied radioactivity.

DOI: 10.1021/acs.est.6b01095
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

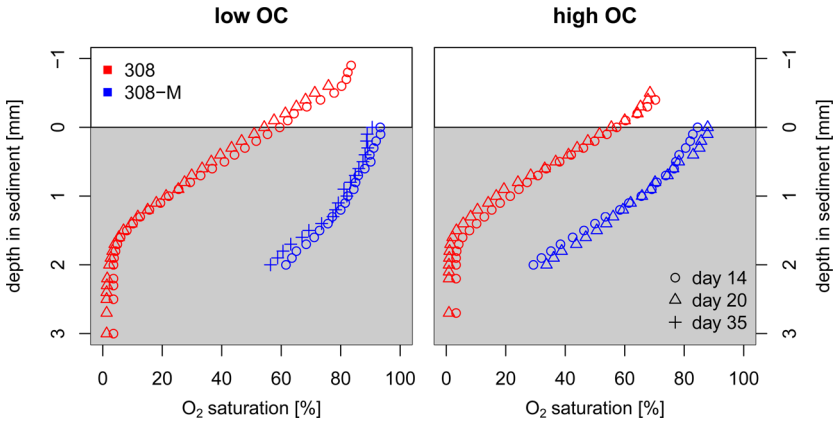

Figure 2. Measurement of oxygenation profile in the upper sediment layers of both high- and low-OC sediment for standard OECD 308 and modified OECD 308 using an oxygen microsensor (vertical precision: 5/100 mm).

one system (i.e., aniline in the high OC sediment) in which this was not the case. Similarly, NER formation across the different systems did not simply follow the S−W ratio. Instead, NER formation was also in almost all cases higher in the two modified systems than in the standard OECD 308 and 309 systems (Figure 1). To describe the relationship between mineralization and NER formation among different test systems, we compared the quotient of CO_2−NER across the systems. For both compounds, the quotient was always higher in modified OECD 308 systems compared to standard OECD 308 (Figure 1), suggesting that contact of the compounds with sediment in the modified OECD 308 system was more likely to lead to mineralization than in the standard OECD 308 system. Beyond the two OECD 308 systems, in tests with low OC sediment, aniline showed a clear trend of an increasing CO_2−NER quotient with decreasing S−W ratio. In the high OC sediment, the trend looked similar, the only difference being that there was no clear difference between the two modified systems. Given the previous knowledge on and observation of rapid abiotic NER formation of aniline when in contact with sediment, these results indicate that purely abiotic interactions with the sediment decrease and biotic degradation increases along the series of standard OECD 308, modified OECD 308, modified OECD 309, and standard OECD 309. The mostly higher mineralization in the two modified systems compared to the two standard systems (Figure 1) for both aniline and pyriproxifen can then be understood as a combination of two effects. Whereas biotic degradation per sediment mass increases, the absolute amount of sediment in the system decreases, leading to maximal degradation in the two modified systems.

The increase of biotic degradation per sediment mass goes along with increased disturbance and, as a consequence, larger fractions of oxygenated sediment in the different test systems. The difference between standard and modified OECD 308 is due to a larger fraction of the total sediment being oxic in the modified OECD 308 system, which was caused by a larger interfacial area, the stirred water phase, and the thinner sediment layer. In contrast, in the standard OECD 308 system, oxygen supply through diffusion is slow relative to microbial respiration, resulting in mostly anoxic conditions. Indeed, enhanced oxygen supply into deeper layers of the sediment could be demonstrated in the modified OECD 308 system compared to standard OECD 308 (Figure 2). Overall, it is likely that the larger fraction of oxic sediment in the modified OECD 308 system led to the observed enhanced degradation. Even though it can be expected that, for the same reasons, standard OECD 308 outperforms the modified system in terms of anaerobic biodegradation, the significance of anaerobic degradation could not be verified with our data because anaerobic degradation is expected to be mostly negligible for the selected compounds. Although anaerobic degradation pathways for aniline are known,[33] rapid aerobic biodegradation and rapid abiotic NER formation within the test systems most likely led to limited availability of aniline for anaerobic degradation. Indeed, the lack of anaerobic degradation is confirmed by the similar metabolite profiles across test systems for the individual compounds (Figure S9−11). There were no metabolites that were detected in considerable amounts in one particular test system but not in the others, indicating that degradation did not proceed through completely different pathways in the different test systems. If anaerobic degradation had played a major role for our test compounds, it should have led to a different metabolite spectrum, at least in standard OECD 308.

