PROCEDURES FOR BIODEGRADABILITY TESTING OF ORGANIC CHEMICAL COMPOUNDS

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Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

Background

In an era of acute awareness of the need for environmental and human health protection and of ever stricter water pollution control measures, there is an increasing need for organic chemicals entering the aquatic environment to be completely degraded to harmless chemicals components such as carbon dioxide, water and inorganic salts. The extent and ease with which this would be possible in the natural environment or in waste treatment plants can be established by means of biodegradability tests.

Three types of standardized aerobic biodegradability tests exist, namely short-term ready or ultimate biodegradability tests, inherent biodegradability tests and simulation tests. The ready biodegradability tests are also called screening tests or ultimate biodegradability tests, and refers to the total breakdown of organic chemicals to carbon dioxide, water and mineral salts. These tests are the most stringent and conditions are such that maximum biodegradation will not necessarily occur. Inherent tests use test conditions which more closely resemble environmental conditions and simulation tests provide information on biodegradation rates in *specific environments under environmentally realistic conditions.*

Biodegradation studies in South Africa mostly involve simulation tests with industrial waste in laboratory scale activated sludge units. Very few laboratories are equipped to carry out standardized, ready biodegradability tests for chemical products. In addition, no tests [apart from the 5-day biochemical oxygen demand (BOD) test] have been established to evaluate the biodegradation of chemical compounds in natural waters. With the increased production of new chemical products which could find their way into the water environment the demand for such testing is rapidly increasing.

Aim

The aim of this project was to evaluate short-term aerobic screening tests used in other countries to test the biodegradability of chemical products and environmental pollutants under typical conditions in the aquatic environment and the modification of the most suitable tests for application in South Africa.

Methodology

Three different aerobic ready biodegradability tests were selected for evaluation. The tests included dissolved organic carbon (DOC) reduction tests (river water and activated sludge inocula); a carbon dioxide (CO_2) production test; and an oxygen (O_2) depletion test. ISO/OECD standard procedures/guidelines were followed. For chemical compound testing the required inorganic nutrients, organic test compounds and microbial inoculum were added to water. The organic concentration of test solutions generally ranged from 2 to 40 mg/ℓ. Water and effluent were tested in the presence and absence of mineral medium and inoculum. Tests were carried out for 28 days in the dark/diffused light at temperatures ranging from 20 to 24° C.

In an attempt to optimize tests a few experiments were carried out under altered experimental conditions *e.g.* various test temperatures, incubation in darkness/diffused light/ambient light, and agitation by means of aeration/shaking/stirring and no agitation in the DOC reduction test;

the use of additional scrubbers to remove CO_2 from air supply, different volumes of $Ba(OH)_2$ in end absorbers, and stirring in addition to aeration in the CO_2 reduction test; and the incubation of BOD bottles in an upright position and inverted with necks in water in the oxygen depletion test.

Three water soluble chemical compounds (aniline, lauryl sulphate and diethylene glycol) and one non-soluble compound (stearic acid) were tested. Water samples were collected from the Apies river and Moreleta stream, and effluent from a paper mill, steel works, and food, cutting oil and pesticide manufacturers. In addition to DOC analysis, TOC, COD and microorganism numbers were analyzed in a few studies to investigate possible improvement of measurement.

Results

Preliminary tests showed that Apies river and Daspoort sewage works (activated sludge and sludge effluent), Pretoria, provided microorganism numbers and contained DOC levels which were within the requirements of the different ISO and OECD tests evaluated. A 0.5 μm membrane filter (SLCR, Millipore) was found to be the most suitable for preparation of samples for DOC analysis, not increasing or adsorbing DOC in samples and providing effective decontamination.

The chemical compounds aniline and lauryl sulphate was found to be ready biodegradable in all the tests. Diethylene glycol was difficult to biodegrade, and showed ready biodegradability only in the DOC reduction test when activated sludge (ISO test) and a low test concentration was used. Stearic acid was ready biodegradable in the oxygen depletion test, but not in the CO_2 reduction test.

Tests on mixtures of aniline (used as reference chemical) and chemical compounds showed that the test concentrations at which studies were carried out were not toxic. Likewise, abiotic degradation could not be demonstrated.

Biodegradability tests on water and effluent showed that the CO_2 production test was positive (ready biodegradable) for all the samples tested (biodegradation: 85 to 359%). The DOC reduction and oxygen depletion tests showed positive results in a few instances. The biodegradation in the DOC reduction test was particularly low when river waters were tested, where reduction from approximately 8 to 3 mg/ℓ C occurred.

No toxicity was detected in water and effluent at the concentrations tested (mixtures with aniline and water/effluent). The effluent from the food industry showed abiotic degradation.

Conclusions and Recommendations

The evaluation of the three types of ready biodegradability tests showed that all the tests have a viable role to play in assessing the ultimate biodegradability of chemical products in local water laboratories. The appropriate method will depend on the chemical to be tested. For example, soluble compounds can be tested in all the tests. The CO_2 production and oxygen depletion tests are more suitable for poorly soluble chemicals. When low concentrations of chemicals need to be tested, for example when test compounds are toxic at the normal levels of 10 and 40 mg/ℓ, the oxygen depletion test will be the most applicable.

The DOC reduction test was found to be the most simple of the tests in terms of preparation, maintenance and data analysis. The test was reproducible, showing little variation between duplicate tests and produced similar biodegradability results during repetitive evaluations.

The DOC reduction test using river water as inoculum was more sensitive, and therefore more stringent, than the test using activated sludge. However, activated sludge was found to be a more suitable source for inoculation as the concentration was determined directly before use, while in the case of river water a standard volume based on previous analysis of the water was used. The use of activated sludge resulted in a microbial concentration in test solutions similar to that found in our surface waters (10^3 to 10^4/mℓ), providing a more realistic test than when river water is used for inoculation.

The CO_2 production test has great potential for chemical product testing, especially when used in combination with DOC reduction analysis (two tests in one). Problems were, however, experienced with too much CO_2 produced by the controls. The variation between duplicates was sometimes also too large. It is recommended to use the actual titrated value for $Ba(OH)_2$ (mℓ) instead of the calculated value to reduce the CO_2 produced by controls to below the required 70 mg/ℓ.

The preparation of the O_2 depletion test was found to be more tedious than that of the other tests in order to ensure that the dissolved oxygen concentration remains constant during sample distribution.

Aeration was found to be a suitable substitute for stirring and shaking of solutions in the DOC reduction test. This type of agitation will also be more cost effective for routine use.
Differences in light intensity in the laboratory did not influence the results of the DOC reduction test. The same should be true for the CO_2 production test. Algae can, however, influence the results in the O_2 depletion test (low concentrations). It is thus, recommended to follow the standard test instructions using complete darkness during incubation in the O_2 depletion test and diffused light for the other two tests. Biodegradation was influenced by temperature indicating that it is important to standardize the temperature for testing. A temperature of between 20 and 25° C, as used in standard tests, should be acceptable. It should, however, be kept in mind that slower breakdown can be expected in the natural environment during the winter period.

Additional scrubbers containing soda lime, placed in front of the normal NaOH and $Ba(OH)_2$ *scrubbers, did not reduce the amount of* CO_2 *produced by control flasks in the* CO_2 production test, indicating that the high CO_2 produced by controls was not due to inadequate removal of CO_2 from the air. Clogging occurred in additional scrubbers, particularly in high humidity, rendering such devices inadequate and dangerous because pressure can build up in flasks.

Studies using 100 mℓ and 200 mℓ $Ba(OH)_2$ in end absorber flasks in the CO_2 production test resulted in similar absorption of CO_2 in control flasks, indicating that the use of larger volumes of $Ba(OH)_2$ in end absorbers did not increase CO_2 concentrations in control tests. Furthermore, larger volumes of $Ba(OH)_2$ did not result in a better absorption of CO_2 in test flasks, indicating that the contact time between bubbles and 100 mℓ $Ba(OH)_2$ in Erlenmeyer flasks was sufficient. Stirring in addition to aeration resulted in an increased rate of biodegradation but did not improve the large variation sometimes noticed between duplicate tests (both CO_2 production and DOC reduction).

Bubble formation in flasks in the oxygen depletion test did not appear to contribute to the large oxygen depletion in controls and blank controls. Similar results were obtained when flasks were standing upright and inverted with stoppers in Milli-Q water.

It is recommended that the standard methods as outlined in ISO and OECD protocols, with certain modifications, are used for local biodegradability testing of chemicals:

DOC reduction test		
Standard procedure (ISO, 1984)	**Modification**	**Explanation**
1. 250 mℓ sample	1. 1 ℓ sample	1. Sufficient sample for frequent DOC analysis
2. Activated sludge/secondary effluent/surface water inoculum	2. Activated sludge inoculum	2. Final microbial numbers similar to that found in surface waters - more realistic test
3. Use membrane filter of 0.2 μm porosity to filter samples for DOC analysis	3. Use 0.5 μm SLCR millipore filters to prepare samples for DOC analysis	3. Does not remove or add carbon to sample
4. Stirring for aeration and mixing	4. Aeration to mix	4. As effective as stirring/shaking and more cost effective
CO_2 production test		
Standard procedure (ISO, 1990)	**Modification**	**Explanation**
1. Use Drechsel bottles as end absorbers	1. Use 250 mℓ Erlenmeyer flasks with tightly sealed stoppers as end absorbers	1. More cost effective and work relatively well
2. Stirring for aeration and mixing	2. Aeration to mix (stirring for poorly soluble substances)	2. As effective as stirring/shaking and more cost effective
3. Activated sludge/secondary effluent/surface water inoculum	3. Activated sludge inoculum	3. Final microbial numbers similar to that found in surface waters - more realistic test
Oxygen depletion test		
Standard procedure (OECD, 1992)	**Modification**	**Explanation**
1. Use 0.05 to 5 mℓ/ℓ activated sludge for inoculation	1. Instead of inoculating 0.05 mℓ/ℓ activated sludge effluent directly, first make a dilution and add a larger volume	1. More accurate
2. Place flasks upside down in water bath	2. Flasks placed upright in a constant temperature cupboard	2. As effective and more cost effective (does not need expensive equipment)
3. Maximum oxygen concentration: approximately 9 mg/ℓ	3. Maximum oxygen concentration: approximately 7 mg/ℓ	3. Saturation

As in standard biodegradability tests, it will be necessary to include, in addition to controls and tests, a test on a reference chemical, an abiotic test and a test to examine possible toxicity, in order to understand and verify results. Although not examined in our study, a test to

examine adsorption (DOC reduction tests) could also be included. Such a test is prepared in the same way as the abiotic test, but inoculum is also added (OECD, 1992). When complex compounds are tested it is recommended that a toxicity test is carried out before the biodegradation test to avoid toxicity. Oxygen uptake tests with activated sludge or bacterial growth inhibition tests (Slabbert, 1994) were found to be useful for this purpose. The OECD guideline (1992) also provides information on toxicity testing. In order to examine abiotic degradation, a sufficiently high $HgCl_2$ concentration should be used ($\geq$10 mg/ℓ) to avoid microbial growth.

Aniline and lauryl sulphate proved to be useful reference chemicals. If chemical products which are expected to be non-biodegradable are tested it could be useful to include a chemical like diethylene glycol which is difficult to biodegrade as example.

Biodegradation studies on water and effluent showed that the best biodegradation was achieved when medium and inoculum, as specified in the standard tests, were added to test solutions.

The evaluation of additional parameters such as TOC, COD and microbial analysis in the DOC reduction and CO_2 production tests on water and effluent showed that TOC results were, in general, in agreement with DOC results. In some instances TOC levels were lower than DOC concentrations, indicating biodegradation (no filtration) before analysis. DOC, therefore, proved to be a more reliable determinand than TOC. In general, COD values showed large fluctuations, particularly for control solutions and when organic concentrations were low, indicating that this determinand is not an accurate measure. However, when samples of high organic content is evaluated, this determinand could supply useful additional information. Microorganism numbers showed large fluctuations and no specific pattern of growth, therefore this parameter is not appropriate to use.

Compared to tests on individual chemicals high biodegradation values (much larger than 100%) were in some instances obtained for the CO_2 production and O_2 depletion tests. Measured DOC and COD values were used to calculate the TCO_2 and TOD, which are in turn used to calculate biodegradability. It is known that the COD is often not as high as the TOD as some chemicals are poorly oxidized in the COD test, resulting in falsely high values for biodegradation (OECD, 1992).

The study indicated that methods developed for the ready biodegradability testing of chemical substances are not so applicable to environmental samples. Further development is required to establish applicable protocols for biodegradability testing of complex samples.

The biodegradability tests evaluated in this study are only applicable to aerobic biodegradation. Anaerobic degradation occurs in sediments of surface waters and in ground water. It is recommended that attention is also given to the development/establishment of anaerobic ready biodegradability tests for local use.

Technology transfer

A paper was presented at the Water Institute of Southern Africa Biennial Conference and Exhibition held in Cape Town from 4 to 7 May 1998. The title of the paper was **Biodegradability testing of organic chemical compounds**. The paper was published in the conference proceedings.

It is also envisaged to publish the data in a peer reviewed journal and to include the methodology in a practical training course.

Archiving

Only the final results are presented in this report. The raw and calculated data will be stored at Building 21, CSIR in written and electronic format.

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Dr SA Mitchell	Water Research Commission (Chairperson, 1994)
Mr FP Marais	Water Research Commission (Secretary)
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GLOSSARY

Acceptable biodegradability - Minimum biodegradation necessary to remove undesirable properties of a compound, *e.g.* foaming or toxicity. This will correspond to primary biodegradation but may vary depending on the circumstances under which products are discharged into the environment

Biodegradability - Conversion of organic chemical compounds, which are potentially water pollutants, to innocuous substances through the action of living organisms

BOD (Biochemical oxygen demand) - Amount of oxygen utilized during a specified incubation period by microorganisms to metabolize a compound

COD (Chemical oxygen demand)- Amount of oxygen utilized during the oxidation of a compound with hot acid dichromate - it provides a measure of the oxidizable matter present

Co-metabolism - The process by which a normally non-biodegradable compound is biodegraded in the presence of an additional carbon source

Complete mineralization - Complete breakdown of a compound to carbon dioxide, water and minerals, including biodegradation of new cells formed

Control - Solution containing only the required inorganic nutrients (called blank test in standard methods)

DOC (Dissolved organic carbon) - Amount of carbon present in organic compounds in aqueous solution

Inherently biodegradable - This means that if given the right conditions, a compound is biodegradable. Also referred to as potential biodegradability.

Primary biodegradability - Biodegradation to the minimum extent necessary to change the structure of a compound resulting in loss of specific property of the compound

Ready biodegradable - Passing certain specified screening tests for ultimate biodegradability. These tests are so stringent that such compounds will rapidly and completely biodegrade in a wide variety of aerobic environments

Reference - Solution containing the required inorganic nutrients and a known organic product (reference compound) which meet the criteria for ready biodegradability

Screening test - Relatively simple batch test for the preliminary assessment of biodegradability, treatability or toxicity of a test compound

Simulation test - Test to predict the rate of biodegradation of a compound under relevant environmental conditions

TOC (Total organic carbon) - Total amount of organic carbon in an aqueous solution

Toxicity - Extent to which a test compound adversely affects microorganisms

Treatability - This is a more practical concept than biodegradability, and expresses the extent to which compounds are broken down during biological treatment without adversely affecting the normal treatment processes. Generally, readily biodegradable compounds are treatable but it is not the case for all inherently biodegradable compounds.

Ultimate biodegradability - The level of degradation achieved when a test compound is completely utilized by microorganisms resulting in the production of water, carbon dioxide, inorganic salts and biomass

1. INTRODUCTION

In an era of acute awareness of the need for environmental and human health protection, there is an increasing demand for organic chemicals entering the aquatic environment to be completely degraded to harmless chemical components such as carbon dioxide, water and inorganic salts. The extent and ease with which this is possible in the natural environment or in waste treatment plants can be established by means of biodegradability tests.

Several biodegradability tests have been developed, evaluated and standardized during the past fifteen years (King, 1981; Means and Anderson, 1981, Gerike, 1984; Blok *et al.,* 1985; De Morsier *et al.,* 1987; Birch and Fletcher, 1991; Nyholm, 1991). Most of the methods measure aerobic degradation. The different types of aerobic tests include i) ready biodegradability tests, also known as screening tests, and which are the most stringent tests ii) inherent biodegradability tests where practical conditions more closely resemble environmental conditions, and iii) simulation tests where laboratory scale activated sludge units are used (Nyholm, 1991). Tests for anaerobic biodegradation are currently receiving attention (ASTM, 1987; Battersby and Wilson, 1988; Birch *et al.,* 1989; and Wang and Latchaw, 1990). This type of degradation occurs in anaerobic treatment plants, sediments and ground water. For biodegradation of chemicals in surface waters, die-away tests are usually applied using natural waters and $\mu g/\ell$ levels of C^{14}-labelled test compounds (Nyholm, 1991; Ribas *et al.,* 1991).

Biodegradation studies in South Africa mostly involve simulation tests on industrial waste in laboratory scale activated sludge units. Very few laboratories are equipped to carry out standardized, ready biodegradability tests for chemical products. In addition, no tests [apart from the 5-day biochemical oxygen demand (BOD) test] have been established to evaluate the biodegradation of chemical compounds in natural waters. With the increased production of new chemical products which could find their way into the water environment the demand for such testing is rapidly increasing.

The aim of this project was to establish short-term aerobic screening tests to assess the ultimate biodegradability of chemical products and organic pollutants under typical conditions in the aquatic environment. In order to achieve the objective, tests used in other countries were reviewed and suitable tests were evaluated in the laboratory by testing different chemical compounds, waters and effluents. Where necessary tests were modified/adapted for local use.

2. LITERATURE STUDY

2.1 Aerobic biodegradability tests

These tests can be divided into three groups (King, 1981; Nyholm, 1991):

(i) Ready biodegradability tests;
(ii) Inherent biodegradability tests; and
(iii) Simulation tests.

A tiered approach is usually followed, starting with the ready biodegradability tests, which are also called screening tests. The idea is that these tests should be simple and cheap and at the same time be stringent. In ready biodegradability tests the test conditions do not always correspond to the optimal conditions under which maximum biodegradation will occur. A positive result indicates biodegradability in most environmental situations (aerobic conditions). A positive result will frequently make further testing unnecessary, but does not rule out a need for further investigation if more quantitative information is required. **Negative results during ready biodegradability tests do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that further testing will be necessary to establish biodegradability.**

A test for inherent biodegradability will be required if ready biodegradability has not been demonstrated. Inherent (potential) biodegradability tests make use of practical conditions which more closely resemble environmental conditions and, therefore, have a high potential for degradation. A positive result does not guarantee biodegradability in natural environments, while a negative result indicates possible environmental persistence.

Simulation tests provide information on biodegradation rates in specific environments under environmentally realistic conditions.

2.1.1 Ready biodegradability tests

A number of standard tests have been adopted or proposed for the ready biodegradability testing of organic chemicals and water/effluents containing such chemicals (King, 1981; Nyholm, 1991). These tests are carried out by adding microorganisms to the organic chemical solution in question. Test solutions are prepared with mineral medium containing trace elements. In some instances the solution is supplemented with essential vitamins or a small amount of yeast. The test compound is added in concentrations ranging from 2 to 100 mg/ℓ and serves as the sole source of carbon and energy. Degradation is, therefore, only possible if the microorganisms can grow on the test material as their primary substrate or if they acquire this ability by adaptation. The test mixture is incubated in the dark or in diffuse light at 20 to 25° C under aerobic conditions for a period of 28 days to allow for total utilization of the test sample (ultimate biodegradation). Except in one test, the closed bottle test, test solutions are continuously aerated, shaken or stirred. Biodegradability is measured in terms of die-away [*e.g.* dissolved organic carbon (DOC)] and/or the production of breakdown products [*e.g.* carbon dioxide (CO_2)]. A degradation curve has an initial part with no or slow degradation which is called the lag phase, and then assumes a sigmoid shape (Nyholm, 1991). The lag phase is often variable and poorly reproduced and is defined as the period from inoculation until degradation has increased to about 10%. Results are reported in terms of percentage biodegradation after time, expressed as percent DOC removal, or in respirometric tests as BOD as a percent of TOD or CO_2 production in percent of TCO_2, where

TOD and TCO_2 are the theoretical oxygen demand and carbon dioxide evolution as estimated from the formula of the compound (Nyholm, 1991).

The different methods currently recommended by the European Economic Community (EEC) are presented in Table 1. The EEC legislative test protocols are based to a large extent on Organization for Economic Cooperation and Development (OECD) test guidelines (OECD, 1981; revised 1992). Extensive studies (CEC Environmental Consumer Protection Service, 1979; OECD, 1979; Gerike and Fisher, 1979; 1981) have shown that there is a poor comparability between the recommended EEC methods as well as a poor reproducibility between individual methods with varying lag times as the main problem. Following the

TABLE 1: Current or proposed European Economic Community screening tests for ready biodegradability testing (Nyholm, 1991)

Name of test	Microbial source	Approximate cell density (cfu[a]/mℓ)	Test substance concentration (mg/ℓ)	Parameter measured	Technical description
Closed bottle[b]	Secondary effluent or soil extract	0.25×10^2	2-10	O_2[c]	BOD test
Japanese MITI[b,d]	Sludge grown on peptone/glucose	$0.2\text{-}1 \times 10^6$ (30 mg SS[e]/ℓ)	100	CO_2 evolution and final DOC	Respirometric test in BOD meter
Modified OECD[b]	Secondary sewage effluent, surface water or soil extract	$0.5\text{-}2.5 \times 10^2$	10-80	DOC	Shake flask test
Modified AFNOR[b,f]	Polluted surface water and secondary effluent (filtered and resuspended)	$5 \pm 3 \times 10^5$	80	DOC	Shake flask test
Sturm[b]	Sludge supernatant	$10^4\text{-}10^5$	5-20	CO_2 evolution and final DOC	Respirometric test in CO_2 scrubbing apparatus
UK-MITI[g]	Sludge	$0.2\text{-}1 \times 10^6$ (30 mg SS/ℓ)	100	CO_2 evolution and final DOC	Respirometric test in BOD meter
Repetitive die-away (proposed method)[h]	Sludge	$0.2\text{-}1 \times 10^6$ (30 mg SS/ℓ)	Approximately 50 (100 mg TOD/ℓ)	O_2 uptake and DOC	Respirometric test in closed bottle with shaking

a Colony forming unit
b Commission of the European Communities (1987a)
c Oxygen
d Ministry of International Trade and Industry
e Suspended solids
f Association Francaise de Normalization
g Commission of the European Communities (1987b)
h Commission of the European Communities (1987c)

recommendations of these studies (Blok *et al.*, 1885) the OECD started to update the tests presented in Table 1 for further harmonization (OECD, 1990). As a result six different tests are now proposed by the OECD (1992) (Table 2).

