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Review

Biodegradation testing of volatile hydrophobic chemicals in water-sediment systems — Experimental developments and challenges

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HIGHLIGHTS

- Biodegradation of volatile chemicals in water-sediment systems using closed setup.
- Complete mass balance obtained for range of volatile chemicals.
- Co-solvent application influences the degradation of test chemicals in closed setup.

GRAPHICAL ABSTRACT
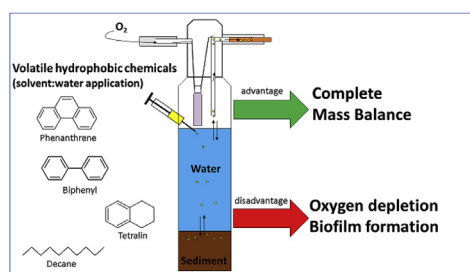
ARTICLE INFO

ABSTRACT

Degradation data are crucial for the persistence assessment of chemicals and they are generated using standard OECD guidelines. The OECD 308 describes a simulation biodegradation test of chemicals in water-sediment systems. This guideline is not applicable for testing highly volatile chemicals and recommends a closed biometer test setup for testing slightly volatile chemicals. However, proper details on system geometries, construction and monitoring of aerobic conditions are not provided. The choice of system geometry and sediment:water ratio influences the partitioning of test chemicals between different compartments (water, sediment and headspace) and can therefore affect their degradation. The guideline recommends the addition of test chemical via aqueous solutions, which however is not possible for hydrophobic volatile chemicals due to their volatilization losses and low solubility. Thus, the use of a co-solvent is necessary for the application of such chemicals but its effects in a closed setup has not been studied. We recently developed an improved closed test setup for testing volatile chemicals in soil. The objective was to adapt this improved test setup to conduct OECD 308 tests using ^{14}C labelled chemicals with different volatilities. Using the adapted test setup it was possible to obtain a complete mass balance even for n-decane and tetralin having the highest Henry's constants of the tested chemicals. However, the use of co-solvent affected the oxygen levels, which in turn affected microbial activity and likely also the degradation of test chemicals. Therefore, the adapted test setup needs further developments for the testing of volatile hydrophobic chemicals.

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Contents

1. Introduction

Biodegradation data are necessary and play a crucial role during hazard and risk assessment of chemicals under different regulatory frameworks (Commission regulation (EU), 2011; Regulation (EC), 2009; Regulation (EU), 2012; Guideline on the environmental, 2006; Revised Guideline, 2009). As a part of the chemical safety assessment under the REACH regulation, an assessment is performed to identify Persistent, Bioaccumulative and Toxic (PBT) and very Persistent, very Bioaccumulative (vPvB) substances. The assessment of persistence (P) is based on data indicating the potential for the chemical to be removed from different environmental compartments through various degradation processes, of which biodegradation is typically a key removal process. Biodegradation data for these regulatory purposes are often generated using tiered laboratory biodegradation tests, according to OECD test guidelines (OECD, 1995; OECD, 2006; OECD, 2017; ECHA, 2017; Kowalczyk et al., 2015). OECD 308 is an example of a higher tiered biodegradation test which has been extensively used to generate degradation kinetics data in water-sediment systems (OECD, 2002a). However, there are certain chemicals whose physicochemical properties render them difficult to test. In particular, volatile hydrophobic chemicals are especially difficult to test, and outside the current scope of the guidelines (Brown et al., 2018; Shrestha et al., 2019; Birch et al., 2017). Hydrocarbons, such as those found in petroleum, are a broad class of chemicals whose physicochemical properties span many orders of magnitude. They are typically hydrophobic and a large subset is also volatile. The combination of hydrophobicity and volatility makes these chemicals particularly difficult to test in aqueous systems like OECD 308 due to challenges dissolving the chemicals in water and avoiding evaporative losses that lead to incomplete mass balances. The standard OECD 308 test requires the application of the test chemicals directly to the water phase and ideally recommends aqueous application solutions. However, this is not possible for these chemicals due to their low solubility in water and losses due to volatilization from water. Alternatively, the guideline allows the use

of solvents like acetone and ethanol, with the stipulation that the total amount of solvent introduced in the test system should not exceed 1% v/v and should not have adverse effects on the microbial activity of the test system. However the effect of co-solvent concentrations on the biodegradation kinetics might be more pronounced in closed tests that minimize the evaporative losses of the solvent, which in turn asks for detailed investigations of the co-solvent effect in the new closed test systems.

The OECD 308 guideline states that it should not be used for chemicals which are highly volatile from water. It is considered applicable to slightly volatile chemicals, although the criteria for defining such compounds are not specified. For testing slightly volatile chemicals, a biometer-type (closed) test setup is recommended, but a detailed description of the system geometries and construction of the test setup is lacking (OECD, 2002a). Honti and Fenner (2015), pointed out the influence of varying system geometries and sediment:water (S:W) ratios on the partitioning and degradation of non-volatile chemicals in such tests. These variables alter the headspace volume in the test setup, which is expected to affect the extent to which volatile chemicals will partition into the headspace and hence be unavailable for degradation.

Other issues related to the sample processing of volatile chemicals have not been explicitly described within the guideline. Losses of parent chemical during sample processing may bias results. Finally, in addition to the existing data treatment and interpretation issues in the context of obtaining robust degradation data from the OECD 308 test (Kunkel and Radke, 2008; Bowmer and Leopold, 2004; Shrestha et al., 2016; Rauert et al., 2014; Honti and Fenner, 2015; Honti et al., 2016; ECETOC, 2010; ECETOC, 2013), accounting for volatilization in these tests is a further potential complication that has not been discussed in the guideline.