Compared to the two OECD 308 systems, the sediments in both standard and modified OECD 309 systems can be expected to be completely oxic, which was in agreement with the observed even higher CO_2−NER ratios for aniline in the two OECD 309 systems. The fact that degradation in the modified OECD 309 system was generally higher than in the standard system is most likely predominantly due to the 10-fold higher amount of sediment used, which also introduced higher amounts of biomass into the system.

Although standard OECD 308 was initially criticized for high NER formation due to its high S−W ratio, NER formation was even higher in modified OECD 308 tests. Except for aniline, in which NER formation and mineralization were observed to be competing processes due to the rapid abiotic NER formation of aniline, NER formation and degradation went hand-in-hand for the other compounds. The observed link between NER formation and biodegradation suggests that similar initial processes (e.g., initial biotransformation reactions) might be involved in both biodegradation and NER formation.[34,35] This would also be in line with recent findings that part of what is assigned as NER with conventional extraction methods is actually radiolabeled carbon that has been incorporated into live biomass.[36−38]

For celecoxib and voriconazole, negligible mineralization and low levels of metabolites were detected in most samples. For these compounds, modified OECD 308 tests always showed the

DOI: 10.1021/acs.est.6b01095
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highest amount of metabolites, indicating advanced degradation in modified OECD 308 compared to the other test systems.

Sorption across Test Systems. Because voriconazole and, to some extent, also celecoxib did not show much degradation, they could be used to study and compare the sorption properties of the different test systems. Figure 3 shows the dissipation of radioactivity from the water in different test systems for celecoxib and voriconazole. Particularly for the more strongly sorbing compound celecoxib, dissipation from the water phase in OECD 308 proceeds gradually over a period of 15 days, whereas in the other systems constant or only slowly decreasing concentrations in water were reached much faster (i.e., 1−10 days). We compared the effective ratio of parent compound in sediment (Ps) to parent compound in water (Pw) at each time point of the test with the ratios that would be expected upon equilibration as derived from independently measured organic carbon−water partition coefficients (K_{OC}) for all four test compounds and both sediments (see pp S13−16 in the Supporting Information for details on K_{OC} determination and values and an estimation of Ps−Pw ratios).

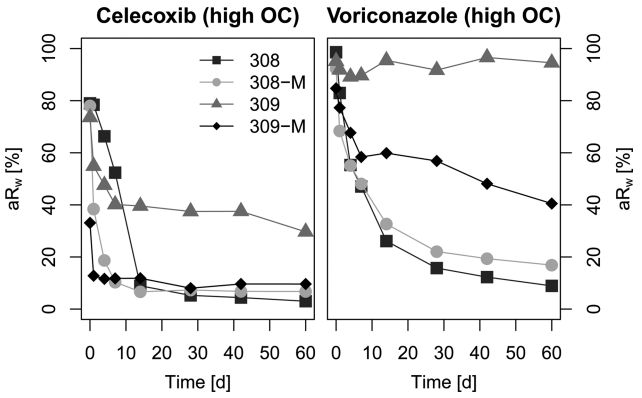

Figure 3. Dissipation of radioactivity from the water phase (celecoxib and voriconazole) in different test systems using fine-texture sediment with high organic carbon content. aRw[%]: percent total applied radioactivity measured in the separated water phase.

First of all, the estimated Ps−Pw ratios for equilibration in Table 2 show that they are expected to vary over 2 orders of magnitude between the different test systems. The observed ratios show that in OECD 308 (standard and modified), these estimated equilibrium values of Ps−Pw are reached only at later time points (42−60 days), if at all. For celecoxib in the standard OECD 308 system, the observed ratios remain below the estimated value, which indicates that equilibration in standard OECD 308 is slow and that the strongly sorbing compound celecoxib has not yet fully penetrated the whole sediment even at test end.