TABLE 2: Ready biodegradability tests proposed by the Organization for Economic Cooperation and Development (Nyholm, 1991)

Name of test	Microbial source	Approximate cell density (cfu[a]/mℓ)	Test substance concentration	Parameter measured	Technical description
Closed bottle	Secondary effluent	$10\text{-}10^3$ (≤5 mℓ/ℓ)	2-10 mg/ℓ	O_2[b]	BOD test
Japanese MITI	Activated sludge	30 mg SS[c]/ℓ	100 mg/ℓ	CO_2 evolution and final DOC	Respirometric test in BOD meter
Modified OECD DOC die-away	Secondary sewage effluent	Approximately 10^2 (0.5 mℓ/ℓ)	10-40 mg DOC/ℓ	DOC	Shake flask
ISO standard method[d]	Activated sludge	$10^4\text{-}10^5$ (15-30 mg SS/ℓ or 50-100 mℓ/ℓ)	10-40 mg DOC/ℓ	DOC	Shake flask
CO_2 evolution test	Sludge supernatant	$10^4\text{-}10^5$	10-20 mg DOC/ℓ	CO_2 evolution and final DOC	Respiration in CO_2 scrubbing apparatus
Respirometric test	Activated sludge	$10^4\text{-}10^5$ (15-30 mg SS/ℓ or 50-100 mℓ/ℓ)	100 mg/ℓ	O_2 uptake	Manometric method

a Colony forming unit
b Oxygen
c Suspended solids
d International Organization for Standardization (ISO, 1984)

2.1.1.1 Evaluation of the recommended EEC methods

Test sample concentration

The concentrations of test substances are far above most expected environmental concentrations, but is necessitated by the limited sensitivity of the test parameters. At such high concentrations toxicity is possible, inhibiting the biodegradation process. In order to avoid toxic effects some of the methods state that the test concentration should be as low as the analytical capability permits. When high concentrations are used difficulty can also be experienced with solubility in case of sparsely soluble compounds. Such problems could be overcame by application of the substance on inert carriers, *e.g.* silica gel or by solubilization with an emulsifier or ultrasonic treatment (de Morsier *et al.*, 1987; Gerike, 1984; Nyholm, 1990), and applying manometric respirometric tests.

Inoculum

The inoculum concentration used by the various tests differs widely. This is also true for the

source and treatment of the inoculum. Activated sludge or secondary effluent are mostly used. In the modified OECD test and the closed bottle test surface water or soil extract is also used. It is important that the inoculum has a high diversity of species. The inocula specified in the different tests have been proved to be sufficiently low to ensure that respiration or carbon release does not interfere significantly with test measurements. For this reason a lower inoculum is used in the closed bottle test.

Usually biodegradation increases with an increased inoculum concentration (Gerike and Fisher, 1979; Painter and King, 1983; Blok and Booy, 1984). The Japanese MITI test is, however, an exception. For this test, sludge is collected from 10 different sources and grown in a peptone/glucose synthetic sewage substrate. Although mixing of inocula from various sources should increase the microbial diversity, cultivation appears to reduce the diversity to an extent where the biodegradation potential is weakened. In comparison only the closed bottle test using a low inoculum and no agitation seems to be more stringent.

In biodegradation tests where the substrate is toxic, lag times are much longer and growth rates comparably smaller. Because only a small part of the inoculum might be specific degraders this concentration becomes critically low, particularly if the inoculated biomass is small. As the inoculum concentration may differ largely between the various tests (more than a factor of 10^4), it is expected that their stringencies will differ substantially (Nyholm, 1991).

2.1.1.2 Evaluation of methods proposed by the OECD

The inoculum used in the tests may be derived from a variety of sources, including activated sludge, sewage effluent, surface waters and soils, or from a mixture of these and may be preconditioned by aeration for 5 to 7 days prior to use (Nyholm, 1991). Preconditioning reduces blank values and might improve reproducibility.

Comparison studies between the different tests showed that the ISO test was the most reproducible, showing a consistent degradation of test substances, while the results of the other tests were scattered (OECD, 1989). The closed bottle test produced the most variable results.

2.1.1.3 General comments on standard ready biodegradability tests

The pre-adaptation of the inoculum to the test substance prior to the test has been proposed by several authors to enhance reproducibility (Larson, 1983; Painter, 1987). This has however gained little support within the EEC and the OECD, because this will result in less stringent testing. This is also the argument against the use of high inoculum concentrations (30 mg suspended solids (SS)/ℓ).

Some studies have shown that in certain instances when screening tests showed negative results, those very chemicals can be biodegraded when added in the C^{14}-labelled form in $\mu g/\ell$ concentrations to natural water samples (Larson, 1983; Shimp and Young, 1987). Such results suggest that current ready biodegradability tests are artificially stringent. Biodegradation at low concentrations probably take place by co-metabolism (Boethling and Alexander, 1979; Nyholm, 1991), because the test compound is now the secondary substrate which is present in much lower concentrations than the natural occurring organic substances serving as the dominant energy and carbon source. Another explanation is that at low concentrations oligotrophs proliferate and that such organisms have a larger variety of pathways for degradation than eutrophs, which grow more easily at high substrate

concentrations (Alexander, 1985). Furthermore, low substrate concentrations rule out the possibility of toxic activity.

2.1.2 Inherent biodegradability tests

There are two standard tests for inherent biodegradability testing:

(i) Zahn-Wellens test

This test has been standardized (DIN, 1984) and is widely used in Germany (Zahn and Wellens, 1974). The test is similar to the ISO standard test (ISO, 1984). Tests are carried out in batch form in 2 ℓ volumes, using 3-4 ℓ reactors. The test makes use of activated sludge as inoculum. A mixture of microorganisms originating from several sources can also be used. Prior to use the activated sludge/microorganism mixture is washed in the test water by means of centrifugation and settling. The sludge is used at a concentration of 1 g SS/ℓ test solution. Test substance concentrations range between 50 and 400 mg DOC/ℓ [100 to 1 000 mg (chemical oxygen demand) COD/ℓ]. Tests are carried out using continuous stirring and aeration and die-away is measured in terms of DOC or COD. The test has a high potential for biodegradation because of the high biomass. Because of the high biomass, blank DOC readings range from 5 to 10 mg/ℓ.

(ii) Semi-continuous activated sludge (SCAS) test

This test involves a one day fill and draw cycle (American Soap and Detergent Association, Biodegradation Subcommittee, 1965). Fresh sludge and a known concentration of the test compound is added once a day after settling of the sludge and decanting two thirds of the supernatant liquor. Activated sludge is used at a concentration of 1-4 g SS/ℓ solution. Continuous aeration is applied and degradation is established by means of DOC analyses. Normally the added sewage is mineralized within 8 h, leaving 16 h for endogenous respiration and degradation of the test compound. This procedure offers great opportunity for adaptation and the test/adaptation period may be as long as six months. For greater sensitivity or to eliminate toxicity C^{14}-labelled compounds (low concentrations) may be used.

If the SCAS test is negative and the possibility of toxicity is ruled out, indications are that the chemical is persistent. No further testing with a simulation test will be required. The Zahn-Wellens test does not show the same adaptability and a negative result will not rule out degradation under adapted conditions. It has been recommended that the Zahn-Wellens test should be carried out with low concentrations of the test chemical (μg/ℓ range) to provide a final proof of environmental persistence (Nyholm, 1991).

2.1.3 Simulation tests

The only officially accepted simulation test so far is a sewage treatment test (Commission of the European Communities, 1987) which is a modification of the OECD confirmatory test for detergents (OECD, 1970). Tests are carried out in laboratory scale activated sludge units. Synthetic (peptone) sewage and a high concentration of test substance is used. The units are operated at a hydraulic retention time of 3 to 6 h, and a sludge retention time of 6 days.

Limitations of the test include:

Data are obtained for only one set of operational conditions;
Synthetic sewage is used;
There is no reinoculation; and
The concentration of the test chemicals are much higher than normally found in municipal sewage (Nyholm, 1991).

Simulation tests are also highly relevant for a variety of other environments (rivers, estuaries, sea, soil). Experiences with simulation tests are relatively limited and agreement on principles and concepts has not yet been reached (Nyholm, 1991). For degradation studies in surface water no official EEC/OECD simulation method has been adopted but die-away tests with natural waters using $\mu g/\ell$ levels of C^{14}-labelled test substances have been used for some time.

Tests for degradation in aerobic sediments using suspended sediment material or sediment cores are also in use.

2.2 Anaerobic biodegradability tests

There is an increasing interest in biodegradation under anoxic and anaerobic conditions. Methods described in literature (Birch *et al.*, 1989) are usually based on anaerobic respirometric methods. They involve the measurement of methane and carbon dioxide which are the final products of anaerobic biodegradation. Gas production is monitored by using gastight syringes with pressure transducers (Shelton and Tiedje, 1984) or sealed serum bottles (Battersby and Wilson, 1988; Birch *et al.*, 1989, Wang and Latchaw, 1990) The most straightforward method to investigate anaerobic biodegradability is the use of C^{14}-labelled substances. In such tests the detection of radioactive methane and carbon dioxide provides evidence of ultimate biodegradation.

Only one standard test has been described. The method determines the conversion of organic substances into methane and carbon dioxide (ASTM, 1987). Tests are carried out in serum bottles, using 10% primary anaerobic digester sludge for inoculation. The test substance is added at a concentration of 50 mg/ℓ as organic carbon.

3. MATERIALS AND METHODS

3.1 Glassware

All the glassware used in contact with test/control samples were carefully cleaned to avoid any trace of organic or toxic matter. The cleaning process was carried out in the following sequence:

Wash with soap and water, rinse with tap water and air dry;
Rinse with chromic acid, rinse with tap water and air dry;
Rinse with acetone, rinse with tap water followed by water prepared via reverse osmosis (RO) (Millipore) and air dry;
Bake in oven at 180°C; and
If required, autoclave.

3.2 Biodegradability tests

Three different aerobic ready biodegradability (also known as ultimate biodegradability) tests were selected for evaluation. The tests included: dissolved organic carbon (DOC) reduction tests; a carbon dioxide (CO_2) production test; and an oxygen (O_2) depletion test. The DOC reduction tests apply to organic compounds which are soluble under the test conditions; which are non-volatile; not significantly adsorbable on glass; and not inhibitory to microorganisms at the concentration selected for testing. The scope of the CO_2 production and O_2 depletion tests are the same as that of DOC reduction tests, except that they are also applicable to insoluble compounds. In principle, the O_2 depletion test may also be used for volatile compounds. The concentration of the organic compounds used in DOC reduction and CO_2 production tests is normally between 10 and 40 mg/ℓ. The O_2 depletion test is applicable to test concentrations of 2 (standard concentration) to 10 mg/ℓ.

3.2.1 DOC reduction test

3.2.1.1 Method based on ISO standard test (1984)

Biodegradation tests were carried out in 3 ℓ Erlenmeyer flasks. Each flask contained 1 ℓ of test/control solution at the start of a test. Test solutions were prepared by adding the required inorganic nutrients (Table 3) and the organic test compounds (10 and 40 mg/ℓ C) to Milli-Q (Millipore) water. Control solutions contained only the inorganic nutrients. Possible inhibitory activity of test compounds was examined by adding a reference chemical (known organic compound, same concentration as test compound) to the test solution. Abiotic degradation (non-biological removal) was examined by adding $HgCl_2$ (1 mℓ of a 1 g/ℓ solution) to test solutions. All the tests/controls were carried out in duplicate. Only one flask each was included to examine inhibition and abiotic degradation.

Activated sludge was used as inoculum. The sludge was taken from the aeration tank at the Daspoort sewage works in Pretoria, which treats predominantly domestic sewage. The sludge was kept aerobic until use on the day of collection. Before use the concentration of suspended solids (SS) was determined. 25 mℓ of the well mixed activated sludge were filtered (Whatman glass microfibre filters) into weighed crucibles (duplicate), dried at 115°C for 3 h, and weighed. The well mixed activated sludge was added to the test and control solutions (except abiotic solution) at a concentration of approximately 30 mg SS/ℓ (10^3 to 10^5 colony forming units (cfu)/mℓ). This volume was always <1% (<10 mℓ/ℓ) to minimize the

TABLE 3: Inorganic nutrients used in the DOC reduction test (ISO, 1984)

Nutrient stock solution	Volume (mℓ) of stock solution added to 1 ℓ test/control solution	Reagent	Concentration[a] (g/ℓ)
A (pH: 7.2)	10	KH_2PO_4 K_2HPO_4 $Na_2HPO_4.2H_2O$ NH_4Cl	8.5 21.75 33.4 2.5
B	1	$MgSO_4.7H_2O$	22.5
C	1	$CaCl_2$	27.5
D (freshly prepared just before use)	1	$FeCl_3.6H_2O$	0.25
E	1	$MnSO_4.H_2O$ H_3BO_3 $ZnSO_4.7H_2O$ $(NH_4)_6Mo_7O_{24}.4H_2O$ $C_{10}H_{12}N_2O_8Fe(III)Na$	0.03023 0.0572 0.0428 0.03685 0.1
F (freshly prepared just before use)	1	Yeast extract	0.015[b]

a Prepared with Milli-Q water

b Per 100 mℓ of water

introduction of organic carbon into solutions (<10% of DOC of test compound). When the SS was low, the activated sludge was first concentrated by settling to reduce the inoculum volume.

Flasks were covered with aluminium foil and maintained at 22±2° C in diffused light (to avoid photodegradation and algal growth). Solutions were gently aerated to allow aerobic conditions and mixing. Samples for abiotic testing were not agitated. Samples for the determination of DOC were taken at the beginning of the test (day 0), at the end of the test (28 days), and at a number of intermediate intervals. When a test solution reached the maximum level of biodegradation before the end of the test, the test was considered to be finished and sampling of the solution was stopped. The DOC of the abiotic solution was determined at the end of the test. In order to compensate for water loss due to evaporation, the volume of the solutions was checked before each sampling and if necessary made up with Milli-Q water to the volume measured after the previous sampling (marked off on flasks). 20 mℓ of solution were taken at each sampling occasion. 15 mℓ of these portions were filtered into stoppered glass bottles using a syringe and a membrane filter (0.5 μm, SLCR, Millipore) which allows minimal adsorption and release of organic carbon. Samples were analyzed immediately. In the few instances where analysis was postponed (no longer than 24 h) samples were kept in the dark at 4° C.

Analysis was carried out with the AQUADOC™ TOC/DOC analyzer which was designed and developed by Dr Ronald van Steenderen of the Division of Water Technology (Watertek), CSIR. The analytical technique used in the AQUADOC™ is based on the ultra-violet photo/catalytic oxidation of organic material to carbon dioxide and the detection thereof by infrared spectroscopy (Standard Methods for the Examination of Water and Waste Water, 1992). The AQUADOC™ was DIN certified at the Technical University of München in April 1992 according to DIN Certification 38409, Part 3.

3.2.1.2 Method based on the modified OECD procedure (1981b)

The test was carried out in the same way and under the same experimental conditions as the method based on the ISO standard test (1984). The only differences were the use of river water as inoculum instead of activated sludge to have a low microbial concentration (final concentration: approximately 10^2 cfu/mℓ); 1 mℓ of nutrient solution A/ℓ of test/control solution instead of 10 mℓ (Table 4); and a higher NH_4Cl concentration (20.0 g instead of 2.5 g) in the nutrient stock solution A. Apies river water, sampled above the discharge point of the Daspoort sewage works, was used as inoculum.

A revised version of the 1981 OECD method appeared in 1992. The ammonium chloride concentration in stock solution A has been reduced to 0.5 g/ℓ, and 10 mℓ of stock solution A instead of 1 mℓ is used for medium preparation. Furthermore, the test uses 0.5 mℓ/ℓ secondary sewage effluent for inoculation (OECD, 1992c).

TABLE 4: Inorganic nutrients used in DOC reduction test (OECD, 1981b)

Nutrient stock solution	Volume (mℓ) of stock solution added to 1 ℓ test/control solution	Reagent	Concentration[a] (g/ℓ)
A (pH: 7.2)	1	KH_2PO_4 K_2HPO_4 $Na_2HPO_4.2H_2O$ NH_4Cl	8.5 21.75 33.4 20.0
B	1	$MgSO_4.7H_2O$	22.5
C	1	$CaCl_2$	27.5
D (freshly prepared just before use)	1	$FeCl_3.6H_2O$	0.25
E	1	$MnSO_4.H_2O$ H_3BO_3 $ZnSO_4.7H_2O$ $(NH_4)_6No_7O_{24}.4H_2O$ $C_{10}H_{12}N_2O_8Fe(III)Na$	0.03023 0.0572 0.0428 0.03685 0.1
F (freshly prepared just before use)	1	Yeast extract	0.015[b]

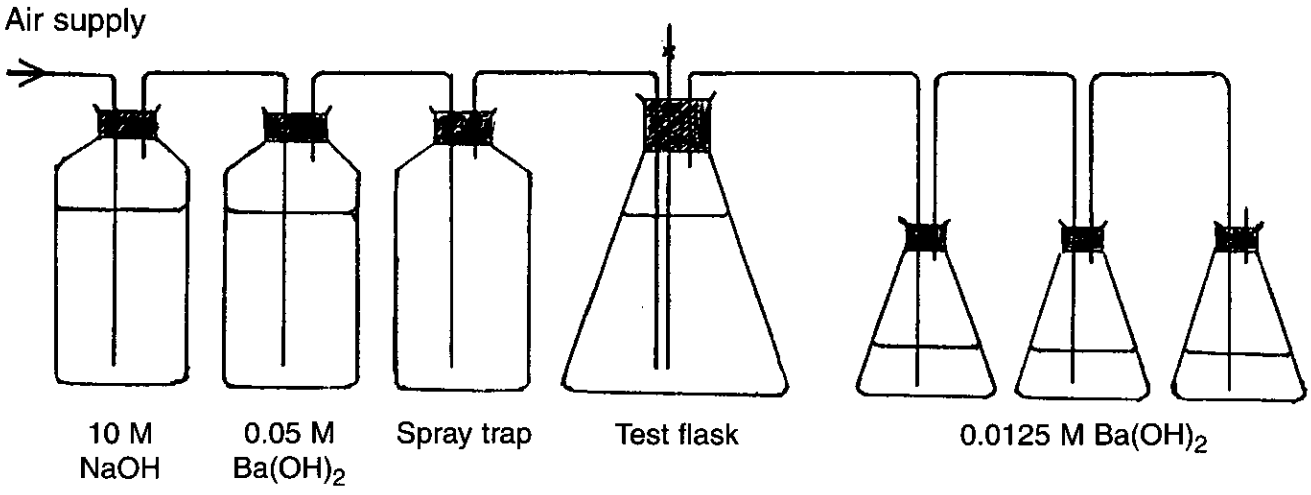
a Prepared with Milli-Q water

b Per 100 mℓ of water

3.2.2 CO_2 production test

The procedure is based on the ISO standard test (1990) and the OECD CO_2 evolution method (1992a). The test is also known as the Sturm test. Biodegradation tests were carried out in 1 ℓ Erlenmeyer flasks (reaction flasks). Each reaction flask was connected to two sets of scrubbing devices (Figure 1). The scrubbing device ahead of a flask ensured that the air used for aeration of test and control flasks was CO_2-free before it enters the flask and the second was used to absorb the CO_2 formed as biodegradation product. Each front-end scrubber consisted of one bottle containing 750 mℓ 10 M sodium hydroxide (NaOH) and a second bottle containing 750 mℓ 0.05 M barium hydroxide [$Ba(OH)_2$]. An empty bottle was placed between the scrubber bottles and the reaction flask to serve as spray trap. The back-

(a)



(b)

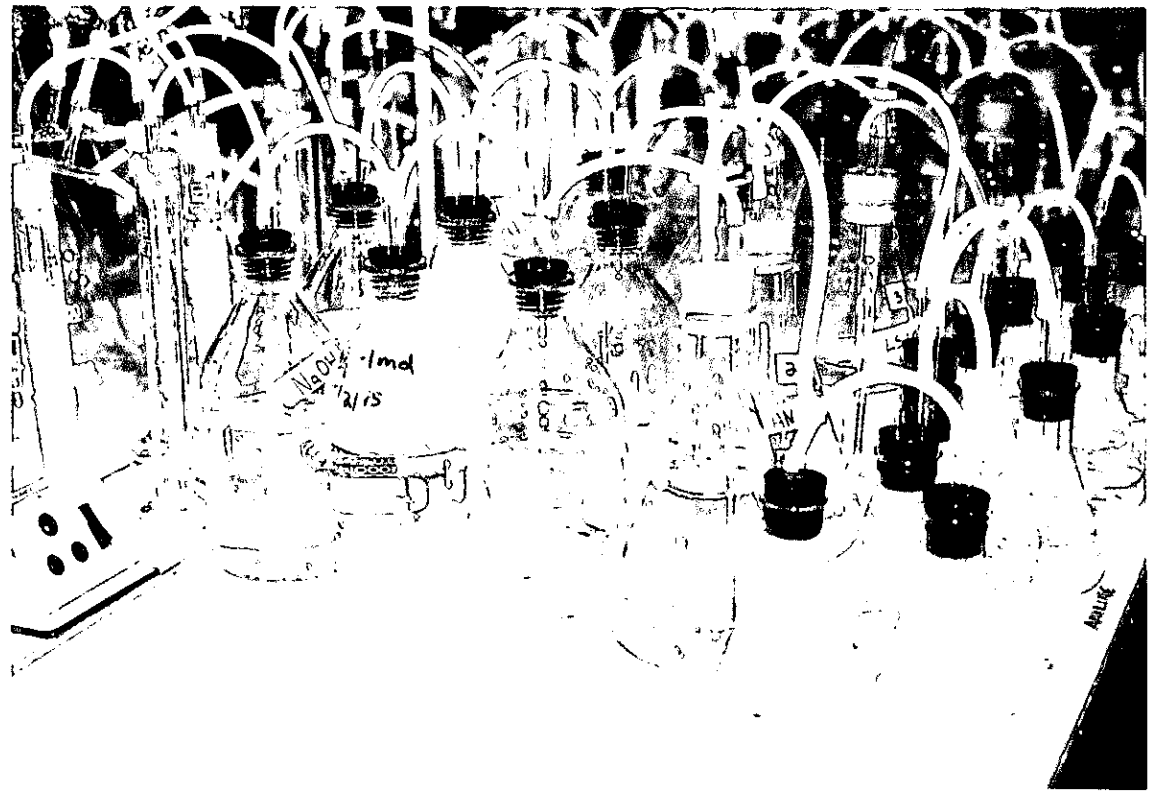


FIGURE 1: Diagram (a) and experimental set-up (b) for the CO_2 production test

end scrubber consisted of three 250 mℓ Erlenmeyer flasks (CO_2-absorbers), each containing 100 mℓ 0.0125 M $Ba(OH)_2$. Milli-Q water through which nitrogen gas was bubbled to remove CO_2 was used to prepare the 0.0125 M $Ba(OH)_2$. All the flasks/bottles were fitted with rubber stoppers containing glass in- and outlets, interconnected with silicon tubing. The ends of the glass tubes were narrowed to ensure small air bubbles. Sintered glass tubes were used in the NaOH bottles only [$Ba(OH)_2$ and reaction mixtures will clog sinters]. Thymol blue was added to the NaOH as indicator (colour change from blue to yellow) for CO_2 saturation (final concentration: 10 mg/ℓ).

