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Improved closed test setup for biodegradation testing of slightly volatile substances in water-sediment systems (OECD 308)

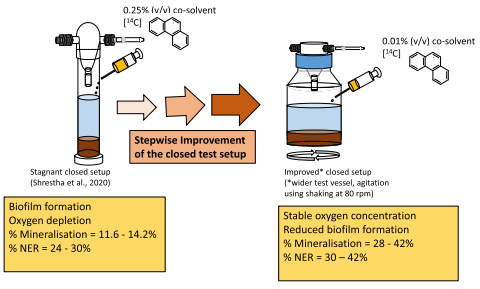
Prasit Shrestha [a,*], Christopher B. Hughes [c], Louise Camenzuli [b], Delina Lyon [d], Boris Meisterjahn [a], Thomas Hennecke [a], Megan Griffiths [c], Dieter Hennecke [a]

[a] Fraunhofer IME-AE, Auf dem Aberg 1, 57392, Schmallenberg, Germany
[b] ExxonMobil Petroleum and Chemical B.V.B.A., Hermeslaan 2, 1831, Machelen, Belgium
[c] Ricardo Energy & Environment, Harwell, OX11 0QR, UK
[d] CONCAWE, Boulevard du Souverain, 165B-1160, Brussels, Belgium

HIGHLIGHTS

- Agitation of the water-sediment sample without/limited sediment resuspension.
- Use of co-solvent for application should be limited while testing volatiles using closed setup.
- Improved closed setup results in stable aerobic conditions and higher degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Standardized biodegradation testing methods, like the OECD 308 Aerobic and Anaerobic Transformation in Aquatic Sediment Systems, generate data on biodegradation required during environmental risk and hazard assessment of chemicals under different European and international regulations. However, difficulties arise when applying the OECD 308 guideline for testing hydrophobic volatile chemicals. Especially the use of a co-solvent (like acetone) as a measure to facilitate the application of the test chemical in combination with a closed setup to reduce losses due to volatilization tend to deplete/restrict the amount of oxygen in the test system. The result is a low oxygen or even anoxic water column in the water-sediment system. Thus, the degradation half-lives of the chemical generated from such tests are not directly comparable to the regulatory half-life values for Persistence assessment of the test chemical. The aim of this work was to further develop the closed setup to improve and maintain aerobic conditions in the water phase of the water-sediment systems for testing slightly volatile hydrophobic test chemicals. This improvement was attained by optimizing the test system geometry and agitation technique to maintain aerobic conditions in the water phase in a closed test setup, investigating appropriate co-solvent application strategy, and trialing the resulting test setup. This study shows that when using a closed test setup for OECD 308 tests, agitation of the water phase overlaying the sediment and the test item application using low co-solvent volume is critical for maintaining an aerobic water layer.

* Corresponding author.
E-mail addresses: prasit.shrestha@ime.fraunhofer.de (P. Shrestha), environment@concawe.eu (D. Lyon).

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1. Introduction

Biodegradation is an important environmental fate process which deals with the partial or complete removal of chemicals by microorganisms. When chemicals fail to degrade in this way, they may persist in the environment, resulting in long term risks to the environment and human health (The 12 Initial POPs under, 2001). Information on biodegradation is required during environmental risk assessment of chemicals under different European and international regulations (Commission regulation (EU), 2011; Regulation (, 2009; Regulation (, 2012; Guideline on the environmental risk, 2006; Revised Guideline on Environmental Impact, 2009; CEPA Canadian Environmental Protection Act, 1999; USEPA Category for Persistent, 1999; USEPA Persistent Organic Pollutants, 2016; METI Chemical Substances Control Law, 2015; USEPA, 2015; REACH, 2011). Under Annex XIII of REACH, biodegradation data are also required for Persistence (P) assessment, where degradation half-lives in different environmental compartments are compared against the corresponding trigger values (Commission regulation (EU), 2011; ECHA (2017a, 2017). (Bio)degradation data for such regulatory purposes are normally generated using standard OECD test guidelines (OECD, 2017; OECD, 2002; OECD, 2004; OECD-Guideline for the testing of, 2002; OECD, 2008). The OECD 308 test guideline is an example of higher tier biodegradation test of chemicals in a water-sediment system (OECD-Guideline for the testing of, 2002).

The OECD 308 test guideline has been widely used for testing chemicals with different physicochemical properties. However, difficulties arise when applying the guideline for testing poorly water soluble and volatile chemicals (OECD-Guideline for the testing of, 2002; Shrestha et al., 2020). When testing slightly volatile chemicals, a closed test setup is recommended by the guideline. Previous work (Shrestha et al., 2020) (Shrestha et al., 2020) developed a closed setup for testing hydrophobic volatile chemicals in a water-sediment system. Application of the hydrophobic volatile test chemicals was performed using a co-solvent:water mixture and the amount of co-solvent (acetone) introduced was limited to $\leq 0.25\%$ (V/V). Complete mass balances were obtained for a range of volatile chemicals (tetralin, decane, phenanthrene, biphenyl). However, the measurement of different test parameters, specifically Dissolved Organic Carbon (DOC) and O_2 concentration in the water phase, highlighted that test conditions within these closed setups were compromised during the test. These changes in test conditions were primarily attributed to the stagnant water layer and high concentration of co-solvent used for application of the test substance. This led to 1) significant depletion in oxygen concentration in the water phase during the test relative to the conditions described for an aerobic test in OECD 308 guideline (7–10 mg O_2/L in the water layer), and 2) thick biofilm layer formation on the air-water surface. Therefore, further improvement of the closed setup was necessary in order to ensure applicability of the OECD 308 test guideline for slightly volatile, hydrophobic test chemicals and comparability of results to other substances tested using the standard OECD 308 guideline.