Recent degradation studies with soil (OECD 307) (OECD, 2002b) and pelagic surface water (OECD 309 (OECD, 2004) like suggest improved closed test setups which makes it possible to generate reliable degradation kinetics data also for highly volatile chemicals (Shrestha et al., 2019; Birch et al., 2017). We wanted to extend the applicability of these recent developments in the context of water-

sediment tests (OECD 308). OECD 308 can be performed as a completely anaerobic biodegradation test using nitrogen streaming of the headspace. The focus of this study was however to perform an 'aerobic' degradation test, where the headspace and water phase is kept aerobic even though an anaerobic sediment zone in the water-sediment system is unavoidable (Shrestha et al., 2016). Our objectives were 1) to use the recently developed closed setup (Shrestha et al., 2019) to conduct water-sediment tests and check its suitability, 2) to check the applicability of the adapted setups to carry out a full scale OECD 308 study for a range of volatile chemicals and understand the underlying processes, and 3) to suggest other appropriate data reliability measures in addition to/instead of the current complete mass balance requirement. First, in order to check the suitability of the adapted test setup, a preliminary test was carried out with a semi volatile test chemical (phenanthrene) and a non-volatile reference chemical (sodium benzoate), which were both tested in a standard flow-through and adapted closed test setup and the results compared. The adapted test setup was then used to conduct full-scale OECD 308 studies for four different chemicals with varying volatility ranging from slightly to highly volatile chemicals (see section 2.1) in two different water-sediment systems. In addition, a non-volatile chemical (benzo[a]pyrene) was tested with the standard flow-through setup. The data obtained were used to check the suitability of the tests for generating reliable biodegradation data for hydrocarbons, and understanding the underlying processes like sorption, volatilization and degradation.

2. Materials and methods

2.1. Test and reference chemicals used

In total five ^{14}C labelled test chemicals and one reference chemical were used during different tests performed within this project: 1) benzo[a]pyrene, ($C_{20}H_{12}$, >98% purity, sP. Radioactivity 3.87 kBq/μg, ARC Inc.), 2) phenanthrene, ($C_{14}H_{10}$, >98% purity, sP. Radioactivity 3.71kBq/μg, Hartmann Analytics), 3) biphenyl, ($C_{12}H_{10}$, >99% purity, sP. Radioactivity 7.98kBq/μg, ARC. Inc.), 4) tetralin, (1,2,3,4-tetrahydronaphthalene, $C_{10}H_{12}$, >98% purity, Hartmann Analytics), and 5) decane, (n-decane, $C_{10}H_{22}$, > 98% purity, BlyChem). The test chemicals were selected to cover a broad range of air-water partitioning and sorption properties with their Henry's law constant (K_H) values following the order decane (K_H 522 000 Pa m^3 mol^{-1}) > tetralin (K_H 138 Pa m^3 mol^{-1}) > biphenyl (K_H 31.2 Pa m^3 mol^{-1}) > phenanthrene (K_H 4.29 Pa m^3 mol^{-1}) > benzo[a]pyrene (K_H 0.0463 Pa m^3 mol^{-1}) and their Koc values following the order benzo[a]pyrene (Koc 208800) > decane (Koc 22270 L/kg) > phenanthrene (Koc 7421 L/kg) > biphenyl (Koc 3019 L/kg) > tetralin (Koc 1068 L/kg) (National Food Institute DTU, 2017). A separate pre-test was performed (See section 2.7) using a reference chemical, sodium benzoate ($C_7H_5NaO_2$, >99% purity, sP. Radioactivity 33.37 kBq/μg, ARC inc.).

2.2. Water and sediment used

OECD 308 can be used to perform biodegradation tests with both freshwater and marine water. This study focuses on freshwater biodegradation tests. Two types of water-sediment were used in the tests as required by the OECD 308 guideline. A sediment with fine texture and high organic carbon (OC) content (Sand: 14.4%, Silt: 64.2% Clay: 21.4%, OC: 2.9% pH: 4.7) was sampled at an artificial lake (Biggesee, 51° 6′ 37″ N, 7° 53′ 45″ E NRW, Germany) and a sediment with coarse texture and low OC content (Sand: 80%, Silt: 10.3%, Clay: 9.8% OC: 0.6% and pH: 6.0) was sampled at a quarry pond (Nesthauser See 51° 44′ 54.55″ N, 8° 40′ 32.46″ E, NRW,

Germany). For the benzo[a]pyrene test, instead of sampling from Biggesee, the fine texture sediment (Sand: 32.5%, Silt: 52.2%, 15.3%, OC: 3.1%, pH: 8.07) was sampled from a different artificial lake (Hennesee, 51°17′40″ N, 8°15′43″ E NRW, Germany). The sediments were sampled from the surface to a depth of 5−10 cm as recommended in OECD 308 and were sieved through 2 mm sieves before sample preparation. The sediment texture and TOC content were determined using standards ISO11277 (Soil quality, 2009) and DIN EN15936:2012−11 (DIN EN 15936:2012−11, 2012), respectively.