Each reaction flask contained 1 ℓ of test/control solution. Tests and controls were carried out in duplicate. Possible inhibition of test chemicals was examined by including one flask containing the test compound and the reference chemical (known organic compound, same concentration as test compound).

Tests were started by adding only the nutrient solutions (Table 5) to Milli-Q water in all the reactions flasks. The control solution was made up to 1 ℓ. Test solutions (40 mg/ℓ C) contained 40 to 120 mℓ less solution to allow for the later addition of stock solutions of test compounds. If chemicals were added directly (insoluble chemicals) the test solution was made up to 1 ℓ. Hereafter, all the flasks were inoculated with activated sludge (30 mg SS/ℓ - 10^3 to 10^5 cfu/mℓ). This volume was always <1% (<10 mℓ/ℓ) to minimize the introduction of organic carbon into solutions (<10% of DOC of test compound). Activated sludge was taken from the aeration tank at the Daspoort sewage works in Pretoria. The sludge was kept aerobic until use on the day of collection. Before use the concentration of SS was determined as described in paragraph 3.2.1.1. When the SS was low, the activated sludge was concentrated by settling, to reduce the inoculum volume.

After mixing, reaction flasks were connected to the scrubber devices. The CO_2-absorbers were left empty. The reaction flasks were aerated with CO_2-free air (flow rate: 50 - 100 mℓ/min) for 24 h to purge the system of CO_2. After the aeration period (next day), the CO_2-absorbers were filled with $Ba(OH)_2$ and reconnected to the reaction flasks while aeration continued. The required volumes of the test compounds (40 mg/ℓ C) were then carefully added to the reaction flasks. Incubation took place for 28 days at 22 ± 2° C in diffused light. The air flow rate was regularly checked (50-100 mℓ/min). The amount of CO_2 released from each reaction flask was measured on day 1, day 28, and at a number of intermediate intervals.

TABLE 5: Inorganic nutrients used in CO_2 production test (ISO, 1990; OECD, 1992a)

Nutrient stock solution	Volume (mℓ) of stock solution added to 1 ℓ test/control solution	Reagent	Concentration[a] (g/ℓ)
A (pH: 7.4)	10	KH_2PO_4 K_2HPO_4 $Na_2HPO_4.2H_2O$ NH_4Cl	8.5 21.75 33.4 0.5
B	1	$MgSO_4.7H_2O$	22.5
C	1	$CaCl_2$	27.5
D (freshly prepared just before use)	1	$FeCl_3.6H_2O$	0.25

a Prepared with Milli-Q water

If a constant level of degradation was obtained before the end of the 28 day test period, a test was considered to be completed.

The amount of CO_2 released and trapped in the $Ba(OH)_2$ was measured by means of a titrimetric method. The CO_2 reacts with the $Ba(OH)_2$ and is precipitated as barium carbonate ($BaCO_3$). The amount of CO_2 produced is determined by titrating the remaining $Ba(OH)_2$ with 0.05 M hydrochloric acid (HCl) [50 mℓ HCl would be needed to titrate 100 mℓ $Ba(OH)_2$].

The CO_2-absorber nearest to the reaction flask was removed for titrations. This was done before significant precipitation of $BaCO_3$ occurred in the second absorber [approximately every second day for the first 10 days (OECD, 1992a) and every 5 days thereafter]. Without stopping aeration, the stoppers on the bottles were loosened starting with the bottle farthest from the reaction flask. By doing this the pressure was released preventing solutions to run into other containers. The remaining two absorbers were rapidly moved one position closer to the reaction flask. A new absorber containing 100 mℓ $Ba(OH)_2$ was placed at the end of the series of flasks. The $Ba(OH)_2$ solution was immediately filtered through a 0.45 μm membrane filter (Millipore) to remove the precipitate. Milli-Q water was used for rinsing. The clear solution containing the remaining $Ba(OH)_2$ was titrated with HCl, using a few drops of a phenolphthalein solution as indicator (1 g dissolved in 100 mℓ ethanol, diluted with 100 mℓ Milli-Q water).

In addition to CO_2, DOC was also analyzed. The samples were taken through a third glass tube in the reaction flask stopper, using a syringe. Samples were prepared as described in paragraph 3.2.1.1. Because of limited evaporation it was not necessary to add water to the solutions in reaction flasks to compensate for water loss. Samples were taken on day 0 and 28, and at a few intermediate intervals.

3.2.3 O_2 depletion test

This method is based on the OECD (1981a) O_2 depletion test and is also called the closed bottle test. The test was carried out in 300 mℓ BOD bottles (280-330 mℓ) with glass stoppers. Each sample was placed in a series of bottles to enable oxygen determinations after various incubation periods. Test solutions contained nutrients (Table 6) and the test compound (2 and 10 mg/ℓ C), while the control contained the nutrients only. In addition to the normal control, a blank control was included in each study which received no inoculum. One bottle of each series was used to examine abiotic degradation.

A sufficient volume of test and control solution to fill a series of bottles (2 to 4 ℓ) was prepared in 5 ℓ glass containers with self-made litre marks. Test and control solutions were prepared by filling the 5 ℓ containers with Milli-Q water to about 75% of the required volume. The remaining water was kept in an additional glass container. The Milli-Q water in all the containers was then strongly aerated by compressed air for 20 min to super-saturate. After standing for 18 to 20 h (next day) at 20° C the water was ready to prepare the test and control solutions. The individual nutrient stock solutions were pipetted into the containers according to the required final volumes. Hereafter, test compounds were added to the respective test containers. Pipette tips were held against the wall of the containers, close to the surface of solutions to avoid the introduction of bubbles. When measuring cylinders were used for adding solutions, the containers were held at an angle so that the liquid ran down the container wall into the solution. Subsequently, all the solutions except the blank control were inoculated with 1 drop/ℓ (pointed pipette - approximately 0.05 mℓ) of filtered activated sludge effluent, taken from the Daspoort sewage works. The effluent was kept aerobic until use on

TABLE 6: Inorganic nutrients used in O_2 depletion test (OECD, 1981a)

Nutrient stock solution	Volume (mℓ) of stock solution added to 1 ℓ test/control solution	Reagent	Concentration[a] (g/ℓ)
A (pH: 7.2)	1	KH_2PO_4 K_2HPO_4 $Na_2HPO_4.2H_2O$ NH_4Cl	8.5 21.75 33.4 1.7
B	1	$MgSO_4.7H_2O$	22.5
C	1	$CaCl_2$	27.5
D (freshly prepared just before use)	1	$FeCl_3.6H_2O$	0.25

a Prepared with Milli-Q water

the day of collection. The effluent was filtered just before inoculation through coarse filter paper (Whatman). The first 200 mℓ of filtrate was discarded. The rest of the filtrate was kept aerobic until inoculation. Finally, solutions were made up to the final volumes with the remaining Milli-Q water. The water was transferred by means of siphoning (bubble-free) using a silicone tube. The tube reached down to the bottom of containers to achieve adequate mixing.

When water insoluble compounds were tested these were dissolved in acetone and then a small volume (2 mg/ℓ C) was pipetted into each of a series of test bottles a few hours before a test started. In addition, the same volume of acetone was introduced into another series of bottles as control test for the acetone test solutions. The bottles were left open for acetone to evaporate. The nutrient solutions with inoculum were prepared in 5 ℓ containers as described above.

Immediately after preparation, the solutions were transferred to the respective series of bottles by means of a tube and siphoning (bubble-free). During transfer, the end of the tube was held in the lower quarter of solutions in the 5 ℓ containers (not touching the bottom). Bottles for abiotic testing received 1 mℓ of a 1 g/ℓ $HgCl_2$ solution. Air was excluded when placing stoppers on bottles. One bottle of each series was immediately removed to determine the dissolved oxygen at day 0. The remaining bottles were incubated at 20±1°C in the dark (Figure 2). Duplicate bottles of each series were removed after various incubation periods, until day 28, for oxygen determinations. Abiotic samples were analyzed at the end of the test. When a test solution reached the maximum level of biodegradation before the end of the test, the test was considered to be finished and analysis was stopped.

Dissolved oxygen was determined by means of a iodometric method following the azide modification of the Winkler test (Standard Methods for the Examination of Water and Waste Water, 1989). The reagents used in the test are presented in Table 7. The method was carried out by adding 1 mℓ $MnSO_4$ solution to the sample in a BOD bottle, followed by 1 mℓ of the alkali-iodide-azide solution, holding pipet tips just above the liquid surface. Bottles were carefully stoppered to exclude air bubbles and mixed by inverting the bottle a few times. When the precipitate (manganese hydroxide floc) has settled to about half the volume of the bottle to leave a clear supernatant, 1 mℓ of H_2SO_4 was added. The bottle was re-stoppered and mixed by inverting until dissolution was complete. 200 mℓ of this solution were titrated

with 0.025 M $Na_2S_2O_3$ solution, after addition of a few drops of starch. Titration was completed when the blue colour disappeared. For titration of 200 mℓ of sample, 1 mℓ 0.025 M $Na_2S_2O_3$ = 1 mg/ℓ dissolved oxygen.

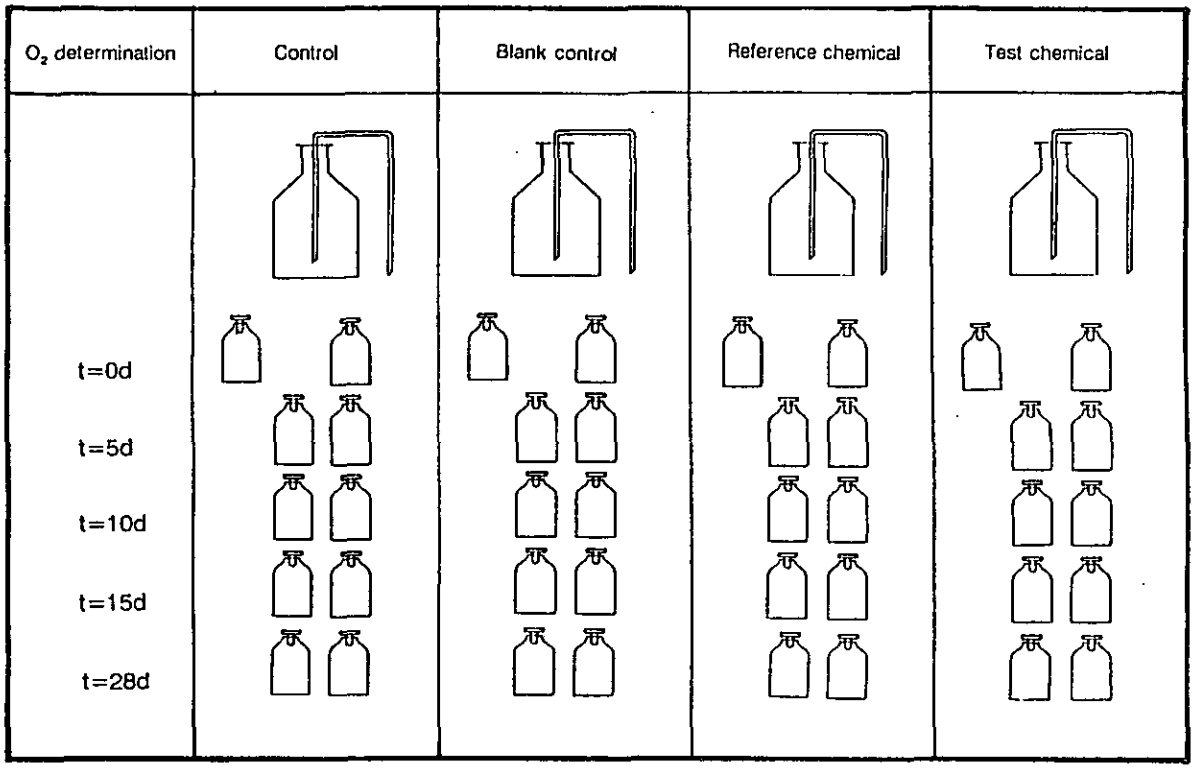


FIGURE 2: Example of bottle arrangement for the oxygen depletion test

TABLE 7: Reagents used for dissolved oxygen determination (Standard Methods for the Examination of Water and Waste Water, 1989)

Solution	Chemicals	Concentration (g/ℓ)[a]
Manganous sulphate	$MnSO_4.H_2O$	364
Alkali-iodide-azide	NaOH NaI NaN_3	500 135 10[b]
Sulphuric acid	H_2SO_4	concentrated
Starch	Soluble starch powder	2[c]
Sodium thiosulphate	$Na_2S_2O_3.5H_2O$ NaOH	6.205 0.4

a Prepared with Milli-Q water
b Per 40 mℓ water, added to 1 ℓ
c Per 100 mℓ of water

The OECD closed bottle test on which our method is based, was revised in 1992. The ammonium chloride concentration was changed from 1.7 g/ℓ to 0.5 g/ℓ. Instead of using a drop of the filtered sewage effluent per litre of medium, the method now specifies the use of one drop (0.05 mℓ) to 5 mℓ of the filtrate/ℓ medium (optimum volume should be established for an effluent). Another change in the method is the omission of the normal control (only blank control used) (OECD, 1992b).

3.3 Data analysis

3.3.1 DOC reduction test

The DOC data were used to calculate the percentage biodegradation at each sampling interval, using the equation:

$$\% \text{ Biodegradation, at time t} = \{1 - \frac{DOCT_t - DOCC_t}{DOCT_0 - DOCC_0}\} \times 100, \text{ where}$$

$DOCC_0$ is the average control DOC concentration at time 0;
$DOCT_0$ is the average test DOC concentration at time 0;
$DOCC_t$ is the average control DOC concentration at time t;
$DOCT_t$ is the average test DOC concentration at time t.

The % biodegradation can be plotted versus time. A typical biodegradation curve is shown in Figure 3 (ISO, 1984). The following parameters can be determined from the graph:

Lag time (t_1) (if sufficient data is available) - time (days) from day 0 until the degradation is 10%;
Maximum level of degradation - approximate level above which no further degradation takes place; and
Degradation time (t_2) - time (days) from the end of lag time t_1 until 90% of the maximum level of degradation has been reached.

The validity criteria given for the ISO (1984) test are as follows:

The difference between the degradation values of duplicate test flasks should be <20%;
The % degradation of a reference chemical (check for inoculum activity) should be ≥80% after 14 days; and
A chemical compound is toxic at the test concentration if the % biodegradation in the toxicity test flasks is <40% in 7 days.

The pass level for chemical substances for ready biodegradability in the ISO test is 80%, as stated for the reference chemical.

In the modified OECD test (1981b; 1992c) the % degradation of a reference chemical and test substance should be ≥70%. The biodegradation has to be reached in a 10-day window within the 28-day test period, counting from the day that 10% degradation (end of lag phase) is reached. The graph which is constructed for the data is the same as the ISO graph (Figure 3). The 10-day window should also be indicated. Additional parameters to be calculated are: the percentage removal at the end of the test, and/or after the 10-day window.

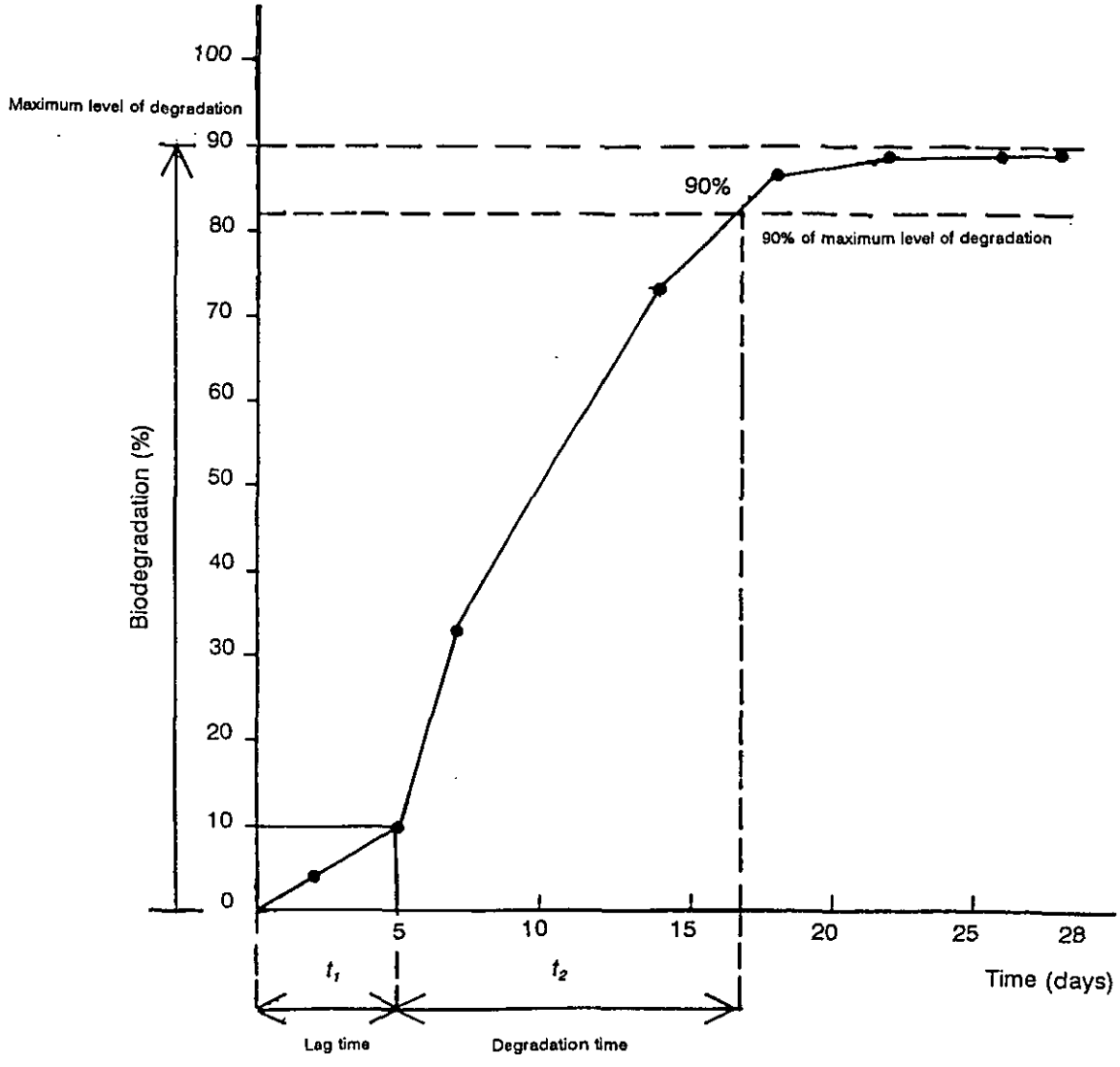


FIGURE 3: Typical biodegradation curve (ISO, 1984)

As in the ISO test, the difference between the biodegradation values of duplicate test flasks should be <20% to be valid. Furthermore, a chemical compound is considered to be toxic at a test concentration if the % biodegradation in the toxicity test flasks is <35% in 14 days (OECD, 1992).

3.3.2 CO_2 production test

Since 1 mmol of CO_2 is produced for every mmol of $Ba(OH)_2$ reacted to $BaCl_2$, and 2 mmol of HCl are needed for the titration of the remaining $Ba(OH)_2$, and given that the molecular mass of CO_2 is 44 g, the amount of CO_2 produced (mg) is calculated as follows (ISO, 1990; OECD, 1992a):

$$\text{mg } CO_2 = \frac{0.05 \times (50 - \text{m}\ell \text{ HCl titrated}) \times 44}{2}$$

= 1.1(50 - mℓ HCl titrated)

These values were calculated for tests and controls for each interval. The amount of CO_2 released by the test compounds during each interval was obtained by subtracting the average control value from the average test value for that interval. The percentage biodegradation for tests for each interval was then calculated using the following formula (OECD, 1992a):

$$\% \text{ Biodegradation} = \frac{\text{mg } CO_2 \text{ produced}}{TCO_2} \times 100$$

TCO_2 is the theoretical amount of CO_2 expressed in mg, and calculated as follows:

$TCO_2 = C \times V \times 3.67$, where

C is the concentration of test compound (mg/ℓ C);
V is the volume of test solution (ℓ); and
3.67 is the conversion factor (44/12) for carbon to CO_2.

The % biodegradation after any time interval (cumulative % biodegradation) was established by adding the % biodegradation calculated for each interval, to that time. The % biodegradation can be plotted as a function of time (see Figure 3). From the curve the lag time (t_1), the maximum level of degradation, the degradation time (t_2) (ISO, 1990), the percentage removal at the end of the test and/or after the 10-day window (OECD, 1992a) can be established (paragraph 3.3.1).

The validity criteria are as follows:

The CO_2 production in the control is insignificant and should not exceed 70 mg/ℓ (usually 30 to 40 mg/ℓ) at the end of the test (ISO, 1990; OECD, 1992a);
The % degradation of a reference chemical (check for inoculum activity) should be ≥50% after 14 days in the ISO test and ≥60% in the OECD test;
A chemical compound is toxic at the test concentration if the % degradation in the toxicity test flask is <25% in 28 days in the ISO test and <25% in 14 days in the OECD test; and
The difference between duplicate values should be <20% (OECD, 1992).

The % reduction in DOC was calculated according to paragraph 3.3.1.