However, the estimated values were already reached at very early time points (0−4 days) in the OECD 309 (standard and modified) systems. This shows that equilibration between the sediment and the water phase occurs rapidly in the stirred OECD 309 systems. However, for later time points, the Ps−Pw ratio continued to increase beyond values expected for equilibration (i.e., factors of 2−5 higher). Moreover, considerably higher amounts of NER formation were observed in the OECD 309 (standard and modified) tests in comparison to OECD 308 (standard and modified) tests for these compounds, which was in contrast to the results for aniline and pyriproxifen. These observations for the OECD 309 systems (standard and modified) indicate that some additional sorption beyond equilibration is evolving over time, paired with unexpectedly high levels of NER formation. Possible reasons for these observations are addressed in the following section.

Nevertheless, the increasing fraction of the compounds in the aqueous phase when going from OECD 308 to modified OECD 308, to modified OECD 309, and, finally, to OECD 309 must have led to an increased bioavailability of the test compounds. This was most likely the second important factor contributing to increased biodegradation along the sequence of test systems.

Shaking versus Stirring in OECD 309. In OECD 309 test systems, particularly those containing the coarse-textured sediment, sediment grinding and erosion of the magnetic stir bars (PTFE) were observed. Therefore, an experiment was carried out to determine the sediment texture of the coarse sediment before and after stirring for 60 days under modified OECD 309 test conditions. A drastic change in the grain size distribution at the end of the stirring experiment was observed (start: 77.5% sand; end: 2.5% sand) (see Table S10). This led to the hypothesis that formation of new surfaces due to sediment grinding during the experiment could explain the increase of sorption and NER formation that had been observed for celecoxib and voriconazole as previously described.

Therefore, the standard OECD 309 test was repeated for celecoxib but with the sediment suspension maintained by shaking (see the Materials and Methods section). When doing

Table 2. Observed Ps−Pw Ratios for Celecoxib and Voriconazole in All Four Test Systems (High OC, Fine Sediment) at Different Time Points (0−60 Days) Compared to Estimated Ps−Pw[b] Ratios upon Equilibration[a]

	celecoxib Ps−Pw					voriconazole Ps−Pw			
time (d)	OECD 308 standard	OECD 308 modified	OECD 309 modified, stirred	OECD 309 standard, stirred	OECD 309 standard, shaken	OECD 308 standard	OECD 308 modified	OECD 309 modified, stirred	OECD 309 standard, stirred
0	0.06	0.08	1.66	0.21	0.09	0.03	0.03	0.07	0.01
1	0.16	0.19	5.82	0.45	0.19	0.24	0.29	0.10	0.03
4	0.36	3.29	8.20	0.61	0.22	0.85	0.58	0.12	0.05
7	0.82	6.73	6.08	0.78	0.21	1.11	0.85	0.21	0.05
14	11.0	16.5	7.62	0.83	0.35	2.82	1.41	0.24	0.06
28	19.9	20.1	12.8	0.89	0.38	4.85	1.94	0.19	0.06
42	26.0	21.6	9.90	0.95	0.39	5.98	1.75	0.31	0.06
60	34.6	28.1	9.35	1.25	0.57	8.89	1.81	0.32	0.05
estimated	90.4	26.8	2.67	0.27	0.27	6.86	1.79	0.17	0.02

[a]See pp S13−16 in the Supporting Information for calculations. [b]Ps: parent residue in the sediment phase; Pw: parent residue in the water phase. For celecoxib, the observed Ps−Pw ratios for the shaken version of OECD 309 are also listed.