In the previous study (Shrestha et al., 2020), a closed flask was used for biodegradation testing in a stagnant water-sediment system. It is known (OECD-Guideline for the testing of, 2002; Shrestha et al., 2020) that stagnant systems lead to reduced diffusion of oxygen from the headspace to the water phase, which is exacerbated in such a closed setup. Additionally, the use of a co-solvent for a volatile test compound in a closed setup meant the co-solvent could not be stripped from the aqueous phase; this led to even lower oxygen levels in the water phase due to biodegradation of the co-solvent.

The aim of this work was to further develop the closed setup to improve and maintain aerobic condition in the water phase of the OECD 308 for hydrophobic and slightly volatile test chemicals, with the following steps:

Step I) Optimization of the test system geometry and agitation technique to maintain aerobic conditions in the water phase in the closed test setup;

Step II) Investigation of an appropriate co-solvent application strategy employing a reduced co-solvent concentration, suitable for testing hydrophobic volatile chemicals;

Step III) Application of the improved test setup and appropriate co-solvent application to conduct a preliminary water-sediment study

Step IV) Conduct a full scale OECD 308 test using the improved test setup.

The obtained results on the degradation, distribution and NER (non extractable residue) formation of phenanthrene using this improved water-sediment system will be discussed and compared with the results from previous work (Shrestha et al., 2020).

2. Materials and methods

2.1. Test materials

The slightly volatile and hydrophobic chemical, phenanthrene (K_H 4.29 Pa m^3 mol^{-1}, Koc 7421 L kg^{-1}) was selected as the test substance for this study. ^{14}C labelled phenanthrene (batch no. SON-1343/15-038-Phe) was purchased from Hartmann Analytik (Braunschweig, Germany). The specific radioactivity of the test item was 3.71 MBq mg^{-1} and the analytical purity was >98%. The application of the test substance was performed using acetone (K_H 3.55 Pa m^3 mol^{-1}) as a co-solvent.

2.2. Water and sediment

In accordance with the OECD 308 test guideline, two different sediments, specifically 1) fine textured with high organic carbon (OC) content and, 2) coarse textured with low OC content were used for this study. The fine textured sediment was sampled at the artificial lake "Biggesee" North Rhine Westphalia (NRW) (51° 4′ 41.96″ N, 7° 50′ 3.1″ E), Germany, whereas the sediment with coarse texture was sampled at quarry pond "Nesthauser" near Paderborn NRW, Germany (51° 44′ 54.55″ N, 8° 40′ 32.46″ E). Sediment was sampled from an upper surface layer to a depth of 5–10 cm. The corresponding water samples were collected from the same locations and sampled at a depth of 30–60 cm beneath the water surface. Three different batches of water and sediment were collected on January 06, 2020 (used for Steps 1 and 2), on May 28, 2020 (used for Step 3) and on September 08, 2020 (for the full-scale main test). The details on sediment characteristics is available in the Supplementary Information (section S1). The sediment samples were transported in a sealed plastic container, wet sieved with a 2 mm sieve, and stored at 4 °C in accordance with OECD 308 guideline before using for water-sediment sample preparation.

2.3. Test setup used

Water-sediment samples test systems were prepared in the test setup by adding a defined volume of sediment (Table 1) and water to achieve a water:sediment volume ratio of 3:1, in accordance with the OECD 308 test guideline. In comparison to the test setup used in Shrestha et al. (2020) (Test Setup 1), two test setups with differing test vessel geometries and agitation conditions (Test Setup 2 and 3) were developed (Table 1). The two types of flasks used were an Erlenmeyer flask (Ø = 10.5 cm, 500 mL) and a cylindrical flask (Ø = 7.5 cm, 500 mL). Two agitation approaches, specifically a) overhead stirring of the water phase using magnetic stirring (setup inspired from previous studies Junker et al., 2010 and Shrestha et al., 2016)and b) shaking of the water-sediment samples in an orbital shaker at 80 rpm, were used in the new test setups. Both the agitation techniques were set making sure that the sediment layer was not disturbed. The new setups included Teflon screw caps (except setup 2 with shaking approach where glass insert cap was used as in setup 1) to ensure no loss of test chemical to adsorption on the inner surface of the screw cap, and acted as an adaptor for overhead stirring (Table 1). Closed setups were designed with Tenax tubes (using a metal Swagelok adaptor) and internal absorption traps (containing 2 N

NaOH) for capturing volatilized and mineralized fractions, respectively. These traps were only used while performing the biodegradation test. All experiments were carried out at 20 °C.

2.4. Oxygen monitoring

The oxygen in the headspace and water phase of control samples was monitored by an optical sensor system (Redflash-Technology, Pyroscience, Germany). The oxygen saturation in the headspace (%) and water phase (mg L^{-1}) were monitored on a regular basis, aerating the samples with 20–30 s of ambient air if oxygen saturation in the headspace and/or water phase was <15%.

2.5. Measurements of other physicochemical parameters

An aliquot of water phase was taken for turbidity and DOC analysis at start and end of each pretest and at 0d, 28d and 103d in the full-scale OECD 308 test. Turbidity measurements were performed using WTW Turb 550 IR and DOC using TOC V $_{CPH}$ analyser (Shimadzu). Additionally, pH and redox measurements of the water phase and the sediment phase was performed at the point of sacrificing the samples.