3.3.3 O_2 depletion test

The O_2 depletion (also called the BOD) in test solutions is calculated at each measurement interval by the following formula (OECD, 1981a):

O_2 depletion (mg/ℓ) = (b - t) - (b - c), where

b is the average blank control oxygen concentration
t is the average test oxygen concentration, and
c is the average normal control oxygen concentration

From these results the % biodegradability is calculated by the formula:

$$\% \text{ Biodegradability} = \frac{\text{mg/}\ell\ O_2 \text{ depletion/mg/}\ell \text{ test substance}}{\text{TOD}} \times 100, \text{ where}$$

The following formula is used to calculate the TOD (theoretical oxygen demand) of a compound with structure $C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ OECD, 1981a):

$$\text{TOD} = \frac{16[2c + 0.5(h\text{-}cl\text{-}3n) + 3s + 2.5p + 0.5na - o]}{\text{molecular weight}}$$

This calculation implies that C is mineralized to CO_2, H to H_2O, P to P_2O_5, and Na to Na_2O. Halogen is eliminated as hydrogen halide and nitrogen as ammonia.

Results can be displayed by plotting the % biodegradation versus time as in Figure 3 to establish the lag time (t_1), the maximum level of degradation, and the degradation time (t_2).

The validity criteria given for the OECD (1981a) test are as follows:

> The oxygen depletion in the blank control should not exceed 0.2 to 0.3 mg O_2/ℓ after 5 days and 0.4 mg O_2/ℓ after 28 days;
> The oxygen depletion in the normal control should not exceed 0.4 to 0.5 mg O_2/ℓ after 5 days and 0.5 to 0.6 mg O_2/ℓ after 15 to 28 days;
> The % degradation of a reference chemical (check for inoculum activity) should be ≥60% within 28 days. This level must be reached within 10 days of biodegradation exceeding 10%, and
> If the oxygen depletion of the inhibition bottle corresponds to the sum of the oxygen depletion of the reference chemical and the test chemical, the test compound is not toxic.

The calculation in the revised bottle test (OECD, 1992b) basically remains the same as that of the 1981 test, except that the normal control values are omitted. The following two validity requirements have become less stringent, namely:

> The oxygen depletion in the blank control should not exceed 1.5 mg O_2/ℓ after 28 days; and
> The residual concentration of oxygen in the test bottles should not fall below 0.5 mg/ℓ at any time.

3.4 Test samples

3.4.1 Organic chemical compounds

The following organic chemicals (analytical grade) were tested:

> Aniline;
> Lauryl sulphate;
> Diethylene glycol;
> and stearic acid.

Stock solutions of 1 g C/ℓ of Milli-Q water were prepared in case of aniline and diethylene glycol (Table 8). Lauryl sulphate was insoluble at this concentration and, therefore, 0.5 g/ℓ C was used. Stearic acid was dissolved in acetone (reagent grade) at a concentration of 1 g/ℓ C for use in the O_2 depletion test. The solutions were kept at 4°C. Stearic acid was weighed off on a glass slide and added directly with the slide to test flasks when using the CO_2 production test.

TABLE 8: Organic chemicals used in biodegradation tests

Chemical	Empirical formula	Molecular weight	Stock solution (g/ℓ C)	Concentration of the chemical (g/ℓ)	mℓ stock solution added/ℓ to have 1 mg/ℓ C
Aniline	C_6H_7N	93.13	1	1.2935	1
Lauryl sulphate	$C_{12}H_{25}O_4SNa$	288.38	0.5	1.0014	2
Diethylene glycol	$C_4H_{10}O_3$	106.12	1	2.2109	1
Stearic acid (O_2 depletion test)	$C_{18}H_{36}O_2$	284.5	1	1.3172	1
Stearic acid (CO_2 reduction test)			40[a]	0.0527	used directly

a mg/ℓ

3.4.2 Water and effluent

Water samples were collected from the Apies river and Moreleta stream. The Apies river water was sampled upstream from the point of discharge of the Daspoort sewage works (designated Apies river 1) and from a bridge (Road 513) near Onderstepoort (designated Apies river 2). The Moreleta stream water was sampled near Eersterust from a bridge on the Baviaanspoort Road.

The following effluents were tested:

Paper mill effluent;
Steel works effluent;
Effluent from a food manufacturer;
Effluent containing cutting oils; and
Effluent containing pesticides.

The first three effluents are currently discharged into the aquatic environment. Paper mill effluent was sampled at Sappi, Springs. The sample was taken after secondary clarification. Secondary clarification takes place in an aeration lagoon for a period of 7 days. The steel works effluent was collected from Iscor and the food manufacturing effluent from Willards, both in Pretoria. The other two effluents were sampled at industries in the Gauteng area.

The composition of Apies river water (sample 1) and paper mill effluent is shown in Table 9.

TABLE 9: Composition of nutrient solutions, Apies river water 1 and paper mill effluent

Determinand	Sample					
	1	2	3	4	5	6
pH	8.0	7.7	7.8	8.0	8.4	7.6
Electrical cond. (mS/m)	79.9	16.1	15.8	73.3	40.1	174
Alkalinity ($CaCO_3$)*	149	11.3	11.2	146	140	291
Calcium (Ca)*	8.0	11.0	11.0	9.0	43.0	20.0
Potassium (K)*	107	11.0	11.0	105	3.0	15.0
Magnesium (Mg)*	2.0	2.0	2.0	2.0	18.0	20.0
Sodium (Na)*	82.0	8.0	8.0	77.0	14.0	224
Ammonia (N)*	5.9	0.8	0.5	1.6	0.3	0.3
Nitrate (N)*	<0.2	<0.2	<0.2	<0.2	1.5	<0.2
Sulphate (SO_4)*	38.0	12.0	13.0	40.0	17.9	149
Chloride (Cl)*	33.1	16.3	16.0	18.9	22.7	182
Total phosphate (P)*	122	13.9	14.1	139	1.0	<0.2
Orthophosphate (P)*	116	13.5	13.4	116	0.2	<0.2
Boron (B)*	<0.1	<0.1	-	-	<0.1	0.148
Iron (Fe)*	<0.03	<0.03	<0.03	<0.03	<0.03	0.23
Manganese (Mn)*	<0.03	<0.03	-	-	<0.03	0.68
Molybdenum (Mo)*	0.021	<0.01	-	-	<0.01	<0.01
Zinc (Zn)*	<0.03	<0.03	-	-	0.25	0.39
COD*	-	-	-	-	19.0	198
DOC*	-	-	-	-	4.6	42.1
Cfu/mℓ	-	-	-	-	$3.2x10^4$	$3.2x10^5$

1 ISO test medium - DOC reduction test
2 OECD test medium - DOC reduction test
3 OECD test medium - O_2 depletion test
4 ISO test medium - CO_2 production test
5 Apies river water
6 Paper mill effluent
* mg/ℓ
\- Not determined

4. RESULTS AND DISCUSSION

4.1 Preliminary examinations

4.1.1 Evaluation of different inoculum sources

An important requirement for biodegradation tests is that the DOC of the inoculum should be low and should not significantly contribute to the DOC of the test sample (*i.e.* <10%). This means if 2, 10 and 40 mg/ℓ C are used in test solutions, the contribution of the inoculum should be <0.2, <1.0 and <4.0 mg/ℓ C, respectively. Table 10 shows that if activated sludge is inoculated at a volume <10 mℓ/ℓ, the DOC will be <0.3 mg/ℓ, which is within the requirement. Activated sludge effluent (O_2 depletion test) is added as 1 drop/ℓ (approximately 0.05 mℓ/ℓ; dilution: >20 000-fold), and will also meet the requirement. River water is usually added at volumes <5 mℓ/ℓ (dilution: >200-fold). This dilution will ensure that the added DOC is within the requirement.

The number of microorganisms in activated sludge, activated sludge effluent and river water was determined by means of spread plates using nutrient agar. Incubation was carried out at 27°C. Table 10 shows that 10^3 to 10^4 cfu/mℓ will be present in test solutions if <10 mℓ/ℓ activated sludge is used (within required number). Likewise, a test sample will contain approximately 10^2 cfu/mℓ if river water is added at a volume <10 mℓ/ℓ. The microorganism numbers in the Moreleta stream and Pienaars river water were about one log lower than that in the Apies river, which meant that the volumes added to samples to obtain 10^2 cfu/mℓ might be too high. The flow in these two streams were also erratic and more prone to changes in numbers. It was, therefore, decided to use Apies river water for inoculation. When activated sludge effluent is used as 1 drop/ℓ of sample, a final concentration of <1 cfu/mℓ (approximately 100-200 microorganisms/BOD bottle) is obtained. Nyholm (1991) mentions that an inoculum concentration of up to 5 mℓ secondary effluent (10^1-10^3 cfu/ℓ) can be used in the oxygen depletion test. Because of low test concentrations, a low inoculum is important in the O_2 depletion test. It was, thus, decided to use this low inoculum to examine the activity of the inoculum. The SS of activated sludge was sufficiently large to use <10 mℓ/ℓ.

TABLE 10: Quality of different inocula

Sample	DOC at t=0 h (mg/ℓ)	DOC after 3 h aeration (mg/ℓ)	SS (mg/mℓ)	Cfu/mℓ
Activated sludge - Daspoort	30	30	4.1 (2.8-4.1)	$1.4x10^6$
Activated sludge effluent - Daspoort	24.8	20.7	-	$6.4x10^3$ ($3.6x10^3$-$2.0x10^4$)
Apies river water	15.1	14.5	-	$5.0x10^4$ ($1.0x10^4$-$5.0x10^4$)
Moreleta stream water	15.5	17.8	-	$6.7x10^3$
Pienaars river water	14.0	12.9	-	$2.5x10^3$

Range given in brackets
\- Not determined

4.1.2 Efficiency of filters for filtration

The filters used to filter samples for DOC analysis should allow minimal adsorption and release of organic carbon. In order to select the most suitable filter Milli-Q water and a 40 mg/ℓ aniline solution were passed through different filters (Millipore). The first and second 10 mℓ of each sample was analyzed. Table 11 shows that, compared to the unfiltered samples, the DOC concentrations increased when filtered through the 0.22 μm filter. This indicated a release of organic carbon. The concentration DOC in the first and second 10 mℓ also differed considerably. The 0.45 μm filter showed only a small increase in DOC concentration. These results are in agreement with the findings of a previous study (Slabbert *et al.*, 1993) that the 0.45 μm filter was more suitable than the 0.22 μm filter. The results obtained with the 0.5 μm filter were similar to the DOC of the unfiltered samples, indicating that this filter was superior to the other two filters. Since very little difference was noticed between the values of the first and second 10 mℓ passing through the filter, it was decided to use the filter directly, without discarding sample. This filter has also been found the most applicable by the analytical laboratory where it is routinely used for DOC analysis.

TABLE 11: Effect of filtration on DOC content of samples

Sample	Filter pore size (μm)	Treatment	DOC (mg/ℓ)
Milli-Q water	0.22	First 10 mℓ	not detectable
		Second 10 mℓ	8.4
	0.45	First 10 mℓ	1.3
		Second 10 mℓ	0.5
	0.5	First 10 mℓ	0.4
		Second 10 mℓ	0.4
	No filtration		0.4
Aniline (40 mg/ℓ)	0.22	First 10 mℓ	63.4
		Second 10 mℓ	52.3
	0.45	First 10 mℓ	46.7
		Second 10 mℓ	46.0
	0.5	First 10 mℓ	45.5
		Second 10 mℓ	44.2
	No filtration		44.6

Table 12 indicates that the removal of microorganisms by all three filters for DOC analysis was acceptable. Filtration was carried out under sterile conditions and 1 mℓ of the filtrate was plated on nutrient agar plates. Incubation was carried out at 27°C.

The analysis on Milli-Q water show a low concentration of DOC (Table 11), indicating that the water will not contribute significantly to the DOC of test samples (<10% of test concentration).

TABLE 12: Removal of microorganisms by different filters

Sample	Filter pore diameter (μm)	Cfu/mℓ
Activated sludge	0.22	0
	0.45	1
	0.5	0
Apies river water	0.22	0
	0.45	1
	0.5	1

4.2 Biodegradation tests on chemical compounds

4.2.1 DOC reduction studies

The biodegradation of the water soluble compounds aniline, lauryl sulphate and diethylene glycol was studied on three occasions using activated sludge (ISO test) and river water (OECD test) as inocula (Table 13). Possible inhibiting activity and abiotic degradation were also evaluated during the first two studies. Samples were taken at more frequent intervals during the first experiment to obtain as much information as possible on the degradation of the compounds. The frequency was later changed to coincide with the intervals given in literature. Because some of the chemicals biodegraded very rapidly, it was useful to include a 4 day sampling interval. In general, the DOC in control solutions were <0.5 mg/ℓ (<10% of test solutions), indicating that the Milli-Q water and inoculum did not contribute to the organic carbon of test solutions. During the first experiment, on day 4, the DOC concentrations in controls inoculated with activated sludge and river water were high (3.2 to 3.6 mg/ℓ). This could have been an analytical error as in some instances test concentrations were also higher than on the previous sampling date. In general, measured test concentrations of individual compounds were relatively close to the calculated concentrations on day 0. In a few instances the measured lauryl sulphate concentrations were lower than the calculated values. This resulted in lower concentrations of chemical mixtures, particularly during the first experiment. On a limited number of occasions, when activated sludge was used as inoculum, the duplicate values of tests differed by >20%. This could have been due to contamination in sample bottles or contamination during filtration.

TABLE 13: Activated sludge and river water inocula

Experiment	Activated sludge			River water		
	mg SS/mℓ	mℓ sludge/ℓ	mg SS/ℓ in solution	cfu/mℓ	mℓ river water/ℓ	cfu/mℓ in solution
1	4.55	6.6	30	7.4×10^4	2	148
2	2.9	9.0	26	-	2	-
3	3.6	8.0	29	3.9×10^4	2	78

Not determined

The % biodegradation of aniline is shown in Table 14 and Figure 4. The results show that the lag time t_1 was ≤1 day. The maximum level of degradation (91 to 100%) was reached between 4 and 10 days with activated sludge and river water as inocula. Only in one instance was biodegradation very slow, namely during the third experiment when 10 mg/ℓ C was tested with the river water inoculum (91% degradation after 21 days). The results obtained with aniline show that this chemical compound is ready biodegradable and meets the requirements of a reference chemical, since the biodegradation was ≥80% within 14 days when using activated sludge (ISO test) and ≥70% when using river water (OECD test). The findings of this study are in agreement with the results reported in literature (Nyholm, 1991). The author presented results for the modified OECD DOC reduction test, showing that aniline was 100% biodegradable within 5 days.

Table 15 and Figure 5 depict the % biodegradation obtained with lauryl sulphate. This chemical compound was also very biodegradable. The lag time was ≤1 day. The maximum degradation with activated sludge as inoculum was reached between 2 and 12 days (90 to 100%). However, when using river water as inoculum the biodegradation was slower, reaching the maximum level between 10 and 28 days (89 to 100%). The results show that lauryl sulphate is a good reference chemical when activated sludge is used as inoculum (≥80% within 14 days). However, when river water was used to biodegrade the 40 mg/ℓ C concentration (three of the six samples), it took longer than the required 10 days to reach 70% biodegradation. The results indicate that the chemical compound requires specific organisms for degradation, which are probably not so abundant in river water.

The results obtained with diethylene glycol are shown in Table 16 and Figure 6. This chemical was more difficult to biodegrade than the other two, particularly when river water was used as inoculum. The lag time when using activated sludge as inoculum was between 1 and 4 days. In general, the maximum level of biodegradation with activated sludge as inoculum was only reached after 21 days (90 to 100%). Results indicate a slower biodegradation at the higher test concentration. The results of the first experiment using 40 mg/ℓ C were erratic, showing 54% degradation at day 4, but only 15 to 35% from days 7 to 14. The results show that diethylene glycol is not a good reference chemical, since in the majority of cases a 80% degradation was reached after 14 days. The chemical compound was not ready biodegradable when river water was used as inoculum. The lag time lingered on for as much as 21 days in some instances. The maximum level of biodegradation achieved in 28 days when testing 10 mg/ℓ C was 55%. This increased to 62% after 35 days. The results of the first experiment are not taken into account because of unexplained high values at days 2 and 7. The result of day 4 is high because of a possible analytical error. When testing 40 mg/ℓ the maximum level of degradation was 30% after 28 days and 58% after 35 days. In the first experiment biodegradation remained at ≤15% over the 28 day period. The results indicate that river water does not contain sufficient numbers of organisms which are specific for the biodegradation of diethylene glycol. The results are in agreement with literature describing the biodegradation of this chemical as erratic. Nyholm (1991) showed two graphs obtained with the modified OECD DOC reduction test. In one instance the lag time was 1 day and an 90% degradation was reached within 19 days. The other graph showed a lag time of 14 days whereafter the biodegradation increased to 80% after 19 days.

Table 17 and Figures 7 present the results obtained with mixtures of chemicals to examine possible inhibiting activity. Aniline was used as reference chemical. Very good graphs were obtained for the aniline-lauryl sulphate mixtures. Lag times ranged from 1 to 2 days. Levels ≥90% were reached between 7 and 21 days. Results obtained with activated sludge and river water inocula were similar. Results show that lauryl sulphate was not toxic at the

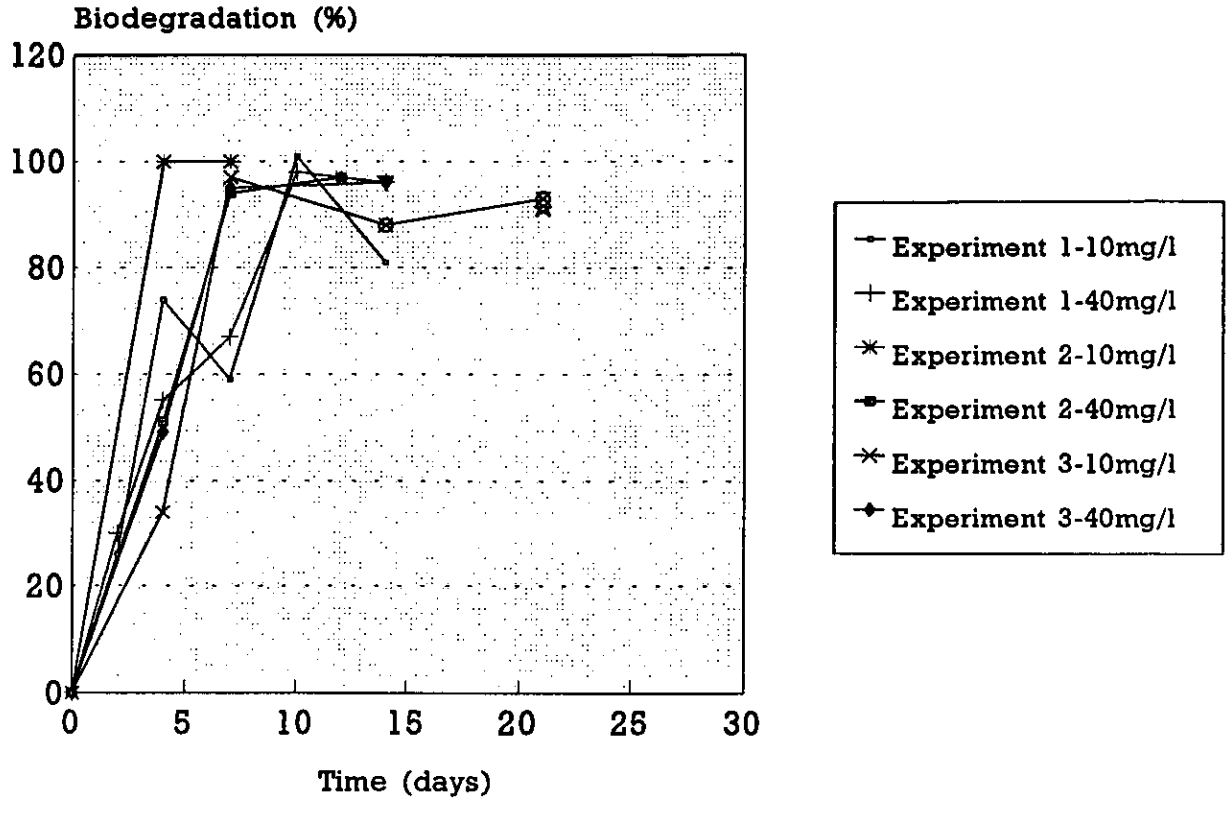
TABLE 14: Biodegradation of aniline[a] (DOC reduction test)

Experiment	Inoculum	Concentration (mg/ℓ)	% Biodegradation after time (days):							
			0	2	4	7	10	12	14	21
1	Activated sludge	10	0	26	74	59	101	-	81	-
		40	0	30	55	67	98	-	96	-
	River water	10	0	28	94	88	97	-	82	-
		40	0	29	60	87	97	-	91	-
2	Activated sludge	10	0	-	100	100	-	-	-	-
		40	0	-	51	94	-	97	-	-
	River water	10	0	-	100	89	-	-	-	-
		40	0	-	54	91	-	94	-	-
3	Activated sludge	10	0	-	34	97	-	-	88	93
		40	0	-	49	95	-	-	96	-
	River water	10	0	-	73	73	-	-	75	91
		40	0	-	53	91	-	-	91	-

[a] Calculated according to paragraph 3.3.1

\- Not determined

(a)