2.3. Water-sediment sample preparation

50 g of sieved sediment (dry weight basis) were filled into a 500 mL cylindrical test vessel (V = 500 mL and Ø = 5.5 cm) followed by the addition of the collected surface water from the respective sampling sites. The different textures of the two sediments lead to considerable differences in volumes occupied by the 50 g sediment. In order to reduce the difference in headspace volume and water phase volume of the two sediment types, a S:W volume ratio of 1:3 was used for the high OC and 1:4 for the low OC sediment sample. The heights of the sediment and water columns were thus 3.5 cm and 10.5 cm for the high OC sediment and 1.6 cm and 6.4 cm for the low OC sediments, respectively. After adding the water, the test vessel was gently shaken by hand to get a plain sediment surface at the water-sediment interface. The suspended particles in the overlaying water phase were allowed to settle down and the samples were pre-incubated for 7 days (12 days for benzo[a]pyrene) without closing the sample bottles at test conditions (20 °C in the dark). After the pre-incubation of the water-sediment sample, ^{14}C-labelled test chemicals were applied to the samples.

2.4. Preparation and application of test chemicals

The 308 guideline states that test chemicals should be applied directly to the overlaying water phase of the water-sediment sample. During method development work it was found that some test chemicals (benzo[a]pyrene, phenanthrene, biphenyl, tetralin) tended to immediately precipitate if applied in a solution of pure acetone to the water phase. In order to avoid this, the application solutions for these chemicals were prepared as acetone-water co-solvent mixtures instead of pure acetone. A further complication was that the use of lower solvent:water ratios would cause the test chemicals to either precipitate or volatilize from the application solution and this had to be avoided. The test chemicals were purchased as solutions dissolved in acetone, the required volume of stock solution was withdrawn for the test and dissolved in acetone and then diluted with UHQ water. The final acetone:water ratios of the application solutions were 1:1 (for benzo[a]pyrene), 1:5 (for phenanthrene and tetralin) and 1:7 (for biphenyl). The use of solvent:water ratio was usually based on the hydrophobicity of the test chemicals. However, for tetralin a higher solvent:water ratio was used in order to prevent its volatilization from the application solution. For decane the stock solution was only available in methanol, and huge losses were observed during preparation of the application solution as a solvent:water mixture. Therefore, in order to prevent the loss of ^{14}C-labelled test chemical during solvent exchange steps, it was decided to apply decane directly using pure methanol while keeping the methanol volume to a minimum. The concentration and purity of each application solution was confirmed using Liquid scintillation counting (LSC) and radio-High performance liquid chromatography (radio-HPLC) analyses prior to application of the test chemicals to the water-sediment samples. The test chemicals were applied directly to the water phase of the water-sediment system. After application, the samples were closed and connected according to their respective

test setups (See section 2.5) and incubated at test conditions.

The starting test concentrations for both (high and low OC) water-sediment samples were set to 0.1 mg/L in the water phase. Due to higher volume of overlying water for the fine texture/high OC sediment compared to the other sediment system, a higher amount of chemical application was necessary to achieve the target concentration. The application volume and the amount of radio-activity applied per sample are listed in Table S18.

2.5. Test setups

A flow-through setup was employed during preliminary tests for phenanthrene and the reference chemical, and for the full-scale 308 test of benzo[a]pyrene. The flow-through setup consisted of the water-sediment samples connected to a series of absorption traps containing 100 mL of different trapping solutions (for details on the used flow-through setup see Shrestha et al., 2019).

The closed incubation test setup was used to perform full scale OECD 308 studies for all test chemicals other than benzo[a]pyrene. The setup was prepared by closing the respective test vessel with a special insert containing a tenax adsorption tube for trapping the volatile fraction and a 6 mL sodium hydroxide (NaOH) for trapping of the mineralized fraction ($^{14}CO_2$) flask as described in previous studies (Shrestha et al., 2019). In order to avoid the loss of test chemical itself due to volatilization, which would have most likely occurred during purging of possibly formed CH_4 from the sediment, the setup was not designed to trap CH_4. The test setup was completely closed using a plastic free lock system (see Fig. S1). Sterile samples were prepared to measure the influence of abiotic degradation. Parameter and solvent control samples were prepared to see the change in pH, O_2, redox and microbial biomass during the incubation period, in the presence and the absence of the solvent used for application of test chemicals. Headspace oxygen monitoring samples (see section 2.6) were also prepared and incubated under identical conditions. For details on the sample preparation of each of these respective samples see S3.

2.6. Headspace oxygen monitoring of samples

In order to monitor oxygen levels in the closed flask test setup and maintain an aerobic water phase, the oxygen concentration in the headspace was monitored using a Fiber optic oxygen meter (Firesting O_2, Pyroscience) without the need to open the vessel. Every week all samples, with exception of the sterile samples, were aerated with pure oxygen through a tube connected to the normally closed sample inlet until 18−20% oxygen saturation in the headspace was reached. The air leaving the sample flask during the aeration procedure was passed through the tenax tube. The aeration procedure was tested on the reference sample prior to application on the test samples. In addition, oxygen concentration was measured in the water phase in the parameter samples at the start, middle and end of the test (See S3).

2.7. Reference samples

In order to facilitate a comparison of degradation results obtained in a closed flask setup versus a flow-through setup, a non-volatile chemical (^{14}C labelled sodium benzoate) was tested under both conditions. Only high OC sediment was chosen for this test and four water-sediment samples were prepared. The samples were pre-incubated for 7 days (at 20 °C in dark conditions) and then applied with the reference chemical. After application two of the samples were connected in a flow-through setup and the other two in a closed flask setup. At regular intervals the flask containing NaOH in both test setups were replaced with fresh NaOH solutions

without sacrificial sampling. An aliquot from the NaOH solution was taken to determine the extent of mineralization using LSC.