2.6. Pre-tests for the improvement of the closed test setup

The water-sediment samples were prepared using a defined amount of sediment and water in different test setups (for detail see individual steps in Table 1), and pre-incubated for 7–10 days at test conditions without completely closing (locks were open) the test vessels. Each test setup was tested in duplicate. The pre-tests were performed using a stepwise approach as described below.

2.6.1. Step I: optimization of test system geometry and agitation conditions

The test setups with wider test system geometries (i.e. test Setup 2 and 3) operated with different agitation (shaking and overhead stirring) were investigated under this step. In total four different test setup variants were investigated (Setup 2-shaken, Setup 2-stirred, Setup 3-shaken, Setup 3-stirred). After pre-incubation, 0.25% V/V of co-solvent (based on the volume of overlying water) was applied to one set of samples. This was equivalent to 0.795 mL and 0.663 mL of acetone in fine and coarse texture water-sediment samples respectively. A corresponding set of samples (Setup 2-shaken, Setup 2-stirred, Setup 3-shaken, Setup 3-stirred) were used as controls, i.e. without co-solvent application. After co-solvent application, each vessel was closed and incubated at test conditions for 39 days. The oxygen saturation, turbidity, DOC, pH and redox were monitored as described in Section 2.4 and 2.5 respectively.

2.6.2. Step II: investigating the influence of reduced solvent (0.01% V/V) application

Under this step, the effect of acetone concentration on dissolved oxygen and biofilm formation observed was investigated. Based on the results of Step I, Setup 3 with orbital shaking at 80 rpm was selected as the most appropriate test setup for further testing. This step included Setup 3 with and without agitation. The stagnant test Setup 1 was also included in parallel. Three sets of samples were prepared, specifically 1) with 0.01% V/V (acetone) application, 2) with 0.25% V/V (acetone) application and 3) control samples without solvent application. After co-solvent application, each vessel was closed and incubated at test conditions for 28 days. The oxygen saturation, turbidity, DOC, pH and redox were monitored as described in Section 2.4 and 2.5 respectively.

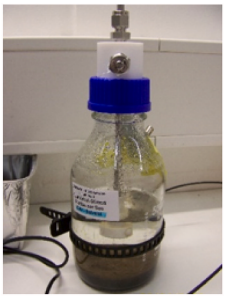
2.6.3. Step III: preliminary biodegradation testing using improved test setup

Water-sediment samples were prepared according to test Setups 1 and 3. The initial ^{14}C-phenanthrene test substance and solvent concentration applied was 0.07 mg/L and 0.01% (V/V), respectively, based on the volume of overlying water. After application, the vessels were immediately closed and incubated under test conditions. In parallel, parameter control samples without test substance were prepared with and without solvent treatment and used for regular monitoring of oxygen in the headspace and water phase and DOC, pH, redox and turbidity of the water phase (after sacrificial sampling). All samples were sacrificed after 7- and 28d of incubation and processed (see Section 2.8).

2.7. Full scale OECD 308 test

Based on the results of the pre-tests, test Setup 3 (as described in Section 2.2 and Table 1) was deemed to be the most suitable test setup for hydrophobic and slightly volatile test chemicals. Therefore, a full-scale OECD 308 test using the improved test setup was carried out. Fresh samples of water and sediment were used for the water-sediment sample preparation. Following pre-incubation of the sample at test conditions for 9 days, ^{14}C labelled phenanthrene was applied with 0.01% (V/V) co-solvent and incubated in test conditions for 103d. The initial test substance concentration for fine and coarse textured sediment samples was 0.082 mg/L and 0.079 mg/L, respectively, considering the volume of overlying water.

Table 1
Test system geometries and agitation conditions of the different test setups used in Step I of this study (SG – Stagnant, SH – Shaken, ST – overhead stirring of water).

	Test Setup 1	Test Setup 2	Test Setup 3
			
Type of flask	Cylindrical	Erlenmeyer	Cylindrical
[a]Vessel diameter Ø [cm]	5.5 cm	10.5 cm	7.5 cm
Sediment amount in dry weight (dW) (g)	50 g	80 g	70–80 g
Sediment height [cm]	Coarse Sed.:2.4 cm Fine Sed.: 3.5 cm	Coarse Sed.:1.4 cm Fine Sed.: 1.9 cm	Coarse Sed.:1.9 cm Fine Sed.: 2.4 cm
Agitation	SG	SH/ST	SH/ST/SG

[a] represents the diameter measurement of the bottom of the flask.

To check for the influence of abiotic processes, sterile samples were prepared (details provided in S2) as well as additional vessels for microbial biomass measurements (DIN EN ISO 14240, 1424), oxygen monitoring and physicochemical parameters (for details see S2).

Parameter and sterile control samples were sacrificed at the start, middle (28 days) and end (103 days) of the test, whereas biotic samples were sacrificed on day 0, 1, 3, 7, 14, 28, 61 and 103 and taken for sample processing and analysis as described in Section 2.8.

2.8. Sample processing and analysis of test samples

At each sampling point, duplicate samples were sacrificed. Before opening the closed test vessels, the headspace was stripped through the Tenax tube using a pump for 5 min while allowing air in from the other end by loosening the screws after 20 s.

2.8.1. Sodium hydroxide traps

After 5 min of headspace stripping, the setup was opened and the NaOH trap was removed. 0.25 mL aliquot of NaOH was taken for liquid scintillation counting (LSC) analysis. If the radioactivity measured in the trap exceeded 5% of applied radioactivity (AR), a confirmation barium chloride test was performed (details on method see S13).

