ECHA note: Sterile controls in biodegradation studies – current status in regulatory testing in persistence assessment under REACH

Discussion document to support PBT Expert Group discussion and guidance development

17 November 2022

Contents

1. Background

In biodegradation studies, determining primary degradation is often important for concluding on persistence. Primary degradation measurement is based on the measuring the concentration of the test substance. Actual concentrations in a test medium (including the liquid and solid phases) can be affected by biotic and abiotic degradation but also by non-degradative phenomena such as the formation of non-extractable residues (NERs), adsorption to the test apparatus, or volatilisation and potential leakage as gas from the test system.

Sterile controls are expected to be helpful for determining primary degradation as they improve the possibility of differentiating the contribution of living organisms to the measured parameters. In addition, they may also be helpful for verifying the maintenance of the test compound in the test system and for estimating the mass balance.

There are differences between the key simulation test guidelines (OECD TGs 307, 308, and 309), with regard to sterile controls, i.e. whether sterile controls are mentioned at all in the TG and whether technical advice for sterile controls is included. This means that sterile controls are not always included in all simulation tests, unless they are specifically required (for example, in ECHA's decisions). If sterile controls are included, the technical implementation may be variable which may have consequences for their usefulness, and hence also for the level of information obtained from the whole study, in terms of the regulatory purpose.

In 2019, the Member State Committee (MSC) discussed the topic 'Sterile controls in simulation tests requests (OECD TGs 307, 308 and 309) under dossier and substance evaluation'[1]. The MSC agreed "not to specify the use of sterile controls when requesting for OECD 309, to specify the use of sterile controls on a case-by-case basis when requesting for OECD 307 and to preferably have a technical discussion at the PBT EG on whether this needs to be specified when requesting for OECD 308". Based on the MSC's request, the PBT EG has further considered the relevance of sterile controls as part of the degradation tests and applicability of the available sterilisation methods in different test media. This discussion document has been prepared to support the PBT EG discussion and the ECHA Guidance on IR&CSA, Chapter R.11 and R.7b update work on sterile controls.

2. Current status

2.1. Test guidelines

Table 1 summarises whether and how sterile controls are included in some of the most relevant OECD test guidelines used in regulatory persistence assessment.

Based on the test guidelines, the sterile controls are used for the following purposes:

- Calculating percentage primary degradation (OECD TG 301A, OECD TG 301C, OECD TG 301E, OECD TG 301F) or primary biodegradation rate (OECD TG 309).
- Calculating percentage abiotic degradation (OECD TG 301A, OECD TG 301B, OECD TG 301E, OECD TG 301F, and OECD TG 310) or abiotic degradation rate (OECD TG 307).
- Estimating whether degradation is predominantly abiotic (OECD TG 309).

[1] MSC-63; meeting minutes available at Meetings of the Member State Committee

2.2. REACH guidance

The current ECHA Guidance on IR&CSA, Chapter R.11: PBT/vPvB assessment mentions sterile controls only in the context of NER formation: "*In contrast, a lack of degradation of the parent compound may be assumed if fast NER formation (with extensive NER formation in several days without any degradation observed) is followed by a period of relative constant levels of NER. This might indicate the fact that the parent compound has become non-extractable, and thus is not readily available to degradation. Information obtained by comparing results from NER formation in sterile and non-sterile soils/sediments can sometimes provide insight into the mechanisms of the process. If NER is only formed at high levels in non-sterile soils/sediments, this may indicate degradation of the parent substance. In this case the formed NER in the non-sterile soil/sediment is unlikely to consist of the parent substance.*"

2.3. Current practice in ECHA's substance evaluation decisions

In ECHA decisions on substance evaluation (SEV), sterile controls have been required in all OECD TG 307 and OECD TG 308 requests adopted by ECHA during the time period reviewed for the present document (i.e. 09/2019 to 11/2021) (Table 8) (Annex 1). Based on the agreed cases, sterile controls have been requested in ECHA decisions on SEV (OECD TG 307 and OECD TG 308), when there are case-specific justifications for that. Text extracts regarding sterile controls from one decision for OECD TG 307 and one decision for OECD TG 308 are included in Annex 2.

From the reviewed set of ECHA decisions on SEV (Annex 1), the following can be summarised:

OECD TG 307 and 308 requests:

- Sterile soil controls/sterile water-sediment controls are included as a requirement in the ECHA decisions on SEV.
- The justifications for requesting sterile controls were as follows:
  - All of the 6 requests (2 cases for OECD TG 307, 4 cases for OECD TG 308) include: "*to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses*;
  - In 5 of the 6 requests, the "abiotic losses" are further specified as "*(e.g. volatilisation, formation of non-extractable residues (NERs));* whereas in one of the cases (a TG 308), it is not specified further;
  - In one case (OECD TG 307), there is an additional justification related to the potential abiotic hydrolysis.
- Some technical advice (including references) for running sterile controls (including sterilisation of soil/water-sediment samples) is included in the ECHA decisions on SEV (see text extracts in Annex 2).
- The ECHA decisions on SEV include that registrants must explain and justify the methods and procedures used for establishing the sterile controls in the study report and determine the efficiency of the sterilisation by measuring microbial biomass.

OECD TG 309 requests:

- The requirement for sterile controls has been included in the decision in 3 of the 4 cases.
- Technical advice for sterile controls in OECD TG 309 has not been included in the decisions

In addition, a sterile purified water control (i.e. without inoculum), in parallel with the sterile control with inoculum, has been requested in one OECD TG 308 and one OECD TG 309 request,

both of which were for volatile substances and the decisions indicate that the tests may be conducted with non-labelled test materials. In these cases, the justification for this additional control has been to minimise NER formation so that the potential losses can be attributed to the loss of the volatile fraction (leakage from test system or sorption to the materials of the test apparatus, e.g. stoppers and tubing). Maintenance of test substances in these controls would enable negligible leakage in the active test bottles to be assumed.

In screening tests, sterile controls with inoculum have been requested in at least three cases, when measurements of the test substance concentrations and/or primary degradation have been required (Table 9) (Annex 1). Additional sterile controls without inoculum have been requested in two of the three cases due to additional case-specific reasons.

3. Sterilisation methods – effects, application and availability

Generally, sterilisation methods for environmental samples (Table 2) can be divided into four main categories based on their mechanism of action:

1) Thermal sterilisation (particularly autoclaving).
2) Sterilisation by irradiation (γ-irradiation, UV irradiation).
3) Chemical sterilisation (e.g. formaldehyde, mercury mercuric chloride, sodium azide).
4) Filter sterilisation.

According to the ECHA Guidance on IR&CSA Chapter R.11, pelagic OECD TG 309 tests for PBT/vPvB assessment should be performed with natural waters with a certain range of suspended matter (SPM)[2]. When the water is filter sterilised, the SPM is expected to be at least partly removed. Filter sterilisation could affect the SPM concentration and consequently the representativeness of the sample with respect to the surface water. It would also affect the comparability of the samples for the viable experiment and for the sterile control (a lower SPM concentration in the sterile controls). Therefore, filter sterilisation is not recommended for surface water simulation tests.

In addition, filter sterilisation cannot be applied for complex environmental samples, and therefore also for OECD TG 309 suspended sediment tests, or for water/sediment or soil tests, other methods need to be considered.

Each method applicable for environmental solids can alter the physical and/or chemical properties of the soil, sediment, and water (including suspended particles) which is undesirable in biodegradation tests where sterile controls are used to compare the dissipation of a substance in a sterile versus non-sterile environment. The changes caused by the sterilisation can affect the abiotic interactions between the substance and the solid phase, particularly by altering the sorption behavior and ultimately affecting, e.g. NER formation.

[2] Between 10 and 20 mgdw/L SPM for simulation tests in freshwater and ca. 5 mgdw/L for simulation tests in marine water.

Table 1. Sterile controls in OECD test guidelines (n.a. = not applicable).

Test type	Test guide-line (year)	Status of sterile controls in test guideline	Technical advice for sterile controls	Advice for use and interpretation of the results of sterile controls
simula-tion test	OECD TG 307 (2002)	the requirement for sterile controls is included in the test guideline but experience has shown that sterile controls are not included in many cases	"To obtain information on the relevance of abiotic transformation of a test substance, soil samples may be sterilised (for sterilisation methods see references 13 and 26), treated with sterile test substance (e.g. addition of solution through a sterile filter) and aerated with humidified sterile air as described in paragraph 35. For paddy soils, soil and water should be sterilised and the incubation should be carried out as described in paragraph 38."	It is stated that the report must include "estimation of abiotic degradation rate under sterile conditions". Two references are given for soil sterilisation methods but those are of no use for the purpose as they do not include any relevant information.
simula-tion test	OECD TG 308 (2002)	not mentioned	n.a.	n.a.
simula-tion test	OECD TG 309 (2004)	integral part of the test	The following advice is given: "sterile control; one or two flasks containing sterilised test water for examining possible abiotic degradation or other non-biological removal of the test substance (symbolised FS). The biological activity can be stopped by autoclaving (121 °C; 20 min) the test water or by adding a toxicant (e.g. sodium azide (NaN3) at 10-20 g/l, mercuric chloride (HgCl2) at 100 mg/l or formalin at 100 mg/l) or by gamma irradiation. If HgCl2 is used, it should be disposed of as toxic waste. For water with sediment added in large amount, sterile conditions are not easy to obtain; in this case repeated autoclaving (e.g., three times) is recommended. It should be considered that the sorption characteristics of the sediment may be altered by autoclaving." "Analyses of transformation products in sterile controls should be considered, if rapid abiotic transformation of the test substance (e.g. hydrolysis) is thought possible." The test report shall contain the following information: information on the method(s) used for establishing sterile controls (e.g. temperature, time and number of autoclavings).	"If degradation has taken place, compare the results from flasks FT with those from flasks FS. If the means of the results from the flasks with test substance (FT) and the sterile flasks (FS) deviate by less than 10%, it can be assumed that the degradation observed is predominantly abiotic. If the degradation in flasks FS is lower, the figures may be used to correct those obtained with flasks FT (by subtraction) in order to estimate the extent of biodegradation." "If the rates of other loss processes than biodegradation are known (e.g. hydrolysis or volatilisation), they may be subtracted from the net loss rate observed during the test to give an approximated estimate of the biodegradation rate. Data for hydrolysis may, for example, be obtained from the sterile control or from parallel test using a higher concentration of the test substance."
ready bio-degrada-	OECD TG 301 (general part)	sterile controls without inoculum to be included "when required";	"When required, check for the possible abiotic degradation of the test substance by determining the removal of DOC, oxygen uptake or carbon dioxide evolution in sterile controls containing no inoculum. Sterilise by filtration through a membrane (0.2-0.45 µm) or by the	

Test type	Test guide-line (year)	Status of sterile controls in test guideline	Technical advice for sterile controls	Advice for use and interpretation of the results of sterile controls
bility	(1992)	sterile controls with inoculum ("adsorption control") required only when DOC removal is measured (and unless adsorption has ruled out).	addition of a suitable toxic substance at an appropriate concentration. If membrane filtration is used, take samples aseptically to maintain sterility. Unless adsorption of the test substance has been ruled out beforehand, tests which measure biodegradation as the removal of DOC, especially with activated sludge inocula, should include an abiotic control which is inoculated and poisoned."	
ready bio-degrada-bility	OECD TG 301A (1992)	optional	"Also, if required, check whether the test substance is degraded abiotically by setting up a flask containing a sterilised uninoculated solution of the substance. Sterilise by filtering through a membrane (0.2-0.45 µm) or by the addition of a suitable toxic substance at an appropriate concentration. "Additionally, if the test substance is suspected of being significantly adsorbed onto glass, sludge, etc., make a preliminary assessment to determine the likely extent of adsorption and thus the suitability of the test for the chemical (see Table 1, p. 4). Set up a flask containing the test substance, inoculum and sterilising agent." In a typical run, the following flasks are used: […] Flask 6 – containing test substance and sterilising agent (abiotic sterile control); Flask 7 – containing test substance, inoculum and sterilising agent (adsorption control); […]	"When a abiotic sterile control is used calculate the percentage abiotic degradation using: $\%\ abiotic\ degradation = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$ where, Cs(o) = DOC concentration in sterile control at day 0, Cs(t) = DOC concentration in sterile control at day t." "If primary degradation measurement is included, % primary degradation is calculated as: $\frac{S_b - S_a}{S_b} \times 100$ "(S_a *and* S_b are the residual amounts of test chemical at end of test in the inoculated test medium and in the sterile control.)
ready bio-degrada-bility	OECD TG 301B (1992)	optional	"Also, if required, check whether the test substance is degraded abiotically by using a sterilised uninoculated solution of the chemical. Sterilise by the addition of a toxic substance at an appropriate concentration." "In a typical run, the following flasks are used:	When an abiotic control is used, calculate the percentage abiotic degradation by: $\%\ abiotic\ degradation = \frac{CO_2\ produced\ by\ sterile\ flask\ after\ 28d\ (mg)}{ThCO_2\ (mg)} \times 100$

Test type	Test guide-line (year)	Status of sterile controls in test guideline	Technical advice for sterile controls	Advice for use and interpretation of the results of sterile controls
			[…] and, preferably and when necessary, also Flask 6 - containing test substance and sterilising agent (abiotic sterile control);"	
ready bio-degrada-bility	OECD TG 301C (1992)	mandatory water control	"No inoculum is added to Bottle 1 which serves as an abiotic control."	"Calculate the percentage primary biodegradation from loss of specific (parent) chemical using the equation given in "Data and Reporting" (p. 7). If there has been a loss of test substance in Bottle 1, measuring abiotic removal, report this and use the concentration of test substance (Sb) after 28 days in this bottle to calculate percentage biodegradation." "When determinations of DOC are made (optional), calculate the percentage ultimate biodegradation at time t using the equation given in 301 A (paragraph 27). If there has been a loss of DOC in Bottle 1, measuring abiotic removal, use the DOC concentration in this vessel at day 28 to calculate the percentage biodegradation." For specific chemical analysis, the following equation $\frac{S_b - S_a}{S_b} \times 100$ is given: (S_a *and* S_b are the residual amounts of test chemical at end of test in the inoculated test medium and in the blank test with water) Primary degradation is calculated for the replicate bottles a1, a2, and a3, respectively.
ready bio-degrada-bility	OECD TG 301D (1992)	not mentioned	no advice	no advice
ready bio-	OECD TG 301E	optional	"In a typical run, the same number of flasks as used in 301 A are	For abiotic degradation (optional) the following equation is given:

Test type	Test guide-line (year)	Status of sterile controls in test guideline	Technical advice for sterile controls	Advice for use and interpretation of the results of sterile controls
degrada-bility	(1992)		used, i.e.: […] and, preferably and when necessary, also "Flask 6 - containing test substance and sterilising agent (abiotic sterile control); Flask 7 - containing test substance, inoculum and sterilising agent (adsorption control)" […] "If abiotic degradation or loss mechanisms are suspected, such as hydrolysis (a problem with specific analysis only), volatilisation, or adsorption, it is advisable to perform a physical-chemical control experiment. This can be done by adding mercury (II) chloride (HgCl2)(1) (50-100 mg/l) to vessels with test substance in order to stop microbial activity. A significant decrease in DOC or specific compound concentration in the physical-chemical control test indicates abiotic removal mechanisms. (If mercury chloride is used, attention should be paid to interferences or catalyst poisoning in DOC analysis)."	$\%\ abiotic\ degradation = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$ " (where $C_{s(0)}$ and $C_{s(t)}$ are the DOC concentrations (mg/l) in sterile control on days 0 and *t*, respectively). For specific chemical analysis (optional) the following equation is given to calculate % primary degradation: $\frac{S_b - S_a}{S_b} \times 100$ (S_a *and* S_b are the residual amounts of test chemical at end of test in the inoculated test medium and in the sterile control.)
ready bio-degrada-bility	OECD TG 301F (1992)	optional	"If measurement of any abiotic degradation is required, prepare a solution of the test substance at, normally, 100 mg ThOD/l which has been sterilised by the addition of a toxic substance at an appropriate concentration." "In a typical run, the same number of flasks as used in 301 A are used, i.e.; […] and, preferably and when necessary, also Flask 6 - containing test substance and sterilising agent (abiotic sterile control)."	For specific chemical analysis (optional) the following equation is given to calculate % primary degradation: $\frac{S_b - S_a}{S_b} \times 100$ (S_a *and* S_b are the residual amounts of test chemical at end of test in the inoculated test medium and in the sterile control.) For abiotic degradation, the following equations are given:

