Technical Report

No 54

Assessment of the Biodegradation of Chemicals in the Marine Environment

August 1993

ISSN-0773-8072-54

Technical Report No. 54

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ISSN-0773-8072-54

Brussels, August 1993

ECETOC Technical Report No. 54

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SUMMARY

Several test methods assessing the biodegradation potential of a chemical has been developed and validated for freshwater situations. Internationally accepted standard methods are now available and the data available covers a wide range of chemicals. In contrast to freshwater situations no generally accepted methods are known to estimate the biodegradation potential of chemicals in marine situations. The literature research on marine biodegradation reveals that apart from a few examples research has focused on degradation of crude oil and petroleum derivatives.

OECD published recently a set of "Marine Ready Biodegradability Tests" which may, to a certain extent, predict the fate of a chemical in heavily polluted estuaries. It is doubtful whether the result obtained from these test guidelines would be representative for open sea or coastal situations where conditions considerably differ from those in freshwater or estuarine situations.

The marine environment is characterised by a low concentration of nutrients (P,N) and organic substrate. Except for the surface microlayer, where large populations of marine microorganisms have been recorded, the bacterial counts are orders of magnitude lower than those observed in freshwater situations. When developing a biodegradation test for marine situations both physico-chemical and biological parameters which are typical for this environment should be maintained. This implies a low test concentration which precludes the monitoring of the fate of a chemical using non-specific analytical methods.

A strategy is outlined and schematically represented as a tier approach to assess the hazard of a chemical which may reach the marine environment. The test strategy is designed to eliminate as far as possible unjustified testing.

Biological processes for biodegradation in freshwater and the marine environment do not differ. Thus, in theory, those chemicals which are readily biodegradable in freshwater will also degrade under marine situations. The available information only supports this conclusion with regard to ready biodegradability.

Marine biodegradability tests should be carried out using test protocols simulating the situation and the receiving environment as closely as possible. Proposals for the design of possible test protocols are described.

It is recommended that, when more knowledge is obtained about marine biodegradation, the strategy should be reconsidered.

SECTION 1. INTRODUCTION

The current approach to determining the biodegradability of chemicals in aquatic systems is focused on freshwater situations and laboratory simulations of waste water treatment plants.

There has been increasing concern about the pollution of estuaries and the sea, particularly the North Sea, from substances discharged directly or carried into the sea by the rivers. Some synthetic chemicals have been identified in seawater and sometimes even in marine organisms. The OECD has produced a set of guidelines which claim to assess the "ready biodegradability" of chemicals in the marine environment. There is some doubt about the value of these tests which do not simulate estuarine or marine environments. There is nevertheless a need to improve our understanding of the fate of chemicals in the marine environment. Accordingly an ECETOC Task Force was constituted with the following terms of reference:

- review the existing information on the biodegradation of chemicals in the marine environment considering both aerobic and anaerobic conditions,
- critically review the test methods currently used to measure biodegradation in the marine environment,
- develop a strategy for investigating biodegradability of chemicals in estuarine and marine environments.

A glossary of terms is included in Appendix A.

A literature review of the published information on the biodegradability of organic chemicals in the marine environment was made using six data bases[1]. Most of the papers retrieved were concerned with either the turnover of naturally-occurring compounds or the biodegradability of two groups of chemicals (hydrocarbons and surfactants). Information on other chemicals appeared to be limited. An overview of the most relevant articles is given in Appendix B.

[1]. Chemical Abstracts (from 1967 onwards; Biosis (from 1969 onwards); Oceanic Abstracts (from 1964 onwards); Aquatic Science and Fisheries (from 1978 onwards); Waternet (from 1971 onwards) and Copendex Plus (from 1970 onwards).

SECTION 2. BACKGROUND

The purpose of assessing the biodegradability of substances is to make a judgement on their disappearance or persistence when they reach the environment. The degradation in the environment is brought about by a combination of physico-chemical and biological processes that participate in the natural geo-chemical cycle. The biological transformations are a result of the interactions of the substances with living organisms and theoretically the materials are exposed to the total metabolic capability of the living world. This cannot be simulated in laboratory test systems and consequently in many cases the biodegradation potential of the real environment is frequently underestimated.

Over the past thirty years, the development of test methods and strategies for assessing the biodegradability of organic chemicals in the aquatic environment has been predominantly concerned with freshwater systems such as rivers, lakes and wastewater treatment plants. This has resulted in a range of standard test methods which have been incorporated into internationally-accepted guidelines and legislation (e.g. OECD, 1981; EEC 1984). Despite efforts to improve those methods only a limited number of procedures are available which reliably predict the fate of substances in real world situations.

In contrast to freshwater, as yet, there are no generally accepted methods for the determination of biodegradability of substances in brackish water or marine situations (seawater). A first draft with a set of guidelines for assessing "ready biodegradation", derived from the freshwater guidelines was recently published (OECD, 1990).

There are several reasons, which explain the imbalance in the development of methods between fresh and marine water environments. On one hand the potential problems of marine pollution were perceived much later than in freshwater, and on the other many more obstacles arise when trying to develop a test method. There is also a variety of marine situations (estuary, coastal, open sea) which affect the composition of biomass, organic carbon content, salinity and mineral composition. So far no conditions simulating and representative for all marine conditions have been suggested or published.

SECTION 3. DIFFERENCES BETWEEN MARINE, ESTUARINE AND LIMNIC ENVIRONMENTS IN RELATION TO BIODEGRADATION OF CHEMICALS

3.1. INTRODUCTION

The hydrosphere comprises a multitude of extremely varied microbial habitats. Although salinity is the most obvious parameter that differentiates marine, estuarine and freshwaters, it is the low concentration of organic substances and inorganic nutrients (especially N and P) which have the greatest effect on the abundance and activity of the marine microorganisms. Marine ecosystems, particularly away from the coast, are more or less static and here the residence time of a chemical can be extremely long. In contrast freshwaters, particularly river systems, are dynamic in that materials like nutrients, substrates and contaminants, are transported through them and the residence time, during which these materials can be utilised, is limited.

Freshwaters include lakes and rivers whose nutrient status vary from oligotrophic to eutrophic. The nature of the soil, rock and vegetation of the catchment area influences the water composition. The temperature of freshwater, depending on depth is subject to a greater seasonal variation than that of seawater.

Estuaries, at the interface of freshwater and marine environments, exhibit a salinity gradient between the river and sea and may even be stratified. The water composition reflects that of the river above and, in the tidal zone, the coastal water. Sedimentation rates are high and therefore sediments are often an important niche in the estuarine environment. As in many freshwater environments there is a dynamic relationship between the sediment and the water column and this is particularly true in estuarine and coastal waters. Frequent disturbance of the sediment, especially in the tidal zones, mixes the microbial populations and releases inorganic nutrients and metabolites from anaerobic regions in the sediment into the water column. These may be further utilised by aerobes and facultative anaerobes in the water column, many of which are attached to suspended sediment particles.

Coastal marine waters differ substantially from the open ocean in salinity and temperature as well as in concentrations of inorganic nutrients and organic substrates due to terrestrial discharges. This depends on the proximity to an estuary and the depth of the coastal shelf. In the open ocean there are also large variations in water composition with increasing depth and hydrographical conditions.

3.2. FACTORS AFFECTING MICROBIAL NUMBERS AND VARIETY

The principle factors that determine the variety and growth of microbial genera include salinity, pH, temperature, dissolved oxygen content, redox potential, concentration of inorganic nutrients and trace elements and the concentration of organic substrates. Light affects the activity of photosynthetic bacteria and algae. Each of these factors vary not only between the major aquatic environments but also within individual niches. They also change diurnally, seasonally and, particularly in the case of estuaries, with the tidal cycles.

3.3. MICROBIAL POPULATIONS

3.3.1. Freshwater and estuarine microorganisms

The autochthonous populations of river, estuarine and coastal water organisms comprise a wide variety of microbial genera but this is enhanced by an allochthonous population from the continuous inflow of soil and wastewater discharges.

The autochthonous population may be considered as the free swimming microbes in the water column, although it may include organisms attached to suspended particles. In many rivers the majority of the microorganisms are free swimming whereas in estuarine waters the majority of bacteria may be attached to suspended particles (Goulder, 1986).

The numbers of viable bacteria in river waters are normally about 10^6/ml in upland rivers and up to 10^7/ml in the lower reaches (Bent and Goulder, 1981; Goulder,1986; Rimes and Goulder, 1986). These are higher than the number found in agricultural land drainage water of between 10^2 and 10^5/ml (Evans and Owens, 1973) which suggests that the autochthonous population accounts for 90%, or more, of the total population. This does not detract, however, from the metabolic importance of allochthonous genera. Thurston (1991) found that the majority of nitrifying bacteria in the Tees estuary originated from runoff rather than growth of an autochthonous population in the estuarine waters. In addition a significant number of heterotrophic viable bacteria (10^7/ml, Kuenemann and Battersby, 1992) are released from sewage treatment plants.

3.3.2. Marine microorganisms

The numbers of bacteria in marine waters are considered to be lower than those in freshwaters (Bartholomew and Pfaender, 1983; Guerin, 1989; Gonzalez *et al*, 1990). In a literature review Austin (1988) concludes that the bacterial population in seawater is generally between 10^3 to 10^6/ml. There are large populations of marine microorganisms at the surface microlayer where up to 10^8/ml

have been recorded. There is a decline in the numbers down the water column so that the average numbers are very low.

Many marine microorganisms are halophilic but others are only halotolerant. In the open ocean most are adjusted to life in oligotrophic conditions in contrast to the eutrophic conditions of many freshwater environments. Microorganisms in marine habitats exist either in a free-swimming state or in association with suspended particles.

3.3.3. Sediment microorganisms

The upper layer of sediment is colonised by many aerobic and facultative anaerobic microorganisms attached to particles that frequently become suspended in the water column whenever the sediment is disturbed.

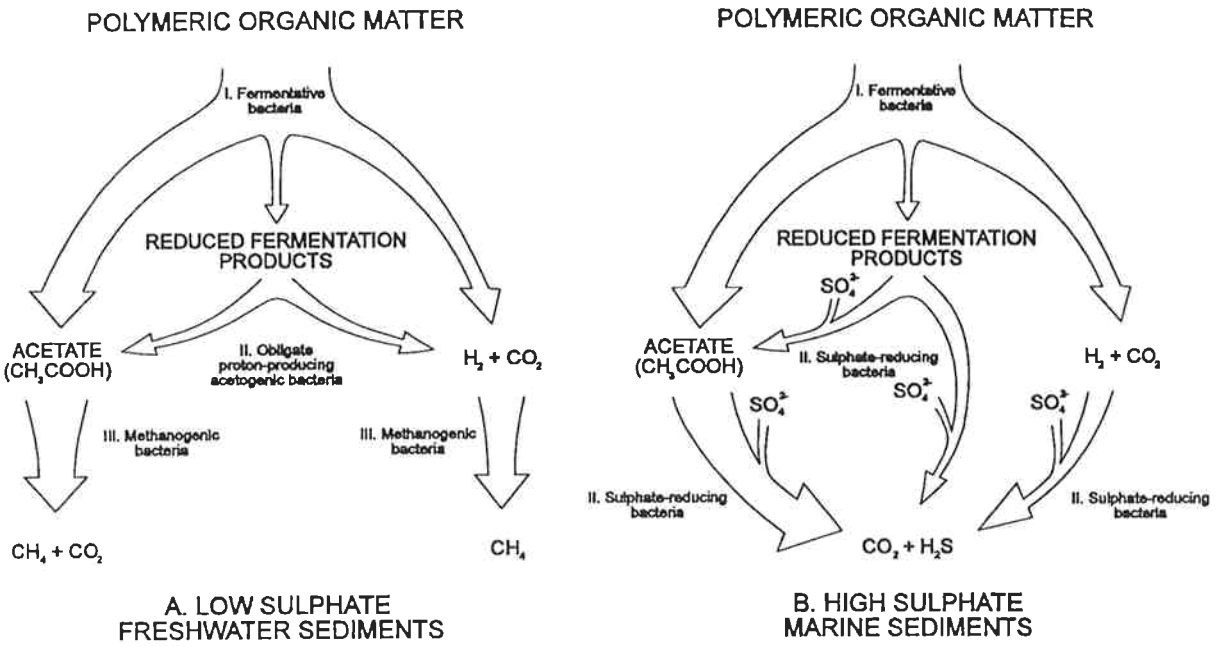
Much of the sediment is anoxic and colonised by four distinct groups of bacteria. Fermentative bacteria convert primary polymeric organic matter into a variety of metabolites, including alcohols, fatty acids, acetate, molecular hydrogen and carbon dioxide. In sediments, low in sulphate (e.g. freshwater), obligate proton reducing acetogenic bacteria convert the reduced fermentation products to acetate, molecular hydrogen and CO_2. Methanogenic bacteria then convert these substrates to methane and CO_2. In sulphate rich marine sediments, sulphate reducing bacteria have a competitive advantage and oxidise the fermentation products via low molecular weight organic acids to CO_2 with the concomitant reduction of sulphate to sulphide (Ward and Winfrey, 1985). These reactions are illustrated in Figure 1 and indicate that the critical stages in breaking down an organic chemical to simple, easily metabolisable compounds are common in both freshwater and marine sediments.