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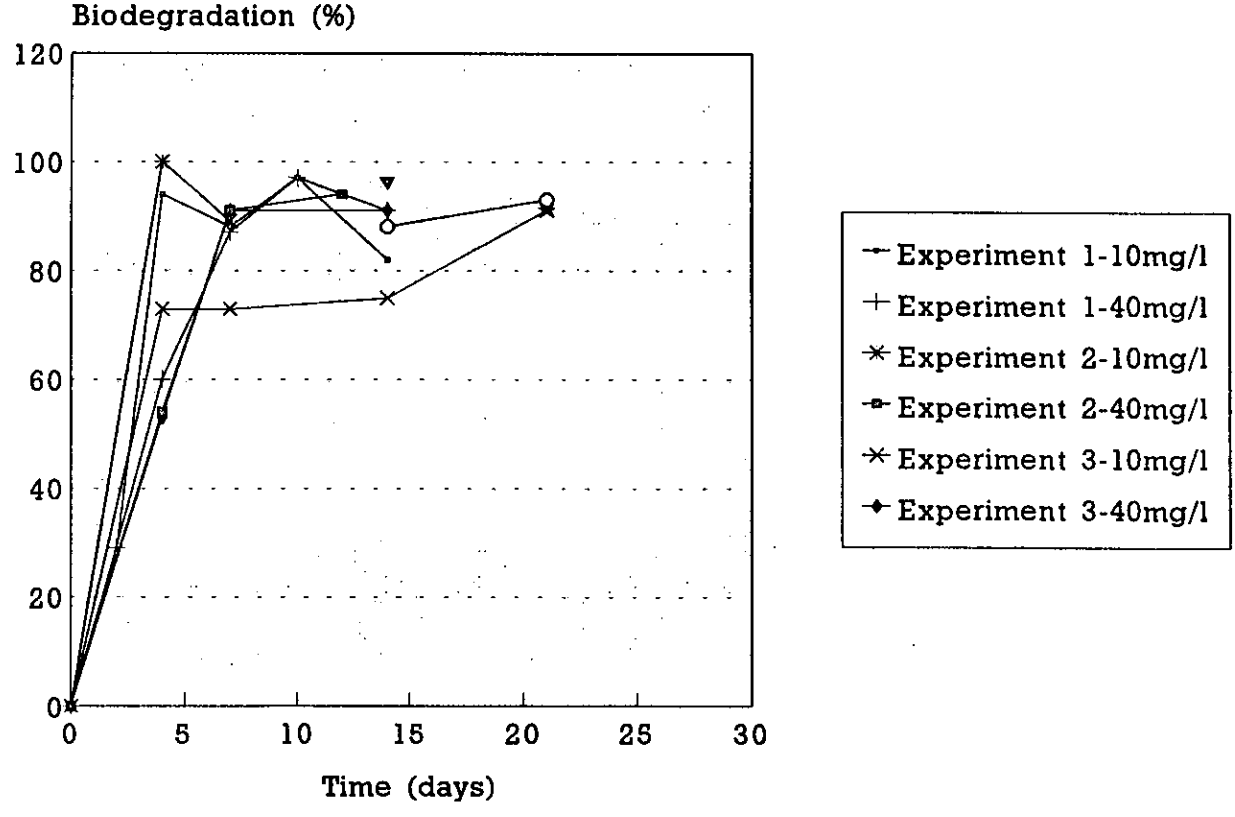


FIGURE 4: **Biodegradation of aniline in the DOC reduction test using activated sludge (a) and river water (b) as inocula**

TABLE 15: Biodegradation of lauryl sulphate[a] (DOC reduction test)

Experiment	Inoculum	Concentration (mg/ℓ)	% Biodegradation after time (days):											
			0	2	4	7	10	12	14	21	22	25	28	35
1	Activated sludge	10	0	95	98	59	103	-	71	-	-	-	-	-
		40	0	70	89	84	99	-	97	-	-	-	-	-
	River water	10	0	52	85	70	95	-	76	-	-	-	-	-
		40	0	39	44	61	66	-	71	-	85	-	90	-
2	Activated sludge	10	0	-	100	100	-	-	-	-	-	-	-	-
		40	0	-	59	77	-	93	94	-	-	-	94	-
	River water	10	0	-	81	89	-	94	-	-	-	-	-	-
		40	0	-	33	40	-	72	75	-	-	-	91	-
3	Activated sludge	10	0	-	79	96	-	-	84	83	-	100	-	-
		40	0	-	78	90	-	-	95	-	-	-	-	-
	River water	10	0	-	66	82	-	-	77	83	-	100	-	-
		40	0	-	56	61	-	-	72	78	-	89	-	92

[a] Calculated according to paragraph 3.3.1

\- Not determined

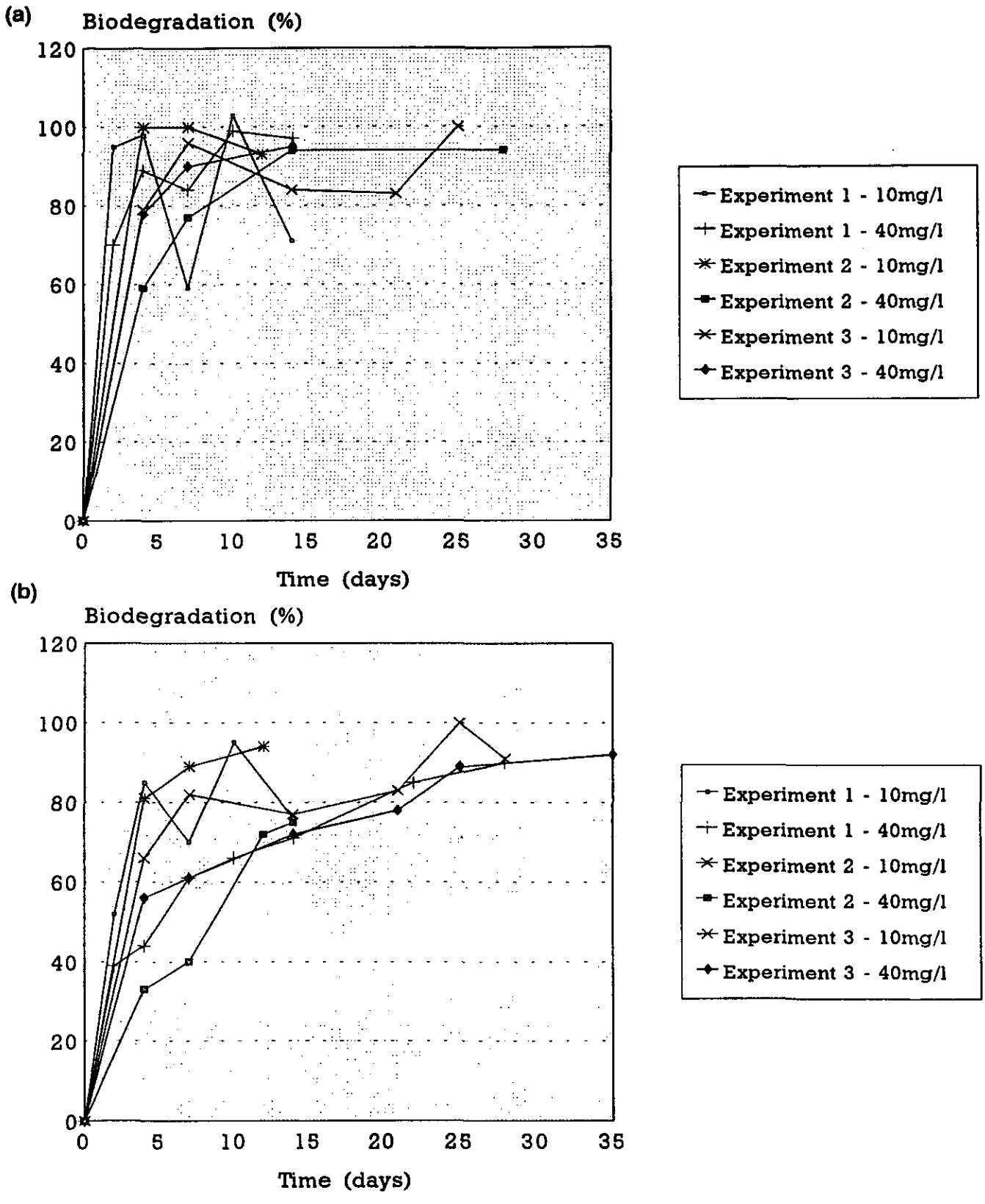


FIGURE 5: **Biodegradation of lauryl sulphate in the DOC reduction test using activated sludge (a) and river water (b) as inocula**

TABLE 16: Biodegradation of diethylene glycol[a] (DOC reduction test)

Experiment	Inoculum	Concentration (mg/ℓ)	% Biodegradation after time (days):										
			0	2	4	7	10	14	21	22	25	28	35
1	Activated sludge	10	0	29	37	95	78	85	-	74	-	87	-
		40	0	+6	54	15	10	35	-	97	-	97	-
	River water	10	0	28	98	63	10	15	-	10	-	42	-
		40	0	+4	15	13	10	9	-	12	-	9	-
2	Activated sludge	10	0	-	11	22	-	68	89	-	90	-	100
		40	0	-	11	13	-	41	85	-	97	-	-
	River water	10	0	-	6	+7	-	+6	26	-	55	-	62
		40	0	-	11	8	-	15	18	-	29	-	58
3	Activated sludge	10	0	-	22	48	-	86	91	-	-	100	-
		40	0	-	15	35	-	76	97	-	-	96	-
	River water	10	0	-	9	22	-	12	11	-	-	44	-
		40	0	-	10	19	-	16	21	-	-	30	-

[a] Calculated according to paragraph 3.3.1

\- Not determined

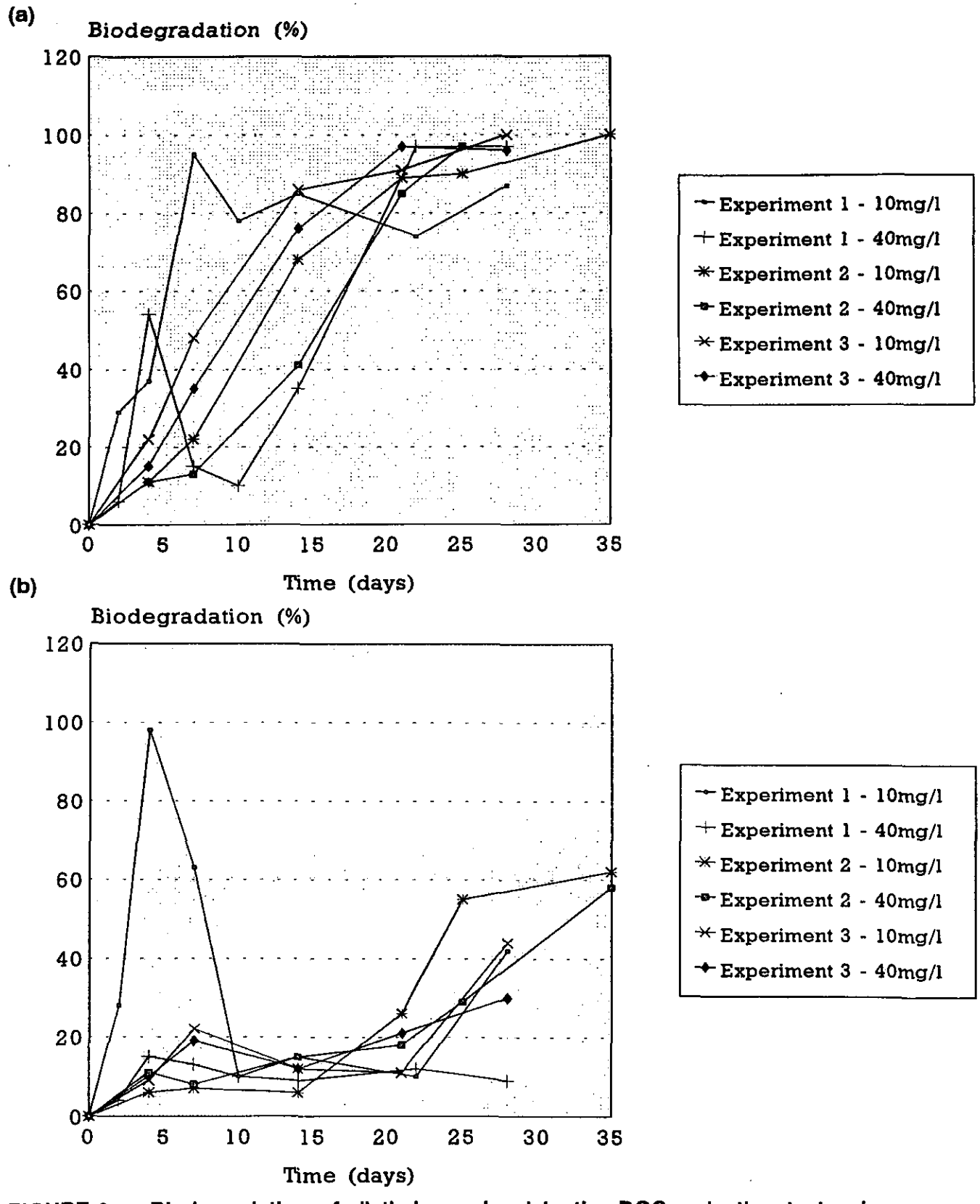


FIGURE 6: **Biodegradation of diethylene glycol in the DOC reduction test using activated sludge (a) and river water (b) as inocula**

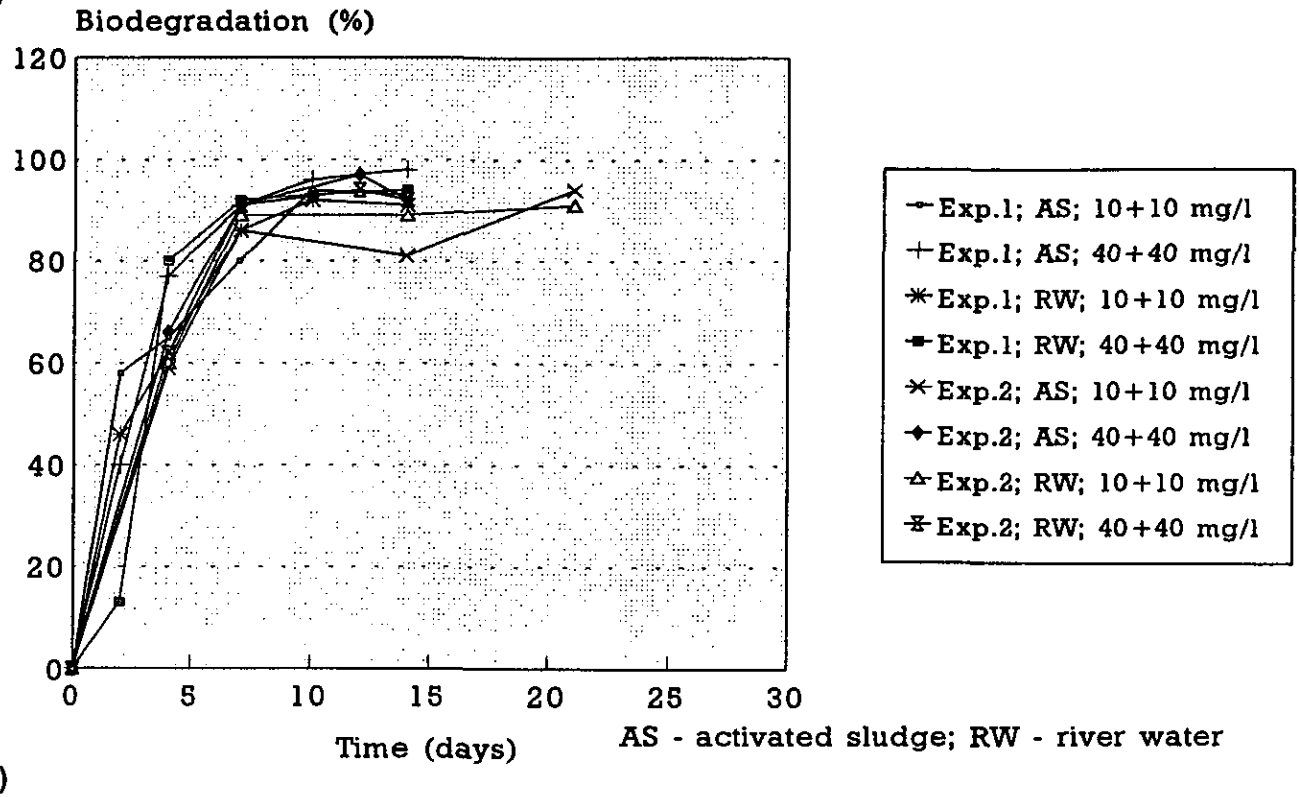
TABLE 17: Biodegradation of mixtures of chemical compounds[a] (DOC reduction test)

Experiment	Inoculum	Mixture of chemical compounds	% Biodegradation after time (days):										
			0	2	4	7	10	14	21	22	25	28	35
1	Activated sludge	10 mg/ℓ aniline plus 10 mg/ℓ lauryl sulphate	0	58	65	80	94	93	-	-	-	-	-
		10 mg/ℓ aniline plus 10 mg/ℓ diethylene glycol	0	37	77	56	76	71	-	87	-	98	-
		40 mg/ℓ aniline plus 40 mg/ℓ lauryl sulphate	0	40	77	91	96	98	-	-	-	-	-
		40 mg/ℓ aniline plus 40 mg/ℓ diethylene glycol	0	20	30	63	51	63	-	90	-	94	-
	River water	10 mg/ℓ aniline plus 10 mg/ℓ lauryl sulphate	0	46	62	86	92	91	-	-	-	-	-
		10 mg/ℓ aniline plus 10 mg/ℓ diethylene glycol	0	35	82	44	49	57	-	83	-	97	-
		40 mg/ℓ aniline plus 40 mg/ℓ lauryl sulphate	0	13	80	92	93	94	-	-	-	-	-
		40 mg/ℓ aniline plus 40 mg/ℓ diethylene glycol	0	19	24	62	50	52	-	66	-	85	-
2	Activated sludge	10 mg/ℓ aniline plus 10 mg/ℓ lauryl sulphate	0	-	59	86	-	81	94	-	-	-	-
		10 mg/ℓ aniline plus 10 mg/ℓ diethylene glycol	0	-	26	50	-	82	92	-	-	-	-
		40 mg/ℓ aniline plus 40 mg/ℓ lauryl sulphate	0	-	66	91	-	92	-	-	-	-	-
		40 mg/ℓ aniline plus 40 mg/ℓ diethylene glycol	0	-	45	63	-	67	94	-	-	-	-
	River water	10 mg/ℓ aniline plus 10 mg/ℓ lauryl sulphate	0	-	60	89	-	89	91	-	-	-	-
		10 mg/ℓ aniline plus 10 mg/ℓ diethylene glycol	0	-	32	43	-	39	61	-	68	-	100
		40 mg/ℓ aniline plus 40 mg/ℓ lauryl sulphate	0	-	62	91	-	92	-	-	-	-	-
		40 mg/ℓ aniline plus 40 mg/ℓ diethylene glycol	0	-	45	63	-	68	89	-	94	-	-

[a] Calculated according to paragraph 3.3.1

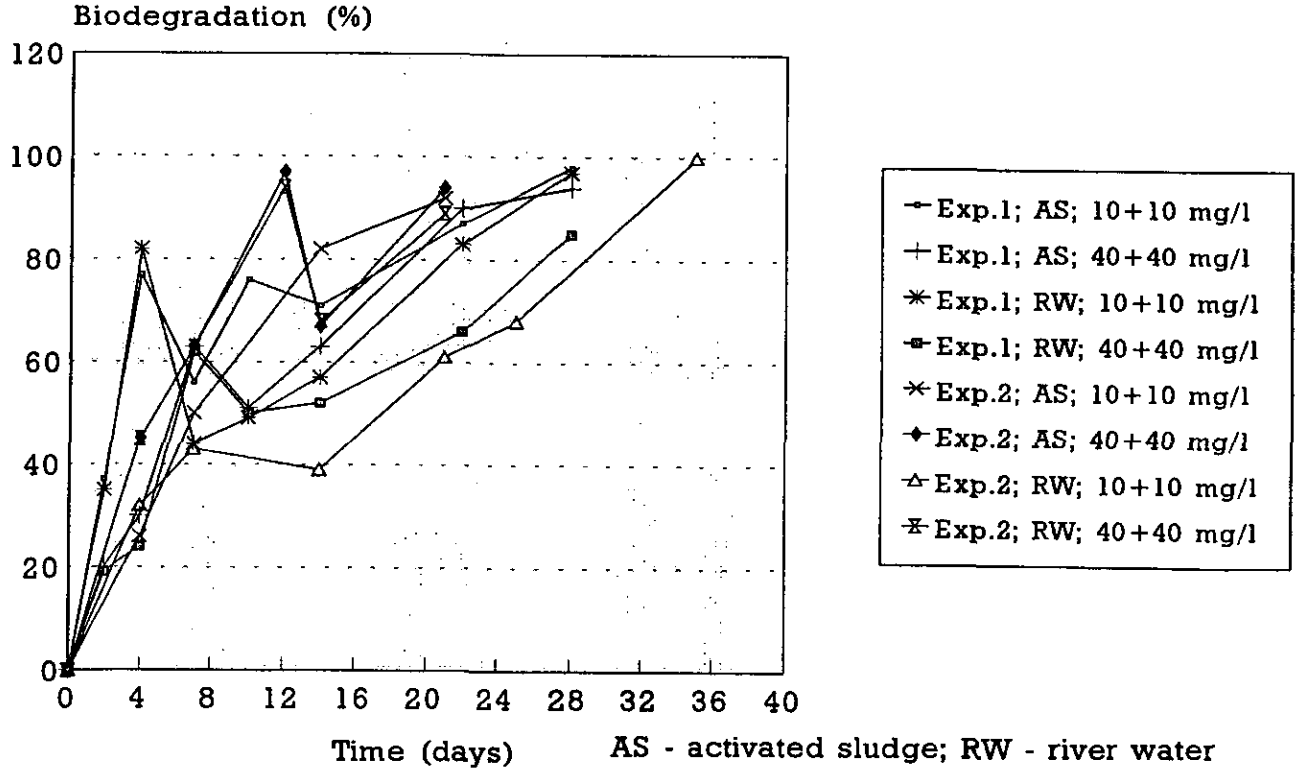
\- Not determined

(a)



AS - activated sludge; RW - river water

(b)



AS - activated sludge; RW - river water

FIGURE 7: **Biodegradation of aniline plus lauryl sulphate (a) and aniline plus diethylene glycol (b) in the DOC reduction test**

concentrations tested, as the % biodegradation was >40% (≥80%) after 7 days. The graphs obtained with the aniline-diethylene glycol mixture show a lag time of 1 day. Maximum degradation (>90%) was reached between 21 and 35 days. No toxicity was detected at the diethylene glycol test concentrations, as biodegradation was >40% after 7 days.

Table 18 shows some degree of degradation in the lauryl sulphate sample (32%). As this was only observed on one occasion the degradation was expected to be due to inadequate sterilization with $HgCl_2$ rather than to abiotic degradation.

TABLE 18: Abiotic degradation of chemical compounds[a] (40 mg/ℓ C) (DOC reduction test)

Experiment	Chemical	% Degradation after time (days):		
		0	**28**	**35**
1	Aniline	0	10	-
	Lauryl sulphate	0	+4	-
	Diethylene glycol	0	+16	-
2	Aniline	0	-	7
	Lauryl sulphate	0	-	32
	Diethylene glycol	0	-	8

a Calculated according to paragraph 3.3.1
\- Not determined

At the end of the first experiment an evaluation was carried out on the number of microorganisms in the different test and control solutions (spread plate). In addition, the filtrates used for DOC analysis were collected and plated to test the efficiency of the filter to remove organisms. The results in Table 19 show that there was approximately ten-times more microorganisms in controls at the end of a test. Test solutions showed a 2 to 3 log increase in numbers when using activated sludge as inoculum. When river water was used as inoculum the increase in test solutions were between 1 and 3 logs. None of the filtrates contained microorganisms, indicating a total removal of cells by the selected filter.