2.8.2. Tenax traps

The Tenax-trap was extracted using 3 × 3 mL acetonitrile. An aliquot of each extract was taken for LSC and further for radio-HPLC analysis.

2.8.3. Water and sediment extraction

Supernatant water from water-sediment test systems was decanted and extracted using petroleum ether (Carl Roth Gmbh) (10% of respective water volume for 3 min) in two cycles. The water extracts were taken for concentration and clean up steps before analysis (See S16) The remaining sediment was transferred into a centrifuge tube and extracted with 4 × 100 mL acetonitrile (1st and 2nd cycle: 30 min shaking, 3rd and 4^{th} cycle: 16 h shaking). The separation of the extracts was performed using centrifugation (1726 g, 10min). Accelerated solvent extraction (ASE) was not performed as a terminal extraction step as some samples at later sampling points showed a recovery of $\leq$ 5% AR. Aliquots from both sediment and water extracts were taken for LSC analysis. If recovery was found to be $\geq$ 5% AR, the extracts were further taken for concentration and clean up steps, followed by radio-HPLC analysis.

2.8.4. Non-extractable residues (NER)

The residual sediment debris obtained after extraction was air dried at room temperature under a fume hood and subsequently combusted using a biological oxidizer Zinsser OX700 to determine the total NER fraction. Three different types of NER have been proposed by Schäffer et al. (2018). Type I NER is the physically entrapped fraction which is potentially remobilizable and poses risk to the environment. Type II NER is the covalently bound fraction and Type III NER is the biogenic fraction; both pose low risk to the environment. Silylation has been proposed as one of the methods for estimating the Type I NER fraction in soil (Kästner et al., 2018) and was utilized in this study for the characterization of Type I NER in sediment residue (for details see SI).

2.8.5. Radio-HPLC

Radio-HPLC analysis was performed using Thermo Fisher Ultimate 3000 coupled with a UV detector (ThermoFisher Ultimate 3000 Photodiode Array detector) and a ^{14}C detector (Raytest Ramona 3, Cell 150 μL). The chromatographic separation was achieved by Phenomenex Luna C18 (2), 150 × 2.0 mm, 3 μm column for the water extracts and Phenomenex Luna C18(2), 150 × 2.0 mm, 5 μm column for the Tenax and sediment extracts. The extracts were analysed for parent test item and metabolites in the radioactive channel. The temperature of the column was maintained at 30 °C. (For details on radio-HPLC method see SI).

3. Results and discussion

3.1. Preliminary tests

3.1.1. Step I: optimization of the test system geometry and agitation conditions

The oxygen measurements in the water phase for the samples from Step I are shown in Fig. 1. Considerable depletion of the oxygen saturation in the water phase was observed in all samples treated with 0.25% V/V acetone, despite the different geometries tested and the different agitation techniques. However, this was expected considering the use of 0.66–0.79 mL acetone, as the complete oxidation of this volume of acetone requires a theoretical oxygen demand (ThOD) of 1.2–1.4 g of oxygen which was significantly higher (factor 45–117) than the total amount of oxygen in the test vessel (see calculation in SI). So, oxygen depletion in the closed system can be attributed to degradation of the solvent which has also been previously reported (Hughes et al., 2020, Shrestha et al., 2020; Leahy and Olsen, 1997; Michaelsen et al., 1992). It is hypothesized that the oxygen-poor conditions in the test system facilitated the formation of an orange biomass tentatively attributed to iron-oxidizing bacteria (Hedrich et al., 2011). Due to agitation of the test system, the orange biomass did not form a biofilm at the air-water interface, but was distributed throughout the water layer (See example picture in S4). However, since the identity of the organisms was not verified, this hypothesis is not confirmed. Overall, aerobic conditions did not improve considerably with the use of wider test system geometries and different agitation conditions when acetone doses $\geq$0.25% V/V were used. It can be assumed that severe depletion of the aerobic conditions would be reached even faster in the stagnant test system. The results of these experiments show that the use of solvent is the main factor contributing to the reduced O_2 concentration in the aqueous phase. Comparison of O_2 saturation in the water phase (Fig. 1) showed that test Setup 3 with the agitation technique “shaken” exhibited relatively higher oxygen saturation compared to the other test setups.

The agitation using shaking approach was more robust, stable, and easy to establish in comparison to the stirred samples. The efficiency of overhead stirring using magnetic stirrers was affected due to the distance between the stir bar and plate. Also, the adjusted rpm in the magnetic stirrer was not consistent across different samples. Further, the use of a higher rpm when stirring could not be employed as this would result in sediment resuspension, which was is to be avoided per the OECD 308 technical guideline requirements.

Turbidity measurements at the end of the test (at 37d) have been given in SI (see S5). Higher turbidity was observed in samples treated with solvent, especially for fine textured sediment, due to the biofilm formation observed. Particularly for the solvent treated samples, the turbidity was inversely related to the DOC measured in water phase (see Figure in S6.4). This further supports the hypothesis that higher turbidity resulted from the formation of a biofilm due to the degradation of the solvent. Lower turbidity was measured in the control samples without solvent treatment, indicating that the agitation technique applied had no or little impact on the sediment resuspension. Furthermore, there was no significant difference in sediment resuspension between the different agitation techniques.

3.1.2. Step II: influence of reduced solvent (0.01% V/V) application

In this step, the effects of reduced solvent concentration (0.01% V/V) on the aerobic condition, biofilm formation and turbidity were investigated using Setup 3. Optical oxygen saturation measurements in the water phase in the fine textured sediment samples are given in Fig. 2.