3.4. SUBSTRATES USED BY MICROORGANISMS

3.4.1. Freshwater substrates

Rivers upstream of urban and industrial areas normally contain little organic material, although following heavy rain they become rich in soil and debris from vegetation. Downstream of these areas, effluent discharges greatly increase the substrate load available to the microbial population. As a result of this increase the biochemical oxygen demand (BOD) of this population often exceeds the oxygen transfer rate. As a result the dissolved oxygen levels may decrease from near saturation to a lower level, or exceptionally even to zero, throughout the water column except for a very shallow surface layer (Berner, 1971; Patrick and De Lanne, 1972; Howarth and Teal, 1979).

Figure 1. Carbon and electron flow models for the decomposition of organic matter in anaerobic sediments (from Ward and Winfrey, 1985)



The thickness of the arrows is proportional to the amount of carbon and electron flow

3.4.2. Estuarine and coastal water substrates

Compounds which are not degraded during the residence time of the material in the river may ultimately be discharged into the sea. The concentrations of substrates in the estuaries and the sea will vary with the input, the freshwater flow into the estuary and coastal water, tidal cycle, season and decay rate. Compounds which are not removed or degraded during the residence time of the compound in wastewater treatment works are ultimately discharged into a river estuary or into the sea. Lung *et al* (1990) estimated that 20 to 30% of all United States publicly owned treatment works (POTW) discharges enter estuaries and coastal waters directly. In the Netherlands, less than 5% of the POTWs discharges directly enter estuaries and coastal waters (de Greef and de Nijs, 1990).

3.4.3. Marine substrates

Primary production of biomass in the marine environment is estimated at between 22-28x10^{15}g C/annum (Zehnder, 1982). The marine environment away from the coastal zones is low in organic substrates and there is also a strong competition between phytoplankton and bacteria for available

nutrients (N and P). Material released by decaying phytoplankton are the major source of organic carbon for marine bacteria. Weisse *et al* (1990) reported that in phytoplankton more than 50% of the primary production is channelled through the microbial loop.

Those chemicals which are not removed under freshwater conditions are normally flushed out of rivers into the sea. The sea and underlying sediments are therefore a major sink of most non-volatile chemicals. Even poorly soluble materials such as PCBs may be found back in the sea. Ernst (1988) demonstrated that several known persistent xenobiotic compounds are found in the North Atlantic, albeit at very low levels (10-150ng/l).

3.5. MICROBIAL ACTIVITY

3.5.1. Freshwater microorganisms

The activity of freshwater microorganisms varies considerably with the quality of the freshwater system. In upland waters the major substrates are from soil and vegetation and activity is normally substrate limited. In contaminated waters activity can be high but is often limited by the oxygen transfer rate and/or the toxicity of influent organic material from domestic as well as industrial sources (Austin, 1983).

3.5.2. Marine microorganisms

In the open ocean Meyer and Morita (1989) claimed that survival of marine heterotrophic bacteria depends on an ability to withstand long periods of nutrient deprivation. Most of the organic matter entering the sea is extensively degraded in surface waters. Consequently levels of carbon below the photic zone are low at 0.4 to 0.7mg/l for dissolved organic carbon (DOC) and 3 to 10µg/l for particulate organic carbon (POC). As this material consists mainly of refractory compounds, metabolic activity is usually low in the marine environment. Zehnder (1982) quotes average ages of deep water DOC between 3,400 and 6,000 years. This DOC originates from natural sources.

3.5.3. Sediment microorganisms

Microbes attached to sediment particles that are regularly suspended in the water column, often classified as part of the water column population, are important in the degradation of organic debris. Present evidence suggests that the particle-associated bacteria may be more metabolically active than their planktonic counterparts in marine environments (Wardell *et al*, 1983).

In anoxic sediment, as described above, the levels of sulphate determine the relative activity of acetogenic, methanogenic and sulphate reducing bacteria. The importance of sulphate reducing

bacteria in sediments has been reviewed by Gibson (1990). The sediments of estuarine and marine waters are the primary habitats of sulphate reducing bacteria. Jørgenson (1977, 1982) found that over 50% of the organic carbon from detritus input to coastal marine sediments rich in sulphate was finally released as carbon dioxide through the activity of sulphate reducing bacteria.

SECTION 4. ASSESSMENT OF EXISTING TEST METHODS

4.1. INTRODUCTION

Experimental systems for studying the fate of chemicals in the marine environment have tended to concentrate on the fate and effects of oil spills or the biogeochemical cycling of organic matter. In recent years attempts have been made to modify existing screening or "ready biodegradability" tests for use with marine inocula and to develop test systems where the biodegradation of a chemical can be studied under conditions which simulate a particular marine environment. These various test methods are reviewed below.

4.2. REVIEW OF EXISTING TEST METHODS

4.2.1. Screening Methods

Screening tests for assessing biodegradability must be applicable to a wide range of chemicals. Hence, non-specific analysis is used and biodegradation is measured as the difference in oxygen consumption, DOC removal or carbon dioxide evolution between vessels containing a known amount of test substance and blanks. The precision of the test is dependent on the size of this difference. A reliable measure of biodegradation requires high concentrations of test substance (e.g. 2 to 100mg/l) as compared to environmentally relevant levels (e.g. <1µg/l). This may affect the biodegradability of the test chemical *in situ*.

Street (1984) proposed that a screening method for assessing the biodegradability of chemicals in seawater was needed, as predictions of environmental fate based on the results of standard "ready biodegradability" tests failed to take into account the lower microbial concentration and reduced species diversity in the marine environment compared with freshwaters. Biodegradation may also be limited by the low concentration of essential nutrients (P and N) in the open sea. Further nutrient limitation becomes an even greater problem when unrealistically high concentrations of test substance are used in screening tests.

Street (1984) compared unamended seawater with standard BOD dilution water (inoculated with secondary effluent) as a medium to assess the biodegradability of organic compounds using the five day BOD test (BOD_5). The rates of biodegradation (oxygen uptake) for sodium acetate, sodium benzoate, sodium dodecyl sulphate and glucose/glutamic acid mixture were similar in both media but the BOD_5 values in seawater were generally 10-20% lower than those in BOD dilution water. These differences were not thought to be due to nutrient limitation as the addition of N and P only

improved the biodegradability of sodium acetate. The author also determined the effect of salinity on the biodegradability of the test compounds in the standard BOD dilution water amended with increasing concentrations of NaCl. Increasing salinity caused a decrease in biodegradability, with biodegradation values at 35g/l NaCl being 60-80% of the freshwater value. The use of a freshwater inoculum pre-exposed to 34g/l NaCl for 6 months did not improve biodegradability under high saline conditions. Three synthetic seawaters (BOD dilution water + 35g/l NaCl; BOD dilution water + a balanced range of salts and BOD dilution water + all major salts found in seawater) inoculated with secondary effluent did not support biodegradation to the same extent as natural seawater. The author concluded that the source of the inoculum was less significant than salinity in affecting the biodegradability of organic chemicals.

Seawater variants of the modified OECD screening test (OECD 301E, a shake flask die-away test based on analysis of DOC), and the closed bottle oxygen consumption test (OECD 301D) were proposed in the mid-1980s. Both methods use nutrient-enriched, coarse-filtered seawater as the inoculated medium and were evaluated in a ring test on behalf of the Commission of the European Communities (Nyholm and Kristensen, 1987). The ring-test used five compounds (sodium benzoate, aniline, diethylene glycol, pentaerythritol and 4-nitrophenol) whose biodegradability had been evaluated in previous interlaboratory studies of "freshwater" tests. The compounds were tested at concentrations of 5-40mg DOC/l in the die-away test and 2-10mg/l substance in the modified closed bottle test. Both tests were performed at 15-20°C and ran from 28 to 60 days. Participants in the ring-test reported problems of high carbon levels or oxygen-demands in their blanks which reduced the precision with which biodegradation could be measured. Similar problems were encountered by Street (1984) as reported above and are a particular problem of coastal waters which are subject to varying inputs of nutrients and micro-organisms from land-based sources.

Most participants in the interlaboratory study found that sodium benzoate and aniline were degradable (i.e. >70% DOC removal, >40% of the theoretical maximum oxygen demand). The lower "pass" level in the closed bottle test was chosen as it was felt that the usual level of 60% was too restrictive due to variability in growth and respiration, and the high blank BOD of seawater. Variable results were reported for the other three model compounds. The relative number of "passes" (as a percentage of the results reported) for the DOC die-away test and the closed bottle test were respectively 44% and 78% for diethylene glycol, 38% and 25% for pentaerythritol and 64% and 22% for 4-nitrophenol. These results are similar to those obtained in other ring-tests of freshwater screening methods. They mainly reflect the differences in the concentration and species composition of the different seawater samples used by participants in this ring test. Similar differences were observed in previous freshwater interlaboratory comparison programmes.

The seawater DOC die-away and closed bottle methods ring-tested in 1984-85 have now been published as draft test guidelines by the OECD (OECD, 1990). The coarse-filtered seawater is supplemented with nutrients using aqueous stock solutions of phosphate buffer plus NH_4Cl, $FeCl_3.6H_2O$, $MgSO_4.7H_2O$ and $CaCl_2$. These additions are identical to the corresponding "freshwater" DOC die-away (OECD 301E) and closed bottle (OECD 301D) tests, except that the seawater version of OECD 301E has one tenth the concentration of buffer and NH_4Cl. Phosphate buffer is needed to replace the natural carbonate-bicarbonate buffer system of seawater, and seawater concentrations of P and N would limit the degradation of the high concentrations of test substance used in these tests. However, the reason for supplementing the seawater sample with $CaCl_2$ and $MgSO_4.7H_2O$ is unclear as a typical seawater normally contains around 400mg Ca/l, 1,800mg Mg/l and 900mg S/l (Ciaccio, 1971).

Kondo *et al* (1988) investigated the biodegradability of 170 chemicals with a screening test called the "cultivation method". Microorganisms from unpolluted river and seawater were grown in 0.1% peptone water and the test chemical. Degradation was measured by specific analysis after 3 days and measures therefore primary biodegradation. Results showed that 103 (61%), 43 (25%) and 24 (14%) chemicals were judged respectively to be poorly (<15% degradation), moderately or easily degradable (>50% degradation). These results are compared with biodegradation data found with the MITI-I method (OECD, 1981) where only 30% of the chemicals were classified as poorly degradable. The authors concluded that the cultivation method was more stringent. For the assessment of the biodegradability of chemicals in the marine environment it is felt that this method is not suitable for several reasons. Firstly, the method does not measure mineralisation and therefore the most essential question on the ultimate fate of the chemical is not addressed. Secondly, the short incubation time of 3 days is not relevant to marine conditions and is too short for many adaptation processes to occur. Finally, the composition of the test medium is far removed from that of seawater.

