OECD GUIDELINE FOR THE TESTING OF CHEMICALS

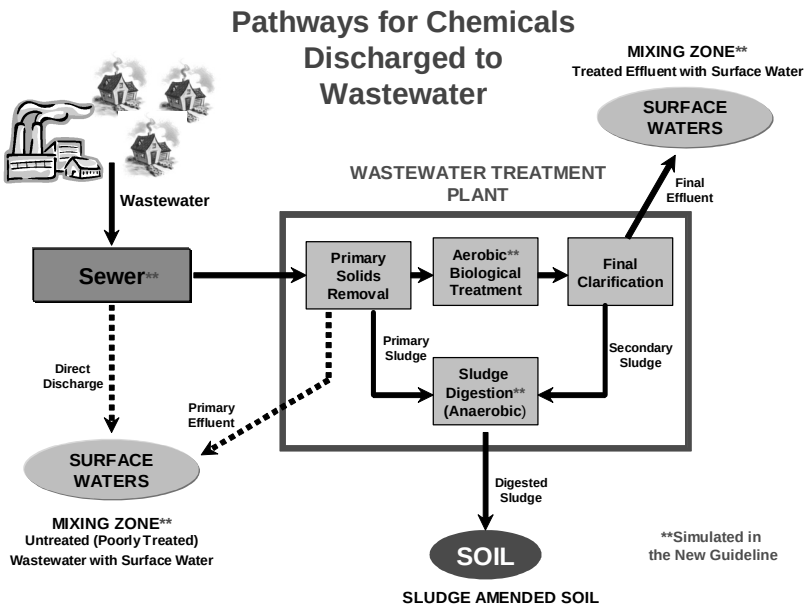
PROPOSED OECD 3xx GUIDELINE

Simulation Tests to Assess the Primary and Ultimate Biodegradability of Chemicals Discharged to Wastewater: Biodegradation in Wastewater, Activated Sludge, Anaerobic Digester Sludge, Mixing Zone for Treated Effluent and Surface water and Mixing Zone for Untreated Wastewater and Surface water

INTRODUCTION

1. This guideline describes methods for determining the extent and kinetics of primary and ultimate biodegradation of organic chemicals whose route of entry into the environment begins with their discharge to wastewater. Personal care, household cleaning and laundry chemicals are typically discarded down the drain as part of their normal use and become consistent components of domestic wastewater. Likewise, pharmaceuticals are excreted or disposed down the drain. Other chemicals may be episodically or continuously discharged to wastewater as a result of manufacturing processes.

2. This guideline consists of five separate but related simulation tests for assessing the primary and ultimate biodegradation of chemicals in wastewater during transit in the sewer, secondary treatment in an activated sludge treatment system, anaerobic digestion of sludge as well as treated effluent in the mixing zone of surface water and untreated wastewater that is directly discharged to surface water. Biodegradation in each compartment can play an important role in determining chemical exposure in interconnected aquatic and terrestrial habitats.



3. The figure at right shows the most common transport pathways for chemicals discharged to wastewater. Wastewater initially enters a sewer, where it may remain for hours or a few days, during its transport to a wastewater treatment plant (WWTP) or environmental release site. In most situations, the wastewater is treated before release, but in some situations the wastewater is released to surface water directly or with only minimal primary treatment. Within a typical WWTP, a portion (40-60%) of the solids is removed during primary treatment. The resulting effluent is then subjected to biological treatment and the solids are removed in a

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final clarifier. The final effluent is subsequently released to surface water. The sludge solids removed during primary treatment and final clarification are most commonly digested under anaerobic conditions if the sludge disposal involves land application.

4. The fraction of the chemical released to the environment in effluent or associated with sludge solids is a function of its partitioning behaviour and its biodegradation rate. Due to chemical residence time and the level of biological activity, the critical opportunities for significant removal through biodegradation are 1) in the sewer, 2) during aerobic secondary treatment and 3) during anaerobic digestion of the sludge. Consequently, these three systems are the most important to simulate for quantifying biodegradation losses during wastewater transport and treatment. Furthermore, the effects of treatment processes extend into the environment at the time of release. Thus, biodegradation in the mixing zones and in the water as it moves away form the point of release is key to understanding downstream dispersion and exposure.

5. The five simulation test methods described are open batch system or closed gas flow-through batch system that include elements from OECD guidelines 301 (1), 303A (2), 309 (3) 310 (4) and 311 (5). The principal objectives of the methods are to 1) measure the rate of primary biodegradation, 2) measure the rate of mineralization, and 3) follow the formation and decay of major transformation products when appropriate. In addition, characterization and quantification of major transformation products may be possible if suitable analytical methods are available.

6. These tests can be designed to assess accurately the biodegradation of both new and existing chemicals (6, 7, 8, 9, 10, 11) released continuously or episodically to wastewater. In some cases, the resulting kinetic constants can serve as input constants for exposure models used for risk assessment. These tests are intended as higher tier tests for assessing the biodegradation of chemicals, which do not biodegrade in OECD screening tests, or for refining biodegradation rates used for an exposure assessment.

GENERAL PRINCIPLES OF THE TESTS

7. Typically, a test chemical, radiolabelled in an appropriate position, is incubated with an environmental sample, which has been freshly collected from a representative field site or maintained in the laboratory under conditions realistically simulating some future environmental condition. Abiotic and biotic treatments are prepared for each test chemical and condition. Biological activity is inhibited in the abiotic control, which is used for estimating mineralization by difference, establishing extraction efficiency, and recovery of the parent molecule and quantifying other loss processes, such as hydrolysis, oxidation, volatilization or sorption to test apparatus.

8. If an analytical method with the required sensitivity is identified, the rate of parent degradation or transformation can be determined using a non-radiolabelled test substance or by following the disappearance of a chemical already in an environmental sample. However, ultimate biodegradation of non-radiolabelled substances cannot be determined unless the biodegradation pathway is well understood and analytical methods with required sensitivity are available for potential metabolites.

9. An environmentally relevant concentration of the test material is dosed to both abiotic and biotic test systems. The prepared treatments are incubated at a relevant temperature with continuous mixing when appropriate. Samples are periodically removed for determination of mineralization and primary biodegradation.

10. Tests can be performed using an open batch system or a closed gas flow-through batch system

where traps are used to capture evolved $^{14}CO_2$ or $^{14}CH_4$. The closed flow-through system is absolutely mandatory for volatile test materials. It is also usually preferred for ^{14}C-lableled test chemicals. Open systems are appropriate for non-volatile ^{3}H test chemicals and for refining the biodegradation kinetics of non-volatile ^{14}C test materials, whose ability to be mineralized has previously been established. In the open system, mineralization to $^{14}CO_2$ ($^{14}CH_4$) can be determined indirectly by measuring the difference in residual radioactivity between samples from the biotic and abiotic treatments following acidification. Similarly, mineralization to $^{3}H_2O$ can be determined indirectly by measuring the difference in residual radioactivity following drying. The open system is not appropriate for use with volatile test materials. In the flow through systems, evolved $^{14}CO_2$ is measured directly in the base traps. In addition, dissolved $^{14}CO_2$ is determined by acidifying samples in a sealed vessel and measuring radioactivity in a base trap contained in the vessel. Under anaerobic conditions, the evolved $^{14}CO_2$ and $^{14}CH_4$ are collected in tandem. The $^{14}CO_2$ is trapped in base and $^{14}CH_4$ is combusted and converted to $^{14}CO_2$, which is subsequently trapped in a similar manner. The choice of test design depends on the type of radiolabel (^{14}C or ^{3}H), the environmental compartment and the properties of the test material.

11. Samples from both treatments are analyzed for total radioactivity, extractable parent and metabolites and radioactivity associated with the extracted solids. The level of parent and metabolites is determined using chromatographic separation and radio-analytical detection methods. The solids remaining from the extraction process are combusted to estimate incorporation into biomass by difference or can be further fractionated to determine uptake into various components of biomass. A complete mass balance of the test system is obtained from the sum total of all fractions at each sampling.

12. The level of parent remaining with time can be analyzed using various decay models to estimate primary biodegradation rates. Likewise, the level of cumulative mineralization can be analyzed using various production models to estimate mineralization rates.

APPLICABILITY OF TESTS

13. The various tests are designed to assess biodegradation during key phases of wastewater transit as well as treatment and environmental release. The choice of tests should be based on the release scenarios and anticipated properties of the chemical in question. In the case of volatile test materials, appropriate modification must be made to quantify losses due to volatilization.

3xxA Biodegradation in a Sewer System Test

14. The purpose of this test is to evaluate biodegradation in raw wastewater under conditions normally found in sewer systems. Performing this test is useful if there is sufficient time in a sewer for the chemical to undergo significant biodegradation and loss. Hence, it is usually most useful for relatively labile chemicals discharged to large municipal sewer systems. In addition, the test provides data that may be used to determine the concentration of a chemical sorbed to primary sludge. Under the conditions of this test, the level of test chemical is at its expected level in wastewater with the biomass level being that normally present in a representative wastewater sample. While oxygen is present, the system is minimally aerated to simulate dissolved oxygen conditions in sewers.

3xxB Biodegradation in Activated Sludge Test

15. The purpose of the activated sludge test is to evaluate biodegradation during a widely used form of biological sewage treatment. It is applicable to any chemical subjected to such treatment and is key to estimating final effluent concentrations. It is generally the first and most important test in the series. This test is characterized by a high level of biomass and a relatively low level of test chemical under well-

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aerated conditions. The activated sludge test can compliment or be a lower cost alternative to the OECD 303A, a dynamic simulation of a wastewater treatment plant which can determine the removal of a test chemical under a specific set of operating conditions (i.e. hydraulic retention time, solids residence time, solids level etc.). The OECD 303A can generate a simple removal number or a comprehensive picture of biodegradation and sorption that occur at steady state during treatment. However, as an alternative to the expense and complexity of running a full scale system, the activated sludge test can generate a first order rate constant for the loss of parent and mineralization that can be used as inputs into a variety of wastewater simulation models to estimate removal under any set of operating conditions.

3xxC Biodegradation in Anaerobic Digester Sludge Test

16. The purpose of anaerobic digester sludge test is to evaluate biodegradation during anaerobic sludge digestion. It is particularly relevant for sorptive chemicals, which partition to primary and secondary sludge. This test is useful for refining the concentration of a chemical present in the sludge leaving a treatment plant as well as demonstrating the potential for anaerobic biodegradation. This test is characterized by reducing conditions, a high level of anaerobic biomass and a level of test chemical based upon expected wastewater concentrations and partitioning behaviour.

3xxD Biodegradation in Treated Effluent-Surface water Mixing Zone Test

17. The purpose of the effluent mixing zone test is to evaluate the biodegradation of the portion of a chemical that passes through treatment and is released in effluent to surface water and can be used to demonstrate that biodegradation occurring in the treatment plant continues in the receiving environment. It is based upon the principle that both the chemical and microbes degrading that chemical are discharged together in effluent. The results of this test can be used to estimate the reduction in a chemical concentration as a result of biodegradation as a volume of water moves downstream from a wastewater treatment plant. This test is characterized by very low levels of both test chemical and biomass under well-aerated conditions. This test differs from OECD 309 in that the surface water is amended with treated effluent and it can be used to evaluate volatile materials. Also, the OECD 309 focuses largely on mineralization whereas this test is designed to evaluate primary and ultimate biodegradation as well as metabolite formation and disappearance of chemicals discharged to wastewater.

3xxE Biodegradation in Untreated Wastewater-Surface water Mixing Zone Test

18. The purpose of this test system is to evaluate biodegradation in untreated wastewater that is directly discharged to surface water. This test is useful for determining the relative biodegradation rate for a chemical compared to other organic components in wastewater. Under the conditions of this test, the levels of test chemical and biomass are based upon their expected concentrations in wastewater-surface water mixing zones. Oxygen is present but at reduced levels due to the high level of organic loading.

INFORMATION ON THE TEST SUBSTANCE

19. In most cases, ^{14}C or ^{3}H radiolabelled test substances are necessary for this test. For radiolabelled materials, additional unlabelled material may be necessary to achieve the needed test concentration. For substances with low specific activities, the sensitivity of the method can be improved in part by increasing the volume of the analytical samples.

20. For ^{14}C, the radiolabel should be localized in the most recalcitrant portion of the molecule to monitor comprehensively metabolite formation and decay. In other cases, it may be more appropriate to position the label in a portion of the molecule whose fate is poorly understood. Regardless, interpretation

of the results must consider the position of the label as it relates to mineralization and the metabolites observed.

21. Tritiated materials can be an alternative to 14C materials, for reasons of cost or practical synthesis. Tritium labelling often results in random or uniform distribution of tritium atoms in the molecule, which must be taken into account in interpreting mineralization and metabolite patterns.

22. Non-labelled test substances can be used to determine the rate of parent degradation or transformation if an analytical method with the required sensitivity is identified.

23. The following information on the test substance is helpful for designing a test:

- solubility in water [OECD 105] (12);
- solubility in organic solvent(s) (substances applied with solvent or with low solubility in water);
- dissociation constant (pKa) if the substance is liable to protonation or deprotonation [OECD 112] (13);
- vapour pressure [OECD 104] (14) and/or Henry's law constant;
- chemical stability in water and in the dark (hydrolysis) [OECD 111] (15).
- environmental concentration, if known or estimated;
- toxicity of the test substance to microorganisms [OECD 209] (16);
- ready [OECD 301] (1) and/or inherent [OECD 302] (17, 18, 19) biodegradability

REFERENCE SUBSTANCE

24. A substance that is normally easily degraded under the test conditions may be useful as reference substance. The purpose of such a reference substance is to ensure that the microbial community in the test system is active. Alternatively, a substance, whose fate in the environment is well understood, may be included as a standard to which the results of the test compound can be compared. While the use of a reference substance is not required, it may provide useful information for the interpretation of the test results.

QUALITY CRITERIA

Validity of the Tests

25. The mass balance from the abiotic treatment is used to confirm the recovery of parent from the test system. It is recommended that an abbreviated pilot die-away study be conducted prior to the definitive test to establish the appropriate extraction system for parent and metabolites. Target recoveries from the test matrix should be 85% -110%; however, these ranges should not be used as criteria for acceptance of the test. If parent recoveries from initial samples taken from the abiotic control are in the targeted range, the sample preparation procedures are suitable for the recovery of the parent compound from the test matrix. Lower than targeted recoveries in the abiotic treatment could be due to poor extraction efficiency, sorption to glassware, or chemical degradation (see below).

26. Total recovery of radioactivity in both abiotic and biotic conditions should normally range from 75 to 115% in each individual sample, and average total recovery for all samples within a treatment should normally range from 85 to 110%. However, these ranges should not be used as criteria for acceptance of the test. If mass balances from the abiotic treatment are in the targeted range but those in the biotic test system are significantly below this range, the lower recovery likely results from the inability to efficiently

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trap $^{14}CO_2$, recover metabolites or the loss of metabolites to glassware or volatilization.

27. If chemical analysis from the abiotic control samples reveals that parent remained intact throughout the experiment, the biodegradation in the biotic treatment can be attributed to microbial activity. If the abiotic treatment indicates degradation of parent over time, interpretation of these results may include a description/explanation of the likely abiotic process that occurred. Comparison between the extent of parent degradation and metabolite formation observed in the two treatments will provide an estimate of the extent of biological versus chemical degradation in the biotic treatment, assuming loss is not an artefact of sample preparation.