For stagnant (SG) samples, a sharp drop in oxygen saturation without any recovery was observed in all treatments, irrespective of solvent concentration, hence highlighting limited oxygen diffusion from the headspace into the water phase. In shaken (SH) samples applied with 0.25% V/V solvent, oxygen was completely depleted in the water phase,

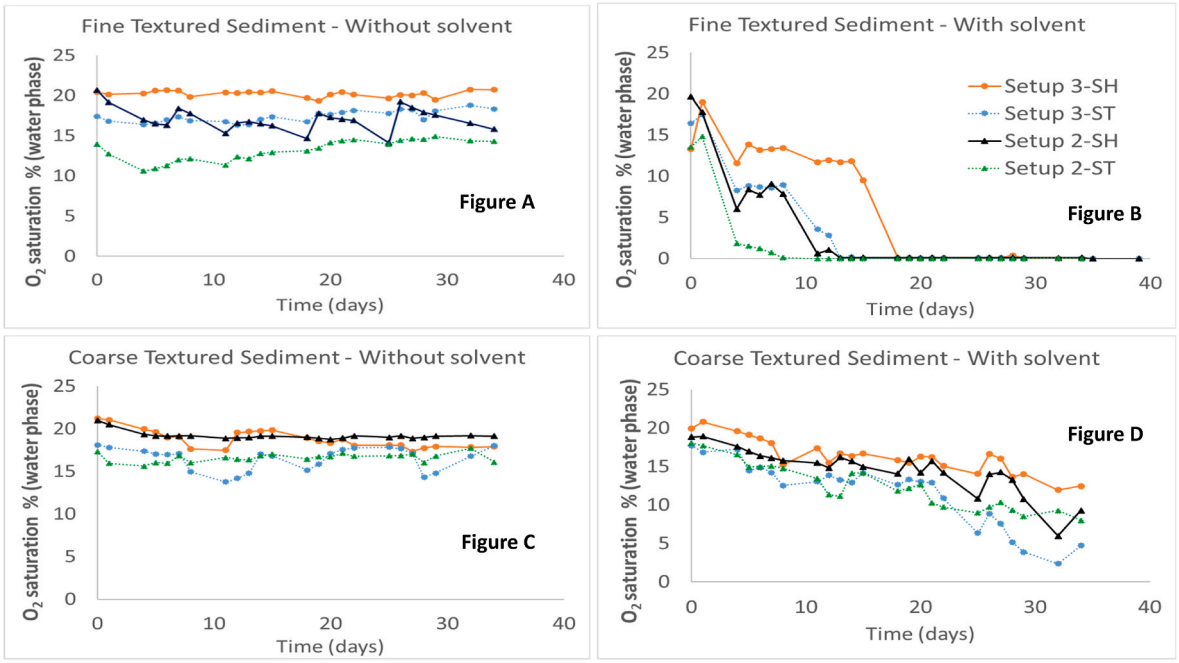


Fig. 1. Oxygen saturation measurements in the water phase using optical oxygen measurements in vessels prepared with fine and coarse textured sediment under Step I. The data plotted are the mean values of the measurements obtained from duplicate samples. Fig. 1A and C shows the samples treated without solvent whereas Fig. 1B and D shows samples treated with 0.25% V/V acetone. Setup 3: cylindrical flask Ø = 7.5 cm, Setup 2: Erlenmeyer flask Ø = 10.5 cm, SH: agitation using orbital shaking at 80 rpm, ST: agitation using overhead stirring of the water phase. The oxygen saturation of 15% measured in the water phase was equivalent to a concentration of 5.9 mg/L of oxygen in the water phase.

confirming findings from step I. However, samples applied with 0.01% V/V solvent only showed an initial decline in oxygen saturation in the first 7 days, after which the concentration remained relatively stable, similar to the control samples. The results clearly show improvement of oxygen saturation in the water phase for agitated samples treated with reduced solvent concentration (0.01% V/V). Biofilm formation was not observed in the samples treated with and without 0.01% (V/V) solvent. A photograph of the samples with different solvent treatment under step II has been shown in SI section S4.

Turbidity measurements at the test start and end (28d) of the test for samples under step II are shown in Fig. 3. As expected, high turbidity was observed in samples treated with 0.25% V/V solvent due to orange biomass formation, described in section 3.1.1. Turbidity measurements for all stagnant and shaken samples not treated with solvent were considerably lower, <3 NPU (nephlometric unit), at the start and end of the test, with the exception of one shaken replicate at test start. This suggests that sediment resuspension in the orbital shaking approach was negligible and comparable with a stagnant setup.

3.1.3. *Step III preliminary biodegradation testing using different test setups*

Preliminary biodegradation testing of ^{14}C-labelled phenanthrene was performed in the Setup 3 (shaken 80 rpm) and in the Setup 1 (stagnant). The test item was applied using a reduced acetone concentration of 0.01% (V/V). Total recoveries ranged between 81 and 99.7% AR for test Setup 3 and 83.8%–101.9% AR for test Setup 1 for both sediment types and at two time points (7d and 28d). For Setup 3, quicker partitioning of the substance from the water to the sediment phase (in both fine and coarse textured sediment) after 7d was observed relative to Setup 1 (see Fig. 4). The quicker partitioning could be attributed to