Shimp and Young (1987) studied the influence of concentration on the biodegradation of benzoic acid in the seawater DOC die-away test using 20mg DOC/l and 50µg DOC/l radio-labelled material. In contaminated estuarine water there was rapid biodegradation at both test concentrations. However, in pristine but nutrient supplemented seawater the biodegradation of 20mg DOC/l benzoic acid was poor and very variable. At 50µg DOC/l degradation was rapid, extensive and less variable.

Both Battersby (1990) and ECETOC (1991) showed that kinetic data obtained from "ready biodegradability" tests are highly dependent on source of inocula and other test conditions and are not suitable to generate environmentaly relevant kinetic parameters.

4.2.2. Simulation Tests

The objective of simulation studies is to be able to extrapolate information obtained in laboratory systems to field situations with a reasonable degree of confidence. The advantages of simulation tests over *in situ* studies are the control of environmental variables, especially use of environmentally relevant low concentration, the possibility of replication and the containment of the test chemical in the laboratory. Other important pathways for the removal of organic compounds such as phototransformation, volatilisation, adsorption on particulates, deposition in sediments and transport through the food chain are not commonly simulated in degradation studies.

Water Column Tests. Wakeham *et al* (1986a) used a 13.3m^3 fibreglass tank filled with sea-water from Narragansett Bay (USA). To simulate tidal mixing in the bay, the tank was mixed four times daily for 2h. The tank was spiked with n-[1-^{14}C]decane. Mass balances calculated after the 2.5-week experiments showed that 70 to 80% of the radio-labelled hydrocarbon had been mineralised to $^{14}CO_2$.

Nyholm *et al* (1992) proposed that biodegradation in the marine water column can be simulated by a shake flask die-away test using 1-30µg/l of a ^{14}C-labelled chemical in seawater without nutrient additions. Degradation over a period of up to 50 days was followed as the loss of residual dissolved radioactivity from samples that had been membrane filtered, acidified to pH <2 and sparged with nitrogen. Results for ten chemicals with different biodegradabilities (e.g. aniline, 4-nitrophenol) indicated that all chemicals which passed the DOC die-away marine screening test (cf. 4.2.1.) were also biodegradable in the simulation test. As for freshwater situations negative results in the screening test did not exclude a positive result in the simulation test.

Rheinheimer *et al* (1990) incubated unamended fresh- and seawater samples for up to 40 days in a shake flask die-away test with different radio-labelled chemicals (phenol, 4-nitrophenol, 2-nitrophenol and others) at a concentration of 1µg/l. They studied the rates and the extent of biodegradation by transferring 10µl samples to closed serum bottle flasks (120µl vol.), acidifying them to pH 2, and collecting the evolving CO_2 in 100µl of ethanolamine in a separate vessel within the serum bottle flask. The authors concluded, that the species degrading phenol and 2-nitrophenol in the marine environment were different from those in freshwater environments.

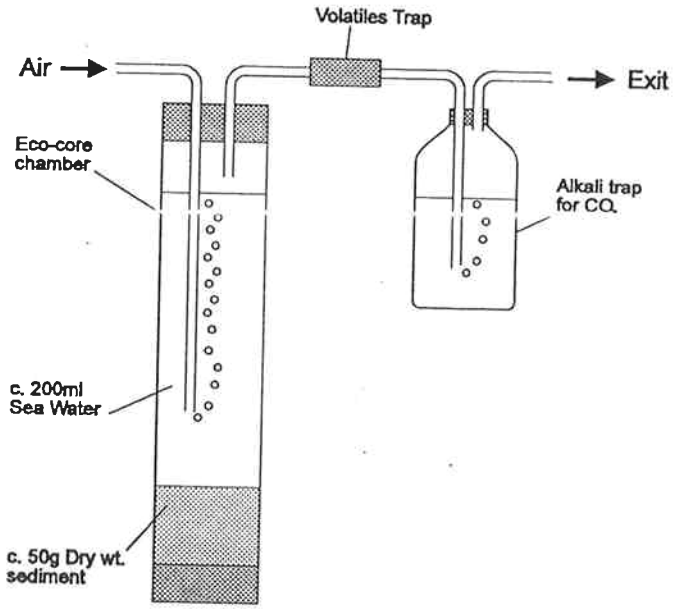
Sediment-water Tests. Simulation tests for estuarine and coastal waters should also take into account the activities of sediment-associated bacteria. These are usually several orders of magnitude more numerous than the free-floating bacteria in the water column and play an important role in the geochemical cycling of nitrogen, sulphur and phosphorus. The physical intermixing of sediments with the overlying water in coastal areas may therefore increase the biodegradation rate.

In addition, aquatic sediments can act as sinks for chemicals (Hamelink, 1980). Adsorption of chemicals to sediments can affect their availability for biodegradation. The degree of adsorption of a chemical in a marine sediment will be dependent on its chemical structure, the mineral composition and the organic carbon content of the sediment particles (ECETOC, 1990).

Several microcosms for simulating the interactions between a chemical and marine sediment are given below.

Static Sediment-water Microcosm Tests. A relatively simple, static sediment/water microcosm has been described by Pritchard *et al* (1979) and is shown in Figure 2. Their "eco-core" microcosm uses an intact core of sediment and its overlying water held in a glass tube.

Figure 2. The basic eco-core biodegradation system of Pritchard et al. (1979).
(ambient pH, 25°C, ^{14}C test compound conc. 150ppb)



The overlying water is gently aerated to mix it without re-suspending the surface sediment and is spiked with ^{14}C-labelled test chemical. Pritchard *et al* (1979) also amended the sample with 0.5mg/l unlabelled chemical and incubated at 24°C. These parameters would have to be lowered if accurate estimations of *in situ* removal rates are required. The amount of radiolabel in the overlying water, as volatile products (exit air passed through a polystyrene resin trap) and as carbon dioxide (exit air passed through an alkali trap) was monitored with time. Poisoned controls (2% v/v

formalin) were run in parallel. At the end of the test, the eco-cores were dismantled and analysed for parent chemical, degradation products and bound residues.

Results for methyl parathion after 32 days incubation indicated that 24% had been mineralised to $^{14}CO_2$ and that 41% of the added radioactivity was present in the overlying water. Degradation products of methyl parathion identified in the overlying water included aminomethyl parathion, 4-aminophenol and 4-nitrophenol. Fifteen percent of the radioactivity was bound to detritus in the sediment and a further 12% was found in the sediment interstitial water. The authors were thus able to account for 92% of the added radioactivity at the end of the study. In the sterile control a mass balance of 90% was achieved with 62% of the added radioactivity being present in the overlying water and 24% in the sediment interstitial water. The principal products extracted from the water were parent material and 4-nitrophenol produced by hydrolysis of the methyl parathion.

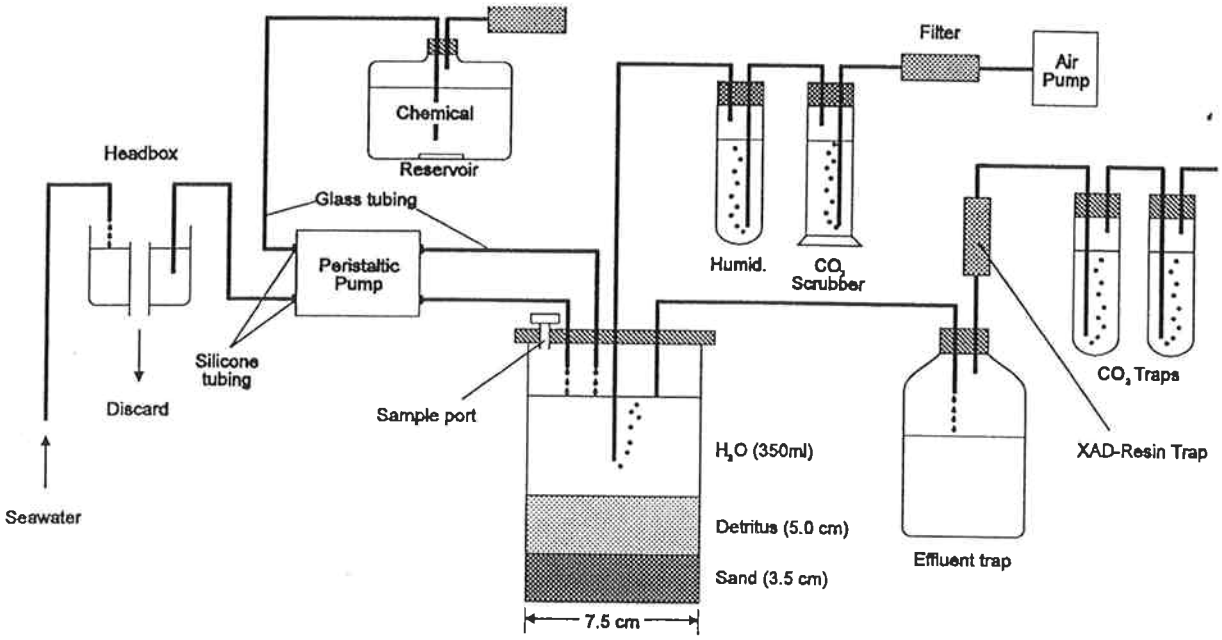
Pritchard *et al* (1979) concluded that an eco-core containing freshly collected, intact sediment and its overlying water gave a better indication of *in situ* processes than systems constructed from a limited number of individual organisms. Prolonged incubation of eco-cores under laboratory conditions decreased the biodegradation of methyl parathion and was attributed to depletion in microbial growth factors during ageing.

Other eco-core studies on biodegradation of chemicals have been reported (Spain *et al*, 1980, 1984). Incubation of intact sediment cores has also been used to study the biodegradation of cellulose fibre waste from a pulp and paper mill and the concomitant stimulation of sulphate reduction in anoxic, marine muds (Malcolm *et al*, 1986).

A mesocosm system for the investigation of the degradation of organotin compounds was described by Adelman *et al* (1990). They used a glass cylinder with a height of 5.5m and 1.8m diameter, in which a 30cm layer of natural marine sediment and 5m layer of seawater was introduced. No supplementary nutrients or biota were added. The radiolabelled test chemical (tributyltin chloride) was added at an environmentally relevant concentration of 0.6µg/l. Periodically, the water column was gently mixed. A complete mass balance of both inorganic and organic radio-labelled carbon was established for all matrices (air, water, sediment). Degradation of tributyltin to monobutyltin could be established for the water column, while the amount transported into sediments was not substantially degraded.

Dynamic Sediment-water Microcosm Tests. Static systems such as eco-cores can be affected by nutrient deficiencies or the build-up of toxic metabolites. These problems can be overcome by the use of a dynamic, flow-through system. A continuous flow sediment-water microcosm has been described by Pritchard *et al* (1979) and is shown in Figure 3.