Sensitivity of analytical methods

28. The limit of detection (LOD) of the analytical method for the test substance and for the transformation products should be $\leq$ 1% of the initial amount added to the test system if possible. The limit of quantification (LOQ) should be equal to or less than 3% of the added concentration.

Results with Reference Substance

29. When a reference substance is included, the results for the reference substance should approximate those anticipated based upon the reasons for its selection.

DATA AND REPORTING

Plot of data

30. For each sample, the exact time of incubation including the time needed to terminate biological activity if applicable is reported. Also for each sampling point, the percentage of the dosed radioactivity recovered as parent, metabolites and associated with solids as well as the cumulative amount of mineralization and the total mass balance are reported. These percentages are plotted against time for both the biotic and abiotic treatments, when appropriate.

Kinetic Analyses (Optional)

31. It some cases, it may be desirable to fit the results from these tests to kinetic models. These models could include decay models for parent and production models for mineralization (e.g. $^{14}CO_2$ or $^{3}H_20$). The most common and useful models for this purpose are first-order models. Most exposure models (e.g. EUSES, SimpleTreat) utilize first-order rates as critical input parameters.

32. A first-order model assumes that the rate constants of a reaction depends solely upon the concentration of the test material. True first-order conditions exist when the test material is below the concentration at which the biodegradative capacity of a system becomes saturated. As the test substance concentration exceeds saturation, the data may still fit a first-order function, but these quasi first-order rates will be slower than a true first-order rate. Such quasi first-order kinetics may arise from a need to test a higher concentration than that occurring *in situ* due to analytical constraints or simply reflect the actual *in situ* situation.

33. When degradation occurs in an exponential manner and the onset of this degradation is not preceded by a lag period during which little or no degradation occurs, it may be possible to fit decay or

production data to a first-order model. Under such circumstances, the percentage of parent remaining as a function of time may be fitted to a simple or two-compartment first order loss function using nonlinear regression methods. Such equations have the form:

$$y = Ae^{-k_1 t}$$

$$y = (Ae^{-k_1 t}) + (Be^{-k_2 t})$$

where *y* equals the percentage of parent remaining at time *(t), A* equals the percentage degraded at first order rate constant k_1, and *B* equals the percentage degraded at the first order rate constant k_2. Such curve fitting can be achieved using nonlinear methods present in commercially available statistical or curve fitting software. The two compartment model is useful when biodegradation is biphasic, consistent with two different pools of test material (e.g. dissolved and sorbed) present in the test system and exhibiting different rates of biodegradation.

34. In a similar manner, mineralization data can be fit to a simple or two-compartment first order production model with the forms:

$$y = A(1 - e^{-k_1 t})$$

$$y = A(1 - e^{-k_1 t}) + B(1 - e^{-k_2 t})$$

where *y* equals the percentage of the material mineralized at time (*t*), *A* equals the percentage mineralized at first order rate constant k_1, and *B* equals the percentage mineralized at the first order rate constant k_2.

35. Under some situations, biodegradation, particularly loss of parent, may occur so rapidly that a true zero time point can not be measured in the biotic treatment In such situations, data from the abiotic treatment may be used to represent time zero for the kinetic analyses.

36. When first-order kinetics are observed, half-lives ($T_{½}$) can be calculated from the estimated first-order rates (k_1 *or* k_2) for each pool (*A* or *B*) using the following equation:

$$T_{½} = -ln2/k$$

37. In some circumstances, the observed data can be fit to a variety of other models, such as Monod or other growth models, which is beyond the scope of the current guideline. Additional detail on biodegradation kinetics can be found in a report from the FOCUS Work Group on Degradation Kinetics (20). Half-life is only relevant for materials exhibiting first-order degradation patterns. In the absence of first-order kinetics, it may be appropriate to report degradation times for 50% (DT_{50}) and 90% (DT_{90}) of the test material if theses levels of degradation are observed during the course of the study. These values can be determined directly or estimated using standard interpolation procedures.

38. When data are fit to a model, the model equation and the software used to fit the model should be reported. The correlation coefficient (r^2), the F value, if available and a plot of the fitted curve with the actual data should be provided. The estimated rate constants (k_1 *or* k_2) and other parameters (*A*, *B*) should be reported with their standard errors.

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Test Report

39. The type of study, i.e. wastewater, activated sludge, mixing zone or anaerobic digester sludge test, must be clearly stated in the test report, which shall also contain the following information, when appropriate:

Test Materials:

- common names, chemical names, CAS numbers, structural formulas and relevant physico-chemical properties of test and reference substances;
- chemical names, CAS numbers, structural formulas and relevant physico-chemical properties of substances used as standards for identification of metabolites;
- purities of and nature of known impurities in test and reference substances;
- radiochemical purity and specific activity of radiolabelled chemicals;
- position within the molecule of radiolabelled atoms.

Environmental Samples:

- source of environmental samples including geographical location and relevant data regarding known prior or existing exposure to the test chemical and related materials;
- logic used to estimate relevant environmental concentration;
- time, date and field conditions relevant to collection;
- temperature, pH, dissolved oxygen (DO) and redox potential as needed;
- suspended solids level, biological oxygen demand (BOD), chemical oxygen demand (COD) and Total organic carbon (TOC) as needed;
- time between collection and use in the laboratory test, sample storage conditions and any pre-treatment of the sample prior to initiating the test;

Experimental Conditions:

- dates when the study was performed;
- amount of test substance applied, test concentration and reference substance;
- method of application of the test substance and associated logic for selection;
- incubation conditions including lighting, aeration type, temperature;
- information on analytical techniques and the method(s) used for radiochemical measurements;
- number of replicates;

Results:

- precision and sensitivity of the analytical methods including the limit of detection (LOD) and the level of quantification (LOQ);
- recovery for each analyte and disposition of dosed radioactivity at each sampling time and treatment in tabular form;
- average mass balance with standard deviation across all time points for each treatment
- procedures and models used to estimate biodegradation rates from the data;
- biodegradation rates and related parameters with relevant standard errors along with correlation coefficients of determination (R^2) and F statistics for the selected models;
- additional characterization or identification of any of major metabolites, where appropriate and possible;
- a proposed pathway of transformation (optional);
- discussion of results.

3xxA BIODEGRADATION IN A SEWER SYSTEM

INTRODUCTION

1. This test is designed to provide rates of primary and ultimate biodegradation for a chemical in raw wastewater during its time in a sewage conveyance system. It is based on a procedure originally published in Matthijs *et al* (6). Wastewater contains a large number of microorganisms, capable of degrading a variety of materials. The test duration is typically short term (≤ 96 hrs) to simulate the residence time in a sewer, but it can be extended to assess the extent to which a chemical can be degraded by wastewater microbes. Performing this test is useful if there is sufficient time in a sewer for the chemical to undergo significant biodegradation and loss. Hence, it is most useful for relatively labile chemicals discharged to large municipal sewer systems. Aeration levels within a sewer can vary widely. To be conservative and simulate the more typical conditions within a sewer, the test is conducted under low dissolved oxygen conditions (≤ 1 mg/L). In order to achieve this but avoid anoxic conditions (e .g. D.O levels < 0.2 mg/L), D.O, or the corresponding oxygen concentration in the test vessel headspace, should be monitored periodically. Air, oxygen, or nitrogen may be added periodically to the test vessels to maintain DO in this range.

2. For existing chemicals consistently present in wastewater, freshly collected wastewater incubated with a tracer level of radiolabelled test chemical will provide the most realistic kinetic parameters regarding the current chemical load. For chemicals not consistently present in wastewater, sufficient test chemical (radiolabelled and unlabelled) should be added to approximate the expected concentration in wastewater during an episodic release or following commercialization of a new chemical. This concentration would reflect the total mass released and the volume of wastewater in which the release is diluted. Approaches for estimating wastewater concentration can be found in Holman (21) and the European Technical Guidance Document (22). In most situations, the chemical and its degrader populations will not be in steady state and the observed kinetics will be quasi first-order or second-order Monod.

GENERAL TEST PROCEDURE

3. The test chemical is incubated with abiotic and biotic wastewater over a period of time under low dissolved oxygen (DO) conditions. Biological activity is inhibited in the abiotic control, which is used for estimating mineralization by difference, determining extraction efficiency and recovery of the parent molecule and quantifying other loss processes, such as hydrolysis, oxidation, volatilization or sorption to test apparatus.

4. If an analytical method with the required sensitivity is identified, the rate of parent degradation or transformation can be determined using a non-radiolabelled test substance or by following the disappearance of a chemical already in wastewater. However, ultimate biodegradation can not be determined unless the biodegradation pathway is well understood and analytical methods with required sensitivity are available for potential metabolites.

5. An environmentally relevant concentration of the test material is dosed to both abiotic and biotic test systems, which are incubated with continuous slow mixing. The biotic samples are incubated in such a way that dissolved oxygen levels remain at or below 1 mg/L, which are typical for sewage. Samples are periodically removed for determination of mineralization and primary biodegradation.

6. Tests can be performed using an open batch system or a sealed, flow-through batch system where

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traps are used to capture evolved $^{14}CO_2$. The closed flow-through system is mandatory for volatile test materials and usually preferred for ^{14}C-lableled test chemicals. Open systems are appropriate for non-volatile ^{3}H test chemicals and for refining the biodegradation kinetics of non-volatile ^{14}C test materials, whose ability to be mineralized has previously been established. In the open system, mineralization to $^{14}CO_2$ can be determined indirectly by measuring the difference in residual radioactivity between samples from the biotic and abiotic treatments following acidification. Similarly, mineralization to $^{3}H_2O$ can be determined indirectly by measuring the difference in radioactivity in a sample following drying. In the flow through systems, evolved $^{14}CO_2$ is measured directly in the base traps. In addition, dissolved $^{14}CO_2$ is determined by acidifying samples in a sealed vessel and measuring radioactivity in a base trap contained in the vessel.

7. Samples from both treatments are analyzed for total radioactivity, extractable parent and metabolites and radioactivity associated with the extracted solids. The level of parent and metabolites is determined using chromatographic separation and when appropriate radio-analytical detection methods. The solids remaining from the extraction process are combusted to estimate incorporation into biomass by difference or can be further fractionated to determine uptake into various components of biomass. A complete mass balance of the test system is obtained from the sum total of all fractions at each sampling.

APPLICABILITY OF THE TEST

8. The method is readily applicable to water-soluble or poorly water-soluble materials, which are non-volatile. It can also be adapted for volatile materials. Typically, ^{14}C or ^{3}H -radiolabelling of compounds is required for the assessment of mineralization. Both radiolabelled and non-labelled compound can be used for the assessment of primary biodegradation.

DESCRIPTION OF THE TEST METHOD

Test Apparatus

9. The volume of wastewater in the test treatments is determined based upon the number and volume of the samples needed for the assessment. Typically, 1 to 2 litres of wastewater are placed into 2 or 4 litre flasks. Ideally, the wastewater is incubated under controlled DO conditions (typically 0.2-1.0 mg/L. This condition can be achieved using an oxygen probe immersed in the wastewater attached to an oxygen controller connected to an actuator valve, which monitors and controls the aeration of the wastewater (see Annex 1). This aeration is balanced against continuous sparging with nitrogen to achieve the targeted DO level. Alternatively, the wastewater can be incubated with stirring but minimum aeration to keep the DO levels low, nitrogen or air can be added periodically to maintain D.O level. In this case, DO readings should be reported at regular intervals.

10. An open test is open to the atmosphere but incubated under conditions that maintain dissolved oxygen levels at the desired level. Flow-through systems are sealed with an appropriate closure containing a sampling port with a valve for removing wastewater samples and connections for influent and effluent gas lines. This closure can be a rubber stopper, but an alternative type of closure may be necessary when working with a volatile hydrophobic test material. When testing volatile compounds, it is recommend that gas lines and sampling tubes consist of inert materials (e.g. Teflon, stainless steel, glass).

11. The head space of the test vessel is continuously purged with gas at a rate sufficient to maintain the wastewater at the desired DO level but not too fast to prevent efficient trapping of CO_2. The test vessel is connected to a series of traps containing potassium hydroxide (e.g. 1.5 N) or other appropriate CO_2 absorbent. An empty trap is usually included in the trapping train as a precaution against back-flow or condensation.

Equipment

12. The following standard laboratory equipment are used:

- miscellaneous glassware and pipettes;
- magnetic stirrers or shaker for continuous mixing of the test flasks;
- centrifuge;
- pH meter;
- solid CO_2 (dry ice)/acetone or liquid nitrogen bath;
- freeze dryer (lyophilizer);
- oven or microwave oven for dry weight determinations;
- membrane filtration apparatus;
- autoclave;
- facilities to handle radiolabelled substances;
- equipment to quantify ^{14}C and ^{3}H in liquid samples and solid samples (e.g. liquid scintillation counter LSC);
- equipment to quantify ^{14}C and ^{3}H in solid samples (e.g. sample oxidizer);
- equipment to trap volatilized ^{14}C and ^{3}H from gas trapping system (in-line activated charcoal trap or equivalent);
- equipment for thin layer chromatography (TLC) or high performance liquid chromatography (HPLC);
- equipment to quantify ^{14}C and ^{3}H for TLC (scanner) or HPLC (in-line detector);
- analytical equipment for the determination of the test (and reference) substance if specific chemical analysis is used (e.g. gas chromatograph, high performance liquid chromatograph, mass spectrometry).

13. The following laboratory equipment is not essential but useful:

- oxygen meter
- oxygen controller with probe and actuator valve.
- COD digestion vials
- Nitrogen ammonia reagent set
- Spectrophotometer

Selection of Wastewater

14. The source of wastewater should be consistent with the objective of the simulation test. For a site specific assessment, the wastewater should be obtained from the specific sewer system in question. For a generic assessment wastewater samples should be predominantly derived from domestic sources. Although difficult to duplicate in practice, the European Technical Guidance Document uses 450 mg/L of suspended solids and 270 mg/L of BOD (biological oxygen demand) as default levels in wastewater (22). In North America, typical wastewaters contain from 100 to 350 mg/L of suspended solids and 110 to 400 mg/L of BOD depending upon its strength (23).

Collection, Transport and Storage of Wastewater

15. The wastewater should be collected from a sewer access point or at the head of a wastewater treatment plant. The temperature of the sample should be noted at the time of collection. Collection containers should allow for adequate ventilation and measures should be taken to prevent the temperature

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of the wastewater from significantly exceeding the temperature used in the test. The wastewater is typically stored at test temperature with continuous slow mixing. Samples should not be stored frozen

Preparation of Test Treatments

16. The freshly collected wastewater should be largely free from coarse particles. Total suspended solids (TSS), COD, pH and NH_3 (optional) should be determined in the wastewater.

17. The preparation of the abiotic treatment is typically performed using a combination of chemical and heat sterilization. A proven approach is to add mercuric chloride (1 g/L) to the wastewater, which is then autoclaved for at least 90 minutes. After cooling, the pH of the abiotic treatment should be measured and adjusted to match that of the biologically active treatment. Alternative approaches to deactivate the treatment can also be used.