4.2.1.1 Effect of different experimental conditions on biodegradation

The standard ISO test uses stirring to agitate test and control solutions, while the modified OECD method uses shaking. The method applied in our study used aeration because this was the most cost effective way to handle a large number of samples. Two sets of experiments were carried out with aniline (40 mg/ℓ C) to compare the effect of different methods of agitation on biodegradation. Tests were carried out with activated sludge and river water inocula. In general, the control values were <1 mg/ℓ C. Furthermore, there were no large differences (<20%) between duplicate results.

TABLE 19: Microbial numbers in test/control solutions at the end of experiment 1

Inoculum	Inoculum (cfu/mℓ)	Sample[a]	Cfu/mℓ after 28 days	Cfu in 1 mℓ filtrate after 28 days
Activated sludge	10^3-10^4	Control[b]	$2.0x10^4$	0
		Aniline	$6.8x10^8$	0
		Lauryl sulphate	$2.1x10^5$	0
		Diethylene glycol	$1.9x10^6$	0
River water	$1.48x10^2$	Control	$4.0x10^3$	0
		Aniline	$3.7x10^4$	0
		Lauryl sulphate	$2.7x10^5$	0
		Diethylene glycol	$2.9x10^4$	0

a Test solution - 40 mg/ℓ C
b Control - 0 mg/ℓ C

Table 20 shows the % biodegradation during the different experimental conditions. The results obtained during experiment 1 were very much the same with maximum degradation (>90%) after 7 days. The second experiment showed a good agreement between the results of aeration and stirring when activated sludge was used as inoculum. The maximum level of biodegradation was reached after 4 days. When shaking was used it took 3 days longer to reach maximum biodegradation. When the sample was not agitated there was a lag period of 4 to 7 days. Maximum degradation was reached after 14 days. When river water was used as inoculum, maximum biodegradation was reached within 3 days when samples were stirred. Samples which were aerated and shaken lagged behind and reached maximum biodegradation after 10 days. When no agitation was used the lag time was between 3 and 4 days, and maximum degradation was reached after 14 days, as was the case with activated sludge. It was found that the temperature of stirring devices increased during the evaluation, which could have contributed to the more rapid degradation in some instances.

The effect of aeration on the degradation of chemical compounds (40 mg/ℓ C; activated sludge as inoculum) is shown in Table 21. For both aniline and lauryl sulphate, the sample which received no inoculum showed a rapid breakdown and was completely degraded within 15 days. This indicated possible abiotic degradation or the introduction of organisms via air. The samples which received $HgCl_2$ were also degraded, raising concern that aeration caused abiotic degradation. The concentration $HgCl_2$ used for sample sterilization was low (1 mg/ℓ, after addition), which means that the degradation could have been due to microbial activity. In the case of diethylene glycol no biodegradation occurred when samples were not inoculated or were treated with $HgCl_2$, indicating that the chemical was difficult to biodegrade if specific organisms were not present. A further study was carried out with a higher concentration $HgCl_2$, using different methods of agitation (activated sludge inoculum). The results in Table 22 indicate that when samples were adequately sterilized (1 mℓ/ℓ of a 10 g/ℓ $HgCl_2$ solution) there was no degradation. The method of agitation did not affect the results.

Biodegradation studies are usually carried out in diffused light or complete darkness to avoid photodegradation and algal growth. Table 23 shows that the results of a study carried out in ambient laboratory light, diffused light and complete darkness were similar. This study was

TABLE 20: Biodegradation of aniline (40 mg/ℓ C), using different methods of agitation[a] (DOC reduction test)

Experiment	Inoculum	Method of agitation	% Biodegradation after time (days):								
			0	1	2	3	4	7	10	12	14
1	Activated sludge	Aeration	0	-	-	-	51	94	-	97	-
		Shake	0	-	-	-	63	91	-	96	-
		Stir	0	-	-	-	63	92	-	95	-
	River water	Aeration	0	-	-	-	54	91	-	94	-
		Shake	0	-	-	-	62	90	-	96	-
		Stir	0	-	-	-	64	92	-	96	-
2	Activated sludge	Aeration	0	+2	10	22	92	93	92	-	-
		Shake	0	3	0	+2	12	99	101	-	-
		Stir	0	1	2	25	97	92	94	-	-
		No agitation	0	5	+1	+2	0	41	79	-	96
	River water	Aeration	0	1	17	22	62	86	88	-	94
		Shake	0	1	0	12	81	85	89	-	91
		Stir	0	6	2	92	93	90	93	-	-
		No agitation	0	1	+2	+2	23	74	88	-	94

a Calculated according to paragraph 3.3.1
- Not determined

TABLE 21: Degradation of aniline, lauryl sulphate and diethylene glycol (40 mg/ℓ C) during aeration[a] (DOC reduction test)

Chemical	Test condition	% Degradation after time (days):					
		0	3	7	15	24	28
Aniline	Inoculum	0	13	82	95	-	-
	No inoculum	0	10	35	97	-	-
	No inoculum plus $HgCl_2$	0	13	18	22	79	85
Lauryl sulphate	Inoculum	0	94	93	97	-	-
	No inoculum	0	43	86	97	-	-
	No inoculum plus $HgCl_2$	0	12	5	9	30	49
Diethylene glycol	Inoculum	0	+3	+4	74	98	97
	No inoculum	0	21	4	12	12	11
	No inoculum plus $HgCl_2$	0	11	1	6	8	8

a Calculated according to paragraph 3.3.1
- Not determined

TABLE 22: Biodegradation of aniline (40 mg/ℓ C) after treatment with $HgCl_2$, using different methods of agitation[a] (DOC reduction test)

Method of agitation	Sample	% Degradation after time (days):				
		0	7	15	21	28
Aeration	Aniline	0	95	93	-	-
	Aniline plus 10 mg/ℓ $HgCl_2$	0	6	4	3	10
Shake	Aniline	0	81	87	-	-
	Aniline plus 10 mg/ℓ $HgCl_2$	0	3	+6	+11	+13
Stir	Aniline	0	95	94	-	-
	Aniline plus 10 mg/ℓ $HgCl_2$	0	5	5	6	1
No agitation	Aniline	0	16	100	-	-
	Aniline plus 10 mg/ℓ $HgCl_2$	0	5	7	5	+4

a Calculated according to paragraph 3.3.1
- Not determined

completed in 8 days. Results might have been different if a chemical was used which took longer to degrade and which had the potential to be photodegraded. It is not expected that algal growth will have a significant effect on the results of the DOC test if not carried out in direct light. Tests carried out at different temperatures (Table 23) showed similar results at 22 and 32°C (total degradation in 4 days). However, when incubation took place at 10°C, maximum degradation was reached after 22 days. There might have been a larger difference between the results at 22 and 32°C if a chemical was tested which took longer to degrade.

TABLE 23: Biodegradation of aniline (40 mg/ℓ C) under different light and temperature conditions[a] (DOC reduction test)

Method of agitation	Test condition	% Biodegradation after time (days):					
		0	4	6	8	10	22
Aeration	Light	0	44	93	92	-	-
	Diffused light	0	30	92	91	-	-
	Darkness	0	21	74	96	-	-
Stir	10°C	0	+10	16	24	28	90
	22°C	0	100	90	92	-	-
	32°C	0	100	91	91	-	-

a Calculated according to paragraph 3.3.1
- Not determined

4.2.1.2 Alternative measurement parameters

The standard ISO and OECD tests use DOC to measure the removal of organic carbon. In

this study COD and microorganism numbers were used in addition to DOC to evaluate their potential as measurement parameters. Tests were carried out on 10 and 40 mg/ℓ aniline during a 7 day period. The DOC concentration of control solutions ranged between 0.5 and 1.0 mg/ℓ. COD values for the control were high and ranged between 19 and 37 mg/ℓ. Figure 8 shows the biodegradation of aniline in terms of DOC and COD. The results for DOC and COD using 10 mg/ℓ aniline were similar, showing a biodegradation of >90%. However, in the case of 40 mg/ℓ aniline, COD measurement only showed a 69% biodegradation as opposed to the 97% obtained with DOC measurement. Microorganism numbers decreased with approximately one log over the 7 day testing period for the control and 10 mg/ℓ aniline. In the case of 40 mg/ℓ a one and a half log increase was noticed (Figure 9).

4.2.2 CO_2 production studies

The biodegradation of the three water soluble chemical compounds aniline, lauryl sulphate and diethylene glycol, and the insoluble compound stearic acid was studied on four occasions. Each experiment also included mixtures of the chemicals to study possible inhibition. Chemicals were used at a concentration of 40 mg/ℓ. Activated sludge was used for inoculation (Table 24). The results of titrations and the amounts of CO_2 produced during the experiments indicated that in general there was very little difference between duplicate control values. In a number of instances there were large differences between the titration values of duplicate test flasks. This could be ascribed to differences in air flow during aeration or because clogging took place (stopping air flow). The titration values obtained with $Ba(OH)_2$

TABLE 24: Activated sludge inoculum

Experiment	mg SS/mℓ	mℓ sludge/ℓ	mg SS/ℓ in solution
1	5.66	5.0	28
2	3.7	8.0	30
3	4.04	7.0	28
4	2.75	9.8	27

in the absorber flasks during the first two experiments were much lower than the required 50 mℓ (background value: approximately 37.5 mℓ). It was later realized that the Ba(OH) was of low grade containing a large amount of impurities. A high grade $Ba(OH)_2$ was used in experiments 3 and 4, obtaining background titration values of between 47 and 48 mℓ when 100 mℓ $Ba(OH)_2$ was used and between 94 and 99 mℓ when 200 mℓ $Ba(OH)_2$ was used. In the first experiment low titration values were recorded in some instances (high CO_2 production), particularly when mixtures of chemicals (80 mg/ℓ concentrations) were tested. This indicated that titrations should be carried out more frequently to ensure that CO_2 is not carried over to the second and third absorber flasks. The first experiment was considered a trail run and was terminated on day 22. According to the ISO method the CO_2 production in the control should be insignificant and not exceed 70 mg/ℓ (usually 30 to 40 mg/ℓ) at the end of the test. Table 25 shows the results obtained when the actual background and the calculated titration values were used to establish the CO_2 production for the experimental period. When the actual background titration values were used for the calculation the stated requirement was met.

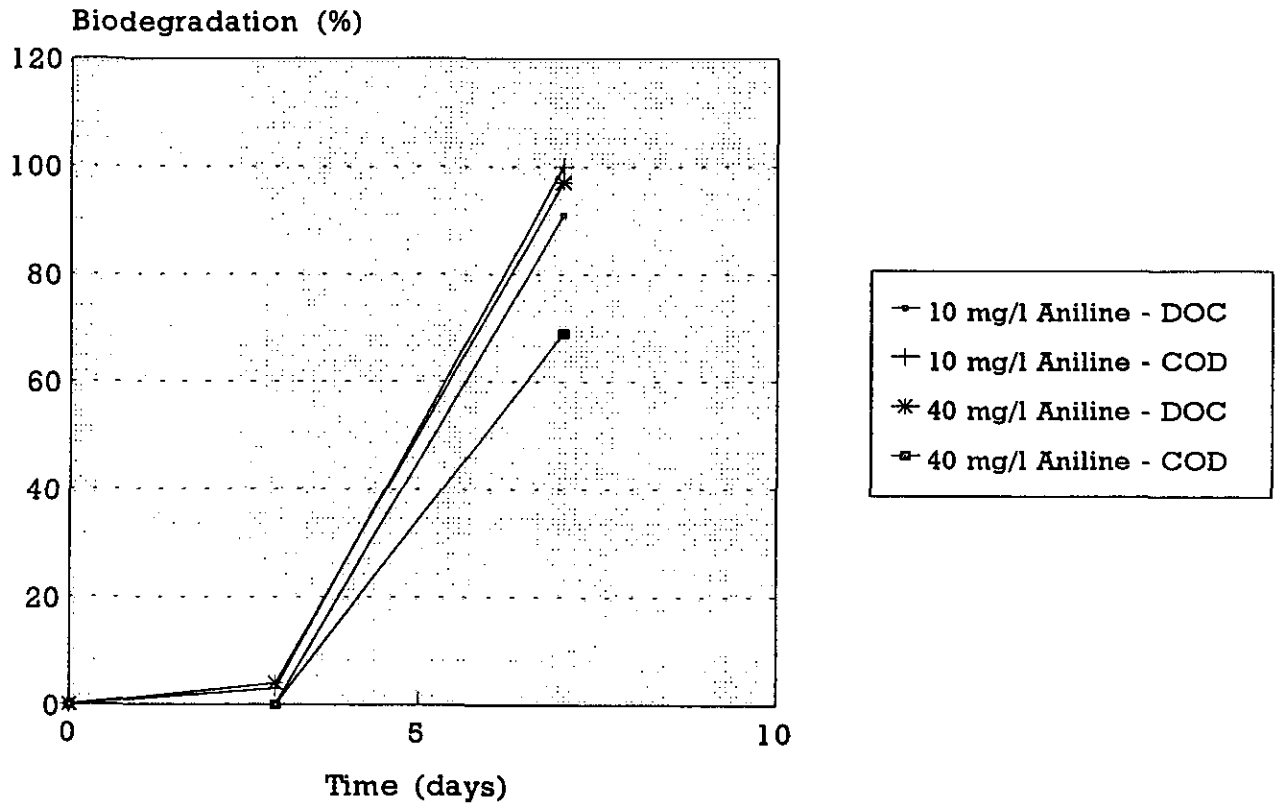


FIGURE 8: Biodegradation of aniline in terms of DOC and COD

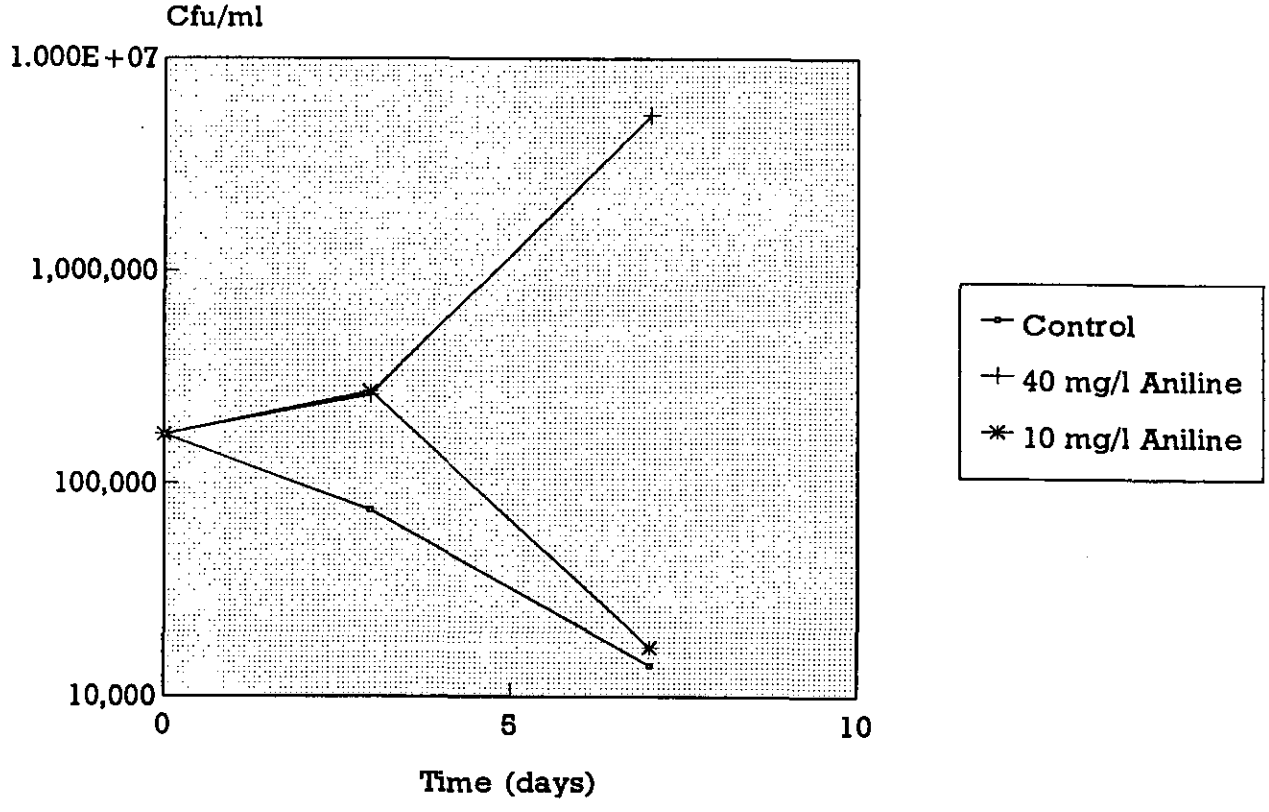


FIGURE 9: Biodegradation of aniline in terms of microorganism numbers

TABLE 25: CO_2 produced by controls during incubation period

Experiment	Actual background value (mℓ HCl)	CO_2 produced (mg)	Calculated value (mℓ HCl)	CO_2 produced (mg)
1	37.5	30.97	50	41.97
2		25.74		44.99
3	47	33.39		69.69
4	96	57.81	100	97.41

The % biodegradation of the different chemical compounds and mixtures of chemicals is shown in Tables 26 to 29 and Figures 10 and 11. The TCO_2 used to calculate the results was 146.8 when 40 mg/ℓ C was used and 293.6 mg/ℓ in case of mixtures of chemicals (80 mg/ℓ C). The results obtained with aniline show a lag period between 1 and 7 days (Figure 10a). The maximum biodegradation at day 28 ranged from 62 to >100%. Except for the first experiment, the results show that this chemical compound is ready biodegradable and meets the requirements of a reference chemical (ISO test), since the biodegradation was ≥50% within 14 days. In general, the results are in agreement with the results reported in literature (Nyholm, 1991), showing ≥80% biodegradation for aniline after 12 days using the Sturm test.

Lauryl sulphate showed a lag time of between 1 and 3 days (Figure 10b). The maximum degradation reached after 28 days ranged from 61 to 100%. 50% degradation was achieved within 14 days in all four experiments, indicating that the chemical is ready biodegradable under the experimental conditions and could be used as reference compound.

Diethylene glycol showed a lag period of between 4 and 12 days (Figure 10c). The maximum biodegradation reached after 28 days ranged from 47 to 67%. In all the experiments the biodegradation after 14 days incubation was <50% (17 to 46%), indicating that the chemical compound is not suitable as reference chemical. The chemical is more difficult to biodegrade than the two other substances, as was also witnessed with the DOC reduction test. As the graphs showed an upward trend it is expected that the product might be fully degraded if the incubation period was increased (which is sometimes done). Nyholm (1991) showed that the maximum level of biodegradation of diethylene glycol in the Sturm test was approximately 70%, which is similar to the results of experiment 3. These levels were obtained after about 14 days, showing a better biodegradability than obtained in our studies.

The results obtained for the insoluble compound stearic acid were much more reproducible that those obtained for the other chemicals (Figure 10d). The lag time ranged from 3 to 4 days. The maximum degradation reached after 28 days ranged from 56 to 80%. As was the case with diethylene glycol the biodegradation did not reach 50% (40 to 46%) within the first 14 days, rendering the chemical not fit as reference compound. The chemical appears to be difficult to biodegrade, but because curves showed an upward trend it is possible that biodegradation might be complete if a longer incubation period was allowed.