2.8. Sample processing and chemical analysis

At each sampling point, two samples were sacrificed and were taken for sample processing steps. For the closed setup, the head-space air was stripped out using a pump and only afterwards the sample bottle was completely opened to sample the flask containing NaOH and tenax tubes for subsequent analysis. The overlaying water phase was carefully removed using a pipette and then extracted (three times) by liquid/liquid extraction in a separating funnel with dichloromethane (in the benzo[a]pyrene and phenanthrene experiments) or petrolether (in biphenyl, tetralin and decane experiments, volume applied in extraction representing 10% of respective water phase volume). The remaining sediment phase was completely transferred to a glass centrifuge tube by rinsing the inner walls of the sample vessels using 80 mL acetonitrile. The resulting mixture was taken for shaking extraction (for details see S11). Extracted sediment material was air-dried and aliquots combusted in an oxidizer (Zinsser OX700) in order to determine the amount of non-extractable residues (NER). Radio-activity in the extracts was determined by LSC. The distribution between the parent and metabolite fractions in the sediment and water extracts was determined either by radio-Thin Layer Chromatography (TLC) or radio-HPLC (for details see S4−S6). For extracts with low concentrations which could not be analyzed using radio-TLC or HPLC, gas-chromatography coupled to mass spectrometry (GC-MS) was used for the analysis of the parent fraction. If necessary, the extracts were concentrated by evaporation of the solvent under a gentle stream of nitrogen.

3. Results

3.1. Preliminary tests

The test with phenanthrene using a flow-through setup (low OC sediment) showed a total mass balance/recovery of just 55.5% after 7 d of incubation (see Fig. 1B). The missing radioactivity was recovered later in the tube connectors used in the flow-through setup that were made of silicone and was identified as being 100% parent. This test clearly illustrates that the flow through test setup is not suitable for semi-volatile test chemicals.

In the closed flask setup, a complete mass balance of 100% after incubation for 14 d with almost no volatilization was observed. This suggested that the volatilization in the flow-through setup was due to continuous exchange of the headspace air, which resulted in stripping of the test chemical from the matrix.

For the reference chemical, sodium benzoate, the observed mineralization in the closed and open incubation setups showed no significant differences (Fig. 1A) with almost 50% mineralization after 19 days for both setups. This suggests that the use of a closed test setup instead of the flow-through setup does not influence the degradation of non-volatile chemicals. However, another important difference between the reference samples and test samples was that no solvent was used for the application of the reference chemical, whereas this was the case for the test chemicals. In the closed flask test applied with the reference chemical, a thin film of biomass floating on the surface of the overlaying water phase was observed. This was not observed during the experiment with the flow-through test setup.

3.2. Full scale 308 studies − mass balances

An average mass balance/recovery of 93.7% ± 8.6 (N = 32) was
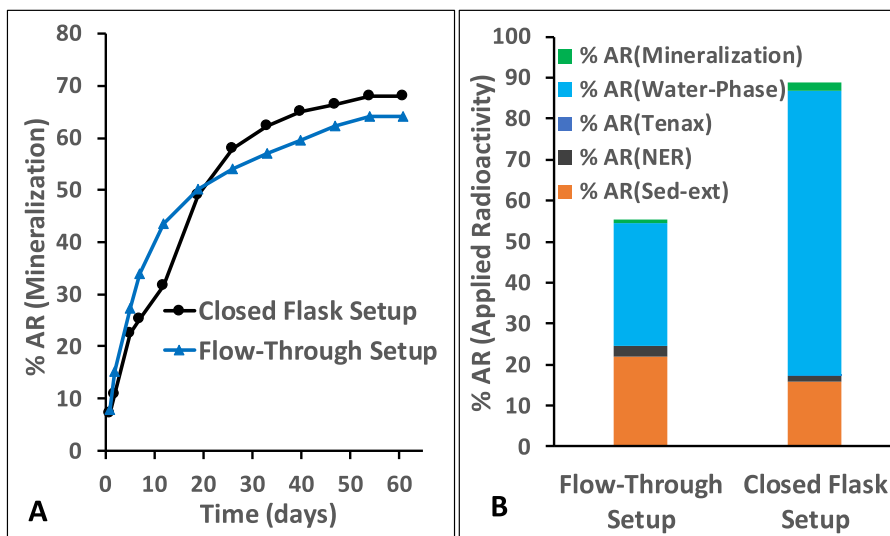
Fig. 1. A) Mineralization of reference chemical (sodium benzoate) observed in high OC sediment in flow-through and closed setup. B) Distribution of phenanthrene in different pools in low OC sediment after 7 days of incubation.

obtained for the non-volatile benzo[a]pyrene study using the flow-through setup. Comparably good recoveries were found in the full scale OECD 308 studies carried out using the closed flask test setup. The average mass balance/recovery observed was $91.3\% \pm 7.3$ (Standard Deviation, S.D., and total no of samples, N = 48), $99.4\% \pm 7.0$ (N = 46), $86.4\% \pm 6.93$ (N = 50) and $93.2\% \pm 9.9$ (N = 44) for phenanthrene, biphenyl, tetralin and decane, respectively. These results suggest that the mass balances obtained using this new optimized test setup, were reliable and reproducible for the range of volatile chemicals tested and in line with the guideline requirements.