Test Substance Preparation

18. Ideally, distilled water should be used to prepare stock solutions of the test and reference substances. When appropriate, an alternative method may be used to solubilize or disperse the test chemical in a manner consistent with its normal entry into the environmental compartment in question. Water-miscible non-toxic solvents may be used when necessary, but attention should be paid to the associated organic load involved with adding organic solvents. Alternatively, the sample may be added in a neat form (i.e. without water) to the test system in a manner that maximizes its even and rapid distribution into the sludge. For materials which are poorly soluble and typically associated with suspended solids in wastewater, it may be appropriate to adsorb the test material onto an inert solid carrier, which is then dosed to the test system. If the test material can not be evenly distributed within the test system prior to the initial sampling point, individual test systems can be prepared that are destructively sampled at each sampling interval.

19. The volume of added stock should be of sufficient capacity to ensure rapid and even distribution of the test material in the treatment and accurate administration of the dose between like treatments. Ideally, when dosing with aqueous solutions, the added volume should be ≥ 2 ml; for non-toxic solvents, < 0.1 ml/ L. If appropriate, dosing solutions may be prepared in advance and refrigerated. The activity of the stock should be checked by LSC.

Test conditions

Test temperature

20. Incubation should take place in the dark (preferred) or in diffuse light at a controlled temperature, which may be the field temperature or a standard laboratory temperature of 20-25°C. Depending upon location, mean annual wastewater temperature ranges from 10 to 20.1° C, with 15.6° C being representative (23).

Agitation

21. To keep the solids in suspension, the test vessels are minimally agitated by means of continuous mixing or stirring.

Test duration

22. The duration of the test should be sufficiently long to assess the biodegradation of the test

chemical during its normal residence time within the sewer system. However, it may be extended longer to obtain additional data points to estimate kinetic constants or to assess the completeness of degradation under the conditions within the test. Conversely, it may be ended before this time if degradation has plateaued.

Number of test vessels

23. At a minimum, there should be a single abiotic and a single biotic test vessel for each test material concentration. While replicates can be prepared for each treatment, more useful kinetic information usually can be gained by increasing the number of time points sampled within a treatment.

PROCEDURE

Dosing

24. At test initiation, the test vessel closure is removed and the test material is quantitatively added directly to the treatment with constant mixing. It is recommended that the dose be administered in a gradual fashion below the air-water interface, to ensure uniform distribution of the test material into the wastewater. The biotic and abiotic treatments are dosed in an identical manner. Generally, the biotic systems are dosed first, followed by the abiotic systems. Exact timing is typically more critical for the biotic *versus* the abiotic treatments for kinetic analyses.

Sampling Schedule

25. Sampling intervals are selected based on existing biodegradation data or the results of a pilot study as no fixed time schedule for sampling is universally applicable. A recommended sampling schedule for a rapidly degraded chemical would be 15, 30 and 60 minutes, with additional samplings after 2, 5, 8, 12 and 24 hours and day 2, 3 and 4. The sampling schedule for slowly degrading chemical should be adjusted so that a sufficient number of measurements are made during the degradation phase.

Measurement of Mineralization

Indirect measurement of $^{14}CO_2$

26. Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood.

27. The samples are bubbled with air for several hours or allowed to stand overnight to allow the dissolved $^{14}CO_2$ to diffuse from the samples. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent of $^{14}CO_2$ is calculated based upon the difference between the total counts in the biotic and abiotic samples.

Direct measurement of $^{14}CO_2$

28. Evolved $^{14}CO_2$: The first base trap in the series is removed and quickly capped. The remaining traps are moved forward in the same order and a fresh trap placed behind the existing traps and the trapping system reconnected as quickly as possible. Replicate subsamples (e.g. 1 ml) from the base trap are removed and transferred to scintillation vials and combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

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29. Dissolved $^{14}CO_2$: Samples (e.g. 10 to 25 ml) are removed through the sampling port of the test flask. They are then placed in vessels (e.g. Bellco Glass Biometer 2556-10250) containing a compartment with an appropriate CO_2 absorbent (e.g. 1.5 N KOH). The vessels are sealed and sufficient acid is added to lower the pH of the samples to < 2 without opening the vessels to the atmosphere (see Annex 1). The samples are allowed to sit for a sufficient length of time (e.g. overnight) to allow CO_2 to diffuse from solution and be trapped from the headspace by the sorbent. Samples of the sorbent are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

Indirect measurement of ^3H_2O

30. Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood.
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31. Half of the samples are immediately analysed directly by LSC for a "wet measurement". The remaining samples are allowed to dry completely to remove the ^3H_2O. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent ^3H_2O is calculated based upon the difference between the total counts in the wet and dry samples and the initial level of radioactivity dosed to the samples.

Measurement of Total Radioactivity in Wastewater

32. Replicate small volume samples (e.g. 1 ml) are analysed directly by LSC to quantify the radioactivity remaining in each treatment over time. These measurements are used to confirm that the recovery of radioactivity from the extracted samples is acceptable and to monitor for volatilization.

Measurement of Parent and Metabolites

Extraction

33. A sample is collected from both the abiotic and biotic treatments. The sample volume is typically ≥ 10 ml. However, the size will depend on the test concentration, specific activity and the sensitivity of the analytical procedures.

34. Various approaches can be used for concentrating and extracting the samples. A proven approach for nonvolatile test chemicals involves flash freezing the samples, followed by lyophilization and extraction of the dried residue with appropriate solvent(s) for parent and metabolites. Flash freezing quickly stops biological activity without hydrolyzing or otherwise altering labile test materials. The dried solids are extracted and the resulting extracts can be concentrated through evaporation and the total radioactivity in each extract is determined by LSC.

35. For volatile test materials, the sample can be passed through a filter and solid phase extraction (SPE) column or SPE disk placed in tandem, which are subsequently eluted with appropriate solvents to recover parent and metabolites. Alternatively, samples can be centrifuged, and parent and metabolites can be extracted from the liquor by solid phase or liquid/liquid extraction. The solids can then be extracted directly or mixed with a drying agent (e.g. sodium sulfate) and allowed to dry prior to extraction with an appropriate solvent system. An alternative is to extract the solids and dry the extract by running the solvent through a column containing a drying agent. In some cases, it may be possible to directly extract the entire aqueous sample with an appropriate solvent system and then filter it to recover biomass solids. The

total radioactivity in all extracts is determined by LSC. Care must be taken in concentrating extracts containing volatile test materials or metabolites. The recovery in the abiotic treatment are used to

36. Other approaches can be utilized, but with all approaches it is important to document recoveries and consider the time involved in terminating biological activity and factor it into the sample times used for kinetic analyses.

Analysis of Parent and Metabolites

37. The relative abundance of parent and metabolites within the extracts can be determined using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) or other separation techniques with radioactivity detection.

38. If sensitive specific analytical methods are available, primary biodegradation can be assessed by measuring the total residual concentration of test substances and metabolites instead of using radioisotope techniques.

Characterization of Metabolites

39. Whenever possible, the chromatographic behaviour of unknown peaks should be compared to that of predicted metabolites, if authentic standards exist. Usually, the quantity and purity of metabolites generated in this test make definitive identification by other direct means impossible. Depending upon chromatographic behaviour, it is usually possible to determine if a metabolite is more or less polar than the parent. This information combined with known biochemical reactions along with when a metabolite appears and disappears in the sequence of biodegradation can form an additional basis for inferring its identity. If necessary, the Kow of major metabolites can be determined by HPLC (e.g. [OECD 117] (24)) using an on-line radioactivity detector.

Measurement of Extracted Solids and Incorporation into Biomass

40. If the extracted samples are filtered, the filter will retain carbonate salts as well as microorganisms from the test system. The filter containing the biosolids is placed into a scintillation vial and acidified to pH $\leq$2 by submerging it in a weak acid solution (e.g. 1 ml of 0.1N HCl). The samples are allowed to sit for sufficient time (e.g. overnight) for the dissolved $^{14}CO_2$ to diffuse from the samples. The samples are analyzed by LSC. In the case of non-filtered extracted solids, they are combusted to determine the level of activity remaining with the solids. The level of radioactivity in the biotic solids above that in solids from the abiotic control typically represents incorporation of radioactivity into biomass. The distribution of this radioactivity among various components of biomass (i.e. nucleic acids, protein, cell wall, etc.) can be determined using a modified Sutherland and Wilkinson procedure (7, 24).

Measurement of Volatilized Radioactivity

41. For volatile test materials, the volatile traps are extracted with appropriate solvents and the radioactivity in the extracts is analyzed by LSC. The relative abundance of parent and metabolites in the extract(s) can be determined as described above.

3xxB BIODEGRADATION IN ACTIVATED SLUDGE

INTRODUCTION

1. This test is designed to assess the extent to which a chemical can be degraded in activated sludge and to provide rates of primary and ultimate biodegradation under the conditions of the test. It is based on a procedure originally published by Federle & Itrich (7). Activated sludge in its various configurations is the most common secondary wastewater treatment process. The usefulness of the measured rates for accurately predicting removal in actual treatment using wastewater treatment models (e.g. SimpleTreat) will be a function of the fidelity of the simulation to actual conditions within an activated sludge wastewater treatment plant.

2. Four factors determine the test material concentration in this guideline:, 1) whether the material is released continuously or episodically, 2) current presence in the environment, 3) expected presence in the environment for a new chemical and 4) analytical sensitivity.

3. When a chemical is already present in the environment in a continuous fashion, the most accurate kinetics are obtained by adding a tracer level of the radiolabelled material to freshly obtained environmental samples. Under these circumstances, the normal ratio of chemical to degraders is not disrupted and the observed rates reflect those occurring in situ.

4. When a new chemical will be released continuously at some future time, the most accurate rates are obtained when the chemical and degrader populations are in a steady state balance. This situation can be achieved by adding a tracer level of test chemical to activated sludge, which has been exposed to the chemical under expected loading and operating conditions in a laboratory continuous activated sludge system (e.g. OECD 303 A).

5. When an existing or new chemical enters the environment in an episodic manner, there is not a normal steady state ratio of biomass to test chemical to disrupt so the test chemical is dosed to freshly collected samples at its expected level in wastewater during a release event. This concentration should reflect the total mass released and the volume of wastewater in which the release is diluted. Approaches for estimating wastewater concentration can be found in Holman (21) and the European Technical Guidance Document (22).

6. Superseding the previous considerations is analytical sensitivity. When it is not possible to use ideal (e.g. tracer) levels of test chemical due to analytical consideration, the lowest possible concentration is employed. At high test material concentrations, biodegradation may be associated with lags related to second–order processes (i.e. growth), which complicate the kinetic analysis. When this standard for simulation is not achieved, observed biodegradation rates may not be fully representative, which should be considered in the interpretation of the results. This factor is particularly important for continuously released chemicals, which often reach steady state conditions in wastewater systems.

GENERAL TEST PROCEDURE

7. The test chemical is incubated with abiotic and biotic activated sludge over a period of time. Biological activity is inhibited in the abiotic control, which is used for estimating mineralization by difference, establishing extraction efficiency and recovery of the parent molecule and quantifying other loss processes, such as hydrolysis, oxidation, volatilization or sorption to test apparatus.

8. If an analytical method with the required sensitivity is identified, the rate of parent degradation or transformation can be determined using a non-radiolabelled test substance or by following the

disappearance of a chemical already in wastewater. However, ultimate biodegradation can not be determined unless the biodegradation pathway is well understood and analytical methods with required sensitivity are available for potential metabolites.

9. An environmentally relevant concentration of the test material is dosed to both abiotic and biotic test systems, which are incubated at a relevant temperature with continuous mixing when appropriate. Samples are periodically removed for determination of mineralization and primary biodegradation.

10. Tests can be performed using an open batch system or a sealed, flow-through batch system where traps are used to capture evolved $^{14}CO_2$. The closed flow-through system is mandatory for volatile test materials and usually preferred for ^{14}C-lableled test chemicals. Open systems are appropriate for non-volatile ^{3}H test chemicals and for refining the biodegradation kinetics of non-volatile ^{14}C test materials, whose ability to be mineralized has previously been established. In the open system, mineralization to $^{14}CO_2$ can be determined indirectly by measuring the difference in residual radioactivity between samples from the biotic and abiotic treatments following acidification. Similarly, mineralization to $^{3}H_2O$ can be determined indirectly by measuring the difference in radioactivity in a sample following drying. In the flow through systems, evolved $^{14}CO_2$ is measured directly in the base traps. In addition, dissolved $^{14}CO_2$ is determined by acidifying samples in a sealed vessel and measuring radioactivity in a base trap contained in the vessel.

11. Samples from both treatments are analyzed for total radioactivity, extractable parent and metabolites and radioactivity associated with the extracted solids. The level of parent and metabolites is determined using chromatographic separation and when appropriate radio-analytical detection methods. The solids remaining from the extraction process are combusted to estimate incorporation into biomass by difference or can be further extracted using a modified Sutherland and Wilkinson procedure (7) to determine uptake into various components of biomass. A complete mass balance of the test system is obtained from the sum total of all fractions at each sampling.

APPLICABILITY OF THE TEST

12. The method is readily applicable to water-soluble or poorly water-soluble materials, which are non-volatile. It can also be adapted for volatile materials. Typically, ^{14}C or ^{3}H -radiolabelling of compounds is required for the assessment of mineralization. Both radiolabelled and non-labelled compound can be used for the assessment of primary biodegradation.

DESCRIPTION OF THE TEST METHOD

Test Apparatus

12. The volume of activated sludge in the test treatments is determined based upon the number and volume of the samples needed for the assessment. Typically, 1 to 2 litres of sludge are placed into 2 or 4 liter flasks. Open batch tests are generally closed with a foam or cotton stopper to minimize evaporative loss of water. Flow-through systems are sealed with an appropriate closure containing a sampling port with a valve for removing MLSS samples and connections for influent and effluent gas lines (see Annex 1). This closure can be a rubber stopper, but glass is recommended when working with a volatile hydrophobic test material. When testing volatile compounds, it also is recommend that gas lines and sampling tubes consist of inert materials (e.g. Teflon, stainless steel, glass).

13. The head space of the test vessel is continuously purged with air or CO_2-free air at a rate sufficient to maintain the activated sludge in an aerobic condition but not too fast to prevent efficient

trapping of CO_2. The test vessel is connected to a series of traps containing potassium hydroxide (e.g. 1.5 N) or other appropriate CO_2 absorbent. An empty trap is usually included in the trapping train as a precaution against back-flow or condensation.

Equipment

14. The following standard laboratory equipment are used:

- miscellaneous glassware and pipettes;
- magnetic stirrers or shaker for continuous mixing of the test flasks;
- centrifuge;
- pH meter;
- solid CO_2 (dry ice)/acetone or liquid nitrogen bath;
- freeze dryer (lyophilizer);
- oven or microwave oven for dry weight determinations;
- membrane filtration apparatus;
- autoclave;
- facilities to handle radiolabelled substances;
- equipment to quantify ^{14}C and ^{3}H in liquid samples and solid samples (e.g. liquid scintillation counter LSC);
- equipment to quantify ^{14}C and ^{3}H in solid samples (e.g. sample oxidizer);
- equipment to trap volatilized ^{14}C and ^{3}H from gas trapping system (in-line activated charcoal trap or equivalent);
- equipment for thin layer chromatography (TLC) or high performance liquid chromatography (HPLC);
- equipment to quantify ^{14}C and ^{3}H for TLC (scanner) or HPLC (in-line detector);
- analytical equipment for the determination of the test (and reference) substance if specific chemical analysis is used (e.g. gas chromatograph, high performance liquid chromatograph, mass spectrometer).