DOI: 10.1021/acs.est.6b01095
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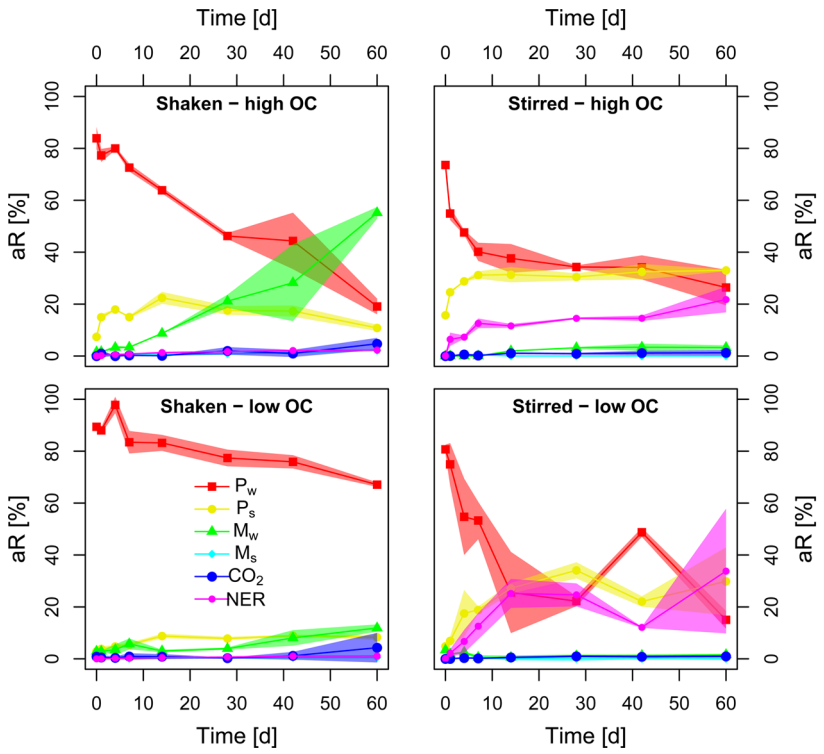

Figure 4. Degradation time series of celecoxib in shaken and stirred version of standard OECD 309 systems for high- and low-OC sediment. The different pools shown are Pw: percent total amount of parent compounds measured in the water phase, Ps: percent total amount of parent compounds measured in the sediment extract, Mw: percent total amount of metabolite measured in the water phase, Ms: percent total amount of metabolite measured in the sediment extract, total CO_2: percent total amount of mineralization measured in the system, and NER: percent total amount of non-extractable residue in the sediment phase. Shaded areas show the standard deviation of the data points, and aR[%] indicates the percent total applied radioactivity.

so, it was observed that sediment suspension in the shaken system was not as efficient as in the stirred system. Especially in the samples with coarse textured sediment, most of the sediment settled immediately at the bottom of the test vessel. Interestingly, the results obtained in the shaken system were clearly different from those obtained in the stirred system. Almost no NER formation over 60 days was observed in the shaken system. Instead, metabolites were formed up to 57.3% ± 2.2% aR in the high-OC sediment and 12.4% ± 1.4% aR in the low-OC sediment after 60 days of incubation (see Figure 4). In addition, the Ps−Pw ratios shown in Table 2 for the shaken OECD 309 were closer to the ratio estimated based on equilibrium partitioning (within ±50% of the estimated value, with the exception of day 60) compared to a factor of 3−4 overestimation in the stirred system.

The differences in NER formation between the shaking and stirring variants of the OECD 309 test supported the hypothesis of enhanced sorption in stirred OECD 309 versions due to freshly exposed sediment surfaces from sediment grinding. At the same time, increased degradation was observed in the shaken system. This observation is consistent with enhanced sorption to new surfaces in the stirred system, considerably reducing the bioavailability of the test compound for microbial degradation. Overall, the starkly different results between the stirred and shaken test system indicate that the options for experimental setup approved by the OECD 309 guideline can highly affect the outcome of the study.

IMPLICATIONS FOR USE OF OECD 308 AND 309 TEST SYSTEMS

Overall, large differences in aerobic degradation were observed between the four test systems investigated. We found that this must have been partly due to larger fractions of the sediment being fully oxygenated with increasing disturbance in the system and partly due to increased bioavailability of the compounds with decreasing absolute amounts of sediment. Because the absolute amount of sediment and hence biomass was decreasing with increasing disturbance of the systems, this led to the two modified systems (308 and 309) typically showing maximum degradation, whereas the standard OECD 308 system, along with the standard OECD 309 system, yielded a more conservative assessment of the compounds' degradation potential. This finding would most likely not hold true for compounds with substantial anaerobic degradation. However, this aspect could not be tested on the basis of our data because anaerobic degradation is expected to be negligible for the test compounds studied.