Figure 11 shows the results obtained with mixtures of chemicals. According to the standard ISO test a compound is toxic at the test concentration if the % degradation in the toxicity test flask is <25% in 28 days. Aniline was used as reference chemical. Very good graphs were obtained for the aniline-lauryl sulphate mixtures (Figure 11a). Lag times ranged from 3 to 4

TABLE 26: Biodegradation of chemical compounds[a] (Experiment 1) (CO_2 production test)

Sample	% Biodegradation at time (days):			
	1	6	9	22
Aniline	0	0	32	54
Lauryl sulphate	0	30	45	61
Diethylene glycol	0	1	7	40
Stearic acid	0	19	37	61
Aniline plus lauryl sulphate	*0*	*24*	*46*	*63*
Aniline plus diethylene glycol	0	6	27	44
Aniline plus stearic acid	1	13	35	52

[a] Calculated according to paragraph 3.3.2

TABLE 27: Biodegradation of chemical compounds[a] (Experiment 2) (CO_2 production test)

Sample	% Biodegradation at time (days):						
	1	6	9		14	21	28
Aniline	0	11	46	51	66	78	89
Lauryl sulphate	1	31	50	51	69	84	91
Diethylene glycol	1	4	8	7	25	57	60
Stearic acid	0	19	33	34	46	58	69
Aniline plus lauryl sulphate	1	24	49	54	68	76	81
Aniline plus diethylene glycol	1	18	32	34	56	66	70
Aniline plus stearic acid	1	22	43	46	60	73	79

[a] Calculated according to paragraph 3.3.2

TABLE 28: Biodegradation of chemical compounds[a] (Experiment 3) (CO_2 production test)

Sample	% Biodegradation at time (days):										
	1	3	6	7	10	13		17	21	24	28
Aniline	14	28	59	79	96	96	107	111	116	117	119
Lauryl sulphate	14	30	40	43	50	53	66	84	100	106	111
Diethylene glycol	0	4	16	23	33	42	46	54	58	60	67
Stearic acid	1	11	17	25	32	34	42	54	61	69	80
Aniline plus lauryl sulphate	0	6	21	38	54	62	63	67	69	70	72
Aniline plus diethylene glycol	0	1	12	21	36	50	59	65	66	67	70
Aniline plus stearic acid	0	0	5	18	31	42	47	53	58	60	63

[a] Calculated according to paragraph 3.3.2

TABLE 29: Biodegradation of chemical compounds[a] (Experiment 4) (CO_2 production test)

Sample	% Biodegradation at time (days):								
	4	7	9	12	14	22	28		
Aniline	-1	0	21	43	50	57	58	56	62
Lauryl sulphate	17	29	42	50	55	60	62	61	61
Diethylene glycol	0	0	2	11	17	34	45	47	47
Stearic acid	9	18	27	35	39	52	57	57	56
Aniline plus lauryl sulphate	11	40	55	60	64	68	71	72	72
Aniline plus diethylene glycol	-1	3	17	32	45	55	60	62	62
Aniline plus stearic acid	3	19	31	46	55	63	69	71	71

[a] Calculated according to paragraph 3.3.2

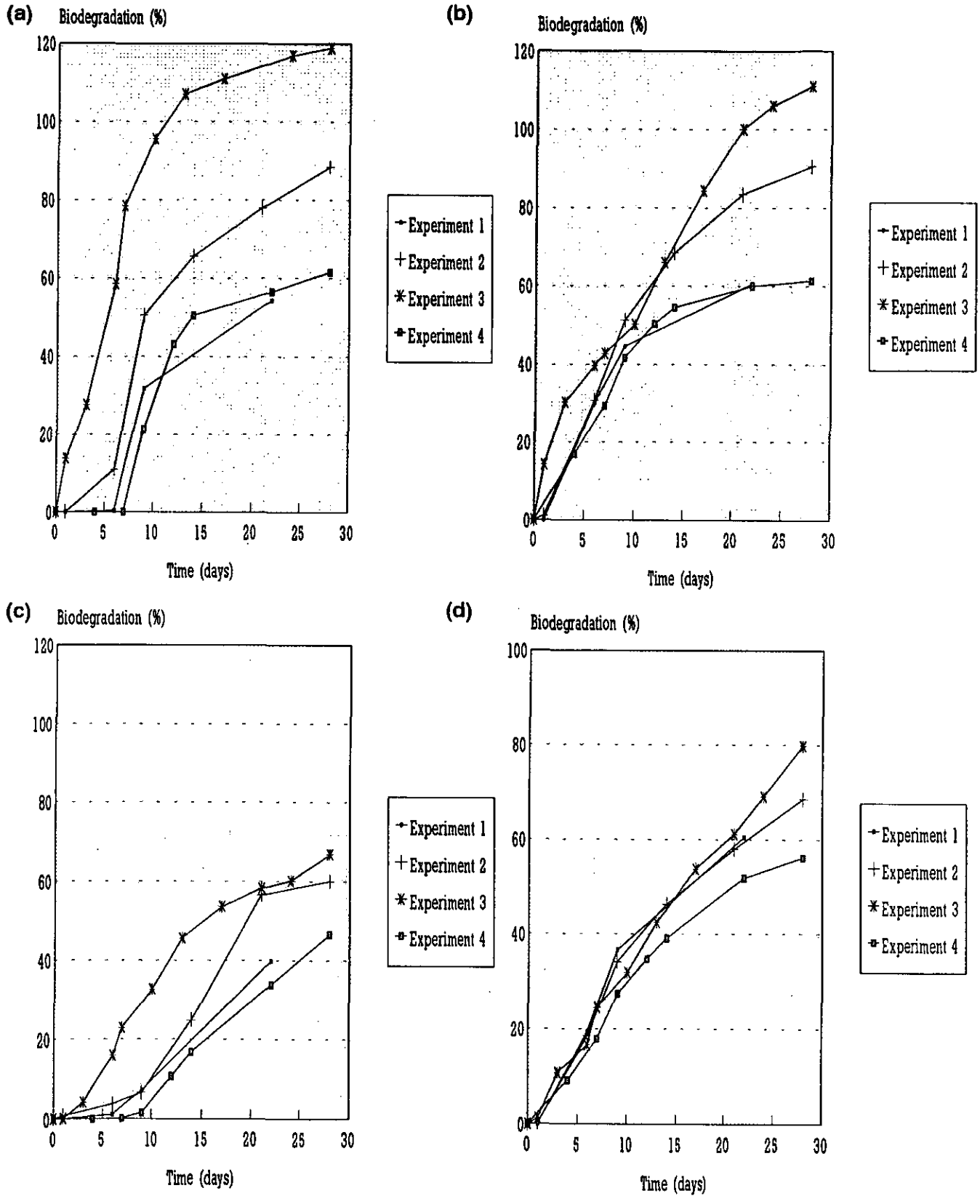


FIGURE 10: Biodegradation of aniline (a), lauryl sulphate (b), diethylene glycol (c) and stearic acid (d) in the CO_2 production test

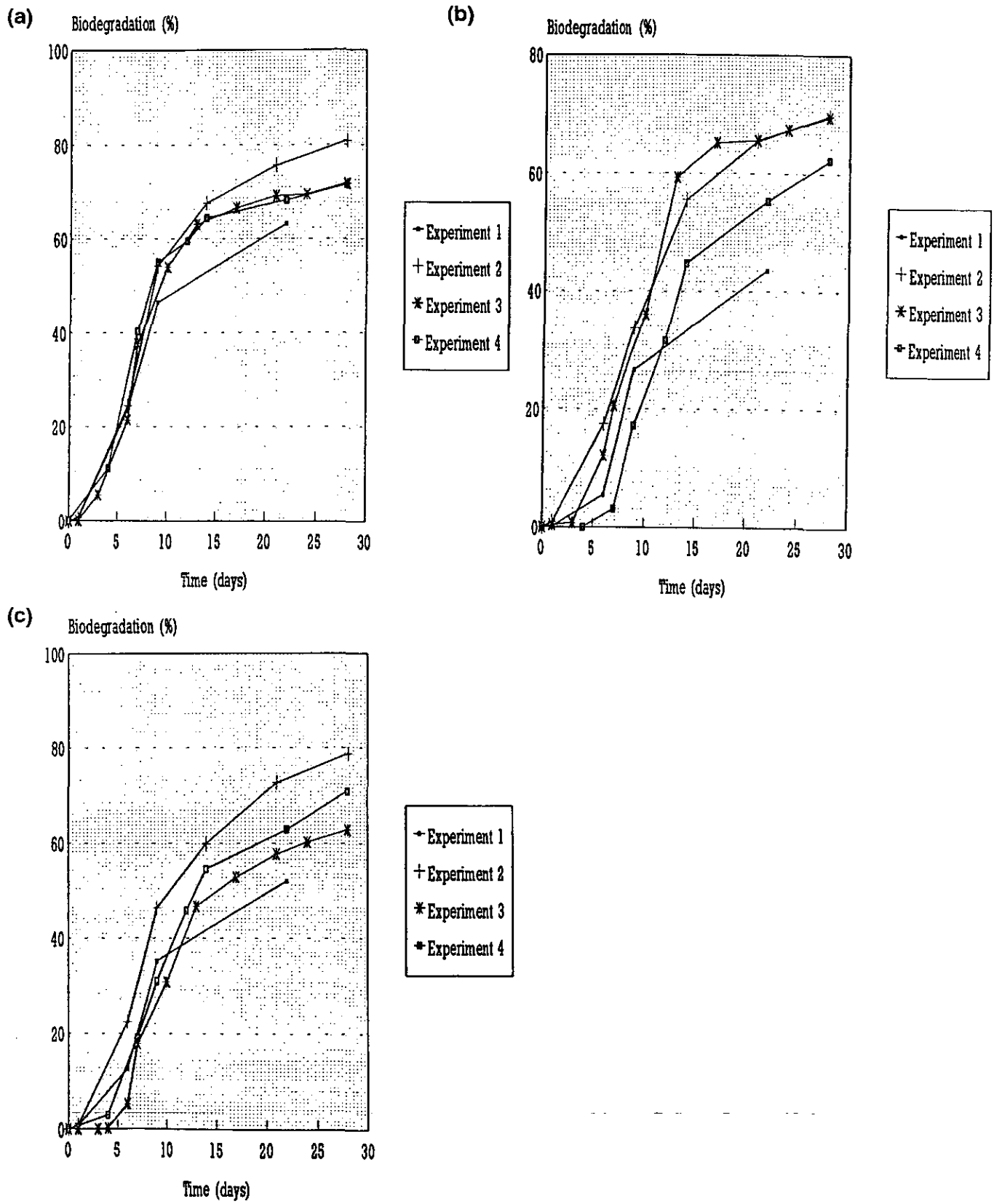


FIGURE 11: Biodegradation of aniline plus lauryl sulphate (a), aniline plus diethylene glycol (b) and aniline plus stearic acid (c) in the CO_2 production test

days. 50% degradation was reached within 8 to 10 days. The maximum values recorded after 28 days ranged from 72 to 81%. Results show that lauryl sulphate was not toxic at the concentrations tested, as the % biodegradation was >25% at the end of the biodegradation period. The graphs obtained for aniline and diethylene glycol (Figure 11b) show a lag time of between 2 and 8 days. The maximum degradation reached after 28 days ranged from 62 to 70%. No toxicity was detected at the diethylene glycol test concentration, as biodegradation was >25% after 28 days. The aniline-stearic acid mixture (Figure 11c) showed a lag period of 3 to 6 days. Maximum degradation after 28 days ranged from 63 to 79%. As these levels were >25%, stearic acid is considered to be non-toxic.

In addition to CO_2 determinations, the DOC reduction for the soluble chemicals was also examined in experiments 3 and 4. Control DOC values were <1 mg/ℓ. In general, duplicate values of tests were similar. The DOC values at day 0 for diethylene glycol in experiment 3 and lauryl sulphate in experiment 4 were considerably lower than the calculated values, 40 mg/ℓ C. The percentage biodegradation is shown in Table 30. In both experiments maximum degradation (>90%) was reached by day 28 for all chemicals and mixtures of chemicals. However the results showed that degradation of lauryl sulphate and diethylene glycol was slow during experiment 3, not reaching 80% degradation within 14 days. The same was found for diethylene glycol during experiment 4 (22% biodegradation after 12 days). The reason why these results were not the same as those of the DOC reduction test is not clear. It could be that the low air flow did not allow sufficient mixing and aeration. The ISO test applies stirring in addition to aeration. It might be necessary to do the same to improve biodegradation rates.

TABLE 30: Biodegradation of chemical compounds[a] (DOC reduction)

Experiment	Sample	% Biodegradation after time (days):				
		0	7	12	15	28
3	Aniline	0	-	-	81	94
	Lauryl sulphate	0	-	-	59	96
	Diethylene glycol	0	-	-	78	94
	Aniline plus lauryl sulphate	0	-	-	93	96
	Aniline plus diethylene glycol	0	-	-	90	95
4	Aniline	0	25	95	-	97
	Lauryl sulphate	0	87	93	-	97
	Diethylene glycol	0	+9	22	-	96
	Aniline plus lauryl sulphate	0	78	92	-	99
	Aniline plus diethylene glycol	0	15	65	-	99

a Calculated according to paragraph 3.3.1
- Not determined

4.2.2.1 Optimization of test

During the first three experiments NaOH and $Ba(OH)_2$ were used for scrubbing, as outlined in the ISO method. NaOH and $Ba(OH)_2$ solutions did not show a large absorption of CO_2 [colour of thymol blue changed slightly to yellow and $Ba(OH)_2$ showed some precipitation], indicating that the concentrations of these solutions were sufficiently high to remove CO_2 efficiently. The results of the studies, however, showed that the CO_2 produced in controls were high when calculated values (instead of actual values) were taken into account (>70 mg/ℓ CO_2). In order to ensure that CO_2 was optimally removed from the air the advice of researchers from the Water Research Centre, Medmenham, UK, was followed to include an additional scrubbing system. The system consisted of a measuring cylinder filled with soda lime granules (approximately 100 mℓ), followed by a measuring cylinder with $CaCl_2$ granules (100 mℓ) to absorb water released during interaction of CO_2, and soda lime and an empty cylinder as trap. These were placed between the compressed air and the NaOH solutions for experiment 4 (Figure 1b). Problems were experienced with clogging of tubes in the $CaCl_2$ cylinders, because of paste formation. Therefore, before the end of the experiment several of these absorbers had to be disconnected to ensure proper bubbling. The system was improved by using glass tubes (length: 30 cm; diameter: 27 mm) which were filled with two thirds soda lime and one third $CaCl_2$. The chemicals were separated by a glass wool plug. Glass wool was also used at the ends before being closed with rubber stoppers containing short glass tubes for connection with the air and the NaOH flask. Silica gel granules were added to the $CaCl_2$ to examine water formation. This system was used when paper mill effluent was tested and worked much better than the measuring cylinders as no clogging occurred (paragraph 4.3.1.2).

One further experiment was carried out to establish whether or not the improved extra scrubbers contributed to optimal removal of CO_2 from the air and, therefore, lower CO_2 values in controls. The ISO method requires the acidification of all reaction flasks (1 mℓ concentrated HCl/ℓ) on day 28 to decompose the carbonates and bicarbonates in test solutions and to drive off the carbon dioxide, while aeration continues for a further 24 h. CO_2 analyses are then carried out on the remaining absorbers on day 29. This was not done in our earlier studies because of uncertainty about the procedure. Acidification was carried out in this experiment.

Table 31 shows that results were of the same order with and without the extra scrubbers (using 100 and 200 mℓ $Ba(OH)_2$ in absorber flasks). In all instances the CO_2 produced when the calculated values (50 and 100 mℓ HCl) were used were >70 mg/ℓ CO_2. The tests where the extra scrubbers were used also exceeded 70 mg/ℓ CO_2 when the actual values (47.5 and 97 mℓ HCl) were used for calculation. The study was carried out during the summer period and as a result of the high humidity continuous clogging of the scrubbers occurred. Because of the discouraging results and the problems with clogging the extra scrubbers were omitted from further tests on water and effluents.

Drechsel bottles are usually used for the absorption of CO_2. These bottles are very expensive. It was, therefore, decided to use Erlenmeyer flasks. However, it was found that contact time between bubbles and $Ba(OH)_2$ in the absorber flasks was very short, which could result in a loss of CO_2. In order to improve this, the third experiment was carried out with $Ba(OH)_2$ in 100 mℓ measuring cylinders. The amount of CO_2 absorbed appeared to be slightly better (Table 28) than in experiments 1 and 2. However, it was very difficult to use cylinders for this purpose, as stoppers did not seal properly and the $Ba(OH)_2$ touched the stoppers. In experiment 4 Erlenmeyer flasks were used again. The flasks contained 200 mℓ $Ba(OH)_2$ instead of 100 mℓ. Higher concentrations CO_2 were produced by the controls (Table 25).

TABLE 31: Control values - different experimental conditions (CO_2 production test)

Experimental conditions	Background value (mℓ HCl)	Interval (days):												Total CO_2 produced
		1	5	7	9	12	15	19	23	28	29			
Additional scrubbers; 100 mℓ $Ba(OH)_2$ in reaction flasks; No stirring	47.5	2.0	9.2	5.9	5.8	7.2	7.0	7.8	8.9	9.5	5.9	2.2	0.9	72.3
	50	4.7	12.0	8.7	8.6	9.9	9.8	10.6	11.7	12.2	8.7	5.0	3.6	105.5
No additional scrubbers; 100 mℓ $Ba(OH)_2$ in reaction flasks; No stirring	47.5	3.3	7.3	5.1	4.9	6.2	7.0	6.6	8.6	6.8	4.7	1.4	1.1	63.0
	50	6.0	10.1	7.8	7.7	9.0	9.8	9.4	11.4	9.6	7.5	4.1	3.8	96.2
Additional scrubbers; *200 mℓ $Ba(OH)_2$ in reaction flasks;* No stirring	97	2.0	9.5	5.1	5.2	7.5	6.8	6.4	5.4	5.8	2.9	0	0	56.6
	100	5.3	12.8	8.4	8.6	10.8	10.1	9.8	8.7	9.1	6.2	3.4	1.3	94.5
Additional scrubbers; 100 mℓ $Ba(OH)_2$ in reaction flasks; Stirring	47.5	3.0	9.9	5.6	6.5	7.0	7.9	8.6	9.4	10.1	6.6	2.1	1.6	78.3
	50	5.7	12.6	8.4	9.3	9.8	10.6	11.4	12.1	12.9	9.4	4.9	4.4	111.5

TABLE 32: Biodegradation of aniline (40 mg/ℓ C) during different experimental conditions[a] (DOC reduction)

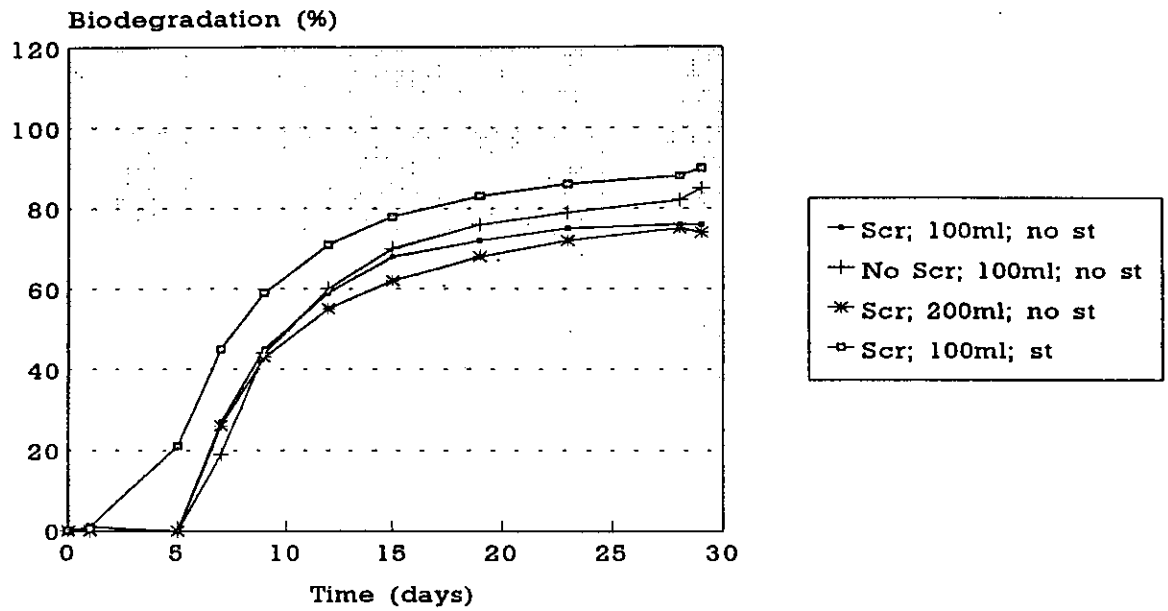
Experimental conditions	% Biodegradation after time (days):		
	0	9	15
Additional scrubbers; 100 mℓ $Ba(OH)_2$ in reaction flasks; No stirring	0	88	90
Additional scrubbers; 100 mℓ $Ba(OH)_2$ in reaction flasks; Stirring	*0*	*87*	*90*

[a] Calculated according to paragraph 3.3.1

As already indicated in a previous paragraph, the use of 200 mℓ $Ba(OH)_2$ instead of 100 mℓ in end absorber flasks resulted in similar absorption of CO_2 in control flasks (Table 31). The results in Figure 12 show a lower biodegradation of aniline when 200 mℓ $Ba(OH)_2$ was used, indicating that the larger volume of $Ba(OH)_2$ did not enhance absorption of CO_2.

Stirring in addition to aeration (Figure 12) resulted in a much more rapid initial biodegradation of aniline and a slightly higher final biodegradation than without stirring. The better mixing of test solution, however, did not improve the variation between duplicate sets of results. The differences were similar to those experienced in previous tests. Table 32 shows similar results in terms of DOC analysis with and without stirring.

Figure 12 shows very little increase in biodegradation on day 29 after acidification of the test samples, indicating that carbonates and bicarbonates were not present.



Scr - Scrubbers; 100ml/200ml - Ba(OH)2; st - stirring

FIGURE 12: Biodegradation of aniline in the CO_2 production test using different experimental conditions

4.2.3 O_2 depletion studies

The biodegradation of the three water soluble chemical compounds aniline, lauryl sulphate and diethylene glycol, and the insoluble compound stearic acid was studied on four occasions. Each experiment also included flasks with $HgCl_2$ to study abiotic activity. During the first three experiments chemicals were used at a concentration of 2 mg/ℓ. (Stearic acid was by mistake added as 6.7 mg/ℓ during the first experiment). A concentration of 10 mg/ℓ was used in experiment 4. A low cell inoculum (1 drop activated sludge effluent/ℓ) was used (Table 10). The sampling frequency was changed during the different experiments to ensure sufficient data. Experiment 1 was terminated after 14 days, as a result of a too small number of flasks.

In general, there was very little difference between the dissolved oxygen concentrations of duplicate results. Control O_2 concentrations ranged between 6.7 and 6.9 mg/ℓ on day 0. Oxygen content in test solutions were generally similar. According to validity criteria (OECD, 1981a) the oxygen depletion in the blank control should not exceed 0.2 to 0.3 mg O_2/ℓ after 5 days and 0.4 mg O_2/ℓ after 28 days. The oxygen depletion in the normal control should not exceed 0.4 to 0.5 mg O_2/ℓ after 5 days and 0.5 to 0.6 mg O_2/ℓ after 15 to 28 days. Experiment 1 showed a depletion of 0.5 mg/ℓ after 4 days and 0.75 mg/ℓ after 14 days. Likewise a too large depletion occurred for the normal control (0.65 mg/ℓ after 4 days and 0.75 mg/ℓ after 14 days). The acetone control showed a depletion of 0.45 mg/ℓ after 4 days and 0.8 mg/ℓ after 14 days. It is expected that these large depletions were due to slight differences in temperature while handling the solutions and bottles. Much lower depletions were observed in the following experiments, and in most instances (particularly experiment 4) the requirements were met. On some occasions values were even slightly higher than on day 0. The acetone control used in the second experiment showed a large depletion (4.55 mg/ℓ) during the 28 day incubation period. It is expected that the acetone did not evaporate before starting the test and that biodegradation took place. The normal control values were used in this case for the calculation of oxygen depletion.

The TOD values of chemicals used to calculate biodegradation are presented in Table 33. The biodegradation of aniline (2 mg/ℓ C) ranged from 92 to >100% after 28 days (Table 34 and Figure 13a). When 10 mg/ℓ C was used the maximum degradation was 27%. This value indicates that the test concentration was too high and oxygen was completely depleted after 7 days, preventing further degradation to take place. The lag time ranged from 1 to 5 days. The results show that this chemical compound is ready biodegradable (when used as 2 mg/ℓ C) and meets the requirements of a reference chemical (OECD test), since the biodegradation was ≥60% within the 10 days after the lag period. The results are in agreement with those presented by Nyholm for the oxygen depletion test (1991).