Selection of Activated Sludge Source

15. The source of activated sludge should be consistent with the objective of the simulation test. For a site specific assessment, the activated sludge should be obtained from the specific wastewater treatment plant in question. For a generic assessment activated sludge should be obtained from a typical wastewater treatment plant receiving predominantly domestic wastewater. If the chemical is currently a component of wastewater entering the wastewater treatment facility or is episodically released to wastewater, freshly collected activated sludge will be ideal for the test.

16. For a new chemical which will be continuously released to wastewater, activated sludge ideally should be obtained from a laboratory scale treatment system such as a porous pot or CAS [OECD 303A] (2), which has been fed wastewater amended with unlabelled test material. The source of the starting sludge, wastewater (influent) and the operating conditions (influent concentration, hydraulic retention time, solids retention time) for the laboratory unit should accurately reflect site specific or generic conditions. In the case of the latter, the European Technical Guidance Document specifies a hydraulic retention time (HRT) of 6.9 hours and a sludge retention time (SRT) of 9.2 days in its generic scenario for wastewater treatment (22). The European Technical Guidance Document also provides guidance on estimating wastewater concentration based upon expected usage volumes. In general, steady state will be reached within 2 to 3 times the SRT after which point the sludge can be used for testing.

Collection, Transport and Storage of Activated Sludge

17. The activated sludge should be collected from a well mixed region of the aeration basin. The temperature of the sample should be noted at the time of collection. Collection containers should allow for adequate ventilation and measures should be taken to prevent temperature of the sludge from significantly exceeding the temperature used in the test. The activated sludge is typically stored at test temperature with continuous aeration. Samples should not be stored frozen.

Preparation of the Test Treatments

Activated Sludge

18. The activated sludge should be sieved through a 2mm screen prior to use. The total suspended solids (TSS) concentration should be measured and if necessary adjusted to the targeted concentration. The European Technical Guidance Document uses a default level of 4000 mg/L in its generic scenario (22). However, 2500 – 3000 mg/L may be more typical for North America (23). The sludge can be diluted with liquor or tap water if the solids concentration is too high. Alternatively if the solids concentration is too low, the solids can be allowed to settle and some of the liquor can be decanted. A final TSS level and pH should then be determined.

19. The preparation of the abiotic sludge is typically performed using a combination of chemical and heat sterilization. A proven approach is to add mercuric chloride solution (1 g/L) to the sludge, which is then autoclaved for at least 90 minutes. After cooling, the pH of the abiotic sludge should be measured and adjusted to match that of the biologically active sludge. Alternative approaches to deactivate the sludge can also be used.

Test Substance Preparation

20. Ideally, distilled water should be used to prepare stock solutions of the test and reference substances. When appropriate, an alternative method may be used to solubilize or disperse the test chemical in a manner consistent with its normal entry into the environmental compartment in question. Water-miscible non-toxic solvents may be used when necessary, but attention should be paid to the associated organic load involved with adding organic solvents. Alternatively, the sample may be added in a neat form to the test system in a manner that maximizes its even and rapid distribution into the sludge. For materials which are poorly soluble and typically associated with suspended solids in wastewater, it may be appropriate to adsorb the test material onto an inert solid carrier, which is then dosed to the test system. If the test material can not be evenly distributed within the test system prior to the initial sampling point, individual test systems can be prepared that are destructively sampled at each sampling interval.

21. The volume of added stock should be of sufficient capacity to ensure rapid and even distribution of the test material in the treatment and accurate administration of the dose between like treatments. Ideally, when dosing with aqueous solutions, the added volume should be $\geq$ 2 ml; for non-toxic solvents, < 0.1 ml/ L. If appropriate, dosing solutions may be prepared in advance and refrigerated. The activity of the stock should be checked by LSC.

Test conditions

Test temperature

22. Incubation should take place in the dark (preferred) or in diffuse light at a controlled temperature, which may be the field temperature or a standard laboratory temperature of 20-25°C.

Agitation

23. To keep the sludge well mixed and in suspension, the test vessels are agitated by means of continuous shaking or stirring. Agitation also facilitates oxygen transfer from the headspace to the liquid so that aerobic conditions can be adequately maintained.

Test duration

24. The duration of the test should be sufficiently long to assess the biodegradation of the test chemical during its normal residence time within an activated plant. Normally, the test period will last 28 days. However, it may be extended longer to obtain additional data points to estimate kinetic constants or to assess the completeness of degradation under the conditions within the test. Conversely, it may be ended before this time if degradation has plateaued.

Number of test vessels

25. At a minimum, there should be a single abiotic and a single biotic test vessel for each test material concentration. While replicates can be prepared for each treatment, more useful kinetic information usually can be gained by increasing the number of time points sampled within a treatment.

PROCEDURE

Dosing

26. At test initiation, the test vessel closure is removed and the test material is quantitatively added directly to the activated sludge with constant mixing. It is recommended that the dose be administered in a gradual fashion below the air-water interface, to ensure uniform distribution of the test material into the sludge. The biotic and abiotic treatments are dosed in an identical manner. Generally, the biotic systems are dosed first, followed by the abiotic systems. Exact timing is typically more critical for the biotic versus the abiotic treatments for kinetic analyses.

Sampling Schedule

27. Sampling intervals are selected based on existing biodegradation data or the results of a pilot study as no fixed time schedule for sampling is universally applicable. A recommended sampling schedule for a rapidly degraded chemical would be 5, 15, 30, 45, 60, and 90 minutes, with additional samplings after 2, 3, 5, 8, 12 and 24 hours. Subsequent samples could be taken after 2, 3, 4, 5, 6 and 7 days and weekly until day 28. The sampling schedule for slowly degrading chemical should be adjusted so that a sufficient number of measurements are made during the degradation phase.

Measurement of Mineralization

Indirect measurement of $^{14}CO_2$

28. Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood. Furthermore, the total solids in the samples should not exceed 30 mg dry weight.

29. The samples are bubbled with air for several hours or allowed to stand overnight to allow the dissolved $^{14}CO_2$ to diffuse from the samples. The samples are combined with a scintillation cocktail that is

suitable for the sample matrix and analysed by LSC. The percent of $^{14}CO_2$ is calculated based upon the difference between the total counts in the biotic and abiotic samples.

Direct measurement of $^{14}CO_2$

30. For rapidly degrading chemicals, it can be difficult to measure accurately the rate of $^{14}CO_2$ evolved due to the rate of the mass transfer of $^{14}CO_2$ from the headspace into the base trap. Under these conditions, it is recommended that indirect measurement of $^{14}CO_2$ be conducted simultaneously with direct measurement.

31. Evolved $^{14}CO_2$: The first base trap in the series is removed and quickly capped. The remaining traps are moved forward in the same order and a fresh trap placed behind the existing traps and the trapping system reconnected as quickly as possible. Replicate subsamples (e.g. 1 ml) from the base trap are removed and transferred to scintillation vials and combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

32. Dissolved $^{14}CO_2$: Samples (e.g. 10 to 25 ml) are removed through the sampling port of the test flask. They are then placed in vessels (e.g. Bellco Glass Biometer 2556-10250) containing a compartment with an appropriate CO_2 absorbent (e.g. 1.5 N KOH). The vessels are sealed and sufficient acid is added to lower the pH of the samples to < 2 without opening the vessels to the atmosphere (see Annex 1). The samples are allowed to sit for a sufficient length of time (e.g. overnight) to allow CO_2 to diffuse from solution and be trapped from the headspace by the sorbent. Samples of the sorbent are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

Indirect measurement of $^{3}H_2O$

33. Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located to a fume hood. Furthermore, the total solids in the samples should not exceed 30 mg dry weight.

34. Half of the samples are immediately analysed directly by LSC for a "wet measurement". The remaining samples are allowed to dry completely to remove the $^{3}H_2O$. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent $^{3}H_2O$ is calculated based upon the difference between the total counts in the wet and dry samples and the initial level of radioactivity dosed to the samples.

Measurement of Radioactivity in Mixed-Liquor Suspended Solids (MLSS)

35. Small volume samples of MLSS (e.g. 1 ml) are analysed directly by LSC to quantify the radioactivity remaining in each treatment over time. These measurements are used to confirm that the recovery of radioactivity from the extracted samples is acceptable and to monitor for volatilization. The total solids in these samples should not exceed 30 mg dry weight to avoid counting efficiency problems.

Measurement of Parent and Metabolites

Extraction

36. A sample of MLSS is collected from both the abiotic and biotic treatments. The sample volume is typically ≥ 10 ml. However, the size will depend on the test concentration, specific activity and the sensitivity of the analytical procedures.

37. Various approaches can be used for concentrating and extracting the samples. A proven

approach for nonvolatile test chemicals involves flash freezing the samples, followed by lyophilization and extraction of the dried residue with appropriate solvent(s) for parent and metabolites. Flash freezing quickly stops biological activity without hydrolyzing or otherwise altering labile test materials. The resulting extracts can be concentrated through evaporation and the total radioactivity in each extract is determined by LSC.

38. For volatile test materials, MLSS can be passed through a filter and solid phase extraction (SPE) column or SPE disk placed in tandem, which are subsequently eluted with appropriate solvents to recover parent and metabolites. Alternatively, samples can be centrifuged, and parent and metabolites can be extracted from the liquor by solid phase or liquid/liquid extraction. The solids can then be extracted directly or mixed with a drying agent (e.g. sodium sulfate) and allowed to dry prior to extraction with an appropriate solvent system. An alternative is to extract the solids and then remove the water from the solvent by running it through a column containing a drying agent. In most cases, it is not efficient to use liquid/liquid extraction to recover parent and metabolites from MLSS. The total radioactivity in all extracts is determined by LSC. Care must be taken in concentrating extracts containing volatile test materials or metabolites.

39. Other approaches can be utilized, but with all approaches it is important to document recoveries and consider the time involved in terminating biological activity and factor it into the sample times used for kinetic analyses.

Analysis of Parent and Metabolites

40. The relative abundance of parent and metabolites within the extracts can be determined using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) or other separation techniques with radioactivity detection.

41. If sensitive specific analytical methods are available, primary biodegradation can be assessed by measuring the total residual concentration of test substances and metabolites instead of using radioisotope techniques.

Characterization of Metabolites

42. Whenever possible, the chromatographic behaviour of unknown peaks should be compared to that of predicted metabolites, if authentic standards exist. Usually, the quantity and purity of metabolites generated in this test make definitive identification by other direct means impossible. Depending upon chromatographic behaviour, it is usually possible to determine if a metabolite is more or less polar than the parent. This information combined with known biochemical reactions along with when a metabolite appears and disappears in the sequence of biodegradation can form an additional basis for inferring its identity. If necessary, the K_{ow} of major metabolites can be determined by HPLC (e.g. OECD 117 (24)) using an on-line radioactivity detector.

Measurement of Extracted Solids and Incorporation into Biomass

43. The extracted solids are combusted to determine the level of activity remaining with the solids. The level of radioactivity in the biotic solids above that in solids from the abiotic control typically represents incorporation of radioactivity into biomass. The distribution of this radioactivity among various components of biomass (i.e. nucleic acids, protein, cell wall, etc.) can be determined using a modified Sutherland and Wilkinson procedure (7, 24).

Measurement of Volatilized Radioactivity

44. For volatile test materials, the volatile traps are extracted with appropriate solvents and the radioactivity in the extracts is analyzed by LSC. The relative abundance of parent and metabolites in the extract(s) can be determined as described above.

3xxC MINERALIZATION AND TRANSFORMATION IN ANAEROBIC DIGESTER SLUDGE

INTRODUCTION

1. This test is designed to assess the extent to which a chemical can be degraded during anaerobic digestion. It also provides rates of primary and ultimate biodegradation under the conditions within a digester. Anaerobic digestion is commonly used to stabilize and reduce the mass of sludge generated by wastewater treatment plants. Biodegradation during anaerobic digestion is particularly relevant for chemicals with a high tendency to partition to primary and secondary sludge. Removal during anaerobic digestion can significantly decrease the level of a chemical present in sludge used as a soil amendment. The test is also easily adaptable for septage to evaluate anaerobic biodegradation in septic tanks.

2. Given that many digesters are operated as batch or plug-flow systems, which have long residence times (30 – 60 days), it is not essential that the chemical and its degrader populations be in steady state at the initiation of a test to generate useful rates for exposure assessments.

3. To simulate conditions associated with episodic release of a chemical, freshly collected digester sludge can be incubated with the maximum concentration of test chemical expected to occur in sludge as a result of periodic releases. Approaches for estimating expected sludge concentrations can be found in Holman (21) and the European Technical Guidance Document (22). For existing chemicals continuously discharged to wastewater, freshly collected digester sludge can be incubated with a tracer level of radiolabelled test chemical or the expected concentration to occur in digester sludge. Usually, sufficient time is available for acclimating new chemicals within the time frame of the test. However, a laboratory anaerobic reactor operated in a draw and fill mode amended with the chemical at its expected concentration in sludge can be considered as an option for generating acclimated sludge. This type of sludge should yield the most accurate kinetic data for a new chemical that will be continuously exposed to wastewater.

GENERAL TEST PROCEDURE

4. The test chemical is incubated with abiotic and biotic digester sludge over a period of time. Biological activity is inhibited in the abiotic control, which is used for estimating mineralization by difference, establishing extraction efficiency and recovery of the parent molecule and quantifying other loss processes, such as hydrolysis, oxidation, volatilization or sorption to test apparatus.

5. If an analytical method with the required sensitivity is identified, the rate of parent degradation or transformation can be determined using a non-radiolabelled test substance or by following the disappearance of a chemical already in wastewater. However, ultimate biodegradation of non-radiolabelled substancescan not be determined unless the biodegradation pathway is well understood and analytical methods with required sensitivity are available for potential metabolites.

6. An environmentally relevant concentration of the test material is dosed to both abiotic and biotic test systems, which are incubated at a relevant temperature under static conditions with only occasional mixing. Samples are periodically removed for determination of mineralization and primary biodegradation.

7. Tests can be performed using an open batch system or a sealed, flow-through batch system where traps are used to capture evolved $^{14}CO_2$ and $^{14}CH_4$. The closed flow-through system is mandatory for volatile test materials and usually preferred for ^{14}C-lableled test chemicals. Open systems are appropriate for non-volatile ^{3}H test chemicals and for refining the biodegradation kinetics of non-volatile ^{14}C test

materials, whose ability to be mineralized has previously been established. In the open system, mineralization to $^{14}CO_2$ and $^{14}CH_4$ can be determined indirectly by measuring the difference in residual radioactivity between samples from the biotic and abiotic treatments following acidification. Similarly, mineralization to $^{3}H_2O$ can be determined indirectly by measuring the difference in residual radioactivity between samples following drying. In the flow through systems, evolved $^{14}CO_2$ is measured directly in the base traps, and $^{14}CH_4$ is combusted into $^{14}CO_2$ which is measured directly in a second set of base traps. As an option, dissolved $^{14}CO_2$ can be determined by acidifying samples in a sealed vessel and measuring radioactivity in a base trap contained in the vessel.

8. Samples from both treatments are analyzed for total radioactivity, extractable parent and metabolites and radioactivity associated with the extracted solids. The level of parent and metabolites is determined using chromatographic separation and when appropriate radio-analytical detection methods. The solids remaining from the extraction process are combusted to estimate incorporation into biomass by difference or can be further fractionated to determine uptake into various components of biomass. A complete mass balance of the test system is obtained from the sum total of all fractions at each sampling.