With respect to the shortcomings of OECD 308 mentioned in the Introduction section, our study thus shows how, through increased disturbance of the systems, conditions can be shifted from mostly anaerobic to (nearly) fully aerobic. Analysis of Ps−Pw ratios over the course of the experiment further confirmed that time to equilibration is considerably reduced in the more disturbed systems. This clearly simplifies interpretation of the experimental outcomes with respect to degradation because phase-transfer processes do not need to be explicitly considered. However, this does not necessarily mean that modified test systems explored here are better-suited for persistence and risk

DOI: 10.1021/acs.est.6b01095
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assessment. Rather, it suggests that redox conditions and degree of disturbance are key factors to increased biodegradation in water−sediment systems and should therefore also be key factors when choosing the testing guideline that most appropriately reflects relevant natural conditions and exposure scenarios for the types of compounds to be assessed. For instance, although 308 is more suitable for reflecting fate in stagnant systems, 309 should be used to represent highly disturbed systems such as turbulent, flowing rivers.

Furthermore, the comparison of results for the shaken and stirred variants of the standard OECD 309 study and the different degradability outcomes obtained for the modified and the standard OECD 309 demonstrated that the current OECD 309 guideline gives too much experimental freedom for the test setup. Our experiments demonstrate that different setups, each in full accordance with the test guideline, yielded very different results in terms of compound behavior. It can be assumed that the option to run the test without sediment (pelagic test) could lead to yet a very different degradation behavior. For the regulatory use of data from OECD 309, this situation does not seem acceptable. We thus strongly suggest that the OECD 309 guideline requires further harmonization and refined specifications to foster scientifically sound and reproducible results.

A third and important corollary of the findings in this study is that some well-known underlying principles seem to manifest themselves across the test systems and compounds investigated, e.g., the dependence of biodegradation on the amount of active biomass and the bioavailable fraction of the compounds. This suggests that data across test systems might be interpretable with a unified model containing a compound-specific aerobic degradation parameter that is valid across all test systems. Thus, trying to find such a system-independent description of biodegradation across all of these systems might also highlight a way forward to derive a biodegradation parameter that is less affected by experimental variations in the OECD guidelines. This has been quantitatively explored in a companion paper.[39]

Finally, we observed that the extent of NER formation varied between compounds and either competed with degradation, as was the case for aniline, or went in parallel with degradation, as was the case for the other compounds. An important implication of this is that systems showing improved degradation are likely to come at the cost of also-increased NER formation. The comparison between the shaken and stirred variants of OECD 309 further showed that NER formation can be increased by disturbing the sediment structure and reducing particle size. This supports concerns raised about how NER formation observed in laboratory studies represents natural environmental conditions. In general, the lack of understanding of processes involved in NER formation makes the interpretation of degradation data difficult. To address this issue, more information is needed on the formation and quality of NER, which is the subject of several ongoing CEFIC-funded research projects (LRI ECO 24 and LRI ECO 25; see http://cefic-lri.org/).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01095.

Additional details on sediment parameters, pictures of different test systems, sediment freezing and active microbial biomass, comparison of microbial biomass, calculation of $^{14}CO_2$ dissolved in the water phase, extraction of the separated water and sediment phases, TLC and HPLC methods, degradation time series, metabolite profiles, variability of results across the test systems, determination of sorption coefficients, calculations for estimating Ps−Pw, comparison of sediment texture, oxygen measurement in the sediment phase, list of physical and chemical properties of the test compounds, and a comparison of observed Ps−Pw. Tables showing properties of sediments used for tests, extraction methods applied, lists of sediment extraction and TLC methods, comparison of ^{14}C mass balances and variability of results, sediment−solution ratios, organic carbon−water partition coefficients, sediment porosity, estimated Ps−Pw of voriconazole and celecoxib, comparison of sediment texture, list of physical and chemical properties, and a comparison of observed Ps−Pw ratio for celecoxib. Figures showing different water−sediment systems, schematics on and comparison of the quantification of active microbial biomass, comparison of methods for measuring microbial biomass, degradation time series, metabolite profiles, and a picture of the control unit for adjusting the oxygen electrode. (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge Cefic LRI for funding the project and the RLT team for providing necessary input and guidance throughout the project. We also thank Pfizer Inc. for the donation of ^{14}C-celecoxib and ^{14}C-voriconazol for conducting our tests. Special thanks go to Angela Bauer, Claudia Knoche, Elena Heusner, Daniel Gilberg, and Pedro Ferreira for contributing their valuable work and necessary guidance during the project. We also give a sincere thanks to all of the participants of the Cefic LRI-ECO18 workshop held at Eawag on October 5 and 6, 2015.

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DOI: 10.1021/acs.est.6b01095
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