3.3. Phase transfer processes

Fig. 2 shows the dissipation from the water phase and volatilization of parent chemicals, during the test in high OC and low OC water-sediment systems. Not shown in the figure, the dissipation of decane from the water phase was extremely rapid in both sediment types in comparison to the other test chemicals and was attributed to its higher volatilization (Fig. 2C and D) and faster degradation. The observed dissipation of the other test chemicals was comparable for both sediment types, despite the differences in volatilization, degradation and sorption properties for these individual
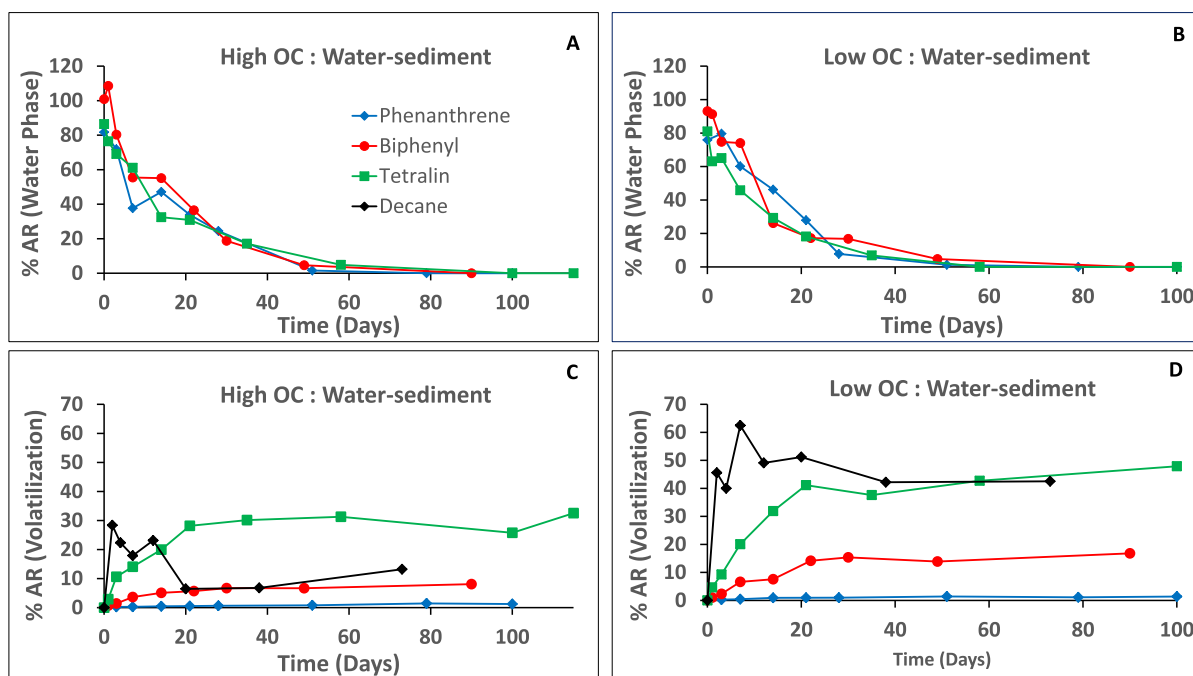
Fig. 2. Different pools of radioactivity (A and B: Water phase dissipation; C and D: Volatilization) resulting from partitioning and degradation processes for all four test chemicals (phenanthrene, biphenyl, tetralin and decane) in the high and low OC content water-sediment systems used during the study. All radioactivity fraction pools are calculated as a percentage of the initial applied radioactivity (%AR).

chemicals. In general, higher volatilization is expected for low-OC sediment systems (Shrestha et al., 2019; Burkhard and Guth, 1981; Chiou and Shoup, 1985; Alvarez-Benedi et al., 1999), which was clearly observed for biphenyl, tetralin and decane (Fig. 2C and D). Contrary to expectations, greater and faster partitioning of test chemicals into the sediment phase from the water phase was observed for the low OC sediment than the high OC sediment. While this may be partially explained by faster degradation in the high OC sediment system, even in cases where mineralization and volatilization between the two systems was comparable (e.g. in the phenanthrene test) the partitioning to sediment was greater and more rapid in the low OC sediment system. This indicates that in such a stagnant system as used for these studies, the dissipation from the water phase to the sediment phase is not only driven by partitioning coefficients but also significantly by the rate of diffusion, as the height of the water column differed by a factor of 1.36 between the high OC and the low OC water-sediment systems. Thus, the choice of system geometry, headspace volume and the height of the water column, as influenced by sediment characteristics and the S:W ratio, is highly significant for the phase transfer processes within the test setup.

Similar to the preliminary tests, no volatilization of phenanthrene was observed in the full studies conducted in the closed setup (see Fig. 2C and D). For the other substances tested, the volatilized fractions were extracted and identified as 100% parent substance by radio-HPLC. Thus, only the parent chemical was volatilized and remained unavailable for degradation. As the measurement of biodegradation is the primary goal of these tests, other measures could be applied to reduce overall volatilization of the test chemical. One possible option, apart from decreasing the headspace volume in the test setup, would be to look at alternative methods of application of test chemicals, such as passive dosing (Birch et al., 2017), or direct application of the test chemical into the sediment phase rather than to the water phase. Direct spiking of the sediment is currently not permitted in the test guideline, but such application techniques have been developed for sediment toxicity testing, and have been suggested as potential solutions to testing challenging substances in the context of OECD 308 studies (Guidance on information, 2017). The methodological challenges associated with direct sediment spiking, and its effects on sorption and biodegradation of test chemical still need further research. In the decane study, a clear sign of re-partitioning of the volatilized fraction from the tenax was observed for the high OC sediment system.