APPLICABILITY OF THE TEST

8. The method is readily applicable to water-soluble or poorly water-soluble materials, which are non-volatile. It can also be adapted for volatile materials. Typically, ^{14}C or ^{3}H -radiolabelling of compounds is required for the assessment of mineralization. Both radiolabelled and non-labelled compound can be used for the assessment of primary biodegradation.

DESCRIPTION OF THE TEST METHOD

Test Apparatus

9. The volume of digester sludge in the test treatments is determined based upon the number and volume of the samples needed for the assessment. Typically, 0.25 to 1 litres of digester sludge, diluted with an anaerobic salts medium, is placed into 0.5 to 2 litre containers. The sludge is diluted to facilitate quantitative removal of sub-samples during the study. Anaerobic sludge must be protected from oxygen at all times including set-up and sampling.

10. Open batch systems are generally closed with a foam or cotton stopper to minimize evaporative loss of water and are incubated inside an anaerobic chamber under a reducing atmosphere. Flow-through test vessels are sealed with an appropriate closure containing a sampling port with a valve for removing sludge samples and connections for influent and effluent gas lines. This closure can be a rubber stopper, but glass is recommended when working with a volatile hydrophobic test material. When testing volatile compounds, it also is recommend that gas lines and sampling tubes consist of inert materials (e.g. Teflon, stainless steel, glass).

11. The flow-through system is a modification of that originally described by Steber and Wierich (10) and later refined by Nuck and Federle (11). An example of a typical system is shown in Annex 1. The test vessels are continuously purged with a flow of nitrogen and connected to a series of traps containing potassium hydroxide solution (1.5 N) or other appropriate CO_2 absorbent. An empty trap is usually included in the trapping train as a precaution against back-flow or condensation. The effluent gases from these traps are mixed with oxygen and passed through a quartz column packed with cupric oxide and maintained at approximately 800°C in a tube furnace to combust methane to CO_2. The gas exiting the combustion column is then passed through another series of base traps.

Equipment

12. The following standard laboratory equipment are used:

- miscellaneous glassware and pipettes;
- magnetic stirrers or shaker for continuous mixing of the test flasks;
- centrifuge;
- pH meter;
- solid CO_2 (dry ice)/acetone or liquid nitrogen bath;
- freeze dryer (lyophilizer);
- oven or microwave oven for dry weight determinations;
- membrane filtration apparatus;
- autoclave;
- facilities to handle radiolabelled substances;
- equipment to quantify ^{14}C and ^{3}H in liquid samples and solid samples (e.g. liquid scintillation counter LSC);
- equipment to quantify ^{14}C and ^{3}H in solid samples (e.g. sample oxidizer);
- equipment to trap volatilized ^{14}C and ^{3}H from gas trapping system (in-line activated charcoal trap or equivalent);
- equipment for thin layer chromatography (TLC) or high performance liquid chromatography (HPLC);
- equipment to quantify ^{14}C and ^{3}H for TLC (scanner) or HPLC (in-line detector);
- analytical equipment for the determination of the test (and reference) substance if specific chemical analysis is used (e.g. gas chromatograph, high performance liquid chromatograph, mass spectrometer).

13. The following specialized equipment may be used:

- anaerobic chamber;
- tube furnaces;
- redox probe and mV meter;

Selection of Digester Sludge Source

14. The source of digester sludge should be consistent with the objective of the simulation test. For a site specific assessment, the sludge should be obtained from the specific digester system in question. For a generic assessment digester sludge should be obtained from a typical single stage or first stage digester receiving primary and secondary sludge from a wastewater treatment plant, receiving predominantly domestic wastewater. If the chemical is currently a component of the wastewater entering the treatment facility or is episodically released to wastewater, freshly collected digester sludge will be ideal for the test. For a new chemical which will be continuously released to wastewater, acclimated sludge from a laboratory anaerobic reactor, may be more appropriate. For a generic assessment, this reactor should simulate the operation of a single stage anaerobic digester, and be semi-continuously fed sludge that consists of combined primary and secondary sludge solids from a wastewater treatment plant receiving predominantly domestic wastewater, which has been amended with test chemical at its expected sludge concentration for approximately 60 days.

Collection, Transport and Storage of Digester Sludge

15. The digester sludge should be collected from the digester in a manner that protects it from oxygen. The use of wide mouth bottles constructed from high-density polyethylene or a similar material,

that can expand, is recommended for the collected of digester sludge. The temperature of the sample should be noted at collection. Sample containers should be tightly sealed. During transport, the temperature of the sample should not significantly exceed the temperature used in the test. The digested sludge is typically stored under the exclusion of oxygen at test temperature. Storage containers should be vented in a manner that releases excess biogas but does not allow ambient air into the container

Preparation of the Test Treatments

Dilution Medium

16. An appropriate volume of the following mineral salts solution should be prepared prior to the test initiation. This solution is autoclaved for 30 minutes with slow exhaust, and allowed to cool overnight in an anaerobic chamber or under an anaerobic atmosphere.

Material	Amount
Potassium phosphate monobasic, KH_2PO_4	8.5 mg/L
Potassium phosphate dibasic, K_2HPO_4	21.8 mg/L
Sodium phosphate dibasic heptahydrate, $Na_2HPO_4 \cdot 7H_2O$	50.3 mg/L
Ammonium chloride, NH_4Cl	20.0 mg/L
Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$	2.2 mg/L
Calcium chloride anhydrous, $CaCl_2$	2.8 mg/L
Ferrous chloride, $FeCl_2 \cdot 4H_2O$	0.25 mg/L
Deionized water	To volume

Digester Sludge

17. The digester sludge ideally should be stored and manipulated inside of an anaerobic chamber. However, other approaches may be utilized to protect the sludge from exposure to oxygen. The digester sludge should be sieved through a 2mm screen. The total solids concentration should be measured.

18. A workable solids level that can be sampled during the study is approximately 25,000 mg/L. If the solids are too high, they can be diluted with the dilution media. Alternatively, if the solids concentration is too low, they can be allowed to settle, the liquor decanted and the sludge can be re-suspended in the dilution media. A final solids level and pH should then be determined.

19. The preparation of the abiotic sludge is typically performed using a combination of chemical and heat sterilization. A proven approach is to add mercuric chloride solution (1 g/L) to the sludge, which is then autoclaved for at least 90 minutes. After cooling, the pH of the abiotic sludge should be measured and adjusted to match that of the biologically active sludge. Alternative approaches to deactivate the sludge can also be used. When preparing the abiotic sludge it should be stored and manipulated in an anaerobic chamber or other another approaches utilized to protect it from exposure to oxygen.

Test Substance Preparation

20. Ideally, distilled water should be used to prepare stock solutions of the test and reference substances. When appropriate, an alternative method may be used to solubilize or disperse the test chemical in a manner consistent with its normal entry into digester sludge. When practical, the dosing

solutions should be equilibrated overnight in a reducing atmosphere to remove dissolved oxygen prior to use. The volume of added stock should be of sufficient capacity to ensure rapid and even distribution of the test material in the treatment and accurate administration of the dose between like treatments. Ideally, when dosing with aqueous solutions, the added volume should be $\geq$ 1 ml; for non-toxic solvents, < 0.1 ml/ L. The activity of the stock should be checked by LSC. For materials which are poorly soluble and typically associated with sludge solids, it may be appropriate to adsorb the test material onto an inert solid carrier, which is then dosed to the test system. If the test material can not be evenly distributed within the test system prior to the initial sampling point, individual test systems can be prepared that are destructively sampled at each sampling interval.

21. As an alternative, the test chemical can be applied to dried inactive sludge solids, which can be mixed into the test system. Water-miscible non-toxic solvents may be used when necessary, but attention should be paid to the associated organic load involved with adding organic solvents. In addition, the test material may be added in a neat form in a manner that maximizes its even and rapid distribution into the sludge.

Test conditions

Test temperature

22. Incubation should take place in the dark (preferred) or in diffuse light at a controlled temperature, which may be the typical operating temperature for an anaerobic digester (35°C), a field temperature or a standard laboratory temperature of 20-25°C.

Agitation

23. To simulate the static conditions which occur within a digester, the test vessels are not usually continuously mixed. During sampling, they should be well mixed to ensure a representative sample. Additionally, they can be gently agitated for a few minutes 2 to 3 times per week.

Test duration

24. The duration of the test should be sufficiently long to assess the biodegradation of the test chemical during its normal residence time within an anaerobic sludge. Normally, the test period will last 60 days. However, it may be extended longer to obtain additional data points to estimate kinetic constants or to assess the completeness of degradation under the conditions within the test. Conversely, it may be ended before this time if degradation has plateaued.

Number of test vessels

25. At a minimum, there should be a single abiotic and a single biotic test vessel for each test material concentration. Additional replicates can be prepared for specific chemical analysis. The additional replicates are maintained under anaerobic conditions but typically not connected to the mineralization apparatus and can be subsampled or sacrificed at a particular sampling point.

PROCEDURE

Dosing

26. At test initiation, the test material is quantitatively added directly to the digester sludge with constant mixing. Dosing must be done in such a manner that the test system is protected from exposure to oxygen. It is recommended that the dose be administered in a gradual fashion below the air-water interface, to ensure uniform distribution of the test material into the sludge. The biotic and abiotic treatments are dosed in an identical manner.

Sampling Schedule

27. Sampling intervals are selected based on existing biodegradation data or the results of a pilot study as no fixed time schedule for sampling is universally applicable. A recommended sampling schedule for a rapidly degraded chemical would be 30, 60, and 120 minutes, with additional samplings after 4, 8 and 24 hours. Subsequent samples could be taken after 2, 4 and 7 days and weekly until day 56. The sampling schedule for slowly degrading chemicals should be adjusted so that a sufficient number of measurements are made during the degradation phase.

Measurement of Mineralization

Indirect measurement of ^{14}C Gases ($^{14}CO_2$ and $^{14}CH_4$)

28. Direct LSC counting of samples is not possible due to the high solids levels in the samples. Therefore, samples are centrifuged and the supernatant analyzed for total radioactivity by LSC and the solids combusted and then analyzed for radioactivity to determine the total radioactivity in the sample. Individual replicate samples (e.g. 1 ml) of digester sludge are collected from each treatment and placed into centrifuge tubes that contain sufficient acid (e.g. 0.025 ml of HCl) to lower the sample pH to < 2. The samples are centrifuged and the supernatant transferred to a scintillation vial, which is allowed to stand overnight for the dissolved $^{14}CO_2$ to diffuse from the samples. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The solids remaining in the centrifuge tube are combusted using a sample oxidizer prior to LSC. The percent of total ^{14}C Gas produced is calculated based upon the difference between the total counts in the biotic and abiotic samples.

Direct measurement of $^{14}CO_2$ and $^{14}CH_4$

29. Evolved $^{14}CO_2$ and $^{14}CH_4$: Direct measurement of $^{14}CO_2$ and $^{14}CH_4$ is possible only in a sealed flow-through batch system with connected base traps. For $^{14}CO_2$, the first base trap in the first trapping train is removed and quickly capped. The remaining traps are moved forward in the same order and a fresh trap placed behind the existing traps and the trapping system reconnected as quickly as possible. Replicate subsamples (e.g. 1 ml) from the removed base trap are transferred to scintillation vials and combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. This process is repeated for the second trapping train to determine $^{14}CH_4$.

30. Dissolved $^{14}CO_2$ (optional): Sludge samples (e.g. 10 ml) are removed through the sampling port of the test flask. They are then placed in vessels (e.g. Bellco Glass Biometer 2556-10250) containing a compartment with an appropriate CO_2 absorbent (e.g. 1.5 N KOH). The vessels are sealed and sufficient acid is added to lower the pH of the samples to < 2 without opening the vessels to the atmosphere (see Annex 1). The samples are allowed to sit for a sufficient length of time to allow CO_2 to diffuse from solution and be trapped from the headspace by the sorbent. Samples of the sorbent are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

Indirect measurement of $^{3}H_2O$

31. Samples (e.g. 8 ml) of sludge are collected from each treatment and placed into centrifuge tubes

that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood. The tubes are mixed and centrifuged. Individual replicate samples (e.g. 1 ml) of the supernatant are placed into separate vials. Half of the samples are immediately analysed directly by LSC for a "wet measurement". The remaining samples are allowed to dry completely to remove the ^3H_2O. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent ^3H_2O is calculated based upon the difference between the total counts in the wet and dry samples and the initial level of radioactivity dosed to the samples.

Measurement of Radioactivity in Digester Sludge

32. Direct LSC counting of samples is not possible due to the high solids levels in the samples. Therefore, samples are centrifuged and the supernatant analyzed for total radioactivity by LSC and the solids combusted and then analyzed for radioactivity to determine the total radioactivity in the sample. Individual replicate samples (e.g. 1 ml) of digester sludge are collected from each treatment and placed into centrifuge tubes. The samples are centrifuged and the supernatant transferred to a scintillation vial. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The solids remaining in the centrifuge tube are combusted using a sample oxidizer prior to LSC.

Measurement of Parent and Metabolites

Extraction

33. A sample of digester sludge is collected from both the abiotic and biotic treatments. The sample volume is typically ≥ 10 ml. However, the size will depend on the test concentration, specific activity and the sensitivity of the analytical procedures.

34. Various approaches can be used for concentrating and extracting the samples. A proven approach for nonvolatile test chemicals involves flash freezing the samples, followed by lyophilization and extraction of the dried residue with appropriate solvent(s) for parent and metabolites. Flash freezing quickly stops biological activity without hydrolyzing or otherwise altering labile test materials. The resulting extracts can be concentrated through evaporation and the total radioactivity in each extract is determined by LSC.

35. For volatile test materials, samples can be centrifuged, and parent and metabolites can be extracted from the liquor by solid phase or liquid/liquid extraction. The solids can then be extracted directly or mixed with a drying agent (e.g. sodium sulfate) and allowed to dry prior to extraction with an appropriate solvent system. .An alternative is to extract the solids and then remove the water from the solvent by running it through a column containing a drying agent. The total radioactivity in all extracts is determined by LSC. Care must be taken in concentrating extracts containing volatile test materials or metabolites.

36. Other approaches can be utilized, but with all approaches it is important to document recoveries and consider the time involved in terminating biological activity and factor it into the sample times used for kinetic analyses.

Analysis of Parent and Metabolites

37. The relative abundance of parent and metabolites within the extracts can be determined using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) or other separation techniques with radioactivity detection.

38 If sensitive specific analytical methods are available, primary biodegradation can be assessed by measuring the total residual concentration of test substances and metabolites instead of using radioisotope techniques.

Characterization of Metabolites

39. Whenever possible, the chromatographic behaviour of unknown peaks should be compared to that of predicted metabolites, if authentic standards exist. Usually, the quantity and purity of metabolites generated in this test make definitive identification by other direct means impossible. Depending upon chromatographic behaviour, it is usually possible to determine if a metabolite is more or less polar than the parent. This information combined with known biochemical reactions along with when a metabolite appears and disappears in the sequence of biodegradation can form an additional basis for inferring its identity. If necessary, the K_{ow} of major metabolites can be determined by HPLC (e.g. [OECD 117] (24)) using an on-line radioactivity detector.