Test type	Test guide-line (year)	Status of sterile controls in test guideline	Technical advice for sterile controls	Advice for use and interpretation of the results of sterile controls
				$O_2\ consumption\ per\ mg\ test\ chemical = \frac{a}{C_o V}$ $\%\ abiotic\ degradation = \frac{a}{C_o V \times ThOD} \times 100$
ready bio-degrada-bility	OECD TG 310 (2006)	sterile controls with inoculum are included in the test guideline; however, unclear if these are mandatory as "if included" is stated in one part of the text	"Vessels (denoted FS) for checking a possible abiotic degradation as (a) plus 50 mg/L HgCl2 or sterilised by some other means (e.g. by autoclaving)." "Bottles representing the checks for inhibition and for abiotic degradation need not be sampled as frequently as the other bottles; day 1 and day 28 would be sufficient."	"59. If there has been a significant increase in the TIC content of the sterile controls (FS) over the test period, then it may be concluded that abiotic degradation of the test substance has occurred, and this must be taken into account in the calculation of D in Equation [2]." Note that Equation [2] is to calculate the Percentage biodegradation (*% D*) based on TIC production) "63. In the same way, a curve for the reference substance, FC, is plotted and, if included, for the abiotic elimination check, FS and the inhibition control, FI. 64. The amounts of TIC present in the blank controls (FB) are recorded as are those in flasks FS (abiotic check), if these vessels were included in the test." 65. […] If in flask FS (abiotic) a significant increase (>10%) in the amount of TIC is observed, abiotic degradation processes may have occurred. "70. […] Record the amount of TIC in the blanks (FB) and in the sterile controls (FS) DOC and/or other determinants, and their percentage removal."
biodegra dability in seawater	OECD TG 306 (1992)	optional	"In a typical run, the following flasks are used: […] Flask 7 - containing test substance and sterilising agent (abiotic sterile control)- optional."	For abiotic degradation (optional) the following equation is given:

Test type	Test guide-line (year)	Status of sterile controls in test guideline	Technical advice for sterile controls	Advice for use and interpretation of the results of sterile controls
			"20. If abiotic degradation or loss mechanisms are suspected, such as hydrolysis (a problem with specific analysis only), volatilisation, or adsorption, it is advisable to perform a physical-chemical control experiment. This can be done by adding mercury (I) chloride (HgCl2)(1) (50-100 mg/l) to vessels with test substance in order to stop microbial activity. A significant decrease in DOC or specific compound concentration in the physical-chemical control test indicates abiotic removal mechanisms. (If mercury chloride is used, attention should be paid to interferences or catalyst poisoning in DOC analysis)." **"Physical-chemical control test (optional)** 28. If the option of using specific analyses is used, a physical-chemical experiment may be performed in order to check whether the test material is removed by abiotic mechanisms, such as hydrolysis or adsorption. A physical-chemical control test may be performed by adding mercury (II) chloride (HgCl2)(1) (50-100 mg/l) to duplicate flasks with test material in order to stop microbial activity. A significant decrease in specific compound concentration in the course of the test indicates abiotic removal mechanisms."	$\%\ abiotic\ degradation = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$ " (where $C_{s(0)}$ and $C_{s(t)}$ are the DOC concentrations (mg/l) in sterile control on days 0 and *t*, respectively). For primary degradation, no specific equation is included. The equations for ultimate degradation include a note that "Similar formats may be used when degradation is followed by specific analysis and for the reference compound and toxicity controls." Sterile controls are not included in those equations.

In this section, we examine the effects of the sterilisation methods on the properties of soil and sediment based on the available literature. Particularly, we will focus on the changes that would be most relevant considering biodegradation tests.

Environmental solids are typically viewed as containing three components that are fundamental to chemical activity (Mackay and Vasudevan, 2012):

1) *Organic matter:* Organic matter is composed of carbon and hydrogen rich polymeric aggregates with large non-polar domains and -COOH and -OH ligand groups. The non-polar domains attract non-polar organic molecules. We consider that changes in this compartment are particularly important considering that many of the (suspected) PBT/vPvB substances are non-polar substances.
2) *Aluminosilicates:* Composed of linked alumina octahedra and silica tetrahedra.
3) *Metal oxides and hydroxides*: Particularly iron, aluminium, and manganese oxyhydroxide minerals, may be crystalline or amorphous with surficial Al, Fe or Mn atoms bound by water or hydroxyl ions.

Electrostatic interactions are major processes that cause sorption of (especially ionic) substances to a solid phase (Margot *et al*., 2015). Changes in soil/sediment pH are therefore an important additional factor to consider, as they can change the pH dependent charge of a solid by deprotonating or protonating -COOH and -OH groups in the receptor sites (Mackay and Vasudevan, 2012). It is worth noting that environmental solids also possess a permanent negative charge, which is not dependent on the pH. For neutral test substances, the changes in the charge of the solids may not be as important, as the sorption is mainly driven by other mechanisms (e.g. hydrophobic interactions) between the sorbent and the substance.

In addition to these factors, properties and composition of the water phase are relevant. For example, changes in dissolved organic carbon (DOC) content may have an effect on the mobility of organic substances (and therefore change the sorption behaviour) by:

1) changing the water solubility of the substance due to interaction with dissolved humic and fulvic acids (Flores-Cespedes *et al*., 2002); and
2) competing in sorption sites in the solid organic matter (Chiou *et al*., 1986).

Additional changes, that might not be as relevant regarding biodegradation studies, are provided in Table 2. Based on this section, recommendations for choosing a sterilisation method are given in Section 5.1.

3.1. Thermal sterilisation (autoclaving)

From the studies reviewed (Table 3), autoclaving seems to be the most commonly used sterilisation method in water-sediment studies, which may be attributed to its relatively cheap cost and good availability in testing laboratories. Autoclaving is also relatively effective at sterilising soil and sediment when the samples are autoclaved two or more times (Lotrario *et al.,* 1995; Tuominen *et al.,* 1994). A typical cycle is 30 minutes, during which samples are exposed to 121 °C saturated steam under high pressure.

Disadvantages are that exposing the soil and sediment samples to these conditions may have significant effects on the mineral phases and geochemistry in soil and sediment by e.g. increasing DOC, affecting metal oxides (Mn, Fe) and changing the soil aggregation state (Otte *et al*., 2018; Lees *et al.,* 2018; Berns *et al*., 2008; Shaw *et al*., 1999). Berns *et al.* (2008) observed an increase of 29 to 37-fold in the DOC content in two soils after autoclaving, while Otte *et al.* (2018) reported a 12-fold increase following autoclaving in total DOC in marine sediment

samples. It is suggested that a substantial fraction of the DOC released from the soil is due to the lysis of micro-organisms, while autoclaving can also release physically trapped organic carbon and detach it from particle surfaces (Berns *et al.,* 2008).

Regarding aggregation state, there have been somewhat conflicting results, where autoclaving has either increased or decreased the surface area of the soil. Lees *et al.* (2018) reported on decrease in surface areas, which was suggested to result due to collapsing pores or clumping of particles. On the contrary, Berns *et al.* (2008) observed an increase in soil surface area due to disaggregation of microaggregates. It was speculated in the study that the differences between studies may be attributed to analytical methods used to determine the aggregation state (mechanical sieving separation versus gentle aggregate fractionation procedure). Nevertheless, it was suggested that the extent of disaggregation is different for each soil and appears to depend on the intrinsic aggregate stability.

Shaw *et al.* (1999) reported that the pH may also change due to autoclaving, which may be due to solubilisation of organic acids. The authors speculate that the magnitude of change will depend on the acidic buffer capacity of the soil.

3.2. Sterilisation by irradiation (γ-irradiation, UV irradiation)

According to McNamara *et al.* (2003) γ-irradiation is a highly effective sterilisation method, which eliminates the soil bacteria when applied at a dosage of 20 – 70 kGy. The correct dosage is dependent on soil type and properties (e.g. soils with higher organic matter content would require higher dosages).

There seems to be a consensus that γ-irradiation is the favourable sterilisation method to produce sterile samples with the least number of changes. Otte *et al.* (2018) stated that "…gamma-radiation can be recommended for pure cultures studies, water, sediment and soil sterilisation for microcosm experiments for quantifying substrate turnover." γ-irradiation was also recommended by Berns *et al.* (2008) for degradation/sorption experiments while Lees *et al.* (2018) stated that it was the best option available for sterilising soil for adsorption/desorption studies.

Authors from both studies observed that γ-irradiation caused less alterations on the physical and chemical properties of the soil compared to other methods tested. However, they advocated a case-by-case basis approach for choosing the best sterilisation method, as the changes observed are dependent on properties of the soil and therefore cannot be generalised.

Some disadvantages of γ-irradiation are that the method is relatively expensive and the availability is more limited compared to the other sterilisation methods, as not every test facility has the capability for γ-irradiation. Therefore, the field samples may need to be sent to external laboratories for sterilisation and during shipment environmental conditions (e.g., temperature) may not be adequately controlled (Otte *et al.,* 2018). γ-irradiation may also have relevant effects on the soil and sediment properties. The method can change the cation exchange capacity (CEC) (Bank *et al.,* 2008), which may impact the sorption behaviour of ionisable chemicals. Increase in DOC concentration has been also reported which has been attributed to the same processes as with autoclaving. Berns *et al.* (2008) observed an increase of 6 and 18-fold in DOC in soil, while in sediments, the increase was about 8-fold (Otte *et al.,* 2018). Effects on metal oxides include release of Mn(II), reduction of Fe(III) to Fe(II) and transformation of ferrihydrite to hematite (Otte *et al.,* 2018).

Regarding soil structure, Berns *et al.* (2008) observed a decrease in aggregation state (increased surface area) in γ-irradiated soils, although the decrease was not as pronounced as in autoclaved soils. In the study, the authors note, that in most studies[3], significant changes in the surface

[3] Based on McNamara *et al.,* (2003).

area or particle size distribution after γ-irradiation were not observed while there was one study where a decrease in clay aggregation stability was observed. Likewise, Lees *et al.* (2018)[4] states that there is little evidence that γ-irradiation would change the soil structure. Nevertheless, as studies exist where changes have been reported, the possibility for soil structure modifications should not be ruled out when using γ-irradiation, especially since it seems to be dependent on the intrinsic soil aggregation stability.

Changes in pH due to γ-irradiation are also reported. Wehr and Kirchhof (2021) cited multiple studies, where γ-irradiation either increased, decreased, or did not affect the pH at a dosage of 20 – 100 kGy. However, the authors observed only small changes in pH (mean change ~-0.03 units) in over 100 soils they sterilised with 50 kGy. The soils were air-dried, which is not advised in the OECD TG 307. As is the case with autoclaved soils, the magnitude of change in pH seems to depend on the properties of the soil.

Besides γ-irradiation, UV irradiation is used as a sterilisation method in many applications such as in waste-water treatment. However, there is little information available on the applicability of UV irradiation on soil and sediment samples. One study was identified, where a UV irradiation-based method was developed and tested for sterilising lacustrine and coastal sediment samples (Chifflet *et al.,* 2019). The method showed 95 % cell mortality for sediment/water samples contained in fluorinated ethylene-propylene (FEP) bottles after one cycle (4 h), and the effectiveness raised to >98 % after four cycles. During the cycle, sediments were stirred every 30 minutes. Regarding geochemical balance, UV irradiation had minor effects on the dissolved trace metal concentrations in sediment samples after one cycle. However, after four cycles, the variability in concentrations was observed. The authors mentioned that although they recommend four cycles for effective sterilisation, the effects on geochemical balance of complex sediment samples would require further investigation.

3.3. Chemical sterilisation

Sterilisation can be achieved chemically by applying toxicants (e.g. formaldehyde (CH_2O), mercuric chloride ($HgCl_2$), sodium azide (NaN_3)) into the samples. Although being a relatively inexpensive method, the general downside of using toxicants is that they leave a chemical residue to the samples which increases hazardous waste. The addition of toxicants may also affect the interactions between the test material and solid phase. For example, mercuric chloride can compete in sorption sites with the test substance (Stephens *et al.,* 2002).

Furthermore, the use of toxicants can interfere with the measurements used for determining biodegradation. Formaldehyde can oxidise to CO_2 biotically[5] or abiotically[6] which could cause interference when quantification of biodegradation is based on CO_2 production or O_2 consumption. Likewise, application of sodium azide to soil may lead to the formation of highly volatile hydrazoic acid (HN_3), which can skew the results of CO_2 evolution measurement if the measurement is based on titration of residual alkalinity from KOH (Rozycki and Bartha, 1981). Similar effect may be applicable when NaOH is used. Obviously, these concerns do not apply when radiolabelled test material is used.

The mode of action for toxicity varies between the toxicants, which can have implications on their applicability. For example, the toxicity of sodium azide is based on hindering the respiration chain of microbes by inhibiting the cytochrome c oxidase. However, fermenting bacteria (anaerobic) whose energy metabolism lacks the respiration cycle, can metabolise and even grow

[4] Citing Lensi *et al.,* (1991).
[5] According to the REACH registration data, formaldehyde is readily biodegradable https://echa.europa.eu/fi/registration-dossier/-/registered-dossier/15858/5/3/1 (accessed 11 July 2022).
[6] In ambient air, formaldehyde is quickly photo-oxidised in carbon dioxide. Source: WHO Guidelines for Indoor Air Quality: Selected Pollutants. Geneva: World Health Organization; 2010. https://www.ncbi.nlm.nih.gov/books/NBK138711/ (accessed 11 July 2022).

in the presence of sodium azide (Otte *et al*., 2018). Thus, sterilisation by sodium azide may be insufficient in tests which include anaerobic conditions (i.e. all anaerobic tests as well as the "aerobic" OECD TG 308 test[7]), unless there is convincing evidence available to *a priori* exclude the possibility for significant anaerobic biodegradation of the test compound.

There is no information available that toxicants would affect the soil/sediment structure, making these potentially attractive options for sterilisation in biodegradation studies. However, changes in pH have been observed in samples treated with sodium azide or formaldehyde.

Lees *et al.* (2018) observed that the pH of the loam soil solution changed by 0.53 units after application of sodium azide. Rozycki and Bartha (1981) reported considerably higher changes in pH from an initial 5.2 to 8.7 at the end of the incubation period (30 days). For the latter case, the sodium azide concentration was 5 % (based on dry soil) compared to 0.01 % in Lees *et al.* (2018).

It is worth to note, that in Lees *et al*. (2018) the application of 0.01 % sodium azide did not inhibit microbial activity, and therefore higher concentration may be required. Regarding formaldehyde, a two-unit pH decrease was observed in formaldehyde treated sediments (1 %) (Tuominen *et al.,* 1994). However, significant pH changes were not observed at lower concentrations (0.2 and 0.4 %) while the microbial activity was still effectively inhibited.

The pH change is dependent on the concentration of a toxicant and soil/sediment buffer capacity (Trevors, 1996 as cited in Lees *et al.*, 2018). Therefore, if these toxicants are used, it should be considered that the relationship between concentration and effect may depend on the type of sample.