Figure 3. Schematic diagram of continuous-flow microcosm (Pritchard *et al*, 1979)



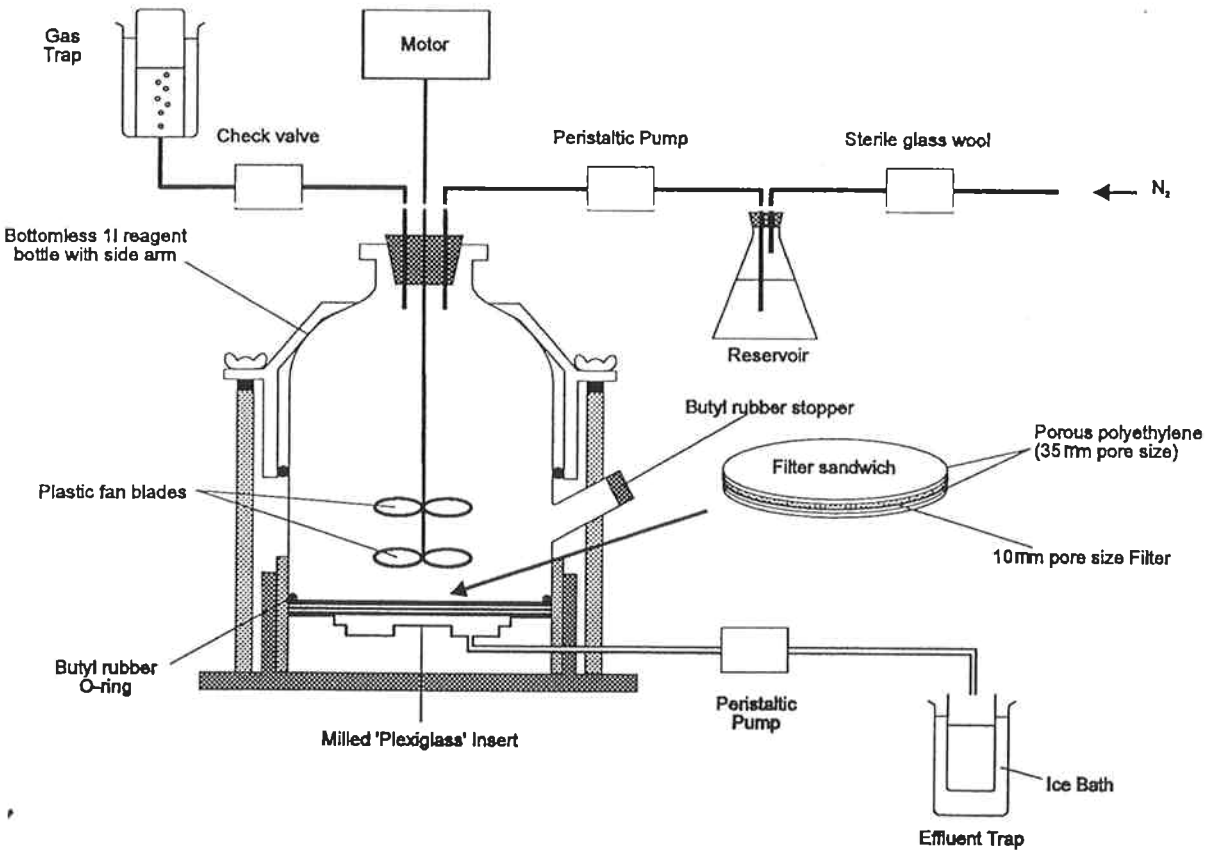
The microcosm was fed continuously with seawater amended with 20µg/l radiolabelled chemical to give a 25 hour turnover time for the contents of the reactor vessel. Controls to check for any abiotic breakdown were prepared by initially adding formalin to the microcosm (2% v/v final concentration), followed by the addition of seawater containing 0.1%v/v formalin. Trapping of the various radiolabelled fractions is similar for the eco-cores (cf. Static Sediment-water Microsm Tests above). Unfortunately, preparation of the microcosm results in disturbance of the sediment and the sediment-water interface, and the microcosm may have to be left unamended with test chemical for some time to enable natural gradients of nutrients, oxygen and redox potential to become re-established.

Based on a study with methyl parathion Pritchard *et al* (1979) concluded that the continuous addition of nutrients, micro-organisms and invertebrates in the seawater feed complicates the interpretation of the data obtained. However, the continuous addition of a low concentration of the test chemical facilitates studies on acclimation and yields quantitative data on the degradation potential of a particular marine environment.

Dynamic Sediment Microcosm Tests. If there are reasonable grounds to assume that a chemical will reside predominantly in anoxic sediments, then it may be appropriate to perform detailed studies on its fate in a system containing anaerobic sediment only. Such a system has been described by

Smith and Klug (1987) and is shown in Figure 4. As the authors investigated the effect of sulphate on microbial metabolism in freshwater (lake) sediments, they measured methane levels in the headspace gas and the concentration of sulphate, alkalinity and volatile fatty acids in the reactor effluent (equivalent to the interstitial water). The sediment reactor system contained 700ml homogenised surface sediment which was stirred and maintained at *in situ* temperature. Influent was pumped into the system at a rate of 2ml/h. Sediment interstitial water was pumped out of the system at the same rate through filters (e.g. porous polyethylene). The flowthrough reactor system could be adapted for testing chemicals with marine sediments using low concentrations of ^{14}C-labelled chemicals.

Figure 4. Diagram of the sediment reactor system (Smith and Klug, 1987)



4.2.3. Possibilities and Limitations of Present Test Methods

For the existing screening tests it is clear that phosphate-buffered, nutrient-supplemented seawater is no longer a "natural" medium with which to assess the biodegradability of a chemical under

marine conditions. The natural variation in the nutrient levels and microflora of seawater samples collected from different locations, or at different times of the year, can cause large variations in the biological activity encountered in seawater screening tests. This will lead to large variations in results and hinder interlaboratory comparisons of data. This is already a recognised problem with existing "ready biodegradability" tests despite efforts to standardise the inoculum by recommending a standard concentration of dry matter from a certain type of wastewater treatment plant (e.g. 30mg activated sludge dry solids/l).

For the above seasons it is clear that marine biodegradability screening tests are not relevant for assessing the fate of a chemical in the marine environment. The latter can be studied using microcosms which simulate a particular marine ecosystem. These allow key variables to be controlled in the laboratory and permit replication and containment of the test chemical. Static systems such as eco-cores are relatively simple to operate and could be described in a format suitable for use as a standard protocol. Dynamic flow-through systems are more complicated to operate but can give a better indication of removal rates *in situ* (Pritchard *et al*, 1979). The use of both types of simulation tests is dependent on the availability of a suitable (i.e. uniformly- or ring-) ^{14}C-labelled test chemical which is representative of the product to be investigated.

4.3. BIODEGRADABILITY DATA FOR MARINE AND ESTUARINE ENVIRONMENT

Considerable research has been conducted on the biodegradation of chemicals in freshwater. By comparison, only a limited number of chemicals have been investigated for their biodegradability in marine or estuarine environments.

The biodegradation behaviour of linear alkylbenzene sulfonate (LAS), one of the major surfactants used in detergent products, has been studied extensively. Laboratory studies at comparatively high concentrations (e.g. 20mg/l) in the marine environment demonstrated primary biodegradation (Cook and Goldman, 1974). Shimp (1989) reported that the rate and extent of LAS mineralisation in estuarine water receiving treated effluent was comparable to that of glucose, with a half-life of less than a week. The test material was radio-labelled in the aromatic ring and degradation of the LAS molecule represented therefore mineralisation and not primary biodegradation.

Similar findings were also reported by Larson *et al* (1983) for Linear Alcohol Ethoxylates (LAEs) in a radio-labelled assay. Mineralisation rates in the estuarine water were also comparable to the degradation rate of glucose, and mineralisation was complete. The rate in estuarine water was approximately four times slower than the rate in freshwater systems.

Shimp (1989) reported that LAS mineralisation in water from a pristine estuarine site is delayed or not observed. This suggests that the history of exposure to the surfactant may play an important role. This process of adaptation has also been observed by a number of other researchers (Spain *et al*, 1980; Pfaender *et al*, 1985; Heitkamp *et al*, 1986; Pfaender *et al*, 1989) and is characterised by a lag time before biodegradation begins. Shimp (1989) suggested that the level of LAS to induce adaptation in the pristine microbial community should be >20µg/l. Unexposed freshwater microbial communities (Rapid Creek, OH, USA) were able to adapt to LAS during the biodegradation test at concentration of 50µg/l. Similar findings have been reported by Pfaender *et al* (1985) on nitrilotriacetic acid (NTA) biodegradation in marine environments.

Robinette (1991) investigated the fate of cationic, anionic and non-ionic surfactants in samples of impacted and unimpacted estuarine waters. Radiolabelled materials were used to assess respiration, cellular uptake and residual material. Adaptation of the microbial community through previous exposure to the chemical is only important for LAS. All other surfactants degraded in a similar fashion as reported for freshwater. In some samples, the author reported incomplete LAS mineralisation, possibly due to the long incubation time (160 days) and elimination of active viable degraders. The prolonged confinement may have biased results because it prevented the exchange of cells and nutrients with surrounding waters.

Adaptation is a prerequisite for LAS and NTA, as both products are rapidly mineralised in pre-exposed estuarine and marine waters. The concentration at which adaptation occurs may also be important. The need for pre-exposure however is chemical specific since the mineralisation rate and extent of ^{14}C LAEs and alkyl (C_{12} or C_{18}) trimethyl ammonium chloride in estuarine and marine waters were not affected by a history of pre-exposure (Pfaender *et al*, 1989). Bauer *et al* (1988) showed that exposure of marine sediments to a particular polycyclic aromatic hydrocarbon (PAH) or benzene results in an enhanced ability of the sediments to subsequently degrade these chemicals. Similar observations were made by Hudak *et al* (1988).

It has been claimed that biodegradation of some organic molecules ceases at low concentrations in freshwaters (Boethling and Alexander, 1979; Alexander, 1981). This could be extrapolated to a possibility that some organic compounds occurring in marine environments could persist due to dispersion and infinite dilution. However, Larson *et al* (1983) did not observe threshold concentrations for NTA and LAEs below which biodegradation was reduced in estuarine systems. Similar findings were reported by Ursin (1985) who claimed that the chemical half-life decreased with decreasing concentrations from µg to ng/l of ^{14}C labelled chemicals such as phenol, N,N-dimethyl formamide and MCPA (2-methyl-4-chloro-(^{14}C-U-ring)phenoxyacetic acid) in freshly collected seawater and marine sediment. In all experiments the degradation, mediated by the natural occurring microbial community, started immediately with the addition of test compounds.

Thus it can be assumed that the necessary enzymes are present or are being produced at all times, albeit in small amounts. These enzymes may not be substrate specific. Ursin (1985) postulated that saturation of the enzyme activity may explain the observed concentration effect at high concentrations. Hwang *et al* (1989) also reported similar concentration dependent kinetics for the degradation of several organic chemicals (e.g. phenol, p-cresol, p-chlorophenol, acetone, and methanol) at low concentrations (0.1-200µg/l).

The practical interpretation of these observations is that an organic chemical present in a waterbody will be mineralised faster with decreasing concentrations. This contrasts with many first-order models for biodegradation kinetics where degradation rate decreases as the test substance concentration falls.

Thus in order to accurately assess the degradation rate in marine environments, it will therefore be essential to test the biodegradability at environmentally relevant (low) concentrations. Only a limited number of studies report biodegradation at environmentally relevant concentrations due to a need for radio-labelled chemicals or specific analytical methods (Vashon and Schwab, 1982; Guerin, 1989; Shimp, 1989; Pfaender *et al*, 1989). Biodegradation activity in the marine environment, assessed *in-situ* by monitoring, may be higher or lower than those exhibited in freshwater systems. The activity is determined to a great extent by the biological, chemical and nutritive status of that environment (Bartha and Atlas, 1977; Fenon and Oliver, 1979; Passman *et al*, 1979; Larson *et al*, 1983; Shimp, 1989).

4.4. COMPARISON OF BIODEGRADATION OF CHEMICALS IN THE MARINE, ESTUARINE AND RIVER ENVIRONMENTS

As described above laboratory tests have indicated that biodegradation in natural or modified seawater is typically slower and less extensive than in freshwater. LAEs were mineralised approximately four times slower in estuarine than in freshwater systems (Larson *et al*, 1983). Similar observations have been described for m-cresol, chlorobenzene and 1,2,4, trichlorobenzene (Bartholomew and Pfaender, 1983), p-nitrophenol (Spain and van Veld, 1983), glutamate, hexadecane, mineral oil (Ward and Brock, 1978), LAS and alkylethoxylate (AE) surfactants (Larson *et al*, 1983; Shimp, 1989) and methyl parathion (Spain *et al*, 1980). In contrast Street (1984) reported that the rates of biodegradation in seawater and freshwater were found to be similar using compounds known to be readily biodegradable, i.e. sodium acetate, sodium benzoate and sodium dodecyl sulfate. In a study using the modified draft OECD screening tests and simulation tests with seawater from four sampling sites (Nyholm *et al*, 1992) assessed the biodegradabilities of 10 chemicals. Compounds known to be biodegradable in freshwater tests were also found to be

biodegraded in seawater tests although degradation rates in the latter tests were generally slower than those recorded in standard freshwater OECD/EEC screening tests.