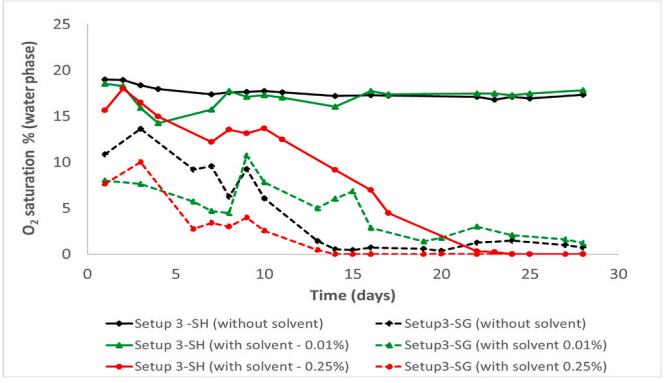


Fig. 2. Oxygen saturation measurements in the water phase in different samples without solvent and treated with 0.01% V/V and 0.25%V/V acetone for fine textured sediment. The figure shows the samples with and without agitation. Setup 3: cylindrical flask with Ø = 7.5 cm, SH: agitation using orbital shaking at 80 rpm. SG: stagnant samples without agitation. An oxygen saturation of 15% measured in the water phase was equivalent to 5.9 mg/L of oxygen concentration in the water phase. The OECD 308 guideline recommends an O_2 concentration of 7–10 mg/L for an aerobic water layer.

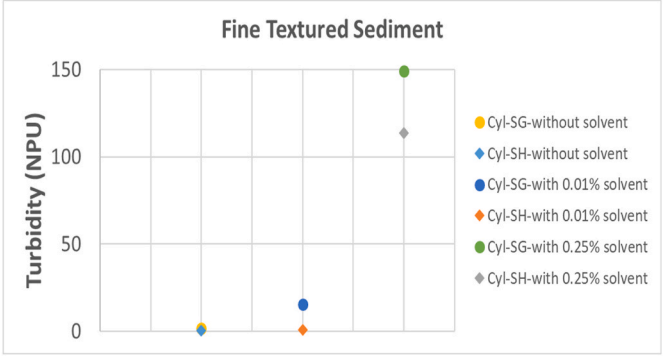


Fig. 3. Turbidity measurements in the water phase of different samples under step II at the test end (after 28d of incubation for fine textured sediment. Setup 3: cylindrical flask with Ø = 7.5 cm, SH: agitation using orbital shaking at 80 rpm. SG: stagnant samples without agitation.

agitation in Setup 3 or/and the use of an increased amount of sediment (70–80 g) in comparison to Setup 1 (50 g). Volatilized fractions measured in Tenax traps were 2.2–4.6% AR and 0.2–1.7% AR for Setups 1 and 3, respectively. The reduced volatilization observed in Setup 3 could have resulted from quicker partitioning of test substance from water to sediment or from faster mineralization of the test substance.

At 28d, higher mineralization and NER formation was observed in Setup 3 in comparison to Setup 1. For fine textured sediment, 36.7% AR mineralization and 37.6% AR NER were observed in Setup 3 in comparison to 16.5% AR mineralization and 6.1% AR NER for Setup 1. For coarse textured sediment, 16.8% AR mineralization and 31.7% AR NER were observed in Setup 3 in comparison to 14.2% AR mineralization and 10.2% AR NER for Setup 1 (see Fig. 4). The higher NER formation in both sediments observed after 28d in Setup 3 was further investigated (see section 2.8.4 and 3.2.1). Higher mineralization was attributed to higher degradation in Setup 3, resulting from improved oxygen saturation in the water phase. The oxygen saturation in the water layer in Setup 3 with agitation was stable and close to the oxygen concentration recommended by OECD 308 guideline for both sediment types (7–10 mg/L).

3.2. Full scale OECD 308 tests

3.2.1. Degradation, distribution and test conditions in water-sediment system

The degradation and distribution of phenanthrene in water-sediment systems using fine and coarse textured sediment is shown in Fig. 5,Tables 3 and 4(in S6). The total recoveries ranged from 81.1 to 112.6% AR for fine textured and 76.1–108.1% AR for coarse textured sediment. Notably, recoveries of <90% were encountered in the samples at later sampling points (28 -103d). However, no recovery problems were observed in sterile samples (See S8) where volatile losses of parent are more likely. These results indicate that the incomplete mass balance in the non-sterile samples was likely not due to loss of parent test item from the test system as incubation conditions were similar for both.

Similar to the pretests (Section 3.1.3), partitioning processes of the test substance from the water to the sediment phase was faster in comparison to previous studies (Shrestha et al., 2020). Almost 56–57% AR could be recovered as sediment extract after 1d and up to 73–75% AR was reached after 7d incubation for both sediment types. After this, a decline in the sediment extractable fraction was observed, likely due to the increase in mineralization and NER fraction.

Mineralization increased throughout the incubation time and reached 41.99% AR for fine and 28.43% AR for the coarse texture sediment after 103d. Higher mineralization in comparison to previous studies (Shrestha et al., 2020) (11.6 and 14.2% AR in fine and coarse textured sediment after 100d of incubation respectively) was observed. Additionally, no considerable deviations between the replicates was observed compared to the previous study (Shrestha et al., 2020). Higher mineralization was attributed to improved aerobic conditions in the water phase. The oxygen monitoring data (S10) also shows stable oxygen concentrations in the water phase for both fine and coarse textured sediment throughout the study. For some fine textured sediment samples, errors in the oxygen monitoring data were observed due to obstruction of the sensor spots by the sediment layer. This obstruction was caused by an increase in the sediment height (by 1.9–2.1 cm) and volume (picture in S9) and was not observed for the coarse textured sediment vessels. Water phase oxygen measurements in the parameter samples using electrodes support, however, that aerobic conditions (7.77–8.11 mg/L without solvent treatment and 6.90–8.11 mg/L with solvent treatment) were maintained throughout the test. Unlike in the preliminary tests, turbidity measurements, especially for fine textured sediment in the full-scale tests, was slightly higher with high deviation between replicates. However, only a few replicates showed higher turbidity measurements when compared to the turbidity measured (1.42–18.8 NU after 28d) in the step 2 stagnant samples. It should be noted that different sediment batches were used between these tests. This suggests that sediment resuspension in the full-scale test for fine textured sediment was slightly higher than in the pre-test. Turbidity measurements for the coarse textured sediment were relatively lower and considerably more consistent throughout the study.