[7] The "aerobic" OECD TG 308 is a mixture of aerobic and anaerobic sediment. The OECD TG states that the "*aerobic test simulates an aerobic water column over an aerobic sediment layer that is underlain with an anaerobic gradient"* (ECHA guidance R.11).

Table 2. Methods used for the sterilisation of water, sediment and soil samples.

Method	Medium/ test type applied	Advantages	Disadvantages	Remarks
Filtration	Water (screening tests)	• Effective way to sterilise water samples. • Leaves no chemical contamination. • Applicable to heat-labile components (Chifflet *et al.,* 2019).	• Not applicable to complex environmental matrices (water-sediment, soil). • Applicability to OECD 309 unclear due to SPM.	

Auto-claving	Water, sediment, Soil	• Relatively cheap and easy to use.
		• Fairly effective in inactivation of microorganisms (more than one cycle may be required) (Otte *et al.,* 2018).
		• Leaves no chemical contamination.
			• One autoclaving cycle may not be sufficient to sterilise the sample (Tuominen *et al.,* 1994).
			• May lead to changes in soil and sediment properties by
			  ○ Increasing DOC (Berns *et al.,* 2008).
			  ○ Changing the aggregation state of soil and sediment causing decrease or increase in soil surface area (Berns *et al.,* 2008).
			  ○ Affecting metal oxides by increasing Mn(II) content and by causing heat-induced crystallisation of Fe (oxyhydr)oxide minerals (Tuominen *et al.* 1994; Otte *et al.,* 2018).
			  ○ Changing pH (Shaw *et al.,* 1999).
			• Releases nutrients (Otte *et al.,* 2018).	

γ - irradia-tion	Water, Soil, sediment	• Effective in inactivation of microorganisms (20 - 70 kGy dosage) (McNamara *et al.,* (2003))).
		• Leaves no chemical contamination.
		• Causes relatively low number of changes in geochemical and mineralogical properties.
			• More expensive compared to autoclaving and NaN_3 (Otte *et al.,* 2018).
			• May lead to changes in mineral phases and affect the geochemistry in soil and sediment by
			  ○ Altering the aggregation state of soil (Berns *et al.,* 2008).
			  ○ Increasing DOC (Berns *et al.,* 2008).
			  ○ Changing the cation exchange capacity (CEC) (Bank *et al.,* 2008).
			  ○ Causing changes in metal oxides (Fe reduction, Mn(II) release, transformation of ferrihydrite to hemitite) (Otte *et al.,* 2018).
			  ○ Changing pH.
			• May increase nutrient availability, particularly nitrogen (Otte *et al.,* 2018).
			• Creates free hydrogen and hydroxyl radicals (Otte *et al.,* 2018).
			• In exceptional cases, radiation might enhance degradation by releasing enzymes. Enzymes remain active after 75 kGy (Otte *et al*., 2018).	

Method	Medium/ test type applied	Advantages	Disadvantages	Remarks
			• Unknown laboratory capacity for widespread applicability of method.	
UV radiation	Water, water-sediment, soil (?)	• Minor effects on distribution of dissolved trace metals (Chifflet *et al*., 2019). • Leaves no chemical contamination.	• Penetration may not be deep enough in sediment and soil samples -> requires stirring. • Limited information available on the applicability on soil/sediment samples.	
Formaldehyde (CH_2O)	Screening test media, sediment	• Effective for inhibiting bacterial activity in sediment (Tuominen *et al.,* 1994). • Does not cause any changes in dissolved nutrient concentrations (Otte *et al*., 2018).	• May be oxidised to CO_2 biotically or abiotically, which can cause interference when quantification of biodegradation is based on CO_2 production or O_2 consumption. • Has influence on pH (decrease pH), depends on formaldehyde concentration.	Harmonised C&L of formaldehyde according to CLP: Acute Tox. 3, Skin Corr. 1B, Skin Sens. 1, Acute Tox. 3, Muta. 2, Carc 1B; H301, H311, H314, H317, H331, H341, H350).
Mercuric chloride ($HgCl_2$)	Water, water-sediment, soil	• Inhibited bacterial activity effectively (Tuominen *et al.,* 1994). • Does not affect the sample pH or dissolved nutrient levels (Otte *et al*., 2018). • Caused minimal changes in the chemical and physical properties (Otte *et al*., 2018).	• May compete in sorption with the test material in soil (Stephens *et al.,* 2002). • May increase NH_4^+ concentration (Tuominen *et al.,* 1994). • May reduce nitrate (Otte *et al.,* 2018).	Harmonised C&L of mercuric chloride according to CLP: Acute Tox. 2, Skin Corr. 1B, Muta. 2, STOT RE 1, Aquatic Acute 1, Aquatic Chronic 1, Repr. 2; H300, H314, H341, H372 **, H410, H361f).

Method	Medium/ test type applied	Advantages	Disadvantages	Remarks
Sodium azide (NaN_3)	Water-sediment, Soil	• Recommended by Otte *et al.,* (2018), as it causes relatively low number of changes in geochemical and mineralogical properties. • Relatively cheap and easy to use.	• Can trigger pH changes. • The toxicity of sodium azide is based on inhibiting the respiration of bacteria. From this follows, that fermenting bacteria can grow in the presence of sodium azide (Otte *et al.,* 2018). Thus, sterilisation by sodium azide may be insufficient in tests which include anaerobic conditions (i.e. all anaerobic tests as well as the "aerobic" OECD TG 308 test). • Application to soil may lead to a formation of highly volatile hydrazoic acid (HN_3), which may skew the results of CO_2 evolution measurements in biometer type flasks where CO_2 measurement is based on titration of residual alkalinity (KOH) (Rozycki and Bartha, 1981). Similar effect may be applicable when NaOH is used, although the authors mention only KOH. • Microbial activity may be partially recovered due to formation of HN_3, which decreases the concentration of NaN_3 in soil (Rozycki and Bartha, 1981).	(Harmonised C&L of sodium azide according to CLP: Acute Tox. 2, Aquatic acute 1, Aquatic chronic 1; H300, H410, EUH032).

Table 3. Examples of sterile controls in studies used for regulatory assessments or in other relevant studies.

Test type	Test substance	Sterilisation method	No. of time points (sterile control/viable experiment)	Remarks	Reference
Surface water simulation tests considered in REACH regulatory processes:					
OECD TG 309	Isopropyl-isopropyl constituents of S-(tricyclo[5.2.1.02,6]deca-3-en-8(or 9)-yl) O-(isopropyl or isobutyl or 2-ethylhexyl) O-(isopropyl or isobutyl or 2-ethylhexyl) phosphorodithioate, non-labelled	Autoclaved twice	2/9	Sterile controls were measured on days 31 and 91 (test duration 91 days). The sterile controls were useful for the assessment. It was concluded that dissipation was taking place to a relevant extent and a biodegradation rate constant could not be determined in a direct manner. It was concluded that comparing the viable with the sterile experiment at the same sampling day and the same concentrations one can see qualitatively that primary biodegradation is hardly taking place if not at all.	ECHA, 2021
Water-sediment simulation tests considered in REACH regulatory processes:					
OECD TG 308 with modifications	Decamethylcyclopentasiloxane (D5); ^{14}C-labelled	Toxicant (sodium azide)	7/9 (aerobic) 7/7 (anaerobic)	It is stated e.g. that The authors further report that under aerobic conditions D5 degradation in non-sterilised samples was significantly faster than that in the chemically sterilised samples, suggesting that the degradation of D5 in the	Unnamed, 2010; ECHA, 2018a

Test type	Test substance	Sterilisation method	No. of time points (sterile control/viable experiment)	Remarks	Reference
				sediment might not be purely abiotic. This observation should be, however, considered with caution because the degradation rate in the non-sterilised samples was also very slow (cf the reported long half-lives above)."	
OECD TG 308	Phenanthrene	Autoclaved twice (2x 15 min at 120 °C)	3/8	Sterile samples were incubated in the same manner as the test samples. They were sampled at the start (0 d), middle (28d), and end (103d) of the study. The data was used to evaluate abiotic losses and inform NER formation.	Shrestha (2022)
Soil simulation tests considered in REACH regulatory processes:					
non-guideline study (internal method)	A mixture of quaterphenyls and terphenyls, non-labelled	Toxicant (0.5 w/w mercury chloride); sterility was checked on week 9 and revealed some microbial growth; after week 9, soil moisture adjustments were performed by adding saturated HgCL2 solution	11/11	Sterile controls were conducted with one (higher) of the two test concentrations, with no replication. Study duration was 32 weeks. Half-lives or DT_{50}s were determined for both viable experiments and sterile controls. It is stated that "as the results point rather to persistence than non-persistence and as sterile controls were used for comparison, the dossier submitter considers that the results can be used	ECHA, 2018b

Test type	Test substance	Sterilisation method	No. of time points (sterile control/viable experiment)	Remarks	Reference
				as there is no risk that lack of mass balance would cause a false positive degradation result."	
OECD TG 307	N,N-dicyclohexylbenzothiazole-2-sulphenamide (DCBS), ^{14}C-labelled	γ-irradiation	3/10	Samples were sterilised by irradiation with γ-rays in order to assess whether abiotic process could be responsible for the degradation of the test item. These sterile samples were not connected to the flow-through system and closed with sterilised stoppers. Sterile samples were sampled on days 0, 28, and 120. Test duration was 120 days.	Unnamed, 2016
Screening tests:					

Test type	Test substance	Sterilisation method	No. of time points (sterile control/viable experiment)	Remarks	Reference
OECD TG 310	Esters of rosin acids with glycerol, non-labelled	toxicant (formaldehyde, 18 500 mg/L)	8/8	A significantly higher decrease in test substance concentration and a higher CO_2 production were observed in the viable experiment compared to sterile controls. Significant decrease in test substance concentration occurred also in sterile controls but this was partly reversible and there was a clear difference to the viable experiment. Sterile controls were necessary for the assessment to demonstrate that the decrease in concentration was due to biodegradation.	Tukes, 2021
Studies published in scientific journals:					
OECD TG 307	Phenanthrene	Soil samples autoclaved twice (2x 15 min at 120 °C)	3/8	The sterile samples were closed with a sterilised stopper and were incubated alongside the non-sterile samples, but they were not connected to the flow through system. They were sampled at the start (0 d), middle (30d), and end (91d or 120d) of the study. The data was used to evaluate abiotic losses and inform NER formation.	Hughes *et al*. 2020

Test type	Test substance	Sterilisation method	No. of time points (sterile control/viable experiment)	Remarks	Reference
OECD TG 307	tetralin, decane; ^{14}C-labelled	autoclaved twice at 121°C for 20 min	3/8-10	Sterile controls were useful to determine the influence of abiotic processes in decrease of test substance concentration. Sterile controls were useful to determine the influence of abiotic processes in decrease of test substance concentration. It was concluded that: 1. Sorption of the test chemical to organic matter in the soil reduced its volatilisation 2. Fast degradation rate of decane reduced its volatilisation. In sterile controls, the distribution into the trap was in line with its Henry's law constant. 3. The NER formation was linked to the biological activity (bio-NER), as almost no NER formed in the sterile controls.	Shrestha *et al*., 2018
OECD TG 308	phenanthrene, biphenyl, benzo[a]pyrene, tetralin, decane; ^{14}C-labelled	autoclaved twice at 121°C for 20 min	no information available	It is not stated whether the sterile controls were useful. Results from sterile controls are not included or discussed in the study.	Shrestha *et al*., 2020

Test type	Test substance	Sterilisation method	No. of time points (sterile control/viable experiment)	Remarks	Reference
OECD TG 308	10 micropollutants; isotope-labelled	autoclaved three times at 121 °C for 20 min	3/8	Sterile controls were useful in the study. Three samplings were conducted (0d, 21d and 40d) (study duration 40 days). Some bacteria were present in the sterile controls. The authors speculated that the bacteria may have survived the autoclaving, or the samples were contaminated during aeration. The composition of the bacterial community at phylum level was completely different compared to non-sterilised sediments.	Coll *et al*., 2020
Modified OECD TG 308	24 pharmaceuticals, 15 pesticides, 3 artificial sweeteners, and 1 industrial chemical; non-labelled	autoclaved twice at 121°C for 20 min	5/10	Sediment and water sterile controls were used to determine sorption behavior and hydrolysis of test compounds. Due to concern for resuming bioactivity, the abiotic experiments were conducted only for 24 days.	Seller *et al*., 2021

Test type	Test substance	Sterilisation method	No. of time points (sterile control/viable experiment)	Remarks	Reference
OECD TG 307	4-n-dodecylphenol, 4-n-dodecylbenzenesulfonic acid sodium salt, 4-n-dodecylbenzyltrimethylammonium chloride; ^{14}C-labelled	γ-irradiation (30 kGy, 60Co) + autoclaving 121°C for 2h	1/2	The sterile vessels were incubated for 14 days, after which samples were harvested. Test duration for non-sterile test was 84 days. Sterile controls were used to distinguish whether NER formation was due to abiotic processes or microbial activity.	Claßen *et al*., 2019

4. Advice for deciding on the inclusion of sterile controls

When sterile controls are required in the test guideline or in a regulatory decision, they must always be included in the test set-up. When there is no obligation from the test guideline or regulatory decision to include sterile controls, they can still be included as a voluntary addition to the test set-up by the study conductor/sponsor, when expected to be useful (Sections 4.2 and 4.3). This section includes advice for considering the relevance of sterile controls as part of the study design.

4.1. Reasons for conducting sterile controls

As indicated above (Section 2.1), the test guidelines indicate that sterile controls can be used to estimate primary degradation or abiotic degradation. In addition, sterile controls can be used for verifying the maintenance of the test material in the test system and for the determination of a mass balance.[8] For these purposes, the result of the sterile control is always used in combination with the result of the viable experiment, with the exception of the abiotic degradation estimation, which is based solely on the sterile control. The three objectives of sterile controls are discussed below.

4.1.1. Determination of abiotic degradation

For the simulation tests, the determination of abiotic degradation using sterile control is mandatory in OECD TG 309, whereas in OECD TG 307 the requirement for sterile controls is included in the test guideline but experience has shown that sterile controls are not included in many cases, and in OECD TG 308 abiotic degradation is not mentioned at all (Table 1).

In the screening tests, the determination of abiotic degradation is either mandatory (OECD TG 301C) (in water control), optional[9] (other screening tests listed in the table, using sterilised uninoculated control), or the status is unclear (OECD TG 310) (Table 1).

It is not specified in the test guidelines why the abiotic degradation should be quantified in biodegradation studies. Therefore, we note that even if abiotic degradation needs to be estimated in some of the test guidelines, the way the results of abiotic degradation are used can be based on a case-by-case consideration, depending on the purpose of the study.

For the purposes of P/vP assessment under REACH, a degradation half-life under relevant conditions or, for screening purposes, the percentage of degradation during a specified time in screening tests, needs to be determined. To demonstrate that there is no P/vP concern, the degradation would need to proceed either to CO_2 and products of biosynthesis or to other transformation products which do not have PBT/vPvB properties. In the viable experiment, both biotic and abiotic processes are expected to operate and, therefore, for the purpose of P/vP assessment, there is generally no need to differentiate abiotic degradation from biotic degradation in studies conducted under relevant conditions. An addition of abiotic degradation observed in the sterile control to the degradation in the viable experiment would lead to an overestimated degradation and, therefore, should generally not be done. Correspondingly, a subtraction would lead to an underestimated degradation.

In some specific cases, differentiation of abiotic and biotic degradation might still be needed even from the PBT perspective, for example, when the degradation starts with an initial abiotic step (abiotic primary degradation) and there is a need to understand the factors affecting the

[8] Although this is not directly stated in the test guidelines, it seems that these objectives are taken into account in the equations for primary degradation in the test guidelines, which in most cases use the result of the sterile control.