Complexation with Ca and Mg cations may alter the biodegradation process of organic complexing agents and surfactants (Madsen and Alexander, 1985; Shimp 1989). It is thought that LAS, particularly at low concentrations, is affected by this complexation reaction. Madsen and Alexander (1985) reported that sewage microorganisms mineralised calcium citrate more rapidly than iron and aluminum citrate or citric acid. Magnesium citrate was degraded at the slowest rate. The same study also reported that sewage microorganisms mineralised calcium NTA, but not aluminium, magnesium, iron or hydrogen NTA complexes. Chemical speciation influences the mineralisation of organic compounds by naturally occurring microbial communities, and it is thought that it may partially explain some of the observed differences between marine (high Ca, Mg contents) and freshwaters for certain classes of chemicals which can form complexes with some cations (Shimp, 1989).

The variation of the extent and rate of biodegradation not only depends on salinity as measured by conductivity but will depend on other physico-chemical properties of the environments (pH and ionic composition) but also by intrinsic differences in carbon and energy flow of the different microbial communities. Shimp (1989) reported that both LAS and glucose mineralisation were comparable under marine (42-60%) and freshwater conditions (60-90%). It is presumed that in the marine environment more material is incorporated into cellular biomass or released as uncharacterised extracellular products. Bartholomew and Pfaender (1983) reported that the estuarine communities incorporate a higher fraction of the substrate carbon into microbial biomass than freshwater communities. Crawford *et al* (1974) made similar observations for 15 amino acids and glucose. Less than 50% of substrate carbon was respired and released as CO_2 by estuarine microbial communities.

Ernst (1988) reported a strong decline in heterotrophic bacterial activity along a salinity gradient of the Weser and Elbe estuaries, and attributed the presence of xenobiotics in marine waters to a decreased biodegradation potential and different sorption characteristics. Street (1984) also argued that a direct causal relationship exists between the concentration of NaCl and the degree of biodegradation using a freshwater inoculum in amended BOD dilution water. This can be explained by the fact that the addition of NaCl will inhibit the action of non-halotolerant microorganisms.

Rheinheimer *et al* (1990) reported decreasing degradability rates of radiolabelled 4-nitrophenol (1µg/l) with increasing salinity comparing different freshwater, estuarine, and seawater samples from the river Elbe and the adjacent estuary and marine zones. As revealed by bacterial cell counts, growth of 4-nitrophenol degrading *Pseudomonas fluorescens* strains was hampered with increasing

salinity. When 2-nitrophenol or phenol were used as substrate, different results were obtained: both chemicals were degraded up to only 50% in freshwater or brackish water whereas in the river Elbe estuary and the North Sea an effective biodegradation (80-90%) with high rates was observed. The authors concluded, that the species degrading phenol and 2-nitrophenol in the marine environment were different from those in freshwater environments.

Using a laboratory static sediment-water microcosm Hunter *et al* (1986) reported that bacterial numbers and species diversity decreased with increasing salinity. In the study it was concluded that NTA acclimatisation at higher salt contents ceased because of salinity stress-induced failure of NTA catabolism. Results of the study indicate that bacteria are unable to acclimatise to NTA at moderate salinities (5-9°/oo).

The available information does not allow for a conclusive comparison of biodegradability between freshwater and saline systems. The available data suggest that:

- a chemical which is readily biodegradable in freshwater will also be biodegradable in marine environments;
- the rate of biodegradation tends to be more concentration-dependent in marine than in freshwater environments;
- in the marine environment more material is incorporated into cellular biomass or released as uncharacterised extracellular products.

Whether these differences result from variations in test design or from biological or physico-chemical factors should be further elucidated.

Theory suggests that chemicals which biodegrade in freshwater are also biodegradable in the marine environment. Data in support of this prediction are only available for a number of marine conditions (cf. Appendix B, Street (1984); Nyholm *et al* (1992)). No data have been published which supports the conclusion that biodegradable chemicals in an inherent or simulation freshwater test would also be biodegradable under marine conditions.

SECTION 5. SUGGESTED APPROACH FOR INVESTIGATING BIODEGRADABILITY OF CHEMICALS IN ESTUARINE AND MARINE ENVIRONMENTS

5.1. INTRODUCTION

It should be noted that, within the marine environment, estuarine and coastal waters are the most important regions since they are subjected to the highest concentrations of materials, support the highest densities of marine organisms and are particularly important as spawning grounds for many species. In contrast, the open ocean supports far fewer marine species and its vastness will protect it from any immediate adverse effects by limiting the concentration by dilution while allowing considerable time for subsequent elimination by biodegradation or by abiotic processes such as photodegradation.

It has never been suggested that a totally different biochemistry exists between the marine and the freshwater environment. It is clear from the foregoing sections that the two environments differ and it can be expected that some differences in the biodegradation of chemicals in freshwater and marine environments might be apparent.

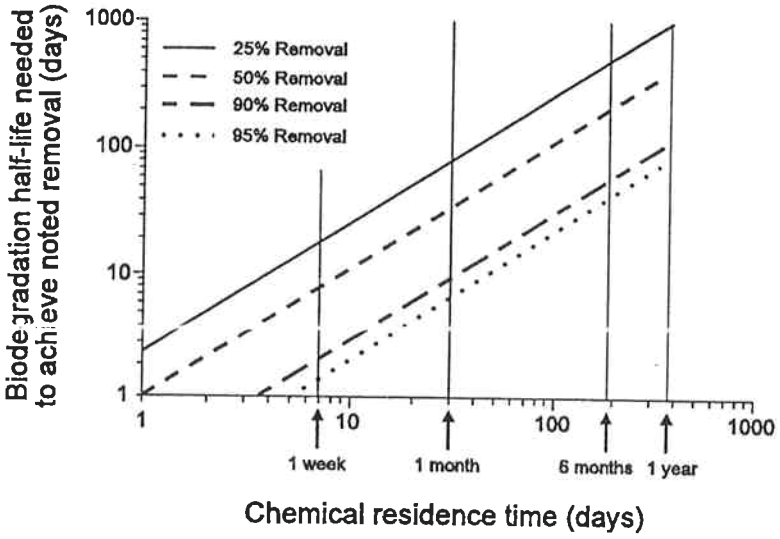
This section attempts to establish whether or not a universally applicable marine biodegradability test is warranted and considers on what basis a reasonably considered decision might be reached. As already discussed the principal attributes of the marine environment cannot be simulated in simple screening tests. Consequently any relevant test is likely to be complex and the need for it and under which circumstances it should be applied, requires careful consideration.

5.2. BIODEGRADATION POTENTIAL OF THE MARINE ENVIRONMENT

Annually thousands of tonnes of organic substances of terrestrial origin reach the sea and have done so for millennia. In contrast to the inert inorganic materials, the concentrations of organic substances in the world's oceans remain extremely low. Consequently marine microorganisms, of necessity, live under intensely oligotrophic conditions compared with freshwater organisms although retention times are considerably longer than in freshwater systems. It would therefore be expected that in the marine environment a slower but more complete biodegradation system would operate than in many freshwater environments and that the biodegradation potential of marine microorganisms is high.

Observations suggest that those materials which are biodegraded in freshwater are similarly attacked in the marine environment. Rates of biodegradation, as might be expected, appear to be slower but would not seem to present a problem in view of the longer residence times. Unlike rivers where chemical retention times may only be in the order of a few days the residence time in the ocean may be orders of magnitude greater. Shimp *et al* (1990) related the residence times in specific environmental compartments to the biodegradation half lives of the chemical (cf. Figure 5). Due to the anticipated long residence times in open oceans, it is expected that (bio)degradation will be a significant removal mechanism, albeit on another time scale than in freshwater.

Figure 5. Critical biodegradation half-lives as a function of chemical residence time at different degrees of removal (Shimp *et al*, 1990)



From the above, a specialised and universally applicable marine biodegradability screening test appears not to be necessary. In general the fate of chemicals in the sea should be predictable from their behaviour (biodegradability) in freshwater systems and knowledge of their physico-chemical properties. In those situations, however, where coastal or estuarine compartments may be exposed to significant amounts of chemicals, specialised seawater biodegradability tests may be required to allow a site-specific hazard assessment. If it is accepted that marine biodegradability data will be required it is therefore necessary to establish a test procedure addressing the particular situation to be investigated (cf. Section 6).

5.3. TESTING STRATEGY FOR ASSESSING MARINE BIODEGRADABILITY

The strategy outlined and schematically represented below as a tier approach is designed to eliminate as far as possible unjustified testing. The approach is based on the required information on which decisions can be made and indicates whether or not a marine test is appropriate and if so which type of test is most suitable.

It is first necessary to consider the mode of use, expected discharge pattern and freshwater toxicity and biodegradability data for the chemical. In those cases where it can be proven that the chemical will not reach the sea directly or indirectly, marine biodegradability studies are not justified.

For all other cases decisions should not be based on an arbitrarily chosen use level but on accepted hazard assessment schemes based on calculated values of PEC (Predicted Environmental Concentration) and PNEC (Predicted No Effect Concentration). If there are indications for a possible hazard further information on the released chemical is required.

It appears from the available data that chemicals passing the OECD freshwater tests of "ready biodegradability" are extensively metabolised in tests made at realistic concentrations in unamended seawater. Consequently chemicals biodegradable in freshwater "ready biodegradability" tests are likewise excluded from further testing and hazard assessment.

Chemicals which are not directly discharged to the sea will reach that compartment via rivers and estuaries. Those chemicals which are not inherently biodegradable in freshwater will pose a greater potential threat to the freshwater environment where concentrations will be orders of magnitude greater than would be realised in the marine environment. For these chemicals a freshwater hazard assessment should thus be made. If there is a freshwater hazard, testing in the marine environment is not necessary as actions should have already been taken for the freshwatwer compartment.

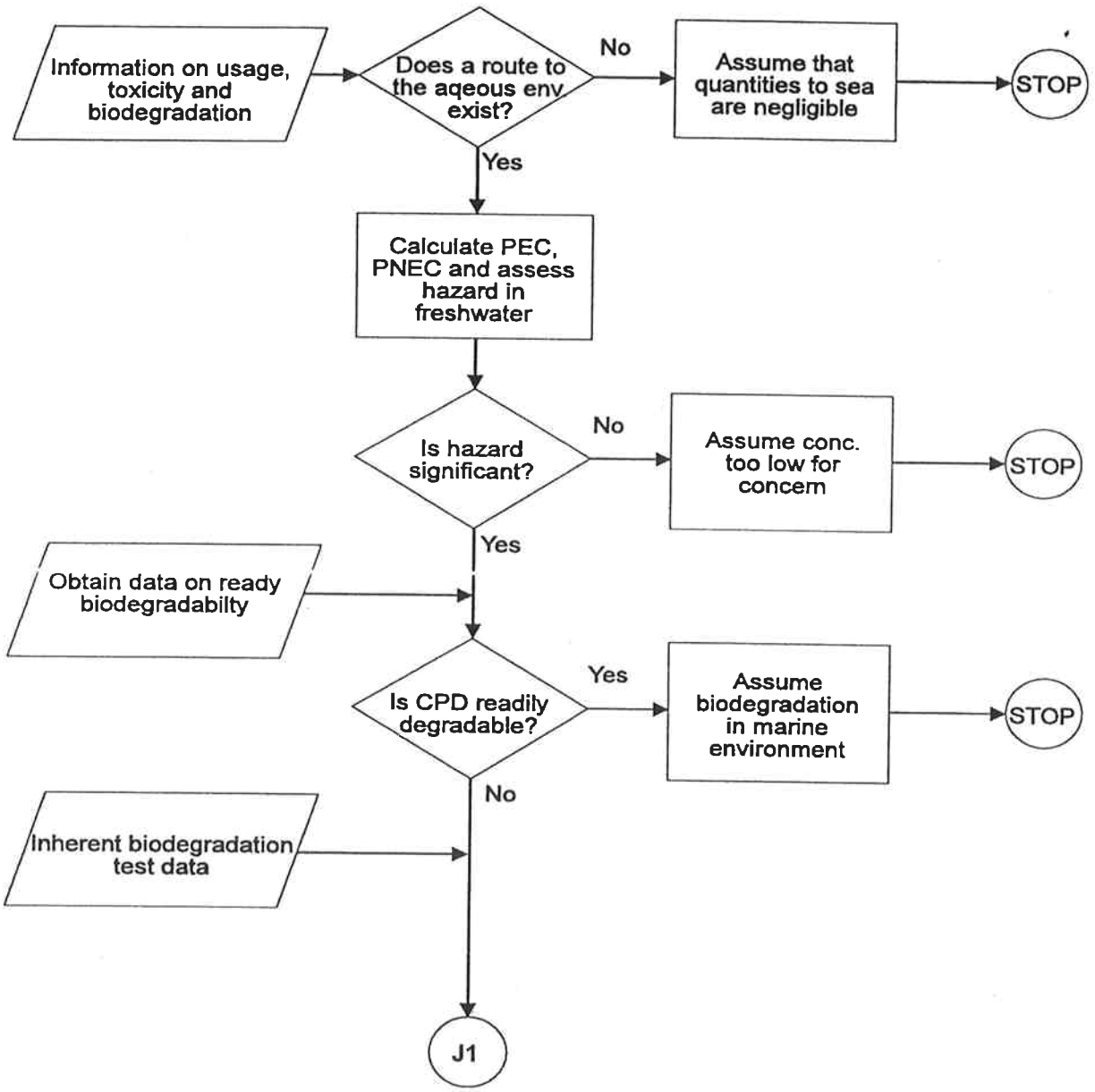
For all chemicals which are inherently biodegradable or for which it is shown that no freshwater hazard is indicated K_{oc} values should be considered.