Almost no volatile fraction (<0.1% AR) was found in Tenax tubes extracts, as observed in the pre-tests (section 3.1.3). An increasing trend of NER formation was observed followed by a gradual decline towards the end of the study. The highest NER formation (35.5% AR) was observed at 28d for fine textured sediment and 61d for coarse texture sediment (47.36% AR). In contrast, NER formation in the sterile samples at the end of the study (103d) was 10.6% AR and 4.36% AR for the fine and coarse textured sediment respectively (see SI section 6.7). Due to high NER formation observed in non-sterile samples using the improved test setup, it was decided to further investigate the type I NER fraction. Silylation was performed with an aliquot of the sediment residue after extraction and the extract was analysed by radio-TLC. The results on the silylation extracts and the amount of identified parent in the silylation extract is given in SI (See S7). The highest amount of parent residue in Type I NER was 12.52 %AR at 14d for fine textured and 9.66 %AR at 28d

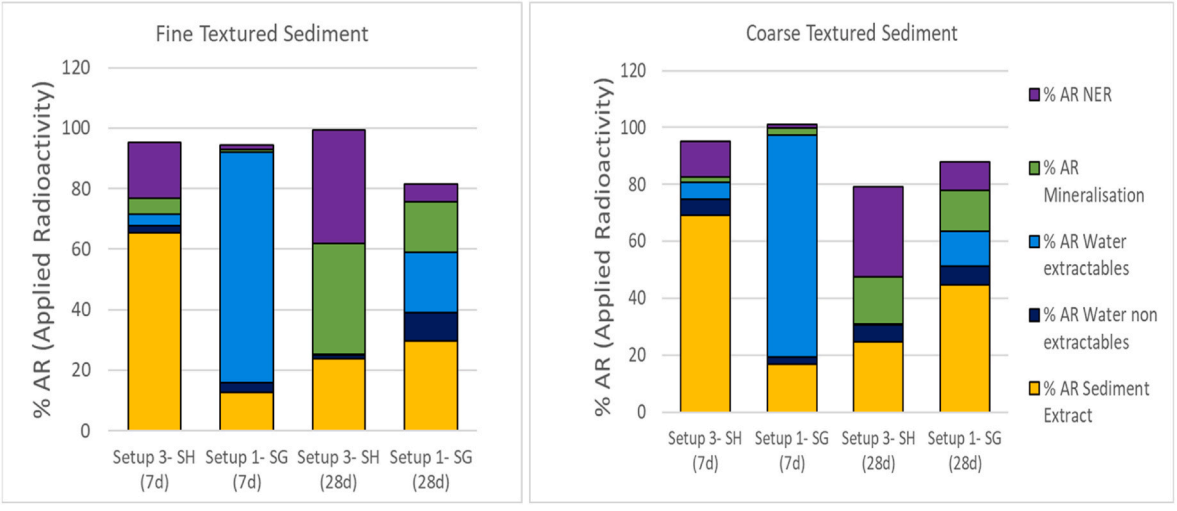


Fig. 4. Distribution of radioactivity and mass balance for the degradation of phenanthrene in the stagnant (SG) test Setup 1 and shaken (SH) test Setup 3 for the fine and coarse textured water/sediment system. The results are given for two sampling points of 7d and 28d.

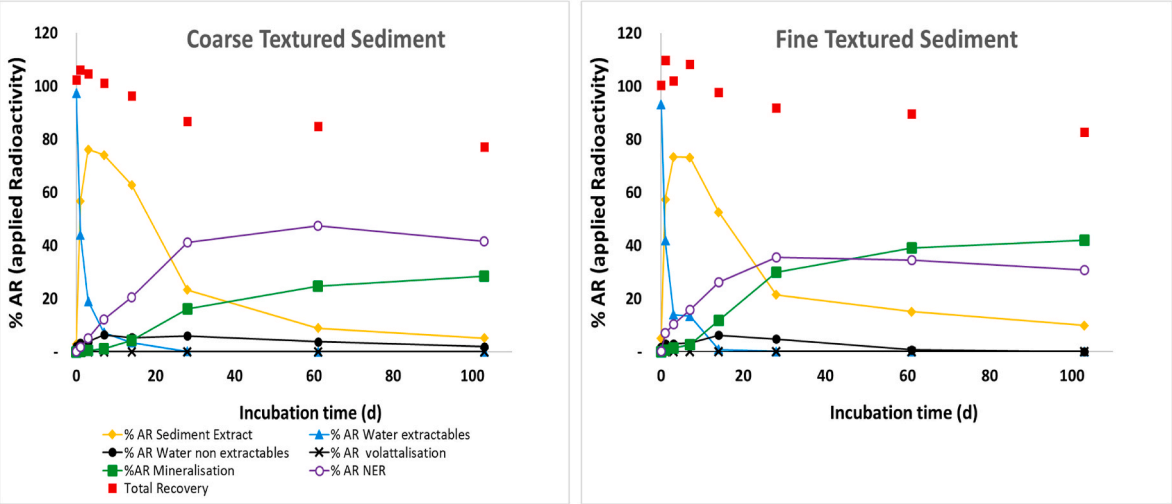


Fig. 5. Degradation and distribution of radioactivity for ^{14}C labelled phenanthrene in fine and coarse textured water-sediment system in a full scale OECD 308 test. Different pools of radioactivity (Sediment extract, water extractable and non extractables, volatilization, NER, Mineralization and total recovery) has been expressed based on the initially applied radioactivity (%AR).