[9] For example, to be conducted "when required" or "if abiotic degradation or loss mechanisms are suspected".

abiotic degradation. In such cases, abiotic degradation occurring in sterile controls can provide valuable information. To demonstrate that degradation occurs, the determination of degradation/transformation products in sterile controls may be necessary. When using the sterile control for these purposes, the potential effect of the sterilisation method on the abiotic degradation should be considered. Due to the sterilisation of the samples, the conditions for abiotic degradation in the sterile controls may differ from those in the viable experiment (see Section 3) and, therefore, the level of abiotic degradation may also differ. Hence, the abiotic degradation observed in the sterile control is not necessarily equal to the abiotic degradation occurring in the viable experiment.[10]

It should be noted that whenever the determination of abiotic degradation is required in the test guideline or regulatory decision, then that requirement must be followed. The present document can only be used as advice regarding the use of the results for the P/vP assessment and not as a justification for omitting the determination of abiotic degradation.

4.1.2. Checking the maintenance of the test substance and mass balance

It is often highly relevant to verify the maintenance of the test substance in the test system (and thus availability for biodegradation) and to accurately determine the mass balance. According to the ECHA Guidance on IR&CSA Chapter R.11, a simulation study should be performed using a radio-labelled substance, whenever feasible. Conducting the test with a radio-labelled test material allows the phase distribution of the test substance carbon, mineralisation and mass balance to be determined. It may also allow testing at lower concentrations than with using a non-labelled test material. Therefore, with radio-labelled test materials, the maintenance of the test substance and mass balance are generally known even without sterile controls. However, in tests conducted with a radio-labelled test material, sterile controls can still be useful for the same purpose, if a complete mass balance cannot be obtained e.g. when a part of the test material cannot be quantified for some reason by ^{14}C measurements (for example due to partitioning to headspace and subsequent adsorption to test apparatus or leakage from the test system).

In addition, there are situations where producing a radio-labelled test material is not possible for various reasons. In tests with non-labelled test materials, it is not generally possible to derive a mass balance to the same level of detail as in tests using radio-labelled test materials. However, sterile controls may to some extent compensate this deficiency by giving information on the maintenance of the test substance in the viable experiment.

If in a test with a radio-labelled or non-labelled test material, the test substance remains in the sterile control throughout the study, it may indicate that the decrease observed in the viable experiment is due to biodegradation. On the other hand, if a decrease in test substance concentration is observed in the sterile control, the decrease in the viable experiment may not be completely due to biodegradation. When using the sterile control for these purposes, the potential effects of the sterilisation method on the fate of the test material, such as the potential difference in the amount of NER formation between the sterile control and the viable experiment should be considered.

4.1.3. Determination of primary degradation

It is often highly relevant to accurately determine primary degradation. This requires that degradation and the non-degradative dissipation processes are differentiated. If there is a decrease in test substance concentration in sterile controls, then the decrease observed in the viable experiment may not be fully due to biodegradation. With non-labelled test materials,

[10]For example, the lack of the "competing" process of biodegradation could lead to higher abiotic degradation in the sterile controls, and the changes in physico-chemical properties (caused by the sterilisation method) could lead to lower or higher abiotic degradation in the sterile controls, than in the viable experiment.

sterile controls may be essential for quantifying the biodegradation and non-degradative dissipation, as determining the biodegradation and maintenance of the test substance in the study would require analytical identification of the transformation products and as the identity of the transformation products is often not known in advance.

With radio-labelled test materials, the biodegradation and maintenance of the test substance may be followed up by measurements (e.g. distribution of ^{14}C in different compartments/extraction steps). However, sterile controls may still be useful as an additional approach even with radio-labelled test materials. If sterile controls indicate the maintenance of a test substance in the system, this could in some cases reduce the need for analytical identification of transformation products or to increase confidence in the primary degradation estimation even when the mass balance for the viable experiment is not complete. Also the difference in NER formation between a sterile control and viable experiment may indicate that primary degradation occurred (Section 2.2).

4.2. Usefulness and added value of sterile controls in simulation tests

In simulation tests, it is considered that sterile controls are useful in the following situations:

(a) Sufficient mineralisation is not occurring to rule out P/vP but the decrease in test substance concentration is sufficient to rule out P/vP for the parent compound assuming that the decrease is completely or partly due to degradation (either biotic or abiotic).

(b) Technical problems are anticipated due to the properties of the test material (e.g. low solubility, volatility, adsorption to test apparatus) with regard the maintenance of the test material in the test system and/or bioavailability. Substances with (suspected) PBT/vPvB properties often have these characteristics.

(c) The test substance forms non-extractable residues (NERs) in such amounts that the interpretation of NERs (either as non-degraded parent, transformation products, or biogenic NERs) affects the conclusion from the study. If NERs are only formed at high levels in non-sterile conditions, this may indicate degradation of the parent substance (See 2.2).

(d) The test is conducted with a non-labelled test material (e.g. in a situation, where radio-labeling is not possible or technically feasible[11]). In this situation, it is more difficult to obtain information on mass balance and maintenance of the test substance in the test system, compared to tests with a radio-labelled test material.

To estimate the relevance of Scenario (a), existing degradation data are generally needed.

The relevance of Scenarios (b) and (c) can be estimated on the basis of physico-chemical properties and/or available data on degradation and other environmental fate processes.

When there is no sufficient data available to assess the relevance of Scenarios (a), (b), or (c), targeted pre-tests conducted under relevant conditions may be useful for deciding on the usefulness of sterile controls.

Sterile controls would likely be useful in simulation tests in the cases (Scenarios (a), (b), (c), and (d)) described above, as sterile controls would provide a more accurate estimation of primary degradation rate and increase the likelihood that the test will enable a conclusion on the P property (not P, P, or vP).

Even if there are cases where sterile controls do not bring added value (e.g. a very low decrease in test substance concentration is seen, or mineralisation is sufficient to conclude "not P"), it

[11] For example cases, see SEV decisions for EC 429-320-2 and 435-790-1.

may be difficult to identify these cases before testing. Pre-tests could be helpful also for this purpose. If there is no need to accurately determine the degradation kinetics (e.g. if the test is conducted only for the purpose of identifying degradation/transformation products), the added value from sterile controls may also be lower. In specific cases, there may still be the need to differentiate between abiotic and biotic transformation, and sterile controls would then be justified[12].

In OECD TG 309 tests, sterile controls are generally always included. Whereas with OECD TGs 307 and 308, the situation is different (Table 1) and experience has shown that for OECD TG 307 and 308 tests, sterile controls are often not included. Figure 1 specifies the situations where sterile controls have added value in OECD TG 307 and 308 studies based on the current discussions.

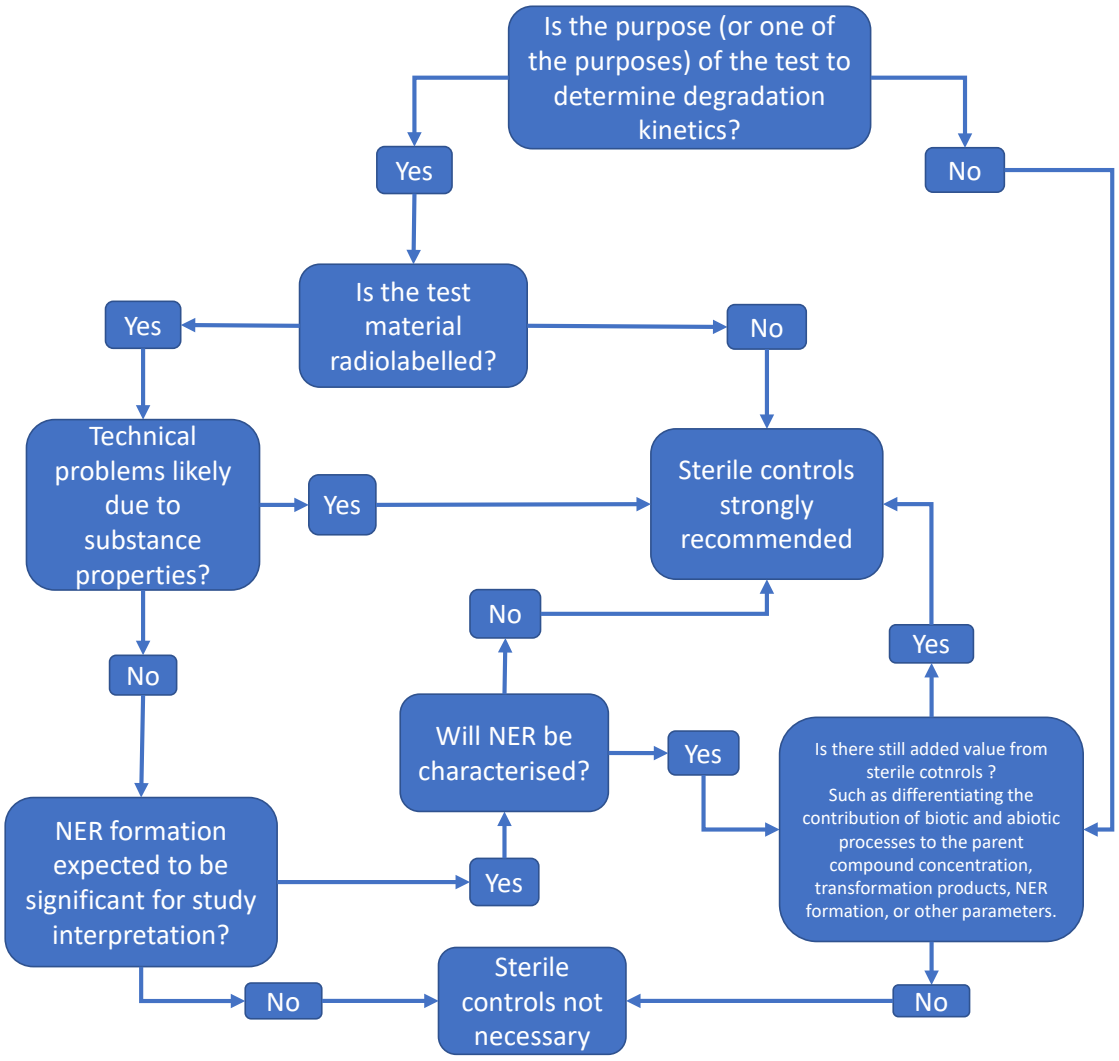

Figure 1. Scheme for estimating the added value from sterile controls in OECD TG 307 and 308 studies for the purpose of persistence assessment. This scheme is made on scientific grounds and should not be used as a reason to omit the inclusion of sterile controls when these are required by the test guidelines or by ECHA's decision.

[12] OECD TG 309 states: *"Analyses of transformation products in sterile controls should be considered, if rapid abiotic transformation of the test substance (e.g. hydrolysis) is thought possible."*

4.3. Usefulness and added value of sterile controls in screening tests

Primary degradation measurement is not mandatory in the screening test guidelines reviewed (Table 1), with one exception[13]. Also, at least under REACH and CLP, the regulatory cut-off values for screening tests are based on ultimate degradation parameters. There are no regulatory criteria for primary degradation in screening tests under REACH and CLP. However, there are still some cases where primary degradation in screening tests could be used as a part of the assessment[14]. In those cases, sterile controls are useful to quantify the contribution of non-degradative phenomena to the decrease in test substance concentration.

In addition, even when primary determination is not in the scope of the study, sterile controls, when accompanied with the measurement of the test substance concentration, may provide information on the maintenance of the test substance in the test system, for example, if volatilisation of the test material and leakage out of the test system are suspected. On the other hand, if sufficient information on the maintenance of the test substance in the test system is obtained by other means, such as by mineralisation exceeding the pass level or by measuring the test substance concentration in the non-sterile test, then sterile control would not necessarily provide added value.

5. Implementation of sterile controls

5.1. Selection of the sterilisation method

The properties of the solid phase may be altered due to the sterilisation methods. Therefore, attention should be paid to the selection of the sterilisation method and how it may affect the properties of the sample.

Based on the studies reviewed it seems that the effect of the sterilisation method on the properties and structure of the solid phase depends on the type of the sediment or soil. Indeed, sediment or soil properties, such as pH, buffer capacity, soil composition, or aggregate stability play a role on what extent the properties are changed. Generally, γ-irradiation would seem to be the best option but even that can cause changes in the test media. Chemical sterilisation seems to be a feasible option, when comparing the changes in the structure of soil/sediment with other sterilisation methods.

The properties of the test chemical, the purpose of the study (e.g. determination of degradation kinetics or identification of degradation/transformation products), and details of test set-up (e.g. radio-labelled or non-labelled test material) should be taken into account when selecting the sterilisation method. Also, whenever sterile control is crucial for determining the degradation rate, the comparability of conditions between viable test and sterile control is important and changes in the properties of the test medium due to the sterilisation method should be minimised. For example, in simulation tests with non-labelled test substances, when significant dissipation is expected due to non-degradative phenomena, the comparability of the sterile control and the viable experiment is highly important.

Table 4 indicates general recommendations for sterilisation methods to be used in different simulation tests. It should be noted that the recommended and alternative methods indicated in

[13] In OECD TG 301 C, primary degradation is calculated from specific chemical analysis made at the beginning and end of the incubation. OECD TG 301 C also includes an abiotic control containing water but no inoculum.

[14] For example, a fast primary transformation to non-PBT/vPvB products could enable to conclude "not P/vP" even when the pass level for mineralisation is not reached. Also, there may be a need to estimate the degradation of individual constituents of a more than one constituent (MOCS) substance.

the table are not necessarily the most optimal or feasible ones for every case, and therefore other methods can be used when justified. A case-by-case consideration is always needed, taking into account the points described above.

Table 4. Recommended sterilisation methods for the samples used in simulation tests.

Test	Recommended sterilisation method(s)	Alternative sterilisation method
OECD TG 309 "pelagic test"	γ-sterilisation or chemical sterilisation[a]	Autoclaving
OECD TG 308 (aerobic study)	For combined and separate sterilisation of the phases: γ-sterilisation or chemical sterilisation[a]	For separate sterilisation of the phases: γ-sterilisation or chemical sterilisation for sediment[b], autoclaving for water
OECD TG 307 (aerobic study)	γ-sterilisation or chemical sterilisation[a]	-

[a] Some toxicants (such as sodium azide and formaldehyde) can affect the pH of the sample and thus are not recommended to be used with ionisable test substances.
[b] Sterilisation with sodium azide may be insufficient in tests which include anaerobic conditions due to its mode of action (for more information see Section 3.3).

5.2. Test set-up and test conditions

In general, the test conditions in the sterile controls for biodegradation testing of chemicals should be as similar to the viable experiment as possible, with the exception that the biodegradation of the test substance should not occur in the sterile controls. The high similarity is important to have a good comparability of the results of the sterile controls to the viable experiment.

This includes at least the following aspects:

- Ideally, there would be a separate sterile control for each studied sample (water, soil, or water-sediment system) and also for each combination of sample and variable studied (e.g. test substance concentrations, methods of test substance application).
- The test vessel type and size, amount of test medium, volume of headspace, traps used to capture volatile compounds, and other features of the test apparatus, should be the same in the sterile control and the viable experiment.
- The number and extent of aeration events and techniques used for aeration should be the same in the sterile control and the viable experiment.
- The number of samplings per test vessel and other possible disturbances, which could cause loss or affect the partitioning of the test substance, should be the same in the sterile control and in the viable experiment.
- The incubation conditions (e.g. temperature, light condition, stationary incubation/shaking/stirring, soil moisture) should be the same in the sterile control and in the viable experiment.

Having a separate sterile control for each combination of sample and variable studied will increase the number of test vessels, the workload, and the requirement for laboratory space. Therefore, the need for a separate sterile control for every sample type and treatment (such as test substance concentration) should be considered on a case-by-case basis, together with the

expected need for time points and the number of samples (e.g. in OECD TG 307, the different soils) to be tested (discussed in Section 5.4).