Measurement of Extracted Solids and Incorporation into Biomass

40. The extracted solids are combusted to determine the level of activity remaining with the solids. The level of radioactivity in the biotic solids above that in solids from the abiotic control typically represents incorporation of radioactivity into biomass. The distribution of this radioactivity among various components of biomass (i.e. nucleic acids, protein, cell wall, etc.) can be determined using a modified Sutherland and Wilkinson procedure (25)**.**

Measurement of Volatilized Radioactivity

41. For volatile test materials, the volatile traps are extracted with appropriate solvents and the radioactivity in the extracts is analyzed by LSC. The relative abundance of parent and metabolites in the extract(s) can be determined as described above.

3xxD BIODEGRADATION IN TREATED EFFLUENT-SURFACE WATER MIXING ZONE

INTRODUCTION

1. This test is designed to evaluate the biodegradation of the portion of a chemical that passes through treatment and is released in effluent to surface water. It can be used to demonstrate that biodegradation occurring in the treatment plant continues in the receiving environment. It also is useful for determining the extent of biodegradation as well as rates of primary and ultimate biodegradation in this environmental compartment. The results can be used to estimate the reduction in chemical concentration resulting from biodegradation as a volume of water moves downstream from a wastewater treatment plant outfall. The test system consists of freshly collected surface water and effluent. The usefulness of the measured rates for accurately predicting downstream exposure will be a function of the fidelity of the simulation to actual conditions in the mixing zone. Along with test material concentration, factors to consider in the designing of this test include the level of solids in effluent and the degree to which effluent is diluted into surface water.

2. To simulate conditions associated with episodic release of a chemical, an appropriately chosen mixture of surface water and effluent is incubated with the concentration of test chemical expected to occur in effluent diluted into surface water during a release event. . In these situations, the chemical and its degrader populations will usually not be in steady state and the observed kinetics will be quasi first-order, or second-order Monod. Approaches for estimating effluent concentrations can be found in Holman (21) and the European Technical Guidance Document (22)

3. For chemicals that are or will be continuously released to wastewater, degrader populations within the treatment plant will become acclimated to the chemical. For existing chemicals continuously discharged to wastewater, surface water with the expected concentration of test chemical and freshly collected effluent under a given discharge scenario will provide the most realistic kinetic parameters. For new chemicals that will be continuously discharged to wastewater, the use of effluent that was exposed to the chemical under simulated activated sludge conditions in the laboratory (e.g. 303 A), will provide the most accurate kinetics.

4. In most circumstances due to analytical considerations, it will be impossible to test at actual surface water concentrations. Consequently, observed biodegradation rates may not be fully representative of those under actual environmental conditions and should be considered in the interpretation of the results.

GENERAL TEST PROCEDURE

5. The test chemical is incubated with abiotic and biotic mixtures of surface water and effluent over a period of time. The ratio of these components is based upon specific or generic scenarios for release of treated effluent to surface water. Biological activity is inhibited in the abiotic control, which is used for estimating mineralization by difference, establishing extraction efficiency and recovery of the parent molecule and quantifying other loss processes, such as hydrolysis, oxidation, volatilization or sorption to test apparatus.

6. If an analytical method with the required sensitivity is identified, the rate of parent degradation or transformation can be determined using a non-radiolabelled test substance or by following the disappearance of a chemical already in wastewater. However, ultimate biodegradation can not be determined unless the biodegradation pathway is well understood and analytical methods with required sensitivity are available for potential metabolites.

7. An environmentally relevant concentration of the test material is dosed to both abiotic and biotic test systems, which are incubated at a relevant temperature with continuous mixing when appropriate. Samples are periodically removed for determination of mineralization and primary biodegradation.

8. Tests can be performed using an open batch system or a sealed, flow-through batch system where traps are used to capture evolved $^{14}CO_2$. The closed flow-through system is mandatory for volatile test materials and usually preferred for ^{14}C-lableled test chemicals. Open systems are appropriate for non-volatile ^{3}H test chemicals and for refining the biodegradation kinetics of non-volatile ^{14}C test materials, whose ability to be mineralized has previously been established. In the open system, mineralization to $^{14}CO_2$ can be determined indirectly by measuring the difference in residual radioactivity between samples from the biotic and abiotic treatments following acidification. Similarly, mineralization to $^{3}H_2O$ can be determined indirectly by measuring the difference in radioactivity in a sample following drying. In the flow through systems, evolved $^{14}CO_2$ is measured directly in the base traps. In addition, dissolved $^{14}CO_2$ is determined by acidifying samples in a sealed vessel and measuring radioactivity in a base trap contained in the vessel.

9. Samples from both treatments are analyzed for total radioactivity, extractable parent and metabolites and radioactivity associated with the extracted solids. The level of parent and metabolites is determined using chromatographic separation and, when appropriate, radio-analytical detection methods. The solids remaining from the extraction process are combusted to estimate incorporation into biomass by difference or can be further fractionated to determine uptake into various components of biomass. A complete mass balance of the test system is obtained from the sum total of all fractions at each sampling.

APPLICABILITY OF THE TEST

10. The method is readily applicable to water-soluble or poorly water-soluble materials, which are non-volatile. It can also be adapted for volatile materials. Typically, ^{14}C or ^{3}H -radiolabelling of compounds is required for the assessment of mineralization. Both radiolabelled and non-labelled compound can be used for the assessment of primary biodegradation.

DESCRIPTION OF THE TEST METHOD

Test Apparatus

11. The volume of the surface water-effluent mixture in the test treatments is determined based upon the number and volume of the samples needed for the assessment. Typically, 1 to 2 litres of surface water are placed into 2 or 4 litre flasks. Open batch systems are generally closed with a foam or cotton stopper to minimize evaporative loss of water. Flow-through systems are sealed with an appropriate closure containing a sampling port with a valve for removing samples and connections for influent and effluent gas lines. This closure can be a rubber stopper, but glass is recommended when working with a volatile hydrophobic test material. When testing volatile compounds, it also is recommend that gas lines and sampling tubes consist of inert materials (e.g. Teflon, stainless steel, glass).

12. The head space of the test vessel is continuously purged with air or CO_2-free air at a rate sufficient to maintain the system in an aerobic condition but not too fast to prevent efficient trapping of CO_2. The test vessel is connected to a series of traps containing potassium hydroxide (1.5 N) or other appropriate CO_2 absorbent. An empty trap is usually included in the trapping train as a precaution against back-flow or condensation.

Equipment

13. The following standard laboratory equipment are used:

- miscellaneous glassware and pipettes;
- magnetic stirrers or shaker for continuous mixing of the test flasks;
- centrifuge;
- pH meter;
- solid CO_2 (dry ice)/acetone or liquid nitrogen bath;
- freeze dryer (lyophilizer);
- oven or microwave oven for dry weight determinations;
- membrane filtration apparatus;
- autoclave;
- facilities to handle radiolabelled substances;
- equipment to quantify ^{14}C and ^{3}H in liquid samples and solid samples (e.g. liquid scintillation counter LSC);
- equipment to quantify ^{14}C and ^{3}H in solid samples (e.g. sample oxidizer);
- equipment to trap volatilized ^{14}C and ^{3}H from gas trapping system (in-line activated charcoal trap or equivalent);
- equipment for thin layer chromatography (TLC) or high performance liquid chromatography (HPLC);
- equipment to quantify ^{14}C and ^{3}H for TLC (scanner) or HPLC (in-line detector);
- analytical equipment for the determination of the test (and reference) substance if specific chemical analysis is used (e.g. gas chromatograph, high performance liquid chromatograph, mass spectrometer).

Selection of Environmental Samples

14. The source of surface water, activated sludge or effluent should be consistent with the objective of the simulation test. For a site-specific assessment, activated sludge or effluent should be obtained from the specific wastewater treatment plant in question. Likewise, the surface water should be obtained upstream from that treatment plant outfall. The ratio of these components should be chosen to simulate a specific flow scenario (e.g. low flow or mean flow). Alternatively, if hydraulic conditions below the outfall are known, the test system can consist simply of samples obtained downstream from the outflow. However, such conditions are variable and hard to reproduce.

15. For a generic assessment activated sludge or effluent should be obtained from a typical wastewater treatment plant receiving predominantly domestic wastewater. Likewise, the surface water should be typical of surface waters into which effluent is released. If the chemical is currently a component of wastewater entering the wastewater treatment facility or is episodically released to wastewater, freshly collected activated sludge or effluent will be ideal for the test. WWTP effluent consists of activated sludge liquor and biosolids, but it can be variable in its solids level and impacted by chlorination or other processes. The best method of simulating this scenario is using filtered effluent and surface water combined at the targeted dilution ratio and separately adding activated sludge biosolids at a targeted concentration. If the effluent is difficult to obtain, the activated sludge can be filtered or centrifuged to remove biosolids, and the liquor and biosolids can then be added at a defined ratio to the test. For a generic assessment, the European Technical Guidance Document assumes effluent containing 30 mg/L of biosolids is diluted 10-fold into surface water containing 15 mg/L of suspended solids (22). Hence, 3 mg of biosolids in 100 ml of filtered effluent or activated sludge liquor and 900 ml of surface water approximates this generic scenario. An additional scenario with 10 mg of biosolids and 330 ml of

filtered effluent or liquor per litre also might be considered to simulate critical low flow conditions that might occur during dry seasons.

16. For a new chemical, which will be continuously released to wastewater, the activated sludge or effluent ideally should be obtained from a laboratory scale treatment system such as a porous pot or CAS [OECD 303A] (2), which has been fed wastewater amended with unlabelled test material. The source of the starting sludge, wastewater (influent) and the operating conditions (influent concentration, hydraulic retention time, solids retention time) for the laboratory unit should accurately reflect site-specific or generic conditions. In the case of the latter, the European Technical Guidance Document (22) specifies an HRT of 6.9 hours and an SRT of 9.2 days in its generic scenario for wastewater treatment. The European Technical Guidance Document also provides guidance on estimating wastewater concentration based upon expected usage volumes. In general, steady state will be reached within 2 to 3 SRTs after which point the biosolids or effluent can be used for testing.

Collection, Transport and Storage of Environmental Samples

17. The activated sludge should be collected from a well mixed region of the aeration basin, the effluent should be collected from the discharge point of the WWTP. Surface water should be collected from a site with known inputs of wastewater. The temperature of the samples should be noted at collection. Collection containers should allow for adequate ventilation and measures should be taken to prevent the temperature of the sample from significantly exceeding the temperature used in the test. The samples are typically stored at test temperature with continuous aeration. Samples should not be stored frozen.

Preparation of Test Treatments

18. The surface water should be characterized by measuring the total suspended solids (TSS), total hardness and pH. A standard plate count and organic carbon analysis are optional. When using activated sludge to represent WWTP effluent, the MLSS is sieved through a 2mm screen, blended (optional) and allowed to settle. The TSS concentration of the liquor is measured. The liquor is added to the surface water at a volume sufficient to achieve the targeted biosolids concentration. If more volume is needed to reach the targeted dilution ratio in the test, filtered or centrifuged activated sludge liquor is added to reach the necessary dilution. If using treated effluent and a targeted activated sludge biosolids concentration, the effluent is filtered and mixed with the surface water at the targeted dilution ratio. The MLSS is prepared as described previously. The pH and TSS of the prepared surface water mixture should be measured. An optional standard plate count and organic carbon analysis can also be performed.

19. The preparation of the abiotic treatment is typically performed using a combination of chemical and heat sterilization. A proven approach is to add mercuric chloride solution (0.1 g/L) to the mixture, which is then autoclaved for at least 90 minutes. After cooling, the pH of the abiotic treatment should be measured and adjusted to match that of the biologically active treatment. Alternative approaches to deactivate the surface water mixture can also be used.

Test Substance Preparation

20. Ideally, distilled water should be used to prepare stock solutions of the test and reference substances. When appropriate, an alternative method may be used to solubilize or disperse the test chemical in a manner consistent with its normal entry into the environment. Water miscible non-toxic solvents may be used when necessary, but attention should be paid to the associated organic load involved with adding organic solvents. Alternatively, the sample may be added in a neat form to the test system in a manner that maximizes its even and rapid distribution into the test treatments. For materials which are poorly soluble and typically associated with suspended solids in effluent, it may be appropriate to adsorb

the test material onto an inert solid carrier, which is then dosed to the test system. If the test material can not be evenly distributed within the test system prior to the initial sampling point, individual test systems can be prepared that are destructively sampled at each sampling interval.

21. The volume of added stock should be of sufficient capacity to ensure rapid and even distribution of the test material in the treatment and accurate administration of the dose between like treatments. Ideally, when dosing with aqueous solutions, the added volume should be $\geq$ 2 ml; for non-toxic solvents, < 0.1 ml/ L. If appropriate, dosing solutions may be prepared in advance and refrigerated. The activity of the stock should be checked by LSC.

Test conditions

Test temperature

22. Incubation should take place in the dark (preferred) or in diffuse light at a controlled temperature, which may be the field temperature or a standard laboratory temperature of 20-25°C.

Agitation

23. To keep the test medium in suspension, the test systems are agitated by means of continuous shaking or stirring. Agitation also facilitates oxygen transfer from the headspace to the liquid so that aerobic conditions can be adequately maintained.

Test duration

24. The duration of the test should be sufficiently long to assess the biodegradation of the test chemical during its normal residence time within the WWTP effluent-surface water mixing zone. Normally, the test period will last 28 days. However, it may be extended longer to obtain additional data points to estimate kinetic constants or to assess the completeness of degradation under the conditions within the test. Conversely, it may be ended before this time if degradation has plateaued.

Number of test vessels

25. At a minimum, there should be a single abiotic and a single biotic test vessel for each test material concentration. While replicates can be prepared for each treatment, more useful kinetic information usually can be gained by increasing the number of time points sampled within a treatment.

PROCEDURE

Dosing

26. At test initiation, the test vessel is opened and the test material is quantitatively added directly to the treatment with constant mixing. It is recommended that the dose be administered in a gradual fashion below the air-water interface, to ensure uniform distribution of the test material into the test medium. The biotic and abiotic treatments are dosed in an identical manner. Generally, the biotic systems are dosed first, followed by the abiotic systems. Exact timing is typically more critical for the biotic versus the abiotic treatments for kinetic analyses.

Sampling Schedule

27. Sampling intervals are selected based on existing biodegradation data or the results of a pilot study as no fixed time schedule for sampling is universally applicable. A recommended sampling schedule for a rapidly degraded chemical would be 5, 30, 60 minutes, with additional samplings after 3, 5, 8, 12 and 24 hours. Subsequent samples could be taken after 2, 3, 4, 5, 6 and 7 days and weekly until day 28. The sampling schedule for slowly degrading chemical should be adjusted so that a sufficient number of measurements are made during the degradation phase.

Measurement of Mineralization

Indirect measurement of $^{14}CO_2$

28. Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood.

29. The samples are bubbled with air for several hours or allowed to sit overnight to allow the dissolved $^{14}CO_2$ to diffuse from the samples. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent of $^{14}CO_2$ is calculated based upon the difference between the total counts in the biotic and abiotic samples.

Direct measurement of $^{14}CO_2$

30. For rapidly degrading chemicals, it can be difficult to measure accurately the rate of $^{14}CO_2$ evolved due to the rate of the mass transfer of $^{14}CO_2$ from the headspace into the base trap. Under these conditions, it is recommended that indirect measurement of $^{14}CO_2$ be conducted simultaneously with direct measurement.