With the exception of decane, the parent fraction in the sediment extract was higher in the low OC sediment for all substances. The higher amounts of parent chemicals extracted from the low OC sediments can be explained by the lower microbial biomass and subsequent degradation observed in this sediment type. Similarly, lower NER formation was seen in high OC sediments, compared to low OC sediments, apart from for benzo[a]pyrene where the NER fractions were similar in both sediments. This might be explained by the shorter overall residence time of test chemicals in high OC sediments due to a) lower fraction of parent chemicals in the sediment phase and b) faster degradation of test chemicals in this sediment type.

3.4. System parameters and observations

In an attempt to maintain aerobic conditions in the water phase of the test setup, the headspace of the closed flask setup was oxygenated every week (See section 2.6). Fig. 3 shows an example of the headspace oxygen depletion with time in the test samples using the closed setup. Almost no oxygen depletion in the headspace was observed for samples treated without solvent. In samples
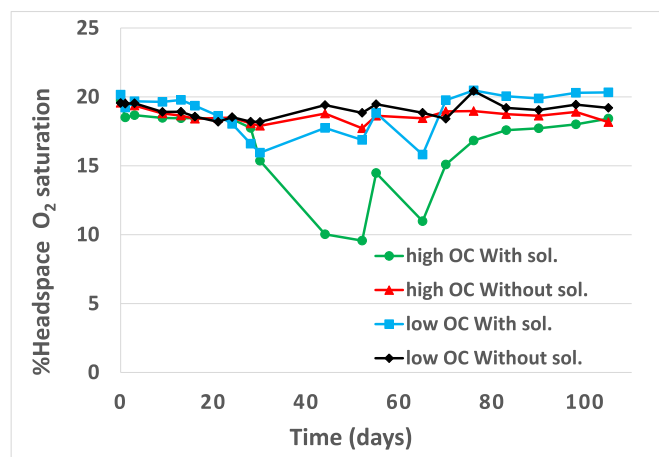

Fig. 3. Oxygen depletion in the headspace for samples treated with and without solvent in a closed flask test setup during a phenanthrene 308 study for both high and low OC sediment.

treated with solvent, oxygen levels in the headspace appeared stable for around 25 days before gradually dropping, with higher oxygen consumption being observed for the samples with high OC sediment, corresponding to the higher microbial activity and degradation observed for this sediment system.

The results shown in Fig. 3, suggests that the oxygenation was able to maintain the aerobic conditions in the headspace of the test setup. However, other evidence indicated that simply maintaining oxygen levels in the headspace was not sufficient to ensure that the water phase remained aerobic (See Fig. 4).

Fig. 4A shows the measured dissolved oxygen concentration after 28 days in the water phase of the parameter samples with and without solvent during different studies. Significant difference (p-value < 0.05, Paired two tailed t-test, $n_1 = n_2 = 4$) was observed in the dissolved oxygen between the samples treated with and without solvent for both sediment types. There was a corresponding high dissolved organic carbon (DOC) content at this time point in the solvent treated samples (see S8), indicating that a considerable amount of solvent remained in the system after around 28 days, and was yet to be degraded. By the end of the test the DOC levels had significantly reduced indicating that the solvent had been removed, most likely through degradation. The reduced dissolved oxygen concentration indicates a clear influence of the use of the solvent on the dissolved oxygen concentration in the water phase during the test. This drop in oxygen concentration in the water phase was attributed to increased consumption of oxygen as a result of the degradation of the solvent. Towards the end of the test (Fig. 4B), significant difference (p < 0.05) in dissolved oxygen between the samples treated with and without solvent was only observed in case of high OC sediment. In particular for low OC sediment a recovery in oxygen concentrations relative to untreated samples was observed in two solvent treated samples (biphenyl and decane low OC). In other samples the dissolved oxygen concentration remained substantially below the level observed in solvent untreated samples.