The importance of sterile controls in the data interpretation depends to an extent on the degradation rate and the relationship between degradation and other dissipation processes. For example, in borderline cases (with respect to the P or vP cut-off value) it may be more important to have accurate data on abiotic processes than in other cases. Thus, the available information on the biodegradability and physico-chemical properties of the test substance should be considered when deciding on the test set-up regarding sterile controls. Also, other aspects of the test set-up should be taken into account when considering the number of sterile controls needed. For example, if information on abiotic losses can be obtained by other means, including, e.g. solvent traps for volatiles or remobilisation of substances adsorbed to the glass surface of the test vessels, this can potentially reduce the number of sterile controls needed.

Regarding the test concentrations, according to OECD TG 309, at least two different concentrations of test substance should be used to determine the degradation kinetics and both concentrations are used to interpret the results. On the contrary, in OECD TGs 307 and 308, one concentration is generally sufficient for determining the degradation kinetics. OECD TG 309 does not explicitly indicate that there should be a separate sterile control for each concentration. However, it is recommended that, in any simulation test, a separate sterile control should be considered at least for those test concentrations which are intended to determine the degradation kinetics. When the purpose of test is not to determine degradation kinetics but, for example, the identification of degradation/transformation products or when a separate test is conducted for this purpose, using higher test substance concentrations, then a separate sterile control may not be necessary.

Regarding the aeration of sterile controls, sterilised air should be used to avoid contaminating the sample. Naturally, aeration of the sterile controls should only be conducted if the viable experiment is handled in the same way.

Additionally, a sterile purified water control can be included in the test set-up of simulation studies. This is a control containing water and the test substance, but without any environmental medium (water/soil/sediment). One possible application for the sterile purified water control is to verify the maintenance of the test substance in the test system. As there is no solid matter which could interact with the test substance in the water control, the loss in the water control represents a higher boundary for the potential loss in the viable experiment. Therefore, at least the amount remaining in the test system in the water control remains in the test system also in the viable experiment. When both a sterile control and a sterile purified control are included in the same study, the proportion of the test substance partitioning to the solid phase can be estimated from the difference of the measured concentration between the two controls.

In screening tests, in specific cases, a sterile control without inoculum may be useful in addition to the sterile control with inoculum. It is probably better to use the same test medium in this control as in the viable experiment, rather than pure water. It is noted that some of the screening test guidelines include a water control with no inoculum, either as a mandatory or an optional part of the test (Table 1).

5.3. Validation of the analytical methods

The analytical methods to determine a test substance concentration often require an extraction step. The extraction efficiency depends on the interaction of the test substance and the solid matter. Also, sample composition, such as the presence of a toxicant, could at least in theory affect the analytical results e.g. due to matrix effects. Therefore, it should be considered that the applicability of chemical analysis may differ in the conditions of the viable experiment and the sterile control. For example, if extraction needs to be used, the extractability of the test substance may depend on the properties of the medium. If a chemical toxicant is used, the potential reactivity of the toxicant with the test substance, or the potential effect on analytical results, need to be considered.

In practice, this means that the response of the analytical method (including extraction, if applicable) should be checked both in the conditions of the viable experiment and sterile control to see e.g. whether the same validation data (including calibration) is applicable for both conditions or whether a correction factor or even a more comprehensive validation of the analytical method would be needed separately for the conditions of the sterile control.

5.4. Measurement frequency (number of time points) and replication

The test guidelines OECD TG 307 and 309 include advice for the number and time schedule of measuring points but without any specific reference for sterile controls in those parts. Therefore, the same advice is considered applicable both for the viable experiment and for the sterile control. For example, OECD TG 309 states that no fixed time schedule for sampling can be stated, as the rate of biodegradation varies. OECD TG 307 states that time intervals should be chosen in such a way that the pattern of decline of the test substance and patterns of formation and decline of transformation products can be established. The same applies also for the sterile controls as it is not possible to recommend any fixed time schedule for sampling because, even though biodegradation is inhibited, the rate of abiotic degradation/dissipation is expected to vary. The number of measuring points and the measurement schedule for the sterile controls should be chosen according to the pattern of decline of the test substance in the sterile controls and taking into account the requirements of the test guideline and the purpose of the study. Thus, the number of measuring points and the measurement schedule for the sterile controls do not necessarily need to be the same between the sterile controls and for the viable experiment.

OECD TGs 307 or 309 do not explicitly require a rate constant or a half-life/DT_{50} to be determined for the sterile controls. However, OECD TG 309 includes a subtraction approach to give an approximated estimate of the biodegradation rate (see also Section 5.6.2). Therefore, in OECD TG 309 studies, whenever the abiotic losses contribute to the decline to such an extent that it affects the interpretation of the study with respect to the P/vP criteria, it is recommended to include a sufficient amount of measuring points also for the sterile controls so that kinetic modelling for the abiotic decrease can be performed. For a sufficient number of measuring points for kinetic modelling, see OECD TG 309 (e.g. paragraph 30) as well as FOCUS (2014) (e.g. Section 6.1.1).

OECD TG 307 requires reporting of "estimation of abiotic degradation rate under sterile controls", which is a less specific requirement compared to the viable experiment[15] and does not specify whether this refers to a rate constant derived from kinetic modelling or some other type of rate estimation. OECD TG 307 does not include a subtraction approach unlike OECD TG 309.

When deciding on the measurement schedule for sterile controls in OECD TG 307 and 308 studies, it is recommended to consider whether kinetic modelling for the abiotic decrease is needed or whether, for instance, the overall extent of abiotic dissipation during the whole study or during a certain time period is sufficient information for the case (see Section 5.6). For a sufficient number of measuring points for kinetic modelling, see the respective test guidelines, OECD TG 307 (e.g. paragraph 46) or 308 (e.g. paragraph 38), as well as FOCUS (2014) (e.g. Section 6.1.1).

When kinetic modelling in accordance with FOCUS (2014) is neither considered necessary for the sterile control results for the purpose of the study nor needed to fulfil the requirements of the test guideline or regulatory decision, the following aspects can be considered when deciding on the measurement frequency.

[15] For the viable experiment, OECD TG 307 requires reporting of "half-life or DT_{50}, DT_{75} and DT_{90} for the test substance and, where appropriate, for major transformation products including confidence limits".

- At a minimum, measurements at the start and end of the study should be performed. This would indicate the total decrease during the study and would be useful in certain cases for obtaining information on the maintenance of the test substance in the vessels. However, this would not give information on the kinetics of the decrease under abiotic conditions.
- Three measurements (at the start, middle and end of study) would give some insight on the kinetics although generally not to a sufficient degree for parameter estimation according to FOCUS (2014).

In addition, when deciding on the measurement frequency, regardless of whether or not kinetic modelling is pursued, it can be considered that at the test initiation it may often be important to see the distribution of the test substance (e.g. to water phase/solids/headspace/volatile traps), under biotic and abiotic conditions. The initial part is also important as the sterility is expected to be the highest in the beginning of the study and as there can be an increase of microbial activity in the sterile control during the study. Thus, depending on the substance, a higher measurement frequency compared to the test guideline recommendations could be useful, particularly in the beginning of the study, to understand the abiotic behaviour (e.g. partitioning/loss) of the substance during the study.

One possibility can be to have a higher frequency of measurements (allowing kinetic modelling in accordance with the recommendations), for certain specific sample types and to have a lower frequency for other samples (e.g. in OECD TG 307 where at least four soils are studied).

It is recommended that the number of replicate samples/test vessels per time point is the same in the sterile control and in the viable test.

5.5. Sterilisation efficiency and effects of sterilisation on physico-chemical properties

5.5.1. Advice for deciding on the need to determine the sterilisation efficiency and physico-chemical changes

When biodegradation of the test substance occurs in the viable experiment, an indication of the effectiveness of the sterilisation can be obtained by comparing the degradation in the viable experiment and in the sterile control. In addition, there may be the need to verify or quantify the effect of sterilisation on microbial activity or physico-chemical properties using relevant microbiological/biochemical measurements. This may not be necessary when it is clear from the results that sterilisation has been successful. This includes the following situations:

- A decrease in test substance concentration occurs both in the viable experiment and in the sterile control and the decrease in concentration in the sterile controls can be attributed to other reasons than biodegradation based on the available data (such as the distribution of the test substance or ^{14}C in the test system).
- A significant mineralisation or decrease in test compound concentration is observed in the viable experiment but not in the sterile control. Under those circumstances, the decrease in test compound concentration in the viable experiment most likely indicates the extent of primary biodegradation.
- A decrease in test substance concentration occurs both in the viable experiment and in the sterile control but there is still a clear difference between the results of the sterile control and viable experiment so that the decrease in sterile control does not affect the conclusion with regard to the P or vP cut-off.

However, if it is suspected that the sterilisation method used would *reduce* the abiotic decrease in test compound concentration compared to the viable experiment, the need for measurements

of the physico-chemical properties should still be considered even in the situations described above.[16]

When a decrease in test compound concentration occurs in the sterile controls and its interpretation (whether it is counted as degradation or as non-degradative dissipation) would have an effect on the conclusion (e.g. when the half-life is close to the relevant cut-off value), it is recommended to determine the efficiency of the sterilisation and the changes in physico-chemical properties due to sterilisation.

When there is no prior knowledge of degradation rate or behaviour of the substance in the test it is difficult to *a priori* exclude the need for information on sterilisation efficiency or physico-chemical characterisation.

5.5.2. Determination of sterilisation efficiency in simulation tests – general remarks

None of the test guidelines explicitly require the determination of efficiency of sterilisation. OECD TG 307 and OECD TG 308 require the determination of microbial biomass during the study. OECD TG 307 includes advice on sterile controls, but it is not stated whether the biomass needs to be measured also from the sterile controls.

The information available for biomass measurements in the simulation test guidelines is summarised in

[16] Theoretically, even in these cases there is still a possibility of false conclusions if the sterilisation decreases the abiotic losses so that the abiotic loss is higher in the viable experiment than in the sterile control. This could occur e.g. if there is a significant change in pH which changes the abiotic behaviour of the test material, or, if the surface area or adsorption capacity of solids is reduced.

Table 5 and Table 6. There is no advice in the test guidelines regarding how the biomass results during the study should be used to interpret the results. Some of the parameters are based on metabolic activity (such as respiratory measurements) or growth (such as plate counts) and some are based on the extraction of carbon derived from biomass (fumigation extraction method) or the detection of biomolecules (such as acridine orange staining).

For determining the efficiency of the sterilisation, a parameter indicating the activity of microorganisms, or the amount of metabolically active microorganisms, should be chosen. Detailed assessment of the relevance of the various biomass parameters in the test guidelines for this purpose is not in the scope of the present document. However, the parameters based on metabolism or growth would seem relevant as they require the presence of metabolically active microorganisms. Regarding the parameters based on extraction of carbon or detection of biomolecules, it should be considered to what extent they correlate with the number of metabolically active cells or whether dead cells also contribute to the result.

There are also methods that are not included in the OECD degradation test guidelines reviewed in this document but which could still be relevant for determining sterilisation efficiency (e.g. thymidine incorporation into DNA (Moriarty, 1990), DMSO reduction assay for soil (Alef and Kleiner, 1989) and sediment (Griebler, 1997) or measurement of ATP (Martens, 2001)).

The activity of the biomass and the number of metabolically active cells are expected to decrease by sterilisation and, therefore, a difference between sterile control and viable experiment in the activity or growth of biomass is expected. This difference should be considerable at least right after the sterilisation. Microbial activity or growth could occur also in the sterile controls (Tuominen *et al.,* 1994) and therefore it is possible that the differences in microbial parameters between the viable experiment and sterile control decrease during the study.

The changes in physico-chemical properties due to the sterilisation could potentially also affect the detection of the microbial parameters (e.g. extraction of carbon). This may need further consideration.

To determine the sterilisation efficiency, it is recommended that the measurements are conducted at least three times: before the sterilisation, at the start of the test (after the acclimation period in OECD TG 308 tests) and at the end of the test. Additional measurement times can be chosen based on the need. For instance, a measurement right after the sterilisation may be useful to see the immediate effect of sterilisation. The measurements should be made from both non-sterilised and sterilised test samples because it is possible that microbial growth or activity occurs also in the sterile control and, therefore, it is important to be able to compare the result also to the viable experiment during the study and not only to the situation before the sterilisation.

It is recommended to use vessels with a test substance (rather than controls without a test substance) to study the sterilisation efficiency. This can be done by taking samples from the biodegradation and sterile control replicates, if possible, or from additional replicates with similar conditions. The basis for including a test substance is that the conditions in the sterilisation efficiency vessels should be similar to the biodegradation vessels/sterile controls, and the presence of the test substance can affect the microbial community particularly when biodegradation is occurring and, therefore, it can also affect the parameters used for sterilisation efficiency determination.

However, if for technical reasons the determination of sterilisation efficiency in the presence of a test substance would turn out unreliable or unfeasible (e.g. if the test substance interferes with the method used for determining sterilisation efficiency), blank controls without a test substance can be used for the purpose although, in that case, conditions are not fully representative to the conditions in the biodegradation assay and in the sterile control.

5.5.3. Determination of sterilisation efficiency in simulation tests – specific remarks for OECD TG 307 and 308 studies

In OECD TG 307 and 308 studies, it may be useful to conduct the mandatory biomass measurement using a parameter which is relevant also for determining the sterilisation efficiency, to avoid the need for employing an additional method and equipment.

In OECD TG 307, the mandatory biomass measurements must be done from separate controls (with no test substance added). In OECD TG 307 studies, even when using the same biomass parameter for both purposes (the mandatory biomass measurement and sterilisation efficiency determination), it is recommended to conduct the sterilisation efficiency measurements from vessels with a test substance.

In OECD TG 308, it is stated that the control units, which are not treated with a test substance, can be used to determine the microbial biomass. In OECD TG 308 studies, even when using the same biomass parameter for both purposes (the mandatory biomass measurement and the sterilisation efficiency determination), it is recommended to conduct the sterilisation efficiency measurements from vessels with a test substance.

For OECD TG 308 studies, in general, the sterilisation efficiency determination should cover both water and sediment phases. The sterilisation efficiency can be determined either for the combined water-sediment systems or separately for the water and sediment phases. The following points should be considered when choosing the approach:

- The technical feasibility and representativeness of sampling should be considered.
- In OECD TG 308, the test substance and transformation products are analysed separately for the sediment and overlying water (and, for that purpose, the surface water should be carefully removed with minimum disturbance of the sediment). Therefore, the use of separate phases may be a feasible option also for the sterilisation efficiency determination.
- The separation of the phases may differ between samples (e.g. sterilised and non-sterilised samples or the different time points) due to differences in the physico-chemical properties affecting e.g. aggregation. Therefore, even when the determinations are done for the separate phases, the results should be estimated for the whole water-sediment system (using the results for the separate phases and their volume or mass). This will enable the comparison between the sterile and non-sterile samples and the different time points to be done at the level of the whole water-sediment system.
- When the sterilisation is done separately for the two phases, it is recommended that the measurements for sterilisation efficiency are done for the two phases separately and that, in addition to the three measuring times described above, an additional measurement is performed right after the sterilisation[17]. When the sterilisation is done for combined phases, either of the approaches for sterilisation efficiency determination can be used.
- When the sterilisation efficiency is determined from separate phases, the determination of the liquid-solid ratios or dry weights (g/l) of (parallel) samples from the water and sediment phases may be useful to inform on the comparability of the results from different samples, due to the possible differences in phase separation.
- When the sterilisation efficiency is determined for samples of the combined water-sediment system, the vessel content should be mixed so that the sample taken is

[17] The reason for the additional measurement is to get specific information for the immediate sterilisation efficiency per phase, as after placing the samples in the incubation vessels, the relevant parameters will then be affected by the sterilisation efficiency of both phases and not only one of them. This is due to the movement of microorganisms between the two phases. This may occur also in the sterilised samples as full sterility may not be achieved.

representative of the whole system. The determination of the liquid-solid ratios or dry weights (g/l) of a (parallel) water-sediment sample used for the sterilisation efficiency determination and of the remaining vessel content may be useful as it would inform on the representativeness of the sample to the whole water-sediment system.