31. Evolved $^{14}CO_2$: The first base trap in the series is removed and quickly capped. The remaining traps are moved forward in the same order and a fresh trap placed behind the existing traps and the trapping system is reconnected as quickly as possible. Replicate subsamples (e.g 1 ml) from the removed base trap are transferred to scintillation vials and combined with scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

32. Dissolved $^{14}CO_2$: Samples (e.g. 25 to 50 ml) are removed through the sampling port of the test flask. They are then placed in vessels (e.g. Bellco Glass Biometer 2556-10250) containing a compartment with an appropriate CO_2 absorbent (e.g. 1.5 N KOH). The vessels are sealed and sufficient acid is added to lower the pH of the samples to < 2 without opening the vessels to the atmosphere (see Annex 1). The samples are allowed to sit for a sufficient length of time (e.g. overnight) to allow CO_2 to diffuse from solution and be trapped from the headspace by the sorbent. Samples of the sorbent are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

Indirect measurement of $^{3}H_2O$

33. Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood.

34. Half of the samples are immediately analysed directly by LSC for a “wet measurement”. The remaining samples are allowed to dry completely to remove the $^{3}H_2O$. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent $^{3}H_2O$ is

calculated based upon the difference between the total counts in the wet and dry samples and the initial level of radioactivity dosed to the samples.

Measurement of Radioactivity in Treatment

35. Small volume samples (e.g. 1 ml) are analysed directly by LSC to quantify the radioactivity remaining in each treatment over time. These measurements are used to confirm that the recovery of radioactivity from the extracted samples is acceptable and to monitor for volatilization.

Deleted: benchmark

Measurement of Parent and Metabolites

Extraction

36. A sample is collected from both the abiotic and biotic treatments. The sample volume is typically ≥ 25 ml. However, the size will depend on the test concentration, specific activity and the sensitivity of the analytical procedures.

37. Various approaches can be used for concentrating and extracting the samples. A proven approach for nonvolatile test chemicals involves flash freezing the samples, followed by lyophilization and extraction of the dried residue with appropriate solvent(s) for parent and metabolites. Flash freezing quickly stops biological activity without hydrolyzing or otherwise altering labile test materials. The extract is filtered to recover the solvent and solids separately. The filter must be compatible with the solvent type (e.g. aqueous or non-aqueous). The resulting extracts can be concentrated through evaporation and the total radioactivity in each extract is determined by LSC.

38. For volatile test materials, the sample can be passed through a filter and solid phase extraction (SPE) column or SPE disk placed in tandem, which are subsequently eluted with appropriate solvents to recover parent and metabolites. Alternatively, the aqueous samples can be extracted with an appropriate solvent system and then filtered to recover biomass solids, assuming sufficient extraction efficiency. The total radioactivity in all extracts is determined by LSC. Care must be taken in concentrating extracts containing volatile test materials or metabolites.

39. Other approaches can be utilized, but with all approaches it is important to document recoveries and consider the time involved in terminating biological activity and factor it into the sample times used for kinetic analyses.

Analysis of Parent and Metabolites

40. The relative abundance of parent and metabolites within the extracts can be determined using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) or other separation techniques with radioactivity detection.

41. If sensitive specific analytical methods are available, primary biodegradation can be assessed by measuring the total residual concentration of test substances and metabolites instead of using radioisotope techniques.

Characterization of Metabolites

42. Whenever possible, the chromatographic behaviour of unknown peaks should be compared to that of predicted metabolites, if authentic standards exist. Usually, the quantity and purity of metabolites generated in this test make definitive identification by other direct means impossible. Depending upon

chromatographic behaviour, it is usually possible to determine if a metabolite is more or less polar than the parent. This information combined with known biochemical reactions along with when a metabolite appears and disappears in the sequence of biodegradation can form an additional basis for inferring its identity. If necessary, the K_{ow} of major metabolites can be determined by HPLC (e.g. [OECD 117] (24)) using an on-line radioactivity detector.

Measurement of Extracted Solids

43. Since the filters will retain carbonate salts as well as microorganisms from the test system, the filter containing the biosolids is placed into a scintillation vial and acidified to pH $\leq$2 by submerging it in a weak acid solution (1 ml of 0.1N HCl). The samples are allowed to sit for sufficient time (e.g. overnight) for the dissolved $^{14}CO_2$ to diffuse from the samples. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The level of radioactivity in the biotic solids above that in solids from the abiotic control typically represents incorporation of radioactivity into biomass.

Measurement of Volatilized Radioactivity

44. For volatile test materials, the volatile traps are extracted with appropriate solvents and the radioactivity in the extracts is analyzed by LSC. The relative abundance of parent and metabolites in the extract(s) can be determined as described above.

3xxE BIODEGRADATION IN UNTREATED WASTEWATER-SURFACE WATER MIXING ZONE

INTRODUCTION

1. In developing regions lacking wastewater treatment infrastructure, it is common for wastewater to be directly discharged to surface waters. This test is designed to simulate these situations and evaluate the biodegradation of a chemical that is discharged to surface water as a component of untreated wastewater. It is useful for determining the extent of biodegradation as well as rates of primary and ultimate biodegradation under such direct discharge conditions. The results can be used to estimate the reduction in chemical concentration resulting from biodegradation as a volume of water moves downstream from a wastewater outfall. As an option, this reduction can be compared against the decreases in other wastewater components such as biochemical oxygen demand (BOD), chemical oxygen demand (COD) or total organic carbon (TOC). The test system consists of freshly collected wastewater and surface water. The usefulness of the measured rates for accurately predicting downstream exposure will be a function of the fidelity of the simulation to actual conditions in the mixing zone. Along with test material concentration, factors to consider in designing this test include dissolved oxygen concentration and the degree to which effluent is diluted into surface water.

2. For existing chemicals consistently present in wastewater, freshly collected wastewater and surface water incubated with a tracer level of radiolabelled test chemical will provide the most realistic kinetic parameters regarding the current chemical load. For chemicals not consistently present in wastewater, sufficient test chemical (radiolabelled and unlabelled) should be added to approximate the expected concentration in wastewater diluted into surface water during an episodic release or following commercialization of a new chemical. Approaches for estimating such an expected wastewater concentration can be found in Holman (21) and the European Technical Guidance Document (22).

3. For low dilution situations, it is best to incubate the mixtures under reduced dissolved oxygen conditions (1 – 4 mg/L) to simulate the DO below a wastewater outfall. In this test, the chemical and its degrader populations usually are not in steady state and the observed kinetics will be quasi first-order, or second-order Monod.

4. The test can be done using a single or two phase design. In the former, biodegradation is evaluated in wastewater, which has been diluted into a given clean or wastewater impacted surface water. In the latter, biodegradation is assessed in wastewater, which has been sequentially diluted into clean and wastewater impacted surface waters. In this test design, the test chemical is dosed into wastewater diluted into clean surface water and disappearance of test chemical and conventional pollutants (BOD, COD, etc) are monitored with time (phase 1). Subsequently, a second dose of test chemical and wastewater is added to the same system to simulate dilution of wastewater into surface water previously polluted by wastewater (phase 2).

GENERAL TEST PROCEDURE

5. The test chemical is incubated with abiotic and biotic mixtures of wastewater and surface water usually under reduced dissolved oxygen (DO) conditions (1 – 4 mg/L) over a period of time. The ratio of these components is based upon specific or generic scenarios for release of wastewater to surface water. Biological activity is inhibited in the abiotic control, which is used for estimating mineralization by difference, establishing extraction efficiency and recovery of the parent molecule and quantifying other loss processes, such as hydrolysis, oxidation, volatilization or sorption to test apparatus.

6. If an analytical method with the required sensitivity is identified, the rate of parent degradation or

transformation can be determined using a non-radiolabelled test substance or by following the disappearance of a chemical already in wastewater. However, ultimate biodegradation can not be determined unless the biodegradation pathway is well understood and analytical methods with required sensitivity are available for potential metabolites.

7. An environmentally relevant concentration of the test material is dosed to both abiotic and biotic test systems, which are incubated at a relevant temperature with continuous mixing. The biotic samples are incubated in such a way that dissolved oxygen levels remain at a reduced level (1- 4 mg/L), characteristic of the situation below a wastewater outfall. Samples are periodically removed for determination of mineralization and primary biodegradation and as an option, the level of other wastewater components (e.g. COD, TOC and ammonia) can be determined concurrently.

8. In the two phase test design, once biodegradation of the test chemical and wastewater components have levelled off, a second dose of test chemical and fresh wastewater is mixed into the existing test system to simulate wastewater being diluted into previously polluted surface water and the sampling process is repeated.

9. Tests can be performed using an open batch system or a sealed, flow-through batch system where traps are used to capture evolved $^{14}CO_2$. The closed flow-through system is mandatory for volatile test materials and usually preferred for ^{14}C-lableled test chemicals. Open systems are appropriate for non-volatile ^{3}H test chemicals and for refining the biodegradation kinetics of non-volatile ^{14}C test materials, whose ability to be mineralized has previously been established. In the open system, mineralization to $^{14}CO_2$ can be determined indirectly by measuring the difference in residual radioactivity between samples from the biotic and abiotic treatments following acidification. Similarly, mineralization to $^{3}H_2O$ can be determined indirectly by measuring the difference in radioactivity in a sample following drying. In the flow through systems, evolved $^{14}CO_2$ is measured directly in the base traps. In addition, dissolved $^{14}CO_2$ is determined by acidifying samples in a sealed vessel and measuring radioactivity in a base trap contained in the vessel.

10. Samples from both treatments are analyzed for total radioactivity, extractable parent and metabolites and radioactivity associated with the extracted solids. The level of parent and metabolites is determined using chromatographic separation and when appropriate radio-analytical detection methods. The remaining solids from the extraction process are combusted to estimate incorporation into biomass by difference. A complete mass balance of the test system is obtained from the sum total of all fractions at each sampling.

APPLICABILITY OF THE TEST

11. The method is readily applicable to water-soluble or poorly water-soluble materials, which are non-volatile. It can also be adapted for volatile materials. Typically, ^{14}C or ^{3}H -radiolabelling of compounds is required for the assessment of mineralization. Both radiolabelled and non-labelled compound can be used for the assessment of primary biodegradation.

DESCRIPTION OF THE TEST METHOD

Test Apparatus

12. The volume of the wastewater-surface water mixture in the test treatments is determined based upon the number and volume of the samples needed for the assessment. Typically, 1 to 2 litres of sample is placed into 2- or 4- litre flasks. Ideally, the wastewater-surface water mixture is incubated under one or more controlled DO conditions (e.g. 1 and 4 mg/L DO). This condition can be achieved using an oxygen probe immersed in the wastewater attached to an oxygen controller connected to an actuator valve, which

controls the aeration of the wastewater (see Annex 1). This aeration is balanced against continuous sparging with nitrogen to achieve the targeted DO level. Alternatively, the wastewater can be incubated with stirring but minimum aeration to keep DO levels at desired levels, nitrogen or air can be added periodically to maintain DO level. In this case, DO readings should be reported at regular intervals.

13. Open systems are generally closed with a foam or cotton stopper to minimize evaporative loss of water. Flow-through systems are sealed with an appropriate closure containing a sampling port with a valve for removing samples and connections for influent and effluent gas lines. This closure can be a rubber stopper, but glass is recommended when working with a volatile hydrophobic test material. When testing volatile compounds, it also is recommend that gas lines and sampling tubes consist of inert materials (e.g. Teflon, stainless steel, glass).

14. The head space of the test vessel is continuously purged with air or CO_2-free air at a rate sufficient to maintain the system in an aerobic condition but not too fast to prevent efficient trapping of CO_2. The test vessel is connected to a series of traps containing potassium hydroxide (1.5 N) or other appropriate CO_2 absorbent. An empty trap is usually included and positioned in front of the absorbent in the trapping train as a precaution against back-flow or condensation.

Equipment

- miscellaneous glassware and pipettes;
- magnetic stirrers or shaker for continuous mixing of the test flasks;
- centrifuge;
- pH meter;
- solid CO_2 (dry ice)/acetone or liquid nitrogen bath;
- freeze dryer (lyophilizer);
- oven or microwave oven for dry weight determinations;
- membrane filtration apparatus;
- autoclave;
- facilities to handle radiolabelled substances;
- equipment to quantify ^{14}C and ^{3}H in liquid samples and solid samples (e.g. liquid scintillation counter LSC);
- equipment to quantify ^{14}C and ^{3}H in solid samples (e.g. sample oxidizer);
- equipment to trap volatilized ^{14}C and ^{3}H from gas trapping system (in-line activated charcoal trap or equivalent);
- equipment for thin layer chromatography (TLC) or high performance liquid chromatography (HPLC);
- equipment to quantify ^{14}C and ^{3}H for TLC (scanner) or HPLC (in-line detector);
- analytical equipment for the determination of the test (and reference) substance if specific chemical analysis is used (e.g. gas chromatograph, high performance liquid chromatograph, mass spectrometer).

15.. The following laboratory equipment is not essential but useful:

- oxygen meter
- oxygen controller with probe and actuator valve.
- COD digestion vials
- Nitrogen ammonia reagent set
- Spectrophotometer

Selection of Environmental Samples

16. The source of wastewater and surface water should be consistent with the objective of the simulation test. For a site specific assessment, the wastewater should be obtained from the specific sewer system in question and the surface water should be obtained upstream from the wastewater outfall. The ratio of these components should be chosen to simulate a specific flow scenario (e.g. low flow or mean flow). Alternatively, if hydraulic conditions below the outfall are known, the test system can consist simply of samples obtained downstream from the outflow. However, such conditions are variable and hard to reproduce.

17. For a generic assessment wastewater samples should be predominantly derived from domestic sources, and the surface water should be typical of surface waters into which wastewater is released. Although difficult to duplicate in practice, the European Technical Guidance Document uses 450 mg/L of suspended solids and 270 mg/L of BOD (biological oxygen demand) as default levels in wastewater (22). In North America, typical wastewaters contain from 100 to 350 mg/L of suspended solids and 110 to 400 mg/L of BOD (23).

Collection, Transport and Storage of Environmental Samples

18. The wastewater should be collected from a sewer access point or at the head of a wastewater treatment plant. The temperature of the sample should be noted at collection. During transport, the temperature of the sample should not significantly exceed the temperature used in the test. The wastewater is typically stored at test temperature with low mixing. No samples should ever be stored frozen.

19. Surface water should be collected from a site with known inputs of wastewater. The temperature of the samples should be noted at collection. During transport, the temperature of the samples should not significantly exceed the temperature used in the test. The surface water is typically stored at test temperature with continuous aeration. No samples should ever be stored frozen.

Preparation of Test Treatments

20. The freshly collected wastewater should be largely free from coarse particles. Total suspended solids (TSS), pH and chemical oxygen demand (COD) should be determined for the wastewater. NH_3, organic carbon and standard plate count are optional analysis. The surface water should be characterized by measuring the total suspended solids (TSS), total hardness and pH. A standard plate count and organic carbon analysis are optional. The wastewater is added to the surface water at a volume sufficient to achieve the targeted dilution of wastewater into surface water. The pH, COD, and TSS of the prepared surface water mixture should be measured. An optional standard place count, NH_3 and organic carbon analysis can also be performed on the mixture.