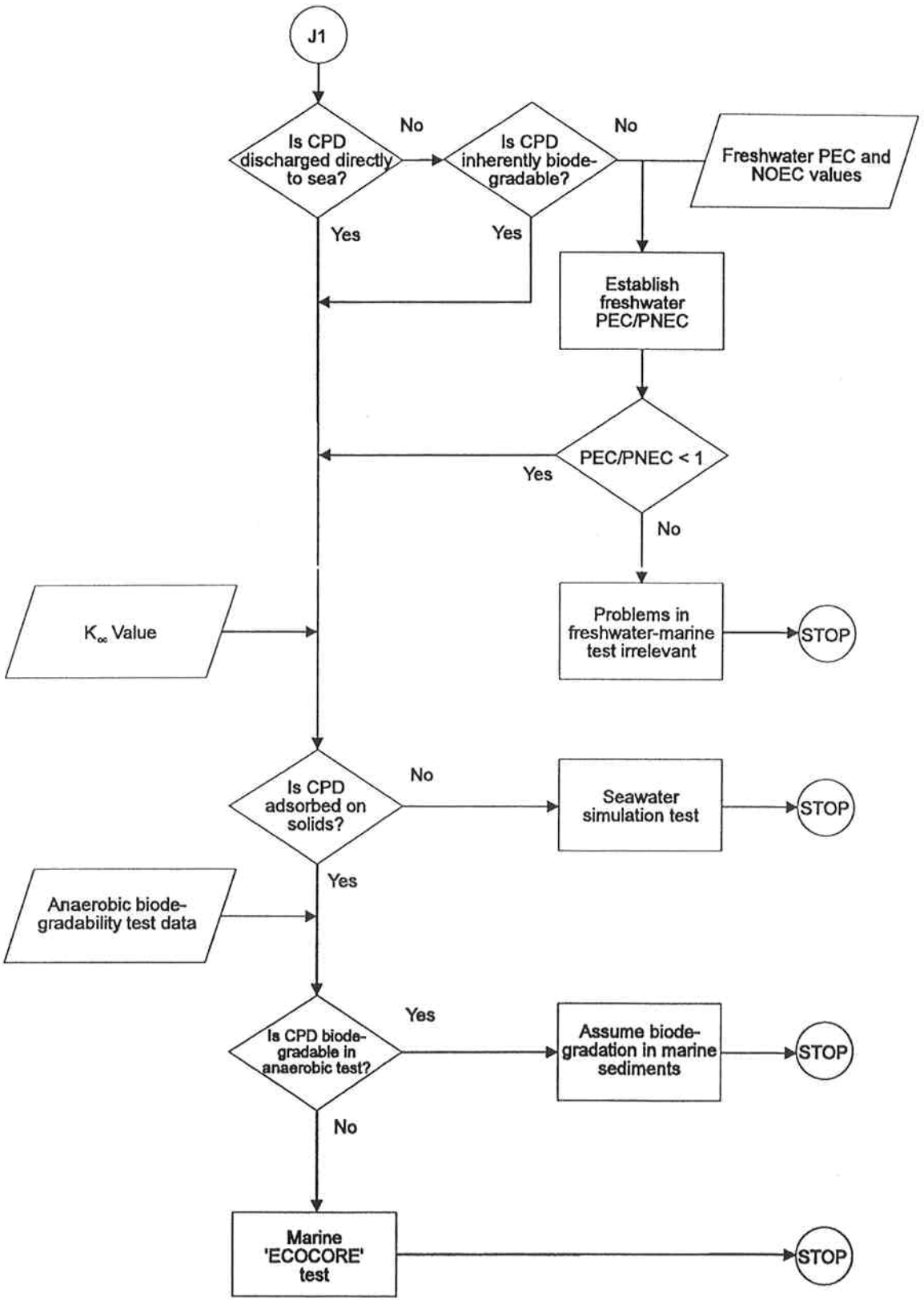
Those chemicals which adsorb strongly on the sediments become potential substrates for anaerobic bacteria since most sediments are anaerobic below the sediment surface. Materials in this group known to biodegrade anaerobically are therefore exempted from further marine testing, as it was shown that the critical stages in breaking down an organic chemical to simple, easily metabolisable compounds are common in both freshwater and marine sediments.

Chemicals retained in the aqueous phase are examined in a seawater simulation test.

Adsorbed chemicals which are not degraded in the anaerobic screening test should be subjected to a marine biodegradation test in the presence of sediment, i.e. a test of the "eco-core" type.

PLANNING DIAGRAM - MARINE BIODEGRADATION TEST




J1
Is CPD discharged directly to sea?
No
Is CPD inherently biode-gradable?
No
Freshwater PEC and NOEC values
Yes
Yes
Establish freshwater PEC/PNEC
PEC/PNEC < 1
Yes
No
K_{oc} Value
Problems in freshwater-marine test irrelevant
STOP
Is CPD adsorbed on solids?
No
Seawater simulation test
STOP
Yes
Anaerobic biode-gradability test data
Is CPD biode-gradable in anaerobic test?
Yes
Assume biode-gradation in marine sediments
STOP
No
Marine 'ECOCORE' test
STOP

SECTION 6. PROPOSAL FOR TEST GUIDELINES

6.1. INTRODUCTION

As shown above there are cases where quantitative information is needed on the extent and rate of biodegradation of an organic chemical in the marine environment. In such circumstances, tests which simulate a particular marine ecosystem are useful and enable many of the variables that affect the fate of a chemical to be studied under laboratory controlled conditions.

The following gives some guidance how a marine or estuarine biodegradation study should be performed, taking into account that such a study should simulate the specific environment for which information is needed. Consequently, only a general guidance for the performance of a marine simulation test can be given, since the investigation must be specific for the chemical and for the environment investigated.

6.2. BASIC REQUIREMENTS FOR TEST PROTOCOLS

From the foregoing arguments it can be concluded that any realistic marine biodegradability test would be facilitated by the use of a ^{14}C labelled test chemical and, if needed, other analytical procedures.

As explained in Section 5 the choice of the most appropriate test will depend on whether the chemical is soluble and remains essentially in the water column or is strongly associated with marine and estuarine sediments. The only approach likely to meet with real success is to carry out tests using natural seawater and ^{14}C labelled test chemicals at environmentally relevant concentrations. It would also be advisable to examine the effect of concentration and to obtain at least some information about the rate of biodegradation.

6.3. SIMULATION TEST FOR SOLUBLE CHEMICALS

For those materials which remain predominantly in the water body a test similar in many respects to the shake-flask die-away procedure could be used to obtain comparable data for marine systems (cf. Section 4). In such a test it would be essential to preserve those characteristics which are peculiar to the considered marine environment.

Since the biodegradation rate in marine conditions is usually lower than in freshwater it would be unreasonable to expect the duration of a test to match those used in freshwater studies and thus tests lasting 3-6 months would be appropriate provided that the volume of test solution is adequate.

Appendix C gives the criteria which are essential for the investigation of the biodegradation of chemical substances in marine or estuarine environments.

6.4. SIMULATION TEST FOR CHEMICALS ASSOCIATED WITH SEDIMENTS

Marine simulation tests should take into account the interactions between the test chemical, the water column and the sediment. Sediment down to a depth of 10-20cm should be sampled as this is the zone where most of the anaerobic biodegradation occurs (Jørgensen, 1977). Such studies should involve the use of intact microcosms (minimally disturbed portions of an ecosystem) which can be studied under laboratory controlled conditions. Comprehensive guidance on the use of sediment/water microcosms, their design, techniques for test substance dosing, sampling, analysis and data interpretation is given in ASTM (1990).

This simple approach fails to consider the fate of chemicals as they are transported from the river systems into the estuarine environment and particularly those compounds which are predominantly associated with sediments.

We would postulate that those chemicals which are biodegradable in anaerobic zones associated with freshwater systems are also biodegraded in anaerobic estuarine and coastal sediments.

SECTION 7. CONCLUSIONS AND RECOMMENDATIONS

Compared to freshwater situations where considerable research has been conducted and a large number of chemicals have been tested only a limited number of attempts have been made to increase the knowledge about the parameters which are critical for the marine biodegradation process.

Although the biochemistry operative in freshwater and marine environments are essentially the same, conditions in the two aquatic regions differ considerably. The most obvious superficial differences are the high salinity and the low concentrations of organic substrates, essential nutrients and microorganisms. These conditions have resulted in many more subtle effects and have produced differences in the marine and freshwater fauna in general and appear to have also influenced the microbiology of the two systems. It is considered that these fundamental differences in the microorganisms and their concentration and the available levels of nutrients must be retained in marine biodegradability tests. The marine variants of the existing "freshwater" OECD screening tests using high high concentrations of chemicals and nutrient enriched saline water are duplications of freshwater tests yielding results equivalent to the freshwater variant. At best they may be representative for processes occurring in estuaries heavily loaded with organic material and nutrients.

Theory suggests that chemicals which are biodegradable in freshwater are also biodegradable in the marine environment. However, the available data only support the correlation of "readily biodegradable" chemicals to be biodegradable under marine conditions. On the other hand, no data have been published which would allow to conclude that chemicals which are biodegradable in an inherent or simulation freshwater test would also be biodegradable under marine conditions.

In those cases where an accurate prediction of the extent and rate of biodegradation of chemicals in specific marine environments is required, biodegradation studies need to be performed under realistic conditions. The concentrations of chemicals, particularly in the marine environment are usually below those that can easily be monitored using conventional analytical methods. It follows therefore that tests which aim to simulate biodegradation in the marine environment must use environmentally realistic concentrations of test chemical and this will probably necessitate the use of ^{14}C-labelled compounds.

Before reaching the open sea most chemicals must pass through estuaries and/or coastal water. These regions are therefore the most at risk. The estuarine environment is extremely complex and the ratio of sediment/muds to overlying water is high. This ratio of sediments/muds will also be an

anaerobic fermentative biodegradation followed by oxidation by sulphate reducing bacteria are important metabolic pathways.

For those materials which are likely to be associated with sediments particularly in estuaries and coastal waters a special test involving sediments is recommended. The eco-core type microcosm test already described in the literature is currently the best available. For materials remaining in the water body a more simple shake-flask die-away test is the most appropriate. For both types of tests environmentally relevant concentrations and temperatures should be chosen and the duration of the experiment needs to take into account the longer residence times in these environments (up to several months).