- If the same method of sterilisation is used for both phases (regardless of whether sterilisation is done for the combined water-sediment system or for the separate phases) and if it can be justified that the measurements for the sediment are sufficient to indicate the efficiency of sterilisation of the whole water-sediment system, then it may be sufficient to determine sterilisation efficiency for the sediment only.

5.5.4. Determination of sterilisation efficiency in screening tests

For screening tests, the test guidelines do not include any advice regarding the monitoring of the sterilisation efficiency. However, negligible or at least a significantly lower mineralisation or decrease in test compound concentration in the sterile control, could indicate successful sterilisation. If more information on the sterilisation efficiency is needed in a screening test, then similar measurements as described above for simulation tests can be considered. Because in screening tests, test substance concentrations are significantly higher than in simulation tests, the presence of a test substance may have a higher influence on the microbial activity in the sterile controls, or on the measurement of sterilisation efficiency.

Therefore, when sterilisation efficiency is determined in screening tests it is recommended to consider using vessels containing the test substance for this purpose, instead (or in addition to) controls without test substance.

5.5.5. Interpretation of the sterilisation efficiency results

Obtaining and maintaining full sterility may be difficult especially with soil and sediment compared to water and in general with longer test durations. The capacity of the microbial community to degrade the test chemical will likely be lost or at least decreased substantially due to the sterilisation even when some microbial activity or viability remains.

Therefore, achieving a full sterility or even a certain level of reduction in total microbial activity compared to the viable experiment should generally not be a prerequisite for the use of the sterile control results for the assessment. Nevertheless, the efficiency of sterilisation should be determined in certain cases (Section 5.5.1) and taken into account in the assessment.

Information on sterilisation efficiency may be used, for example, when considering whether the subtraction approach (Section 5.6) of using the sterile controls is appropriate. In some cases, a clear difference in the measured parameters (e.g. test substance concentration, mineralisation, or formation of degradation/transformation products) between the viable experiment and sterile control would be useful for the assessment, even when some microbial activity occurs in the sterile control.

In specific cases where the abiotic degradation needs to be quantified and differentiated from biotic degradation, it may be more important to achieve and demonstrate a high-level sterility in the sterile controls than on other occasions where sterile controls are used (see also Sections 4.1.1, 4.2, and 5.6.1).

Table 5. Advice given in the simulation test guidelines OECD TG 307, 308, and 309 regarding biomass measurements.

Test guideline	Advice regarding biomass measurements	Methods	Interpretation/use of biomass results
OECD TG 307	Microbial biomass of the soils should be characterised (for aerobic studies only). Microbial biomass to be determined initially, during and at the end of the aerobic studies, from untreated sample (no test substance added). Longer incubation periods should be justified in the test report and accompanied by biomass measurements during and at the end of these periods.	Microbial biomass should be determined by using the substrate-induced respiration (SIR) method or alternative methods.	No advice given except that for soil used for the determination of the transformation pathway, a microbial biomass of at least 1 % of total organic carbon is recommended.
OECD TG 308	Microbial biomass to the sediment should be determined at post-handling and at the start and end of the test. The control units (not treated with test substance) can be used to determine the microbial biomass of the sediment and the total organic carbon of the water and sediment at the termination of the study. Determination of microbial biomass of the water is not mentioned.	The methods indicated are microbial respiration rate method, fumigation method or plate count measurements (e.g. bacteria, actinomycetes, fungi and total colonies) for aerobic studies; methanogenesis rate for anaerobic studies.	No advice given.
OECD TG 309	Biomass measurement is optional from surface water. Biomass measurements during the study are not mentioned.	Microbial biomass (e.g. acridine orange direct count or colony forming units). Chlorophyll-a concentration as a specific estimate for algal biomass.	No advice given.

Table 6. Methods for biomass measurements in OECD TG 307, 308 and 309.

Method	Test guideline	Remarks	References cited in test guideline
Substrate-induced respiration	OECD TG 307		Anderson and Domsch, 1978; ISO 14240-1 and 2, 1997.
Alternative methods	OECD TG 307		Page *et al*., 1982
Microbial respiration rate method	OECD TG 308		Anderson and Domsch, 1985
Fumigation-extraction method	OECD TG 308		ISO-14240-2, 1997
Plate count measurements	OECD TG 308		none
Methanogenesis rate	OECD TG 308	relevant to anaerobic tests	none
Acridine orange direct count	OECD TG 309		none
chlorophyll-a concentration	OECD TG 309	as a specific estimate for algal biomass.	none

5.5.6. Determination of changes in physico-chemical properties caused by sterilisation methods

The determination of changes in physico-chemical properties caused by sterilisation can be based on the same parameters that are required in the corresponding test guidelines[18], or other relevant parameters.

The physico-chemical properties are recommended to be measured from both non-sterilised and sterilised samples, preferably using vessels with a test compound (as also recommended above for the determination of the sterilisation efficiency). These determinations are not required in the test guidelines and, therefore, additional replicates may need to be set up for this purpose unless other vessels (the biodegradation test/sterile control vessels or the sterilisation efficiency vessels) can be used for this purpose[19].

It is recommended that the relevant parameters are measured at least three times: before the sterilisation, at the start of the test, and at the end of the test.

[18] OECD TG 308 indicates that pH and redox potential should be determined at the start of acclimation, start of the test, during the test, and at the end of the test, for both water and sediment. In addition, O_2 concentration in the water should be measured at the same frequency. OECD TGs 307 and 309 do not include required measurements of physico-chemical properties during the study, with the exception of the anaerobic and paddy OECD TG 307 studies, where pH, oxygen concentration, and redox potential need to be measured.

[19] OECD TG 308 requires physico-chemical properties to be determined from samples without the test substance. However, when the changes in physico-chemical properties due to sterilisation need to be determined, this is recommended to be done from vessels with the test substance also in OECD TG 308.

5.6. Interpretation and use of results

5.6.1. Determination of abiotic degradation

When there is a need to determine abiotic ultimate degradation (as a percentage of the initial amount), the advice given in the test guidelines can be used. The determination for abiotic degradation in the test guidelines is mostly based on parameters reflecting mineralisation (respirometry, DOC, or residual activity). Degradation observed in the sterile control is considered to represent abiotic degradation. However, abiotic ultimate degradation is unlikely for most substances and therefore, this calculation is probably relevant only rarely, at least for the purpose of P/vP assessment under REACH.

For determining primary abiotic degradation, there would be a need to differentiate the contribution of abiotic degradation from abiotic non-degradative phenomena to the decrease in concentration observed in the sterile controls. This could require the determination of degradation/transformation products (see also Section 4.1.1) and/or quantification of the non-degradative dissipation e.g. by using traps for volatile compounds.

5.6.2. Determination of primary degradation

For determining the percentage of primary degradation, the screening test guidelines advise to subtract the decrease in concentration in the sterile control at the test end from the decrease in the viable experiment at the test end. The difference in the concentrations is divided by the concentration at the end of the sterile test.

The simulation test guideline OECD TG 309 advises that if rates of other loss processes are known (e.g. hydrolysis or volatilisation), they may be subtracted from the net loss rate observed during the test to give an approximated estimate of the biodegradation rate. As discussed above (Section 4.1) it is considered that, at least for REACH purposes, normally subtraction of abiotic degradation (such as hydrolysis) should not be performed whereas the non-degradative processes should be subtracted. These two main approaches, i.e. the subtraction approach and the comparison approach, are outlined below in more detail.

Subtraction approach

The subtraction approach means that the decrease in test substance concentration observed in the viable experiment is corrected by subtracting the decrease observed in the sterile controls. This approach is proposed in OECD TG 309 when degradation has taken place and the degradation in the sterile controls is lower than in the viable experiment.

OECD TG 309 advises to compare the means (the *residual activity* of test substance for ^{14}C-labelled substances or the *residual concentration* for non-labelled substances) of the flasks of the viable experiments and sterile controls and if the means deviate by less than 10 %, it can be assumed that the degradation observed is predominantly abiotic. The OECD TG 309 indicates that if the degradation in the sterile controls is lower (so that the deviation is 10 % or higher), the subtraction approach may be used.

OECD TG 309 only mentions degradation (but not other dissipation processes) in the context of this 10 % threshold. We consider that this 10 % threshold is not relevant for P/vP assessment, due to the following reasons:

- There is no need to differentiate biotic and abiotic degradation for comparison with P/vP criteria.
- Using a threshold of 10 % (or any other threshold) to exclude the use of the subtraction approach does not seem justified in the case of other dissipation processes either. Using the threshold of 10 % would mean that, in certain cases, even a small difference in residual concentrations in viable experiment vs sterile control (e.g. differences of 10 %

or just below 10%) would lead to a change in the calculation approach and thus a large difference in the obtained half-life and DT_{50}.

Therefore, it is proposed that the subtraction approach can be used regardless of the magnitude of the difference observed between the viable experiment and the sterile controls.

A procedure to use the sterile controls to correct the data in the viable experiment is described below. This procedure is based on OECD TG 309 with modifications.

1. For *residual concentration*, the procedure for the subtraction approach (described in points 2 and 3 below) should be applied. For *residual activity*, the subtraction approach should generally not be used because, for the purpose of P/vP assessment, there is generally no need to differentiate biodegradation and abiotic degradation. Therefore, for *residual activity,* half-life and DT_{50} determined from the viable experiment should be used without correcting for sterile controls[20].
2. Use the figures for the sterile controls to correct those obtained in the viable experiment (by subtraction) in order to give an approximated estimation of the primary biodegradation half-life (HL_{bio}) or DT_{50} (DT_{50_bio}). There are two ways for the correction:
    (a) Correct the residual concentration for every time point and perform kinetic analysis for the corrected data.
    (b) Perform kinetic analysis separately for the viable experiment and for the sterile control and calculate a primary biodegradation rate constant by subtracting the rate constant of the sterile control from the rate constant of the viable experiment.

    Regarding the choice between the two options above, we note that option (b) is in accordance with the approach in OECD TG 309 (paragraph 49) and, therefore, should be used in OECD TG 309 studies, if feasible. However, the subtraction of rate constants is appropriate only when SFO kinetics occur. If the decrease does not follow SFO kinetics (either in the viable experiment or in the sterile control or both), then option (a) should be considered.
3. Use the approximated estimation of the primary biodegradation half-life (HL_{bio}) or DT_{50} (DT_{50_bio}) for comparison with the P/vP cut-off values. Three different scenarios can be identified:
    (a) Both HL and HL_{bio} are below the relevant cut-off value (P or vP). The conclusion from the test would be clear (e.g. parent substance not P).
    (b) Both HL and HL_{bio} are above the relevant cut-off value (P or vP). The conclusion from the test would be clear (e.g. parent substance fulfils the criterion).
    (c) HL_{bio} is above the relevant cut-off value but HL is below it. In this case, the sterile control has a decisive effect on the conclusion and therefore the potential effect of the sterilisation method on the result should be carefully considered (see also Section 5.5.1).

    In addition, if a part of the primary degradation in the viable experiment is due to abiotic degradation, the subtraction approach may lead to underestimated degradation. Therefore, under Scenario (c) above, the abiotic degradation in the sterile controls can be used to refine the half-life or DT_{50} in the viable experiment. This can be done by estimating abiotic primary degradation by determining transformation products in the

[20] The difference in the recommendations for *residual concentration* and *residual activity* are based on the facts that the decrease in residual activity is always due to degradation (as the residual activity is determined based on $^{14}CO_2$ production) whereas the residual concentration can be affected also by other phenomena than degradation.

sterile controls and, in, the case of radio-labelled test substance, also from the decrease in *residual activity* in the sterile controls.

Comparison approach

In the comparison approach, the results of the viable experiment are not corrected with the results of the sterile control. The kinetic analysis is performed separately for the viable experiment and for the sterile control. The results (e.g. rate constants and half-lives) of the viable experiment and sterile controls are used as individual pieces of evidence in the assessment of the study. The half-lives from the viable experiment and sterile control can be compared to each other. However, in this approach, only the half-life derived from the viable experiment (rather than the approximated biodegradation half-life as in point 2 in Section 5.6.2) is used for the comparison to the P/vP cut-off values.

It is also possible to compare the percentages of the residual test material concentration in the viable experiment and in the sterile controls at a certain time point.[21]

Choosing between the subtraction approach and the comparison approach

It is currently difficult to give advice for the choice between these two approaches. For the soil and sediment studies there are more uncertainties regarding the subtraction approach, as there is more interaction between the test substance and the solid phase compared to water studies and partitioning to solid phase and abiotic NER formation may have a big effect on the shape of the concentration curve.

The sterilisation methods can affect these interactions and consequently also the shape of the curve. Direct comparison or subtraction of results from the viable experiment may therefore not be feasible or at least should be interpreted with caution, particularly in soil and sediment studies. It could be also informative to use both approaches (the subtraction approach and the comparison approach) in the same study. Therefore, when the comparison approach is used, an approximated biodegradation half-life could still be calculated (similarly as in the approach described above in point 2(b) under Section 5.6.2) for information, even if the comparison to P/vP cut-off values would be done primarily based on the half-life derived from the viable experiment alone. The measurement frequency should also be taken into account.

5.7. Specific issues regarding sterilisation in water-sediment tests

5.7.1. Separate vs combined sterilisation of water and sediment

OECD TG 308 includes two phases (sediment and water), which are sampled separately from the field, and placed in test vessels in the laboratory. Sterilisation can be conducted either for the combined water-sediment systems or separately for the two phases. We consider that currently there is no sufficient information for recommending either of the two approaches (sterilising water and sediment separately or together) over the other one on scientific or technical grounds[22].

Therefore, we consider that either of the approaches can be used. It is worth noting, however, that a pH decrease and trace metal transfer between phases have been recorded during sediment resuspension experiments which could be linked to abiotic processes (Layglon *et al.,* 2021). This should be taken into account when combined water-sediment is mixed during sterilisation or when the water and sediment are combined after separate sterilisation.

[21] This approach was used in the SVHC identification of EC 401-850-9 (ECHA, 2021).

[22] For example, it is not known whether a separate sterilisation would lead to better overall sterilisation efficiency, or a lower disturbance of the physico-chemical properties compared to sterilising the water and sediment together, or, to a faster stabilisation of the system.

5.7.2. Acclimation period and the timing of sterilisation

As two phases are included in the OECD TG 308 test, an acclimation period is required before the start of the test[23]. Even if the OECD TG 308 test guideline does not include any consideration of sterile controls, it is recommended that an acclimation period is applied also for the sterile controls as the purpose of the acclimation period is to reach a reasonable stability of the system before the start of the test in terms of pH, oxygen concentration in water, redox potential of the sediment and water, and macroscopic separation of the phases.

Even though biological activity may affect these properties, also abiotic processes, such as the physical settling of suspended matter, are significant for the physico-chemical parameters of water and sediment. Therefore, acclimation period is relevant also for the sterile controls. The acclimation period for sterile controls should be carried out exactly under the same conditions as the actual study period for the sterile controls. This includes, for instance, the requirement that the sterilised water-sediment system should be placed in the actual test bottles before the start of the acclimation period.

The sterilisation of the water-sediment systems is expected to cause mixing of the water and the sediment phases or at least some level of disturbance of the sediment phase. If that separate sterilisation of the water and sediment phases is conducted, the two phases will be placed in the same vessel after the sterilisation, and the system needs to be stabilised before the start of the actual test period. Therefore, there should be an acclimation period after the sterilisation, regardless of the chosen sterilisation approach (water and sediment separately or combined).