Fig. 4C and D shows the measurement of redox potential in the water phase for solvent treated and untreated samples during and at the end of the test. The solvent treated samples show, with the exception of the biphenyl low OC test, a considerable drop of redox potential in solvent treated samples in comparison to the untreated samples. Similar to the oxygen concentration, towards the end of the test the measured redox for solvent treated samples is in most cases more comparable to untreated samples. Redox
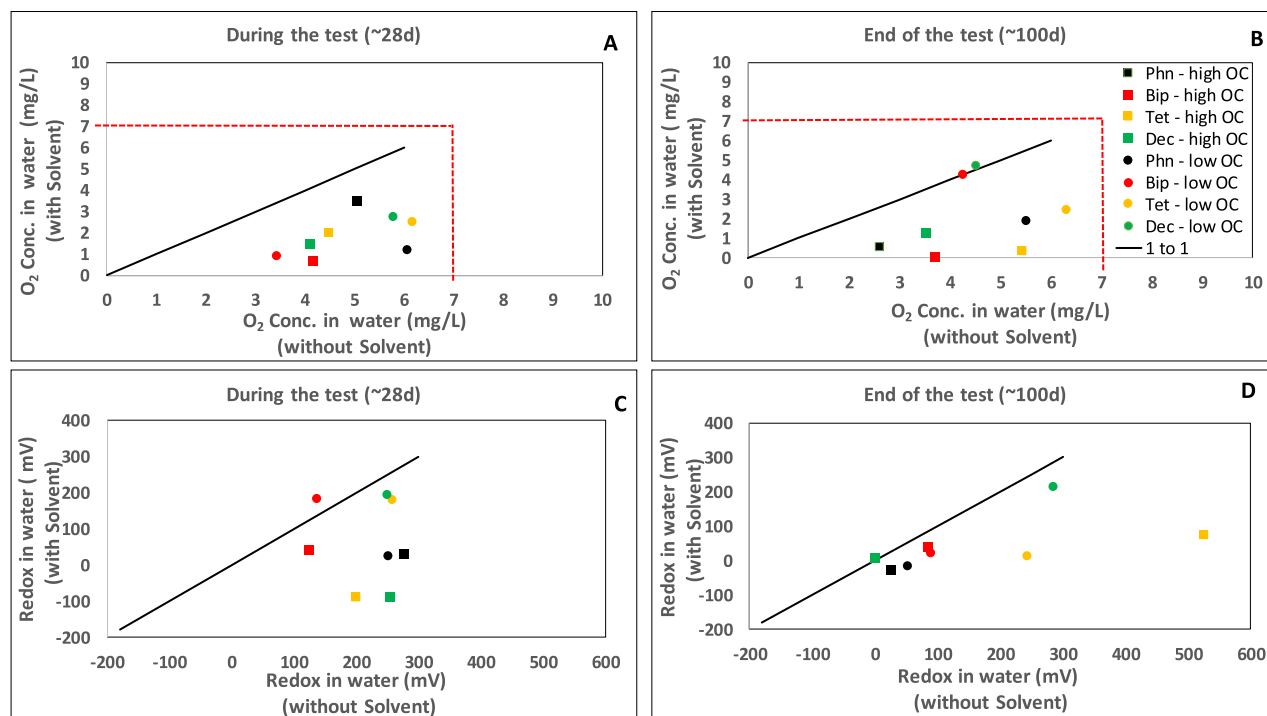
Fig. 4. Oxygen concentration in the water phase measured in high OC and low OC parameter samples with and without solvent treatment for each study at two time points: during the test (Fig. 4A) and at the end of the test (Fig. 4B). An oxygen concentration of 7−10 mg/L in the water phase is recommended for aerobic tests in the OECD308 (illustrated by the red lines). Redox potential measured in the water phase of the high OC and low OC parameter samples with and without solvent treatment for each study at two time points: during the test (Fig. 4C) and at the end of the test (Fig. 4D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

measurements of the bulk sediment were also performed, and these generally indicated that sediment redox potential was less influenced by the treatment of test systems with solvent (see S8). This is perhaps not surprising since the sediment layer is expected to be largely anaerobic under normal circumstances anyway.

In the case of the benzo[a]pyrene test in the flow-through setup, no data are available to compare the parameters of solvent treated vs untreated samples, but it is notable that the dissolved oxygen concentration and redox potential in the water phase of these solvent treated samples remained stable i.e. 5.83 mg/L (during), 5.51 mg/L (end) for high OC and 6.65 mg/L (during), 6.32 mg/L (end) for low OC sediment. These results suggest that the flow-through system was more effective at maintaining aerobic conditions in the water phase, even with the introduction of a solvent. It is likely that the flow of air aided both the exchange of oxygen at the air-water interface and the removal of solvent from the system through stripping.

A further observation in these experiments was the formation of a biofilm on top of the overlaying water (see Fig. S2). This was observed in all solvent-treated samples using the closed setup after 15−30 days of the incubation period, except for sterile samples. The biofilm was neither observed in the untreated samples from the closed setup, nor in the flow-through tests. This biofilm was further supportive evidence that the solvent used in the application of the test substances was impacting the test conditions, which in turn affected the microbial activity and hence the degradation of the test substance itself. The accumulation of the biofilm at the air-water interface was likely due to the depletion of oxygen in the water phase, meaning that the majority of degradation was taking place at the air-water interface. The temporal development of the biofilm coincided with the first indications of oxygen depletion in the headspace of closed systems, suggesting that it was this biofilm

which was responsible for depletion of oxygen in the headspace. The OECD 308 guideline states that, where used, solvents should not have adverse effects on the microbial activity of the test system. However, it appears that this was the case for the closed setup experiments.

According to the OECD 308 guideline, an oxygen concentration of 7−10 mg/L in the water phase is considered a typical aerobic water phase in such tests. Our results show that during these tests, the dissolved oxygen concentration in the water phase for samples with and without solvent were lower than the aerobic conditions recommended in the guideline, with concentrations in solvent-treated samples being significantly lower. Additionally, all tests using the closed setup demonstrated a decline in dissolved oxygen concentration between the start of acclimation and the start of the test. In several cases (particularly in high OC samples) dissolved oxygen concentration continued to decline over the course of the tests, even in cases where no solvent was added (Fig. 4). These results suggest that, despite regular oxygenation of the sample in the headspace, the oxygen from the headspace does not easily diffuse into the water phase in these closed test systems. This was likely due to the lack of air flow over the water surface, used in flow-through systems, but could also have been hindered by the formation of the biofilm, which could have potentially acted as a barrier for the diffusion of oxygen into the water phase and would also likely consume oxygen itself.

In terms of the necessity to use a solvent, application of such hydrophobic volatile chemicals is almost impossible without a co-solvent as the test chemicals volatilize immediately while preparing the application solution in water. Furthermore, for application of test chemicals using co-solvent, these solvents cannot be evaporated in such a closed flask setup due to the evaporation of test chemical along with the solvent. The K_H of the co-solvent acetone

(3.55 Pa m^3 mol^{-1}) (National Food Institute DTU, 2017) was lower than that of all test chemicals except benzo[a]pyrene (see section 2.1).