It is emphasised that marine biodegradation tests are not recommended for all chemicals. Available data suggest that those chemicals which are readily biodegradable in freshwater will similarly degrade under marine conditions. Chemicals which are not inherently biodegradable in freshwater will pose a greater potential threat to the freshwater environment where concentrations will be an order of magnitude greater than would be reached in the marine environment, and thus no marine testing is warrented. For the remaining materials a strategy is proposed to select the most appropriate and relevant test conditions. It is evident that in the proposed strategy an aquatic hazard assessment is required which takes into account the predicted environmental concentration depending on the tonnage of the chemical produced.

It is recommended that, when more knowledge is obtained about marine biodegradation, the proposed strategy should be reconsidered.

APPENDIX A GLOSSARY OF TERMS

AEROBIC:	presence of free oxygen from the air.
ALLOCHTONOUS:	imported material or organisms from outside a defined ecosystem.
ANAEROBIC:	absence of free oxygen from the air.
ANOXIC:	without molecular oxygen.
AUTOCHTONOUS:	material produced or organisms grown within a defined ecosystem.
AUTOTROPHIC:	the process of the formation of organic matter by CO_2 fixation.
BRACKISH WATER:	a mixture of salt and fresh water.
CATABOLISM:	metabolic release of energy from the degradation of organic substrates.
DETRITUS:	particulate organic matter formed as a result of conversion of biological material.
EUTROPHIC:	rich in nutrients.
FACULTATIVE ANAEROBES:	microorganisms able to grow both in presence and absence of oxygen.
FERMENTATIVE:	anaerobic transformation of organic materials to acids or alcohols.
FRESHWATER:	water containing <1‰ total dissolved salts.
HALOPHILIC:	organisms with an obligate requirement for saline conditions.
HALOTOLERANT:	freshwater organisms that will tolerate and grow in saline conditions.
HETEROTROPHIC:	the process of the formation of organic matter on the basis of organic carbon and inorganic nutrients.

INTERSTITIAL WATER: water filling the free spaces between bottom sediment particles (pore water).

LIMNIC ENVIRONMENT: freshwater environments such as rivers and lakes.

METHANOGENIC BACTERIA: bacteria that produce primarily methane during conversion of fermentation end products.

OBLIGATE PROTON REDUCING ACETOGENIC BACTERIA: bacteria producing acetate, H_2 and CO_2 during fermentation processes.

OLIGOTROPHIC: nutrient deficient, cf. Eutrophic.

PHOTIC ZONE: zone where the light intensity is high enough to allow for photoreactions.

PRIMARY BIODEGRADATION: biodegradation step leading to the loss of one or more chemical properties of the original compound.

PRIMARY PRODUCTION: amount of biomass formed by autotrophic processes.

SALINITY: is defined as total quantity of inorganic solids in g/kg seawater when all carbonate has been converted to oxide, the bromine and iodine replaced by chlorine and all organic matter completely oxidised. It is expressed in parts per thousand (ppt or ‰) and has no units. In practice, salinity is now determined by measuring the electrical conductivity relative to a standard seawater and converting to salinity using tables prepared from laboratory correlations between conductivity and salinity.

SEAWATER: is a complicated solution and is characterised by a total amount of dissolved solids (TDS) ≥ 25‰. A significant feature is that while the TDS varies from place to place, ratios of the more abundant components remain almost constant. Some of the more abundant components are Cl^- (55% of TDS), SO_4^{2-} (7.7%), Na^+ (30.6%), Mg^{2+} (3.7%) and K^+ (1.1%) (Pickard, 1979).

SULPHATE-REDUCING BACTERIA: anaerobic bacteria that obtain energy by the dissimilatory reduction of sulphate to sulphide.

RESIDENCE TIME: is the mean time a substance remains in a compartment. It is obtained by dividing the amount of the substance in the compartment by the flux of that substance into the compartment. For example the residence time of water in the ocean is typically 3,550 years and 11 days in the atmosphere.

APPENDIX B. OVERVIEW OF RELEVANT LITERATURE ON BIODEGRADATION IN THE MARINE AND ESTUARINE ENVIRONMENT

Key points from papers are presented in tabular format. Data is presented by author in alphabetical order.

The following key applies to the column headed 'Experimental Method'

Type		Environment		Conditions	
L	Laboratory	F	Freshwater	A	Aerobic
F	Field	M	Marine	AE	Anaerobic
M	Microcosm	E	Estuarine		

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Atlas and Roubal (1978)	L	M/E	A	^{14}C detection ^{14}C-most-probable number enumeration	Crude oil, Pristane, Benzanthracene, Napthalene.	- (no information)	No specific correlation between numbers of hydrocarbon utilisers and hydrocarbon biodegradation potential.	Non-nutrient limited biodegradation followed order hexadecane > napthalene >> pristane > benzanthracene.
Ballerini and Vandecasteele (1982)	L	M	A	Fermentation	Light crude oil.	-	Little or no biodegradation of asphaltenes and resins. Saturated fraction first to undergo biodegredation.	In continuous culture increase in dilution rate leads to increase in biodegradation of hydrocarbons.
Bartholomew and Pfaender (1983)	L	E	A	Heterotrophic uptake kinetics	m-Cresol chlorobenzene, 1,2,4 trichlorobenzene, NTA.	Freshwater site exhibited the highest uptake rate, with somewhat lower rates at the estuarine site.	Although lower, rates at the marine site were relatively uniform throughout the year (i.e. no temperature effect).	-
Bauer *et al* (1988)	M	M	AE	^{14}C	Glucose, anthracene	-	infauna stimulates microbial activity.	A polychaete *Capitella capitala* stimulated microbes.
Bopp *et al* (1981)	L	E	-	model estuary	alkanes	-	C/3 & C/4 alkanes initial half time a few days but increased order of magnitude after 15-30d.	-
Button *et al* (1981)	L	M	AE	^{14}C-Detection	various	-	-	Method for measurement of kinetics/rates
Cheatam *et al* (1977)	L	M	A	GLC	aromatic hydrocarbons	-	Aromatic hydrocarbons persist in sea but some degrade.	Biodegradation different for mono- and di-nuclear hydrocarbons.
Colwell *et al* (1980)	F	M	A	enumeration bacteria	wood		Many different organisms involved.	Bacterial attack on wood and pilings.

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Colwell and Tabor (1976)	F	M	A	enumeration bacteria bacterial activity	-	-	Can estimate deep sea activity.	-
Davies and Tibbets (1987)	F	M	AE	Chambers	oil	-	PAH slow, Aliph. rapid.	PAH remain in sediment.
de Kreuk and Hanstveit (1981)	L/F	F,M	A	Batch and continuous culture. 1.5l plastic bags. ^{14}C-Detection, MPLC	Chlorophenols	Results variable, trend is for degradation in sea water to be slower than freshwater.	Biodegradation in sea water is influenced by N and P content, less by temperature.	-
Desmarquest (1981)	L	M	A	enumeration bacteria	Hydrocarbons	-		Hydrocarbons ranked.
Deveze (1976)	L	M	A	Various	Refinery effluent	-	Degradation potential in sea water appears high for most constituent hydrocarbons.	-
Dibble (1976)	L	M	A	Analysis (GC) Shake flask	Crude oil	-	Ferric oleate, in combination with paraffinised urea and octyl phosphate, is suitable for treating floating oil slicks.	Addition of N and P increases biodegradation.
Fusey and Oudot (1984)	F	M/E	AE	-	light crude	-	Elimination of aliphatic more rapid than aromatics.	Asphalt rather persistant.
Gauthier and Clement (1977)	F	M	A	Toxicity after biodegradation	oil/detergents	-	Tox. is not reduced by marine organisms cf. activated sludge.	Marine and terrestrial microorganisms compared.
Gibbs (1977)	L	M	A	Respirometric	Oil	-	Relation between oxidation rate and nutrient supply observed.	-
Griffith *et al* (1977)	F	M	A	enumeration bacteria	hydrocarbons	-	microbial activity higher in sediment than water.	Seasonal availability of organic nutrients important.

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Guard and Coleman (1980)	F	M	-	Analytical	Petrol. resid.	-	Metabolites are indicators for biodegradation.	-
Hatfori *et al* (1975)		F,M			Phtalate esters	-	-	no abstract.
Hattori *et al* (1988)	L	E/R	AE	Specific	TBT/DBT	F: fast degradation. E: var. degradation.	Degradable in both types of water.	Source of estuarine water influence degradability.
Hervouet and Lapailleur (1981)	L	M	A	Adaptation of AFNOR	organic compounds	-	DOC most suited.	-
Hinga *et al* (1987)	L	M	AE	^{14}C-Detection	TBT	-	Degradable.	Elimination by biodegradation sedimentation volatilisation first order kinetics.
Hon-Nami *et al* (1979)	F/L	M/E/F	AE	Monitoring	LAS	F & E > M	-	Degradation dependent on C-chain length.
Horowitz and Atlas (1977)	L	M	AE	Flow through	Oil	-	Microbial population and degradation increases by addition of N and P.	-
Hunter *et al* (1986)	L	E	AE	shake-flask; mixture of fresh and seawater with added minerals; loss of parent compound	nitrilo acetic acid	degradation decreased when seawater fraction was increased above 9‰ S	-	Different salinities (1-17.5‰) produced by mixing of fresh and seawater.
Kennicutt (1988)	L	M	A	Various	Light crude oil	-	Rate of biodegradation decreased with increasing carbon number. Straight chain alkanes were degraded more rapidly than branched.	Undegraded (stable) chemical properties (fingerprints) are suggested as unique and sensitive indicators to determine the source of microbially-altered hydrocarbons in the environment.

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Koenig and Ward (1984)	L	E/F	AE	Various	Alkanes	-	UMK a chlorophyllous algae degraded.	Degraded by algae.
Kondo *et al* (1988)	L	F/M	A	the cultivation method (description in a previous report)	170 chemicals	-	In comparison with MITI-method this method was found to be more stringent.	Written in Japanese (English translation available).
Krstulovic and Sobot (1977)	F	M	A	enumeration bacteria	organophosphorus compounds	-	Density + distribution of bacteria discussed.	Based on distribution of bacteria.
Laporte *et al* (1982)	L	M	A	enumeration bacteria	COD	-	1g COD/m^3/day max flow.	Importance of nitrogen cycle shown.
Larson and Games (1981)	L	F	A	CO_2 evolution	LAE various chain length	-	Under environmentaly relevant concentration no difference of degradation of short and long chain.	freshwater.
Larson *et al* (1983)	L,F	E	A	^{14}C detection	detergent chem. NTA, LAE	Degradation of high levels of LAE may be limited by nutrient availability in estuarine water	Trace levels of NTA and LAE are efficiently metabolised by native micro-flora in fresh and estuarine systems. No threshold concentrations, below which biodegradation.	Freshwater and estuarine environment compared.
Larson and Ventullo (1986)	L	E	A	CO_2 evolution	NTA	No significant difference observed	natural estuarine water; different DOC, temperature, salinity did not affect degradation.	S-range: 4-17ppt 2-11ng/l.
Lee and Levy (1988)	L,F	M	A	-	Petroleum	Maximum rates are not obtained due to limited availability of O, N and P	Hydrocrabon degraders are ubiquitous throughout oceans.	N, P enrichment is most promising for enhancing biodegradation in oil spills.

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Lee *et al* (1987)	L	E	A	loss of parent compound	tribultyltin	-	removal mainly by microalgae.	-
Leightly (1980)	F	M	A	enumeration bacteria	Wood	-	Marine fungi involved.	Fungi are primary invaders.
Nagata and Kondoh (1978)	L	M	A	Loss of parent compound, specific analysis	Crude oil	-	Degraded by marine bacteria. Correlation between number of bacteria and extent of degradation.	Addition of vitamins increased biodegradation but not N- or P-nutrients.
Nyholm and Kristensen (1992)	L	M	A	Screening OECD	Na-benzoate, Aniline Pentaerythritol, Di-ethyleneglycol, 4-Nitrophenol	-	Result comparable to fresh water Incubation time; extended biodegradation.	Importance of nutrients.
Nyholm *et al* (1992)	L	M	A	shake-flask, closed bottle and shake-flask "simulation" tests	Na-acetate, Na-benzoate, 4-Nitrophenol, Aniline, 4-Chloroaniline, Diethylene glycol, Pentaerithritol, Maleinhydracide PMM, Desmethylmethyl-parathion	-	No difference in biodegradability between screening and simulation test.	-
Olivieri *et al* (1976)	L, F	M	A	Specific analysis	Oil	-	Addition of Mg NH_4 PO_4 enhances biodegradation.	Importance of nutrients.
Olson and Brinckman (1986)	L	M	A	shake-flask; loss of parent compound	Tributyltin	-	-	No nutrients added.
O'Neill (1977)	L	M	A	enumeration bacteria	Oil	-	Many species of oil degraders.	Methods for isolation of cultures.
Oudot (1984)	L	M	A	semi-continuous culture, GC/MS	Crude oil	-	Saturated hydrocarbons degraded more rapidly (74%) than aromatics (50%), asphaltenes (30%) and resins (25%).	Biodegradation potential greater in continuous than batch culture.