Considering that biological activity is expected to be very low in the sterile controls, the duration of the acclimation period for the sterile controls (after sterilisation) could potentially be shorter than for the viable experiment. However, this would need to be demonstrated by measurements (sufficient stability should be reached).

5.7.3. Biologically active time of the samples between field sampling and test start

For the sterile controls there is a need to have an acclimation period after the sterilisation (Section 5.7.2). If the sterilisation is conducted before the acclimation period and the actual test is started after the acclimation period (Option 1)
Table 7,

Figure 2), the biologically active time between field sampling and start of the test will be shorter for the sterile control samples than for the viable samples. Microbial activity after field sampling may cause changes in the properties of the water and sediment. As the change in conditions from the environment to start of the laboratory test is significant[24], also changes in microbial activity could be significant during this period. These changes are likely time dependent. Microorganisms can affect adsorption of chemicals due to hydrophobic interactions between chemicals and extracellular polymeric substances or the lipophilic cell membrane and

[23] Regarding the acclimation period OECD TG 308 states: "A period of acclimation should take place prior to adding the test substance, with each sediment/water sample being placed in the incubation vessel to be used in the main test, and the acclimation to be carried out under exactly the same conditions as the test incubation (see paragraphs 32 and 33). The acclimation period is the time needed to reach reasonable stability of the system, as reflected by pH, oxygen concentration in water, redox potential of the sediment and water, and macroscopic separation of phases. The period of acclimation should normally last between one week and two weeks and should not exceed four weeks. Results of determinations performed during this period should be reported."

[24] Including filtration and sieving of the sediment, mixing the water and sediment, the potential storage, and the acclimation period.

due to electrostatic interactions between positively charged groups of the chemical and the mainly negatively charged surfaces of microorganisms (Margot *et al*., 2015).

Any sterilisation method will affect the physico-chemical properties of the sample. A differing biologically active time between field sampling and start of the test would be an additional difference between the viable experiment and the sterile control. It is not known whether this difference is significant compared to the changes caused by the sterilisation. To minimise the differences in physico-chemical properties between the samples, the biologically active time from field sampling to the test start should ideally be the same for the samples used for the viable experiment and sterile controls. However, the need for an acclimation period after the sterilisation (Section 5.7.1) has to be taken into account. A potential solution that would avoid the difference in the biologically active time is to use two acclimation periods for the sterile controls (one before the sterilisation and one after that) (Option 2) (Table 7, Figure 2). This would mean that the actual test period with the sterile control would be started later than that for the viable experiment.

It is recommended to use option 2, so that the biologically active time between field sampling and the test start is the same for all samples. However, if it can be justified that the difference in the biologically active would not be significant compared to the effect of sterilisation, then options 1 and 2 are considered equally applicable.

Table 7 describes the situation where the sterilisation is applied to the combined water-sediment systems. When the sterilisation is applied separately to the water and sediment phases there are two options: in option 1, sterilisation before mixing the sediment and water in the incubation flasks and, in option 2, sterilisation after the first acclimation period. For the latter, the water and sediment phases are separated, then sterilised, and combined again in the incubation flasks.

Table 7. Options for the timing of sterilisation of the samples for the sterile controls in OECD TG 308 water-sediment simulation tests.

Option	Description	Pros	Cons	Remarks
1	Samples for the sterile controls are sterilised before the acclimation period.		Difference in biologically active time from sampling to test start between samples for viable experiment and sterile controls.	Actual test period would start at the same time for the viable experiment and sterile control.
2	Samples for the sterile controls are sterilised after the (first) acclimation period, then a 2nd acclimation period is run for the sterilised samples.	Similar biologically active time from sampling to test start between samples for viable experiment and sterile controls.		Actual test period would start at different times for the viable experiment and sterile control due to the 2nd acclimation period. The results can still be presented in parallel for the viable experiment and the sterile controls, both starting from their respective day 0. Maybe the 2nd acclimation period could be shorter than the first one, if sufficient stability can be demonstrated. In that case, the time between sterilisation and test start would be shorter than in option 1

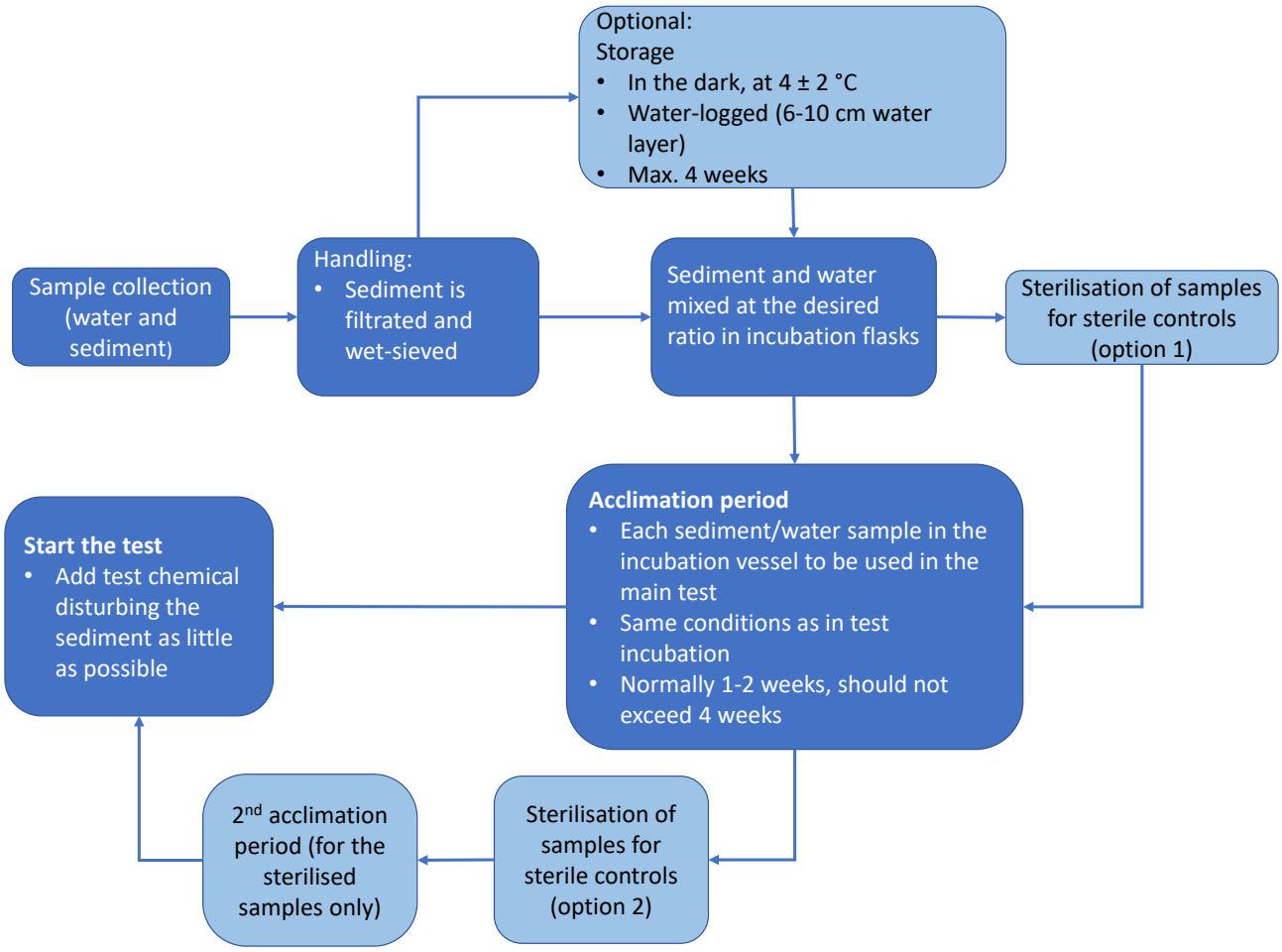

Figure 2. Steps between field sampling and test start in OECD TG 308 water-sediment simulation test, with two proposed options for the timing of sterilisation of the samples for the sterile controls. This scheme concerns situations where sterilisation is applied to the combined water-sediment system.

5.8. Comparison of sterile controls to other potential test refinements

Sterile controls could be considered a part of the "toolbox" of approaches/test refinements (including e.g. the quantification and characterisation of NERs, or traps to quantify volatilised compounds) that can be used to make the results of P/vP testing more unequivocal in terms of concluding on the P/vP property.

In certain cases, adding several of these approaches in the same test could result in the best outcome but it should be further assessed whether there are overlaps between the added value brought by the different approaches.

6. References

Full reference	Year	Remarks
Alef K and Kleiner D (1989) Rapid and sensitive determination of microbial activity in soils and in soil aggregates by dimethylsulfoxide reduction. Biology and fertility of soils, 8(4), 349-355.	1989	
Bank TL, Kukkadapu RK, Madden AS, Ginder-Vogel MA, Baldwin ME, Jardine PM (2008) Effects of gamma-sterilisation on the physico-chemical properties of natural sediments. Chemical Geology, *251*(1-4), 1-7.	2008	
Berns AE, Philipg H, Narres HD, Burauel P, Vereecken H and Tappe W (2008) Effect of gamma-sterilisation and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. European Journal of Soil Science, 59 : 540-550	2008	
Chifflet S, Quemeneur M, Barani A, Angeletti B, Didry M, Grégori G, Pradel N (2019) Impact of sterilisation methods on dissolved trace metals concentrations in complex natural samples: optimization of UV irradiation. MethodsX, *6*, 1133-1146.	2019	
Chiou CT, Malcolm RL, Brinton TI, Kile DE (1986) Water solubility enhancement of some organic pollutants and pesticides by dissolved humic and fulvic acids. Environmental science & technology, *20*(5), 502-508.	1986	
Claßen D, Siedt M, Nguyen KT, Ackermann J, Schaeffer A (2019) Formation, classification and identification of non-extractable residues of ^{14}C-labelled ionic compounds in soil. Chemosphere, *232*, 164-170.	2019	
Coll C, Bier R, Li Z, Langenheder S, Gorokhova E, Sobek A (2020) Association between aquatic micropollutant dissipation and river sediment bacterial communities. Environmental science & technology, *54*(22), 14380-14392.	2020	
ECHA (2018a) Member State Committee support document for identification of dodecamethylcyclohexasiloxane (D6) as a substance of very high concern because of its PBT and vPvB properties (Article 57 D&E) adopted on 13 June 2018.	2018	The document includes information e.g. on the OECD TG 308 study on decamethylcyclopentasiloxane (D5) (EC 208-764-9).
ECHA (2018b) Member State Committee Support Document. Substance name: Terphenyl, hydrogenated. EC Number: 262-967-7. CAS number: 61788-32-7. Adopted on 1 June 2018.	2018	

ECHA (2021) Member State Committee Support Document. Substance name: S-(tricyclo[5.2.1.02,6]deca-3-en-8(or 9)-yl) O-(isopropyl or isobutyl or 2-ethylhexyl) O-(isopropyl or isobutyl or 2-ethylhexyl) phosphorodithioate. EC Number: 401-850-9. CAS number: 255881-94-8. Adopted on 14 December 2021.	2021	
Flores-Céspedes F, González-Pradas E, Fernández-Pérez M, Villafranca-Sanchez M, Socias-Viciana M and Ureña-Amate MD (2002) Effects of dissolved organic carbon on sorption and mobility of imidacloprid in soil. Journal of environmental quality, *31*(3), 880-888.	2002	
FOCUS (2014) Generic guidance for Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration. Report of the FOCUS Work Group on Degradation Kinetics.18 December 2014. version 1.1, 440pp.	2014	
Griebler C (1997) Dimethylsulfoxide (DMSO) reduction: a new approach to determine microbial activity in freshwater sediments. Journal of microbiological methods, 29(1), 31-40.	1997	
Lees K, Fitzsimons M, Snape J, Tappin A and Comber S. (2018) Soil sterilisation methods for use in OECD 106: How effective are they?, Chemosphere, Volume 209, 2018, Pages 61-67, ISSN 0045-6535,	2018	
Lotrario JB, Stuart BJ, Lam T, Arands RR, O'Connor OA and Kosson DS (1995) Effects of sterilisation methods on the physical characteristics of soil: implications for sorption isotherm analyses. Bulletin of environmental contamination and toxicology, *54*(5), 668-675.	1995	
Layglon N, Misson B, Gallois N, d'Onofrio S, Lenoble V, Mounier S, Omanović D and Garnier C (2021) Negligible microbial heterotrophic quantitative contribution onto trace metals remobilization during marine sediment resuspension - insights from a Mediterranean urbanized bay. Marine Chemistry*, 234, 103981.*	2021	
MacKay AA and Vasudevan D (2012) Polyfunctional ionogenic compound sorption: challenges and new approaches to advance predictive models. Environmental science & technology, 46(17), 9209-9223.	2012	
Hughes CB, Brown D, Camenzuli L. Redman A, Arey JS, Vione D, Wang N, Vaiopoulou E (2020) Can a chemical be both readily biodegradable AND very persistent (vP)? Weight-of-evidence determination demonstrates that phenanthrene is not persistent in the environment. *Environ Sci Eur* 32, 148 (2020). https://doi.org/10.1186/s12302-020-00427-1		
Margot J, Rossi L, Barry DA and Holliger C (2015) A review of the fate of micropollutants in wastewater treatment plants. Wiley Interdisciplinary Reviews: Water, *2*(5), 457-	2015	

487.		
Martens R (2001). Estimation of ATP in soil: extraction methods and calculation of extraction efficiency. Soil Biology and Biochemistry, 33(7-8), 973-982.	2001	
Moriarty DJW (1990) 6 Techniques for Estimating Bacterial Growth Rates and Production of Biomass in Aquatic Environments. In *Methods in microbiology* (Vol. 22, pp. 211-234). Academic Press.	1990	
Otte J, Blackwell N, Soos V, Rughdft S, Maisch M, Kappler A, Kleindienst S and Schmidt C (2018) Sterilisation impacts on marine sediment---Are we able to inactivate microorganisms in environmental samples?, FEMS Microbiology Ecology, 94 (12): 10.1093femsec/fiy189	2018	Supplementary data available in journal webpage includes e.g. an overview table of sterilisation methods highlighting their advantages and disadvantages
Rozycki M and Bartha R (1981) Problems associated with the use of azide as an inhibitor of microbial activity in soil. Applied and environmental microbiology, *41*(3), 833-836.	1981	
Seller C, Özel Duygan BD, Honti M and Fenner K (2021) Biotransformation of Chemicals at the Water–Sediment Interface— Toward a Robust Simulation Study Setup. ACS Environmental Au*,* 1(1), 46-57.	2021	
Shaw LJ, Beatonb Y, Glover LA, Killham K and Meharg AA (1999) Re-inoculation of autoclaved soil as a non-sterile treatment for xenobiotic sorption and biodegradation studies. Applied Soil Ecology, *11*(2-3), 217-226.	1999	
Shrestha P, Hughes CB, Camenzuli L, Lyon D, Meisterjahn B, Hennecke T, Griffiths M, and Hennecke D (2022) Improving closed test Setup for biodegradation testing of slightly volatile substances in water-sediment systems (OECD 308). Submitted to Chemosphere 2022.	submitted	
Shrestha P, Meisterjahn B, Klein M, Mayer P, Birch H, Hughes CB and Hennecke D (2018) Biodegradation of volatile chemicals in soil: separating volatilization and degradation in an improved test setup (OECD 307). Environmental science & technology, 53(1), 20-28.	2018	
Shrestha P, Meisterjahn B, Hughes CB, Mayer P, Birch H, and Hennecke D (2020) Biodegradation testing of volatile hydrophobic chemicals in water-sediment systems Experimental developments and challenges. Chemosphere, 238 (January 2020).	2020	
Stephens KD, Farenhorst A and Fuller LG (2002) Effect of soil sterilisation by mercuric chloride on herbicide sorption by soil. Journal of Environmental Science and Health, Part B, 37(6), 561-571.	2002	

Tukes (2021) Substance Evaluation Conclusion as required by REACH Article 48 and Evaluation Report for Resin acids and rosin acids, hydrogenated, esters with glycerol. EC No 266-042-9, CAS No 65997-13-9. 8 July 2021. Evaluating MS: Finland.	2021	
Tuominen L, Kairesalo T and Hartikainen H (1994) Comparison of methods for inhibiting bacterial activity in sediment. Appl Environ Microbiol. 1994;60(9):3454-3457.	1994	
Unnamed (2010). Biodegradation in water: sediment simulation testing. Study report summary included in the ECHA dissemination site for decamethylcyclopentasiloxane (EC 208-764-9). Available at: https://echa.europa.eu/fi/registration-dossier/-/registered-dossier/14807/5/3/ (accessed 01 November 2022)	2010	
Unnamed (2016). Biodegradation in soil. Study report summary included in the ECHA dissemination site for N,N-dicyclohexylbenzothiazole-2-sulphenamide (EC 225-625-8). Available at: https://echa.europa.eu/registration-dossier/-/registered-dossier/14313/5/3/4 (accessed 15 November 2022)	2016	
Wehr JB and Kirchhof G (2021) Gamma irradiation with 50 kGy Has a Limited Effect on Agronomic Properties of Air-Dry Soil. Soil Systems, 5(2), 28.	2021	

Annex 1.