There are several possible options for enhancing the aeration in the closed test setups described in this work. The OECD 308 guideline permits stirring of the water phase to facilitate the exchange of oxygen into the water phase but in the typical flow-through test setup this is not conventionally applied. This would not only promote oxygen diffusion but would also likely affect diffusion and partitioning of the test chemical throughout the water and sediment phases (Shrestha et al., 2016; Honti et al., 2016). Furthermore, depending on the intensity of stirring this could introduce changes in oxygen conditions in the sediment layer. These factors are all likely to have an influence on the degradation process and thus outcomes. Therefore, for reasonable evaluation, stirring of the water phase - if used in a closed setup - should be standardized and reported. Additionally, the contactless sensor spots used to monitor the oxygen in the headspace could in principle also be used to monitor oxygen in the water phase. Another alternative would be to use a system geometry with a larger diameter to provide a larger contact area in the water-sediment and water-air interface to promote the diffusion of oxygen.

3.5. Degradation

The degradation time series for all ^{14}C labelled test chemicals in both high and low OC water sediment systems are shown in Figs. S3-S12 in SI. The complete mass balance obtained in all these studies proves, no considerable losses of test chemical or the mineralized fraction (CO$_2$/CH$_4$). Overall, higher mineralization was observed in the high OC sediment compared to the low OC sediment for all test chemicals except for phenanthrene where similar mineralization was observed in both sediment systems and benzo[a]pyrene where no mineralization was observed (See Figs. 3–4, Figs. S9-10). As expected, the mineralization of decane was much faster than observed for the other test chemicals in both sediments. Higher mineralization and degradation in high OC sediment was attributed to higher microbial biomass in the sediment (See Table S7). This is also supported by the levels of oxygen depletion observed in the headspace of the reference samples (see Fig. 3). Additionally, for volatile chemicals volatilization was relatively lower in the high OC-sediment resulting in higher fraction of parent chemical remaining in the sediment that could be available for degradation. As a general phenomenon, a high deviation between the replicates in the context of mineralization and sediment extractable fraction was observed frequently for all studies using the closed setup. These deviations between the replicates did not coincide with similar differences in total recovery or incomplete mass balances between the replicates, and they were counter-balanced by higher amounts of radioactivity found in other compartments, e.g. in extracts or the volatile fraction. Additionally, this phenomenon was more pronounced in the high OC sediment where higher degradation was observed. This suggests that the replicates did not behave similarly in terms of degradation where much higher mineralization was often observed in one replicate relative to the other.

The reduced levels of oxygen in the closed setup with solvent, is likely to have adversely impacted the degradation of the test chemicals relative to a test where the higher oxygen levels are maintained (e.g. in a flow through system).

3.6. Analytical challenges for volatiles

In our studies, we observed losses of test chemical during the sample processing steps, and these had to be repeated. The use of ^{14}C labelled test chemicals allowed a check of the procedural recovery of the test chemicals at each sample processing and analytical step. Incomplete procedural recoveries have a direct impact on the results of analysis and can lead to underestimation of the amount of parent substance in kinetics calculations, directly impacting the outcome of the test. Therefore, based on our experience, it is recommended to report the procedural recovery of the test chemical during different sample processing steps, especially when dealing with volatile chemicals.

4. Conclusions and implications

Considerable losses of phenanthrene were observed (K_H 4.29 Pa m^3 mol^{-1}) in a flow-through setup due to volatilization. We therefore suggest including a K_H cutoff value in the OECD 308 guideline for choosing between a flow-through setup and a closed setup. Our tests showed that with the use of an adapted closed setup, losses of test chemicals due to volatilization can be avoided. However, this test setup appeared to demonstrate some limitations compared to the flow-through setup in cases where test substances were introduced with a co-solvent, which was necessary for testing of hydrocarbons due to their poor solubility and volatile nature.

The adapted closed setup appeared to have poor exchange of oxygen between the headspace and water phase, which may have been affected by the lack of air flow over the water surface. Of greater concern was the influence of the solvent on the test system, which led to significant oxygen depletion in the water phase compared to parameter samples and the formation of a biofilm at the air-water interface. The test guideline states that the use of a solvent should not have significant adverse effects on the microbial activity in the system, and recommends a dissolved oxygen concentration of 7−10 mg/L in the water phase of an aerobic test, which is far higher than the levels observed in our tests. It therefore does not appear that the current design is suitable for reliably performing an OECD 308 test under aerobic conditions if test substances are applied with solvent, and that the biodegradation potential of these substances is likely underestimated under such circumstances.

It is recommended that additional research be performed to further improve the adapted closed setup. For example, overhead stirring of the water phase and modifying the geometries of the test system to increase surface area at the air-water and water-sediment interfaces appear to be promising options to improve oxygenation of the system (Shrestha et al., 2016). Additionally, the application of solvent free dosing techniques such as passive dosing would be useful in the context of water-sediment tests. Our research also demonstrates that system geometries, in particular headspace volume and height of water and sediment column are important factors influencing the partitioning of volatile chemicals within the test system, which will also influence degradation.

Despite the issues found with oxygen levels, it is promising that complete mass balances were obtained for a wide range of volatile chemicals using the adapted closed setup. However, it was also found that loss of volatile chemicals might occur during sample processing and analytical steps. Reporting of procedural recovery for sample processing steps is therefore recommended as an additional validity criterion, which might further improve the overall reliability of the data generated in these tests.

Declarations of interest

None.

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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.2019.124516.

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