21. The preparation of the abiotic treatment is typically performed using a combination of chemical and heat sterilization. A proven approach is to add mercuric chloride ($HgCl_2$ = 0.1 g/L) to the mixture, which is then autoclaved for at least 90 minutes. After cooling, the pH of the abiotic treatment should be measured and adjusted to match that of the biologically active treatment. Alternative approaches to deactivate the surface water mixture can also be used.

Test Substance Preparation

22. Ideally, distilled water should be used to prepare stock solutions of the test and reference substances. When appropriate, an alternative method may be used to solubilize or disperse the test chemical in a manner consistent with its normal entry into the environment. Water-miscible non-toxic solvents may be used when necessary, but attention should be paid to the associated organic load involved

with adding organic solvents. Alternatively, the sample may be added in a neat form to the test system in a manner that maximizes its even and rapid distribution into the test treatments. For materials which are poorly soluble and typically associated with suspended solids in wastewater, it may be appropriate to adsorb the test material onto an inert solid carrier, which is then dosed to the test system. If the test material can not be evenly distributed within the test system prior to the initial sampling point, individual test systems can be prepared that are destructively sampled at each sampling interval.

23. The volume of added stock should be of sufficient capacity to ensure rapid and even distribution of the test material in the treatment and accurate administration of the dose between like treatments. Ideally, when dosing with aqueous solutions, the added volume should be $\geq$ 2 ml; for non-toxic solvents, < 0.1 ml/L. If appropriate, dosing solutions may be prepared in advance and refrigerated. The activity of the stock should be checked by LSC.

Test conditions

Test temperature

24. Incubation should take place in the dark (preferred) or in diffuse light at a controlled temperature, which may be the field temperature or a standard laboratory temperature of 20-25°C.

Agitation

25. To keep the solids in suspension, the test vessels are minimally agitated by means of continuous mixing or stirring.

Test duration

26. The duration of the test should be sufficiently long to assess the biodegradation of the test chemical during its normal residence time within the wastewater-surface water mixing zone. Normally, the test period will last 28 days. However, it may be extended longer to obtain additional data points to estimate kinetic constants or to assess the completeness of degradation under the conditions within the test. Conversely, it may be ended before this time if degradation has plateaued.

Number of test vessels

27. At a minimum, there should be a single abiotic and a single biotic test vessel for each test material and test material concentration. While replicates can be prepared for each treatment, more useful kinetic information usually can be gained by increasing the number of time points sampled within a treatment.

PROCEDURE

Dosing

28. At test initiation, the test vessel closure is removed and the test material is quantitatively added directly to the treatment with constant mixing. It is recommended that the dose be administered in a gradual fashion below the air-water interface, to ensure uniform distribution of the test material into the wastewater. The biotic and abiotic treatments are dosed in an identical manner. Generally, the biotic systems are dosed first, followed by the abiotic systems. Exact timing is typically more critical for the biotic versus the abiotic treatments for kinetic analyses.

Sampling Schedule

29. Sampling intervals are selected based on existing biodegradation data or the results of a pilot study as no fixed time schedule for sampling is universally applicable. A recommended sampling schedule for a rapidly degraded chemical would be 15, 30 and 60 minutes, with additional samplings after 2, 5, 8, 12 and 24 hours and day 2, 3 and 7 and weekly thereafter. The sampling schedule for slowly degrading chemicals should be adjusted so that a sufficient number of measurements are made during the degradation phase.

Measurement of Mineralization

Indirect measurement of $^{14}CO_2$

30. Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood.
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31. The samples are bubbled with air for several hours or allowed to sit overnight to allow the dissolved $^{14}CO_2$ to diffuse from the samples. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent of $^{14}CO_2$ is calculated based upon the difference between the total counts in the biotic and abiotic samples.

Direct measurement of $^{14}CO_2$

32. Evolved $^{14}CO_2$: The first base trap in the series is removed and quickly capped. The remaining traps are moved forward in the same order and a fresh trap placed behind the existing traps and the trapping system reconnected as quickly as possible. Replicate subsamples (e.g. 1 ml) from the base trap are removed and transferred to scintillation vials and combined with a scintillation cocktail that suitable for the sample matrix and analysed by LSC.

33. Dissolved $^{14}CO_2$: Samples (e.g. 10 to 25 ml) are removed through the sampling port of the test flask. They are then placed in vessels (e.g. Bellco Glass Biometer 2556-10250) containing a compartment with an appropriate CO_2 absorbent (e.g. 1.5 N KOH). The vessels are sealed and sufficient acid is added to lower the pH of the samples to < 2 without opening the vessels to the atmosphere (see Annex 1). The samples are allowed to sit for a sufficient length of time (e.g. overnight) to allow CO_2 to diffuse from solution and be trapped from the headspace by the sorbent. Samples of the sorbent are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC.

Indirect measurement of $^{3}H_2O$

34 Individual replicate samples (e.g. 1 ml) are collected from each treatment and placed into separate vials that contain sufficient acid (e.g. 1 ml of 0.1N HCl) to lower the sample pH to < 2 and located in a fume hood.

35. Half of the samples are immediately analysed directly by LSC for a “wet measurement”. The remaining samples are allowed to dry completely to remove the $^{3}H_2O$. The samples are combined with a scintillation cocktail that is suitable for the sample matrix and analysed by LSC. The percent $^{3}H_2O$ is calculated based upon the difference between the total counts in the wet and dry samples and the initial

level of radioactivity dosed to the samples.

Measurement of Total Radioactivity in Wastewater-Surface water Mixture

36. Replicate small volume samples (e.g. 1 ml) are analysed directly by LSC to quantify the radioactivity remaining in each treatment over time. These measurements are used to confirm that the recovery of radioactivity from the extracted samples is acceptable and to monitor for volatilization.

Measurement of Parent and Metabolites

Extraction

37. A sample is collected from both the abiotic and biotic treatments. The sample volume is typically ≥ 10 ml. However, the size will depend on the test concentration, specific activity and the sensitivity of the analytical procedures.

38. Various approaches can be used for concentrating and extracting the samples. A proven approach for nonvolatile test chemicals involves flash freezing the samples, followed by lyophilization and extraction of the dried residue with appropriate solvent(s) for parent and metabolites. Flash freezing quickly stops biological activity without hydrolyzing or otherwise altering labile test materials. The resulting extracts can be concentrated through evaporation and the total radioactivity in each extract is determined by LSC.

39. For volatile test materials, the sample can be passed through a filter and solid phase extraction (SPE) column or SPE disk placed in tandem, which are subsequently eluted with appropriate solvents to recover parent and metabolites. Alternatively, it may be possible to extract directly aqueous samples with an appropriate solvent system and then filter it to recover biomass solids. The total radioactivity in all extracts is determined by LSC. Care must be taken in concentrating extracts containing volatile test materials or metabolites.

40. Other approaches can be utilized, but with all approaches it is important to document recoveries and consider the time involved in terminating biological activity and factor it into the sample times used for kinetic analyses.

Analysis of Parent and Metabolites

41. The relative abundance of parent and metabolites within the extracts can be determined using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) or other separation techniques with radioactivity detection.

42. If sensitive specific analytical methods are available, primary biodegradation can be assessed by measuring the total residual concentration of test substances and metabolites instead of using radioisotope techniques.

Characterization of Metabolites

43. Whenever possible, the chromatographic behaviour of unknown peaks should be compared to that of predicted metabolites, if authentic standards exist. Usually, the quantity and purity of metabolites generated in this test make definitive identification by other direct means impossible. Depending upon chromatographic behaviour, it is usually possible to determine if a metabolite is more or less polar than the parent. This information combined with known biochemical reactions along with when a metabolite

appears and disappears in the sequence of biodegradation can form an additional basis for inferring its identity. If necessary, the Kow of major metabolites can be determined by HPLC (e.g. [OECD 117] (24) using an on-line radioactivity detector.

Measurement of Extracted Solids and Incorporation into Biomass

44. The extracted solids are combusted to determine the level of activity remaining with the solids. The level of radioactivity in the biotic solids above that in solids from the abiotic control typically represents incorporation of radioactivity into biomass.

Measurement of Volatilized Radioactivity

45. For volatile test materials, the volatile traps are extracted with appropriate solvents and the radioactivity in the extracts is analyzed by LSC. The relative abundance of parent and metabolites in the extract(s) can be determined as described above.

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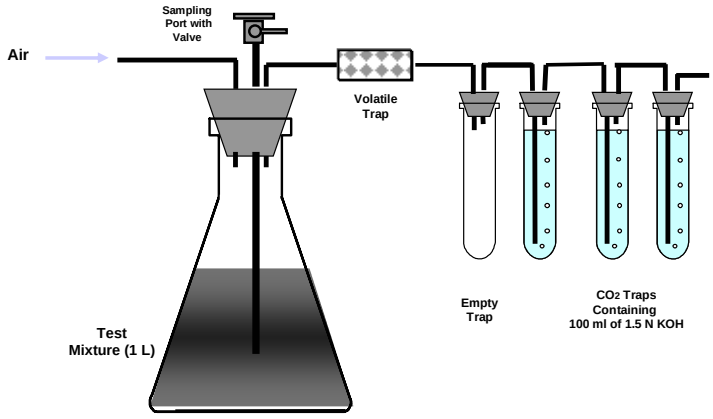
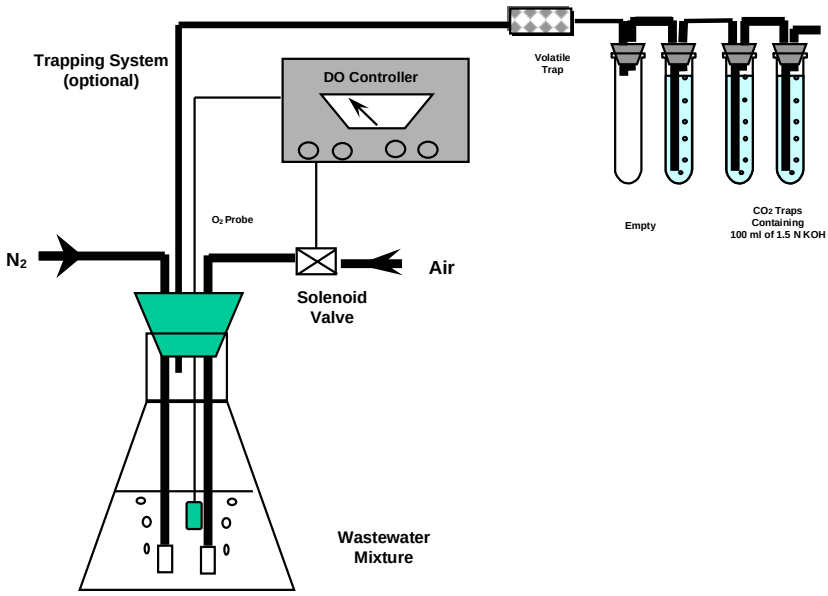
1 **ANNEX 1**

Example of Flow-Through Test Set-Up



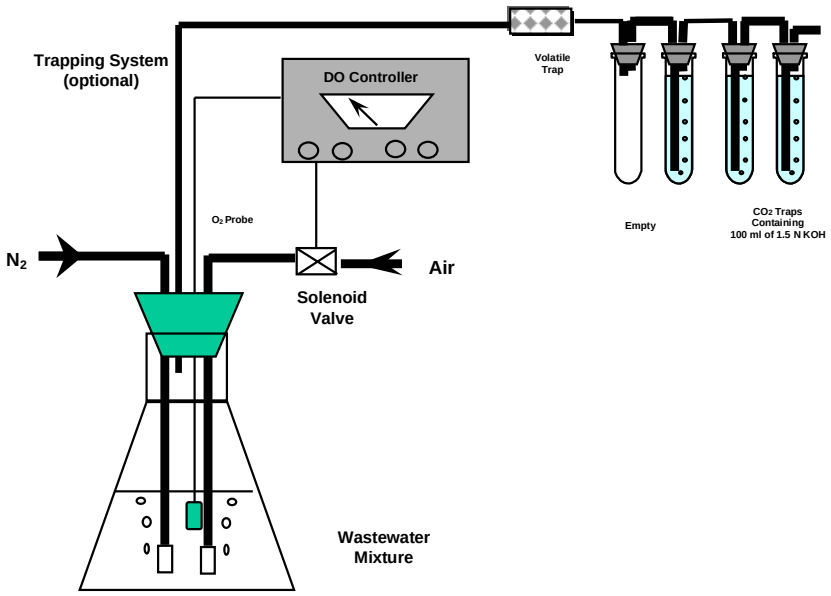
2

Example of Controlled Dissolved Oxygen Flow-Through Test Set-Up

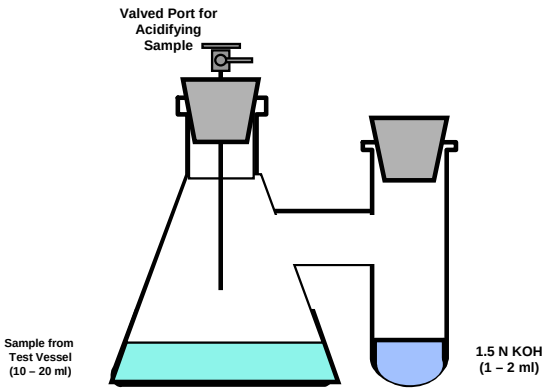


3

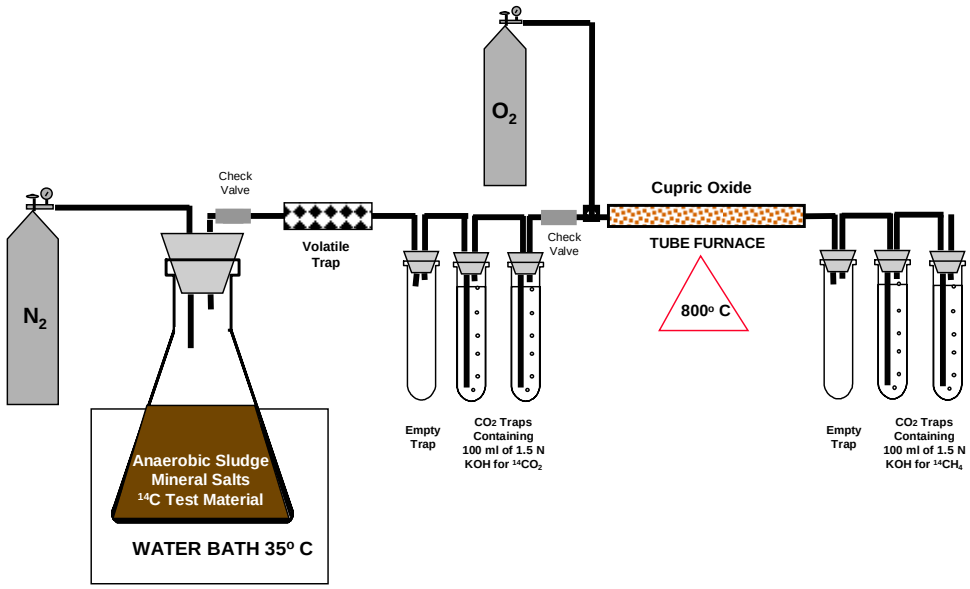
Example of Controlled Dissolved Oxygen Flow-Through Test Set-Up



System for Recovering Dissolved $^{14}CO_2$



Example of Flow-Through Anaerobic Test Set-Up



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2