Table 8. ECHA's substance evaluation decisions adopted until 11/2021 with requests for simulation tests, where requirements for sterile controls have been specified.

Substance name	EC number	Type of test	Type of sterile controls specified	Date of adoption; deadline for data submission	Justifications for requiring sterile controls
3-ethoxy-1,1,1,2,3,4,4,5,5,6,6,6-dodecafluoro-2-(trifluoromethyl)- hexane	435-790-1	OECD TG 308; radiolabelled or non-labelled test material	sterile water-sediment controls, sterile purified water controls	09/2020; 04/2023	**sterile water-sediment controls**: "…must be included in the test to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses (e.g.volatilisation, formation of non-extractable residues (NER))." **sterile purified water controls**: "If the test substance is not radiolabelled, it will not be possible to differentiate whether the observed losses in a sterile water-sediment control are due to leakage of the volatile fraction or due to formation of NER. In that case, including also a sterile control containing only purified water, without sediment addition, will further help the interpretation of the results as the NER formation is minimised, and hence, any potential losses are assumed to be due to leakage of the test substance. If in a sterile purified water control the test substance is maintained in the test bottles, then it can be assumed that negligible leakages are occurring in the water-sediment test bottles. Therefore, if non-labelled test material is used, sterile purified water controls must also be included in the test."
Alkanes, C16– (branched), C20- (branched) and C24- (branched) l	700-992-1	OECD TG 309; OECD TG 308 (request conditional to OECD TG 309); radiolabelled test material	sterile surface water controls, sterile water-sediment controls	11/2021; 12/2023	**sterile surface water controls:** "The inclusion of sterile controls is important to determine to what extent the decrease of the test substance is due to potential contribution of abiotic losses." **sterile water-sediment controls** "Sterile water-sediment controls must be included in the test to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses (e.g. volatilisation, formation of NERs)."
A mixture of: propan-2-one-O,O'(methoxyvinylsilandiyl)dioxime; propan-2-one-O-	458-680-3	OECD TG 307	sterile soil controls	06/2021; 12/2022	**sterile soil controls: "**…must be included in the test to determine to what extent the test substance decrease is due to biotransformation, or to potential abiotic losses (e.g.volatilisation, formation of NER), In addition, as the Substance is known to undergo abiotic hydrolysis in pure water and as adsorption to soil may

Substance name	EC number	Type of test	Type of sterile controls specified	Date of adoption; deadline for data submission	Justifications for requiring sterile controls
(dimethoxyvinylsilyl)oxime; propa n-2-one-O,O',O"-(vinylsilantriyl)trioxime					decrease the rate of hydrolysis, the sterile controls will be useful for estimating the rates of abiotic hydrolysis in the studied soils."
Di-tert-butyl 3,3,5-trimethylcyclohexylidene diperoxide	229-782-3	OECD TG 307	sterile soil controls	06/2021; 09/2023	**sterile soil controls:** "Another PfA suggested to include sterile controls in the test to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses (e.g. volatilisation, formation of non-extractable residues (NER)). ECHA agrees with the PfA and modified the draft decision accordingly."
Methylethylketone peroxide trimer	429-320-2	OECD TG 309: radiolabelled or non - radiolabelled test material	sterile surface water controls, sterile purified water controls	06/2021; 06/2023	"To make a full evaluation of the fate of the MEK peroxide trimer, constituent of the Substance, a mass balance is a prerequisite. […] as you commented that no contract laboratory was willing to generate radiolabelled peroxide trimer due to its potential explosive properties, non-radiolabelled material can be used provided […] If no or negligible degradation (less than 30%) is observed in the OECD TG 309, an analytical procedure showing stable parent MEK peroxide trimer concentrations would be sufficient. Carbon dioxide formation from degradation of the MEK peroxide trimer will however be very difficult to quantify as well as abiotic losses and NER formation. Therefore**, sterile controls (as required by OECD TG 309)** must be included in the test to determine to what extent decrease in MEK peroxide trimer is due to biotransformation or to potential abiotic losses (e.g. volatilisation, formation of non-extractable residues (NER)). By using non-radiolabelled test material, it will not be possible to differentiate whether the observed losses in a sterile control (sterilised surface water including the natural SPM content) are due to loss of the volatile fraction (leakage from test system or sorption to the materials of the test apparatus, e.g. stoppers and tubing) or due toformation of NER. In that case, including also a **sterile control containing only purified water, without addition of surface water,** will further help the interpretation of the results as the NER formation is minimised, and hence, any potential losses are assumed to be due to loss of the volatile fraction of

Substance name	EC number	Type of test	Type of sterile controls specified	Date of adoption; deadline for data submission	Justifications for requiring sterile controls
					the test material. If in a **sterile purified water control** no loss due to sorption/leakage of the volatile fraction occurs then it can be assumed that negligible losses due to these reasons are occurring in the active test bottles. Therefore, if non-radiolabelled test material is used, sterile purified water controls must also be included in the test."
N-[4-[(9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthryl)amino]phenyl]acetamide	267-636-0	OECD TG 309, OECD TG 308 (request conditional to OECD TG 309); radiolabelled test material	sterile surface water controls (OECD TG 309); sterile water-sediment controls (in OECD TG 308)	02/2021;09/2023 (deadline set in Board of Appeal decision A-007-2021))	**sterile surface water controls**: no justification included **sterile water-sediment controls**: "… must be included in the test to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses (e.g. volatilisation, formation of non-extractable residues (NER))."
Oligomerisation and alkylation reaction products of 2-phenylpropene and phenol	700-960-7	OECD TG 309, OECD TG 308 (request conditional to OECD TG 309); radiolabelled test material	sterile water-sediment controls (in OECD TG 308)	12/2020; 09/2022	**sterile water-sediment controls**: "…must be included in the test to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses."

Table 9. ECHA's substance evaluation decisions with requests for ready biodegradation tests, where requirements for sterile controls have been specified.

Substance name	EC num-ber	Type of test	Type of sterile controls specified	Date of adoption; deadline for data submissio n	Justifications for requiring sterile controls
Resin acids and Rosin acids, hydrogenated, esters with glycerol	266-042-9	OECD TG 310 (with several specifications, such as analytical determination of the concentrations of the test substance)	not specified	02/2017; data submitted and SEV conclusion published	"…if primary degradation measurement is used for the conclusion, sterile control experiment is necessary to verify the contribution of abiotic phenomena including adsorption processes."
Resin acids and Rosin acids, hydrogenated, esters with pentaerythritol	264-848-5	OECD TG 310 (with several specifications, such as analytical determination of the concentrations of the test substance)	sterile controls with and without inoculum *	12/2020; 05/2022	Sterile controls as defined in the OECD TG 310 (i.e., including inoculated test medium) and sterile controls with test medium but without inoculum must be included to verify the contribution of abiotic phenomena including adsorption processes and hydrolysis to any observed removal of the test substance. The reason for the requirement of sterile controls with test medium but without inoculum is to determine whether the extractability of the test material changes during the study (in the absence of inoculum) and whether abiotic hydrolysis occurs under the conditions of the test. In the OECD TG 310 study with glycerol monoesters, there was a significant decrease of the monoesters also in the sterile controls and it remains unclear whether this was e.g. due to hydrolytic enzymes present in the inoculum (which may have been still active), or whether this was due to the possible effects of the toxicant7 on the extractability or analysis of the monoesters. In addition, CO_2 production in the study may be lower than in the studies performed on the glycerol monoesters, due to the potentially lower degradability of pentaerythritol compared to glycerol, a lower concentration of the monoesters compared to the test substances used for the glycerol monoesters, and the

Substance name	EC num-ber	Type of test	Type of sterile controls specified	Date of adoption; deadline for data submissio n	Justifications for requiring sterile controls
					likely presence of di-, tri, and tetraesters in the test substance. Therefore, it may be more challenging to quantify to what extent the decrease in the monoester concentration is due to biodegradation. The inclusion of two different sterile controls is expected to help in this quantification.
Bis(2-ethylhexyl)amine	203-372-4	OECD TG 301D (with several specifications, such as analytical determination of the concentrations of the test substance)	sterile control without inoculum and sterile control with (sterilised) inoculum	11/2021 08/2022	The test must include a sterile control containing no inoculum and a sterile control with (sterilised) inoculum, both prepared and treated similarly to the test vessels, to verify whether there are losses from the test system due to adsorption or even volatilisation.”

*The decision stated: ”two sterile controls must be included: 1) sterile controls as defined in the test guideline (i.e., with inoculated test medium) and 2) sterile controls with test medium but without inoculum”

Annex 2.

Text extracts from published SEV decisions.

EC 229-782-3, request for OECD TG 307, with radiolabelled test substance:

"Sterile controls

Another PfA suggested to include sterile controls in the test to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses (e.g. volatilization, formation of non-extractable residues (NER)). ECHA agrees with the PfA and modified the draft decision accordingly.

In this context, ECHA notes that it is important to ensure that test conditions in the sterile controls and the active test bottles are as equal as possible. A precondition for conclusion on degradation is that other removal processes are not assessed as degradation. With this aim it is necessary to compare processes observed in sterile controls with those observed in the active test bottles under comparable test conditions.

Therefore, other specifications of the sterile control bottles, such as the headspace volume, sampling times, analytical measurements as well as any potential cause of disturbance (such as aeration events) that might affect the distribution of the test substance or that could cause leakage, must be the same as in the active test bottles, to ensure comparability.

The OECD TG 307 includes instructions for a sterile control but does not include specific advice on soil sterilisation methods. The OECD TG 307 refers to two references for soil sterilisation methods (OECD, 1993; Stenberg et al., 1996). However, the eMSCA checked these references and found no information on sterilisation of soil samples. Therefore, you are advised to consider relevant publicly available information, such as the articles by Lees et al. (2008) and Berns. et al (2018) for technical guidance on soil sterilisation methods. Considering the importance of the integrity of the soil to produce meaningful results for comparison to unsterilised conditions, ECHA recommends to use methods that have the least impact on the mineral phases and the geochemistry of the soil.

Berns et al. (2008) studied the effect of two common soil sterilisation methods (gamma radiation and autoclaving) on two different types of agricultural soils. They concluded that the choice of the sterilisation method strongly depends on the type of study or research questions being asked. For degradation experiments, gamma-sterilized soils are better suited as control soils than autoclaved soils, because they are physically and chemically less altered by the process of sterilisation.

Lees et al. (2018) assessed autoclaving, γ-irradiation, and sodium azide as soil sterilisation methods for use in adsorption/desorption studies. They reported that autoclaving destroyed the soil structure, therefore potentially affecting its sorption behaviour while sodium azide changed the pH of the loam soil solution by 0.53 pH units. Γ-irradiation exhibited least disruption to the tested soils physico-chemical properties. The authors concluded that γ-irradiation was the best available method for sterilising soils in preparation for sorption-desorption experiments, but advocated for a case-by-case basis approach for choosing the best sterilisation in different soil types.

In conclusion, you must explain and justify the method and procedure used for establishing the sterile controls in the study report, and determine the efficiency of the sterilisation by measurements of microbial biomass. OECD TG 307 indicates that the microbial biomass must be measured initially, during and at the end of the aerobic studies. Finally, ECHA notes that communication with the eMSCA is possible in case you wish to have a mutual discussion on the preparation of the sterile controls."

EC 229-782-3, request for OECD TG 308, with radiolabelled test substance:
(Note that in this case the OECD TG 308 is a conditional request, to be performed should it prove technically unfeasible to perform the water degradation test)

"*Furthermore, sterile water-sediment controls must be included in the OECD TG 308 test to determine to what extent the test substance decrease is due to biotransformation or to potential abiotic losses (e.g. volatilisation, formation of non-extractable residues (NER)).*

ECHA notes that it is important to ensure that test conditions in the sterile controls and the active test bottles are as identical as possible. A precondition for conclusion on degradation is that other removal processes are not assessed as degradation. With this aim it is necessary to compare processes observed in sterile controls with those observed in the active test bottles under comparable test conditions.

Therefore, other test specifications of the sterile control bottles, such as the headspace volume, sampling times, analytical measurements as well as any potential causes of disturbance (such as aeration events) that might affect the distribution of the test substance or that could cause leakage, must be the same as in the active water-sediment test bottles, to ensure comparability.

OECD TG 308 does not include instructions for a sterile control. However, OECD TG 309 gives guidance on the preparation of sterile water controls as well as sterile controls containing water with sediment added in large amounts. Furthermore, ECHA notes that the OECD TG 308 test (ECHA, 2018) for decamethylcyclopentasiloxane (EC 208-764-9), as well as other published water-sediment degradation simulation studies (e.g. Liu et al. 2013; Shrestha et al. 2016, 2020) included sterile controls and can provide guidance on the preparation of sterile controls. In these studies the sterilisation was done either by the addition of sodium azide, autoclaving or both. In addition, in another publication (Otte et al. 2018) different methods for sterilisation of marine sediment were compared.

The selection of the sterilisation method and time to perform the sterilisation in the sterile water-sediment controls, e.g. before or after the acclimation period specified in the paragraph 31 of OECD TG 308, may have an effect on the sediment properties. Based on Otte et al. (2018), thermal sterilisation, gamma radiation and chemical sterilisation have all advantages and disadvantages. Considering the importance of the integrity of the sediment phase to produce meaningful results for comparison to unsterilised conditions, ECHA recommends to use methods that have the least impact on the mineral phases and the geochemistry of the sediment. OECD TG 309 indicates that the sorption characteristics of the sediment may be altered by autoclaving. According to Otte et al. (2018) autoclaving and gamma radiation lead to a large increase in dissolved organic carbon and have impacts on the mineral phase, while chemical sterilisation seems to be the method that would likely have the least impact on the geochemistry of the sediment phase. However, it should be noted that chemical sterilisation may also affect some sediment properties, e.g. triggering changes in pH.

In conclusion, you must explain and justify the methods and procedure used for establishing the sterile controls in the study report, and determine the efficiency of the sterilisation by measurements of microbial biomass. OECD TG 308 indicates that the microbial biomass of both water and sediment must be measured at post-handling, test start and test end, and mentions methods for that. Finally, ECHA notes that communication with the eMSCA is possible in case you wish to have a mutual discussion on the preparation of the sterile controls."