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Pfaender and Bartholomew (1982)	L	F, M, E	A	^{14}C-Detection	Cresol, CLB Trichlorobenzene NTA	Degradation rates depending on water sample		In assays with elimination, mineralisation not always confirmed.
Pfaender *et al* (1985)	L	E	A	^{14}C-Detection	NTA	-	NTA degraded rapidly by bacteria pre-exposed to NTA.	-
Pritchard *et al* (1987)	L	E	A	^{14}C Shake-flask microscosm	Para-chlorophenol (PCP)	-	Rates were slower in estuarine waters.	Addition of detrital sediment resulted in a rapid mineralisation as evidenced by $^{14}C\ CO_2$ evolution.
Quiroga *et al* (1989)	L	M	A	Batch reactor specific analysis	Anionic surfactants	-	Degradation temperature dependent.	No influence of salinity. Polluted sea water improves degradation.
Reichardt *et al* (1981)	L	M	A	^{14}C-Detection Batch cultures unamended sea water	Biphenyl (BP) Chlorobiphenyls (ClBP)		Biodegradation rates were in the order: BP>2-ClBP>4-ClBP>3-ClBP .	No significant build-up of partially degraded products.
Robinette *et al* (1989)	L	E	A	CO_2 evolution	Surfactants	-	Degradable 40-60%.	In non preadapted system longer lag phase.
Rontani *et al* (1987)	L	M	A	GLC, GC/MS	n-Nonylbenzene	-	Photo-oxidation and biodegradation may be closely linked for aromatic hydrocarbons.	The action of light in relation to biodegr. can in some cases be beneficial.
Saltzmann (1981)	L	M	A	-	Aromatic hydrocarbons	-	-	Biodegradation rate in marine sediments.
Seligman *et al* (1986)	L	M	A	Microcosm ^{14}C-TBT	TBT	-	TBT degraded to DBT and MBT t 1/2=7 to 15 days.	-

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Shimp and Young (1987)	L	M/E	A	Comparison of OECD-DOC die-away test and ^{14}C test both method with mineral supplemented seawater	Na-benzoate, Benzo c acid	-	Biodegradation at 20mg/l more variable than at 50µg/l.	
Shimp (1989)	L	E	A	^{14}C-Detection	^{14}C Tridecyl-benzene sulfonate (LAS)	-	Pre-exposure to LAS increases numbers of LAS degraders and biodegradation rate.	-
Spain *et al* (1980)	L	E	A	^{14}C-Detection	Methyl parathion p-nitrophenol	Pre-exposed river populations degraded methyl parathion and p-nitrophenol more rapidly	Numbers of nitrophenol degrading bacteria 4 to 5 order of magnitude during adaptation in freshwaters. Salt marsh populations did not adapt to degrade methyl parathion nor p-nitrophenol.	-
Steinman (1976)		F/M		$^{14}CO_2$ was collected in 0.2ml ethanolamine within the 300ml test vessel	Urea and Uric acid	Optimal growth of urea and uric acid degrading bacteria was obtained with fresh and brackish water but not with seawater	Urea degradation 0.07-1.98µgC/l.h Uric acid degradation 0.0014-0.106µgC/l.h.	Investigation on the quantitive distribution of urea and uric acid decomposing bacteria in the Western Baltic Sea.
Street (1984)	L	M	A	BOD testing with natural seawater nutrient supplemented seawater	Sodium acetate, Socium benzoate, Sodium dodecyl sulphate, Glucose/glutamic acid mixture	The degree of biodegradation reached within the test period was found to be lower in seawater than in fresh water	Inoculum source is less significant than the salinity effect in influencing the degree of biodegradation under marine conditions.	-

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Taylor *et al* (1981)	L	F,E,M	A	Cultivation Respirometry GC	Phthalic acid esters were degraded in laboratory tests in freshwater and marine media	o-Phthalic acid and its esters were degraded	Variety of bacteria exist in some marine regions that can degrade o-phthalic acid and its esters.	Some bacteria from marine sources grew also in freshwater media.
Taylor and Amador (1988)	L	M	A	Growth studies with isolated bacteria on different compounds. Degradation of compounds measured via spectra	m-, and p-Phtalate dipicolinate	Na+ dependent oxidation of growth substrates. Transformation of pyridine compounds via phthalate hydroxylases	A new metabolic route for the transformation of pyridine compounds.	Transformation into hydroxylated compounds Na+-dependent oxidation; relevant in so far that specific compound degradation is reported.
Thain *et al* (1987)	L	M	AE	shake-flask; loss of parent compound	Tributyltin, Dibutyltin	Differences in degradation rates attributable to temperature differences	-	No nutrients added; including suspended matter.
Ursin (1985)	L	M	A	CO_2 evolution	Phenol; dimethyl-formamide; methyl, chloro phenoxyacetic acid	-	Increase of degradation at decreasing concentrations.	Natural seawater including sediment; environmental temperatures.
Vashon and Schwab (1982)	L	E	A	CO_2 evolution	LAE, LAES	Only experiments with estuarine water	Degradable.	First order Kinetics.
Wakeham *et al* (1986a, b)	L	M	AE	^{14}C Detection		-	71-82% mineralised in 2 weeks.	1d residence expected in summer in Narragansett Bay.
				Mesocosm	Volatile Hydrocarbons: Octadecane, Decane	-		70-80% mineralised in 5 weeks.

Authors (year)	Experimental Method			Method Description	Chemicals tested	Difference Biodegr. Freshwater Marine	Conclusions	Specific Information
	Type	Env.	Cond.					
Ward and Brock (1978)	F	M	A	^{14}C Detection	Oil, Hexane, Glutamate	no freshwater experiments performed	Salinity reduces rates for freshwater organisms.	Doubts about HC degradation in saline waters.
	F	M	AE	Monitoring	Hydrocarbons	-	>20% salinity inhibits biodegradation.	Degradation dependent on salinity.
Wunderlich (1986)	L	M	A	TOC or DOC, O_2 consumption, test duration of 60 days	dispersants	Rates were considerably slower than in freshwaters	Both dispersants were readily degraded by marine bacteria.	-

APPENDIX C CRITERIA FOR THE INVESTIGATION OF BIODEGRADABILITY IN MARINE OR ESTUARINE ENVIRONMENT

This guidance gives the criteria which are essential for the investigation of the biodegradation of chemical substances in marine or estuarine environments. It provides the elements for the practice of a site specific biodegradation study, which may include water with suspended particles and sediments (oxic and anoxic). A detailed test protocol for such a study should contain these elements, but needs much more specific instruction on the performance, the use of the specific environmental media, test compound application, duration of test etc..

C.1. PREREQUISITES

The following information should be obtained, before the test is initiated:

- characterisation of environmental test medium / media (where applicable): pH, temperature, salinity, oxygen content, concentration of dissolved and particulate organic carbon, nutrient salts, redox potential, grain size, light radiation;
- characterisation of the test compound, i.e. its physico-chemical specifications, including partition coefficients (e.g. octanol/water) in order to evaluate which environmental medium is most likely involved during biodegradation processes;
- concentration range of the test compound to be expected in the environment.

C.2. DESIGN FEATURES OF A SIMULATION TEST

C.2.1. Size of test vessels

The size of appropriate test vessels can vary depending on the nature of the environmental media used, but test vessels with volumes of approx. 200ml up to 5l are most commonly used for this type of test (Shimp, 1989; Ursin, 1985; Hunter *et al*, 1986; Lee *et al*, 1987; Lindgaard, Jorgensen and Nyholm, 1988; Robinette, 1991). They must be large enough to allow for intermediate sampling.

C.2.2. Test media

The test media for a marine or estuarine simulation test is taken most appropriately from the site or environment, for which information on biodegradation has to be obtained. In case of a less well

defined source of environmental contamination or diffuse introduction of a chemical into the aquatic environment, environmental media from different sources and of differing nature (different salinities, temperatures, organic carbon concentrations etc.) may be sampled. The environmental medium / media can be water or the combination of water and sediments.

C.2.3. Collection of test media

Water should be collected by sampling methods, which are non-destructive for the microbiota, e.g. hand bucketing or non-destructive pumping. If the water at the sampling site is stratified this should be taken into account and water samples taken from different depths. If the water needs to be filtered to remove excess suspended matter, it should be recognised that the microbiota which may degrade the test compound, can be in excess of 50µm in size. For test systems which include bottom sediments, it is important to collect the sediment from the sampling site, as intact as possible, to ensure that the redox gradient and benthic microbiota community is maintained.

Plastic (e.g. acrylic) core tubes can also be used although they should not leach plasticisers into the test system. Sediment/water cores can be taken at intertidal zones by hand, and underwater by either a diver or using a variety of frame-mounted corers (plastic core tubes). The latter being capable of collecting samples from depths down to 5,000m (Herbert, 1988).

Core sampling is the best way to collect sediments. The size and depth of the core cannot be predetermined. Sediment samples from depths of 10 to 20cm will usually include the relevant biological activity (Spain *et al*, 1980). The samples have to be transported from the field into the laboratory as fast as possibly, under conditions which preserves the environmental temperature and the integrity of the samples.

C.2.4. Application of test substance and incubation procedures

The method of addition of the test compound can vary with the test system (e.g. flow-through or static, with or without sediments). The first decision one has to make, is the concentration of the test substance. Environmentally relevant concentrations of the test compounds should be used in simulation tests. Since the biodegradation process may only be initiated above a certain threshold concentration a range of concentrations may be necessary. Low concentrations of the test chemical typically require the use of radiolabelled isotopes. This also facilitates the determination of a mass balance of the test chemical. Consideration has to be given to the carbon atom that needs labelling in a complex molecule in order to give unequivocal results of mineralisation. Dosing may be by direct addition of the test substance to the culture vessels and frequent or continuous mixing. This particularly applies when bottom sediments are not included in the test. In an eco-core or

microcosm where the structural integrity of the bottom sediments is required, mixing of the test compound with the media should proceed by diffusion, enhanced by gentle rotation of small paddles to simulate the natural environment. A flow-through system may be the most appropriate. Adequate time should be given to allow for distribution of the test chemical before making actual measurements of degradation. Control vessels should be used in which the test media are sterilised before adding test substance in order to differentiate abiotic degradation. Incubation should be in conditions, which are as close to the natural environment as possible, particularly with reference to temperature, light intensity and duration, and salinity. Nutrient content is also important. The levels of nutrient should not exceed the natural ranges, but to avoid nutrient limitation they may have to be replenished during a long term study.

C.2.5. Sampling and observation

Hydrographical parameters like temperature, salinity, pH value, organic carbon and nutrients should be measured regularly. Additional parameters such as redox potential and particle size should be analysed in sediments. Samples for the analysis of the residual test compound and its degradation products should be taken at the start of the test and at regular intervals during incubation. For longer term tests a sampling frequency of once per week is appropriate. Sampling of sediments may cause irreversible disturbance of the structure, and it may be deemed necessary to set up specific replicate cultures for each analysis.

C.3. DATA EVALUATION

There is no general guide for the interpretation of results. However, the determination of rate constants may be one way of calculating half-lives. Consideration should be given to the fact that test substances may be irreversibly bound to sediments and/or only partial degradation to intermediate metabolites may occur.

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