for coarse textured sediment. This suggests that a considerable fraction of NER was also either Type II or Type III NER. The high observed mineralization (41.99 %AR for fine and 28.43 %AR for the coarse texture sediment) also corroborates the formation of Type III NER (Kastner et al., 2014; Nowak et al., 2011, 2013; Poβberg et al., 2016; Trapp et al., 2017).

3.2.2. *Degradation kinetics*

Degradation kinetics were calculated based on data from the full scale 308 test. Only Degradation half-lives ($DegT_{50}$) for the total system, i.e. the total degradation from both water and sediment phases, was calculated for this study, since it is considered a more robust and reliable parameter for degradation instead of compartmental specific half-lives (Honti and Fenner, 2015; Rauert et al., 2014; Shrestha et al., 2016; Honti et al., 2016). The $DegT_{50}$ was calculated for this study using the CAKE software version 3.2 (Tessella Ltd., 2015). Four different models were used for fitting the degradation data (i.e., SFO = Single First Order, DFOP = Double First Order in Parallel, HS: Hockey Stick and FOMC = First Order Multi Compartment). When calculating the DegT50, three different scenarios were considered for NER treatment.

a) $DegT_{50}$ based on the fraction of extractable parent compound, considering the NER-content as degraded;
b) $DegT_{50}$ based on the sum of extractable parent and the total activity determined in silylation extracts;
c) $DegT_{50}$ based on the sum of extractable parent and the identified fraction of parent compound in the silylation extracts.

The $DegT_{50}$ calculated from all three different scenarios using the four fitting models is provided in S14. The hockey stick (HS) models showed the best performance as indicated by the small chi (Commission regulation (EU), 2011)-errors (full results in SI). The $DegT_{50}$ ranged from 15.6d (scenario a) - 20d (Scenario b) and 19d (scenario c) - 21.5d (scenario b) for different NER treatment scenarios using the HS model for fine and coarse textured sediment, respectively.

4. Implications

This study shows that when using a closed test setup for OECD 308 tests, agitation of overlaying water and the test item application using low co-solvent volume is critical for maintaining an aerobic water layer. When using closed setups, the OECD 308 guideline recommends stirring of the water layer without causing any sediment resuspension. This stirring of the water layer is necessary for faster oxygen diffusion from headspace to water layer which is necessary to maintain aerobic water conditions during the duration of the test. However, this requires special test equipment which is not standard and might not be easily adapted by regular labs. We have demonstrated in this study that orbital shaking (at 80 rpm) of the water-sediment system could also be applied for maintaining an aerobic water layer with limited/no sediment resuspension. This approach could be easily established in regular labs and can be standardized.

Although the purpose of the improved closed test setup developed in this study was to test slightly volatile chemicals, no considerable volatilization (for e.g >5% AR) was observed for phenanthrene. Lower volatilization was attributed to faster degradation and rapid partitioning of the test item into the sediment phase using the improved test setup. Hence, the question if the improved test setup can be applied for other highly volatile and slowly degrading test item is a subject of future research.

Our studies have shown that the use of different closed setup modifications has significant impact on the outcome of OECD 308 studies. Modifications are necessary in some cases to maintain test conditions required by the guideline. In other cases, modifications can also reflect different environmental scenarios, influencing partitioning and positively or negatively impacting degradation. For example, we observed significant differences in phenanthrene degradation with tests performed with 0.01% v/v solvent application with different setups. So, expert judgement is required to check if data obtained from such tests is fit for fate and risk assessment.

Standard testing methods, like the OECD test guidelines, facilitate global comparison of chemicals assessment data. This paper offers insights into test system modifications required to test slightly volatile substances in the OECD 308 test system. These modifications allow the comparison of test results to half-lives for non-volatile substances. Under EU REACH, the comparison of derived half-lives to persistence criteria results in the assessment of chemical persistence. Therefore, it is critical that utilized test methods generate outcomes that are comparable across all classes of chemicals.

Author contribution

Prasit Shrestha: Conceptualization, Methodology, Data curation, Investigation, Writing- Original draft preparation. **Christopher B. Hughes.**: Conceptualization, Supervision, Data curation, Investigation, Writing- Reviewing and Editing, Project administration. **Louise**

Camenzuli: Supervision, Investigation, Writing- Reviewing and Editing, Project administration. **Delina Lyon**: Project administration, Investigation, Writing- Reviewing and Editing. **Boris Meisterjahn**: Conceptualization, Supervision, Investigation, Writing- Reviewing and Editing. **Thomas Hennecke**: Methodology, Investigation, Data curation, Visualization. **Megan Griffiths** Investigation, Data curation, Writing-Reviewing and Editing. **Dieter Hennecke**: Conceptualization, Supervision, Funding acquisition, Investigation, Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138294.

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