TABLE 33: Calculated TOD of test chemicals[a]

Aniline	Lauryl sulphate	Diethylene glycol	Stearic acid
2.4052	1.9974	1.5077	2.9244

a According to the formula in paragraph 3.3.3

Table 34 and Figure 13b show that lauryl sulphate (2 mg/ℓ C) was totally biodegraded within 4 to 5 days (biodegradation >100%). When 10 mg/ℓ C was tested the maximum degradation was 34%. As in the case of aniline, this value indicates that the test concentration was too high and oxygen was completely depleted after 4 days, preventing further degradation to take place. The lag time for lauryl sulphate was ≤1 day. The chemical was found to be ready biodegradable (when used as 2 mg/ℓ C) and meets the requirements of a reference chemical (OECD test), since the biodegradation was ≥60% within 10 days after the lag period.

The % biodegradation of diethylene glycol is shown in Table 34 and Figure 13c. The results were erratic and fluctuated between 0 and 25%. There was no real difference between the results of the 2 and 10 mg/ℓ C, and at the end of experiments the oxygen levels still ranged from 4.8 to 6.3 mg/ℓ. The results show that diethylene glycol was non-biodegradable in this test. The results were in agreement with the findings of Nyholm (1991) for the oxygen depletion test. In the study the biodegradation was approximately 30% after 28 days.

TABLE 34: Biodegradation of different chemical compounds[a] (Oxygen depletion test)

Experiment	Sample	% Biodegradation after time (days):											
		0	4	5	7	8	11	12	14	15	19	20	28
1	Aniline[b]	0	14	-	-	71	-	-	83	-	-	-	-
	Lauryl sulphate[b]	0	94	-	-	121	-	-	111	-	-	-	-
	Diethylene glycol[b]	0	-5	-	-	-2	-	-	3	-	-	-	-
	Stearic acid[c]	0	24	-	-	31	-	-	30	-	-	-	-
2	Aniline[b]	2	-	74	-	67	-	-	-	-	98	-	92
	Lauryl sulphate[b]	-3	-	114	-	123	-	-	-	-	175	-	144
	Diethylene glycol[b]	0	-	5	-	-3	-	-	-	-	25	-	7
	Stearic acid[b]	0	-	45	-	51	-	-	-	-	74	-	82
3	Aniline[b]	4	-	47	-	-	-	85	-	-	-	107	114
	Lauryl sulphate[b]	-5	-	145	-	-	-	154	-	-	-	150	150
	Diethylene glycol[b]	3	-	10	-	-	-	3	-	-	-	-2	10
	Stearic acid[b]	-2	-	114	-	-	-	97	-	-	-	110	117
4	Aniline[d]	3	4	-	27	-	27	-	-	27	-	-	27
	Lauryl sulphate[d]	-1	34	-	32	-	33	-	-	32	-	-	32
	Diethylene glycol[d]	1	2	-	1	-	2	-	-	2	-	-	12
	Stearic acid[d]	0	9	-	15	-	21	-	-	19	-	-	21

[a] Calculated according to paragraph 3.3.3
[b] 2 mg/ℓ C
[c] 6.67 mg/ℓ C
[d] 10 mg/ℓ C

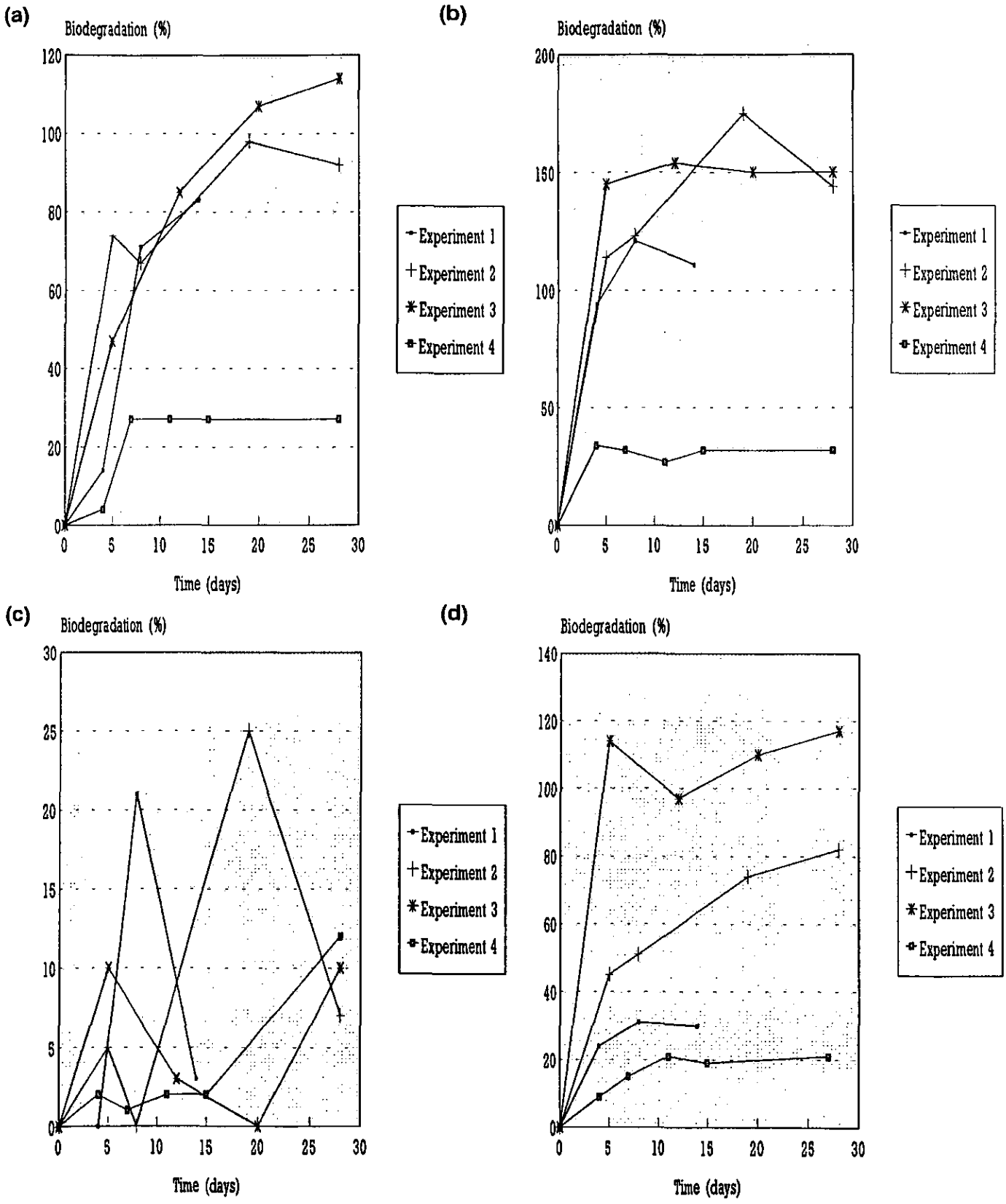


FIGURE 13: Biodegradation of aniline (a), lauryl sulphate (b), diethylene glycol (c) and stearic acid (d) in the oxygen depletion test

The results of the % biodegradation of stearic acid is shown in Table 34 and Figure 13d. The results of the two experiments where 2 mg/ℓ C were used showed a large variation. In experiment 2 the maximum biodegradation after 28 days was 82%. Experiment 3 showed a maximum degradation of >100% at 28 days. When 6.7 (Experiment 1) and 10 mg/ℓ C (Experiment 4) were tested the maximum degradation levels were 30 and 21%, respectively. As in the case of aniline and lauryl sulphate these values indicate that the test concentrations were too high. Constant levels of degradation was obtained after 8 to 10 days. By this time the oxygen was completely depleted, preventing further degradation to take place. The lag time for stearic acid ranged between 1 and 5 days. The results of the two experiments suggest that stearic acid is ready biodegradable since a degradation of ≥60% was obtained within 10 days after the lag time. This chemical would, however, not be suitable as reference chemical due to the large variation between results.

The oxygen concentrations of bottles sterilized with $HgCl_2$ are shown in Table 35. In general, there were not large differences between the results at time 0 and at the end of the experiments, indicating that abiotic degradation did not take place. The largest depletion noticed was 0.7 mg/ℓ for the normal control. These changes could be attributed to temperature fluctuations. The $HgCl_2$ concentration was very high (about 30 mg/ℓ), which means that it is unlikely that bacterial growth caused the depletion. This can be illustrated with the acetone result of the second experiment. When $HgCl_2$ was added no change took place in oxygen content, but during the test the oxygen was depleted by 4.55 mg/ℓ. During the last experiment when 10 mg/ℓ C was used, increases in oxygen content was noticed. The reason for this is not clear.

The 1981 OECD methodology followed in this study states that between 4 and 5 mg/ℓ oxygen should be left in bottles at the end of tests to ensure degradation, if the oxygen concentration at time 0 is 9 mg/ℓ. To achieve this residual oxygen, the concentration of test chemicals is limited to a starting concentration of 2 mg/ℓ C. We used the stated 2 mg/ℓ C test concentration in experiments 1 to 3, but because of a low starting oxygen concentration (approximately 6.9 mg/ℓ instead of 9.0 mg/ℓ) the residual oxygen concentration after 28 days incubation was very low or depleted (aniline: 0.7 to 2.0 mg/ℓ; lauryl sulphate: 0.2 mg/ℓ; and stearic acid: 0.05 to 1.65 mg/ℓ). This indicates that a lower residual concentration should be considered for local studies. The test concentration of 2 mg/ℓ did not seem too high, as in general, an acceptable degradation was already obtained before oxygen was depleted. A value >100% biodegradation is possible when the TOD of a chemical is considerably smaller than the oxygen depletion value (*e.g.* lauryl sulphate). This can be rectified by using a slightly higher test concentration (*e.g.* 2.5 mg/ℓ C).

4.2.3.1 Optimization of test

The bottles used in our studies dried out around the stoppers which resulted in the formation of air bubbles in bottles. This was noticed after only a few days of incubation. The presence of bubbles could influence oxygen concentration. Because problems were experienced with fluctuating values, particularly in case of controls, it was necessary to avoid this accumulation of air in bottles. The problem was solved by placing bottles upside down in a clean flat container with necks immersed in Milli-Q water. No bubbles formed during the incubation period. Table 36 shows that the arrangement did not improve oxygen depletion in control and blank control flasks. Results obtained for aniline were similar whether flasks were upright or upside down with necks immersed in Milli-Q water (Figure 14). Much better results were obtained for controls and blank controls in a second experiment where flasks were upright and upside down (Table 37). No improvement could, however, be noticed in results when

TABLE 35: Degradation of different chemicals in the presence of $HgCl_2$

Sample	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Oxygen concentration (mg/ℓ) at time (days):							
	0	14	0	28	0	28	0	28
Control	6.8	6.8	6.8	6.6	6.9	6.2	6.9	7.0
Blank control	6.8	6.7	6.8	6.6	6.9	6.6	6.9	6.8
Acetone control	6.8	6.7	6.8	6.7	6.8	6.4	6.7	6.9
Aniline	6.8	6.3	6.7	6.5	6.7	6.2	6.1	7.4
Lauryl sulphate	6.8	6.7	6.9	6.8	7.1	6.8	7.0	7.8
Diethylene glycol	6.8	6.7	6.8	6.7	6.8	6.6	6.7	7.0
Stearic acid	6.8	6.4	6.8	6.5	6.9	6.6	6.6	7.7

TABLE 36: Dissolved oxygen concentration in bottles after various incubation periods

Sample	Position of flasks	Average oxygen concentration (mg/ℓ) at time (days):			
		0	6	15	28
Control	Upright	6.9	6.3	6.3	5.9
	Inverted with stopper in water	6.9	6.3	6.3	5.6
Blank control	Upright	6.9	6.7	6.6	5.9
	Inverted with stopper in water	6.9	6.6	6.6	5.9
2 mg/ℓ aniline	Upright	6.9	2.8	2.3	1.3
	Inverted with stopper in water	6.9	2.9	2.2	0.8

TABLE 37: Dissolved oxygen concentration in controls and blanks

Sample	Position of flasks	Average oxygen concentration (mg/ℓ) at time (days):			
		0	5	15	28
Control	Upright	6.9	6.7	6.5	6.8
	Inverted with stopper in water	6.9	6.8	6.7	6.5
Blank control	Upright	6.8	6.6	6.6	6.5
	Inverted with stopper in water	6.8	6.7	6.7	6.6

flasks were upside down with necks in water. It, therefore, appears that bubble formation in flasks is not causing the fluctuating oxygen values in controls and blank controls.

The OECD method recommends the use of one drop of activated sludge/ℓ of sample. This can result in large variations in cell numbers in parallel solutions. In order to have a more accurate volume, 0.05 mℓ/ℓ was used in the last two experiments. The inoculation was further improved by first diluting activated sludge effluent and then adding a larger volume to test solutions (2 mℓ/100 mℓ water, and then adding 10 mℓ to 4 ℓ).

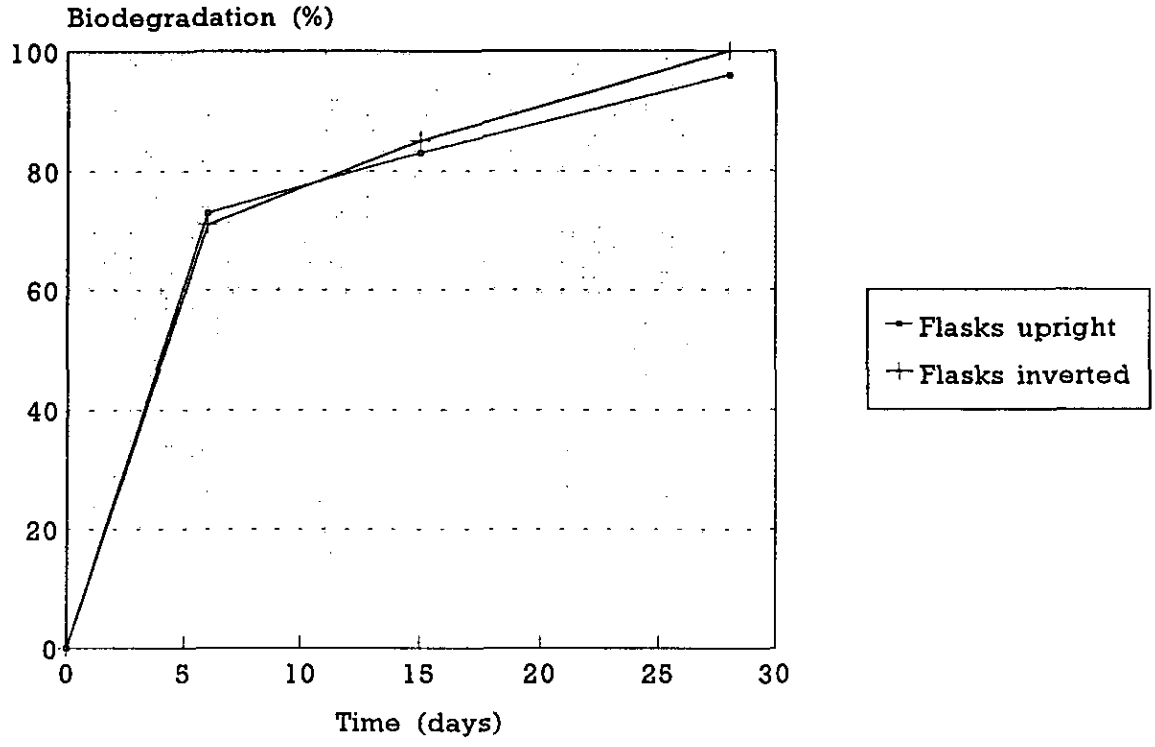


FIGURE 14: Biodegradation of aniline in the oxygen depletion test using different experimental conditions

4.3 Biodegradation tests on river water and effluent

4.3.1 Optimization of tests

Apies river water 1 and paper mill effluent were tested on a number of occasions to optimize tests for water and effluent testing. Table 9 shows that some of the nutrient concentrations in the river water and effluent were higher than those in test media (for example calcium, magnesium, sodium, sulphate, and chloride), others were lower (*e.g.* phosphates), and some were similar. The organism concentrations were similar to the numbers used in the ISO DOC reduction and CO_2 production tests, but much larger than numbers required in the OECD DOC reduction and O_2 depletion tests. The DOC concentration of Apies river water 1 was <10 mg/ℓ, while paper mill effluent contained a DOC of between 40 and 50 mg/ℓ. In order to establish optimum experimental conditions tests were carried out on samples with and without test medium and inocula.

4.3.1.1 DOC reduction test

The first experiment on Apies river water was carried out on the water alone (DOC: 7.0 mg/ℓ), water with inocula (river water and activated sludge) but without additional media, water with media (OECD and ISO) but without inocula, and water with inocula and media. Possible inhibition was evaluated by adding aniline (40 mg/ℓ C) to river water. Aniline also served as reference chemical. In general, the DOC concentrations in control solutions were <0.5 mg/ℓ. In a few instances values ranging from 0.5 to 1.2 mg/ℓ were noticed. The variation between duplicate tests was generally small (<20%).

The % biodegradation of the river water is shown in Table 38 and Figure 15. The results obtained with the river water alone, the water plus the OECD medium, water plus the river water inoculum and water plus the inoculum and OECD medium, showed a large fluctuation (up and down) during the 28 day period. The highest biodegradation of the river water alone was reached on day 7 (51%) and that of the other three samples on day 21 (56 to 68%). The biodegradation values decreased after day 20 and were between 41 and 45% on day 28. When the activated sludge and ISO medium was used, much better results were obtained. Biodegradation increased within 5 days to between 56 and 66%. At day 14 degradation was at its maximum (60 to 72%). Degradation decreased over the next 14 days to end at between 44 and 60%. The best biodegradation was obtained when activated sludge and ISO medium were added to the river water. However, results suggest that the organic compounds in the river water were not ready biodegradable as the recommended 80% level for the ISO test or the 70% level for the OECD test was not reached. DOC values ranged from 2.0 to 4.0 mg/ℓ after biodegradation. Precipitation occurred in solutions where media were added. This was particularly high when ISO medium was used.

The results in Table 39 show that both inocula had high activity (biodegradation of aniline >80% in 14 days). Furthermore, the Apies river water did not contain toxic components, since biodegradation was larger than the specified 40% after 7 days incubation. The concentration of aniline was high compared to the DOC of river water. More realistic results might have been obtained if aniline was added at a lower concentration (*e.g.* 10 mg/ℓ C).

The second experiment on Apies river water was conducted using river water alone, water with OECD and ISO media, water with activated sludge inoculum and water with activated sludge and ISO medium. Toxicity was evaluated by adding aniline to the river water in the absence of inoculum and medium, and with inoculum and medium. $HgCl_2$ was added to river water to evaluate abiotic degradation. The DOC concentration for the river water was 8.8 mg/ℓ. In addition to DOC, the samples were analyzed for TOC, COD filtered, COD unfiltered, and microorganisms (Table 40). DOC and TOC values were similar and biodegradation was therefore only calculated for DOC. COD (filtered and unfiltered) for river water samples were low at the start of the experiment and were higher later. No calculations could, therefore, be made. The results obtained with DOC analysis are shown in Figure 16. The best biodegradation results were obtained with inoculum and ISO medium (86% after 10 days). This was followed by the water with nutrients (64 to 67% after 28 days). The lowest biodegradation was obtained with river water alone and river water with inoculum (34 to 38% after 28 days). The biodegradation obtained under the different experimental conditions were in agreement with those of the first experiment, but without the fluctuation. The biodegradation of the river water plus inoculum and nutrients met the requirements of ready biodegradability (>80% in 14 days). Precipitation occurred in the samples where media were added, which could have interfered with the results. Microbial numbers in the control decreased over time (Figure 29). The numbers in test samples increased and thereafter decreased.

TABLE 38: Biodegradation of river water during different experimental conditions[a] (DOC reduction test)

Inoculum	Sample	% Biodegradation after time (days):						
		0	3	5	7	14	21	28
River water	River water plus inoculum	0	29	44	49	40	56	45
	River water plus nutrients[b]	0	31	51	49	46	68	43
	River water plus inoculum and nutrients[b]	0	29	55	26	44	62	41
Activated sludge	River water plus inoculum	0	33	56	56	60	56	44
	River water plus nutrients[c]	0	39	58	61	66	64	45
	River water plus inoculum nutrients[c]	0	34	66	65	72	70	60
No inoculum	River water	0	40	43	51	39	50	44

[a] Calculated according to paragraph 3.3.1
[b] OECD medium
[c] ISO medium

TABLE 39: Biodegradation of mixtures of river water and aniline[a] (40 mg/ℓ C) (DOC reduction test)

Inoculum	Sample	% Biodegradation after time (days):						
		0	3	5	7	14	21	28
River water	Aniline	0	14	93	89	93	-	-
	River water plus aniline	0	17	80	85	86	92	92
Activated sludge	Aniline	0	+1	19	94	98	-	-
	River water plus aniline	0	8	87	87	93	91	92
No inoculum	River water plus aniline	0	24	79	82	85	89	89

[a] Calculated according to paragraph 3.3.1
- Not determined

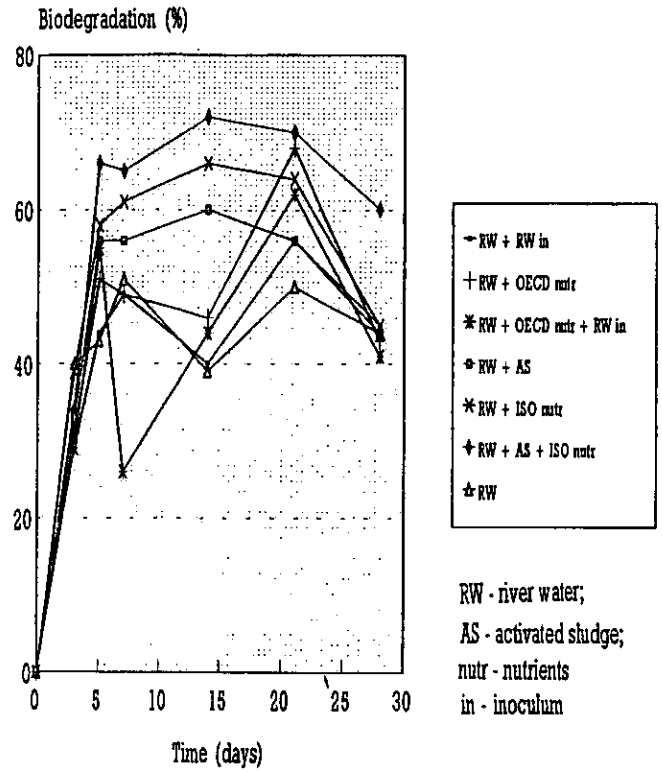


FIGURE 15: Biodegradation of river water in the DOC reduction test (Experiment 1)

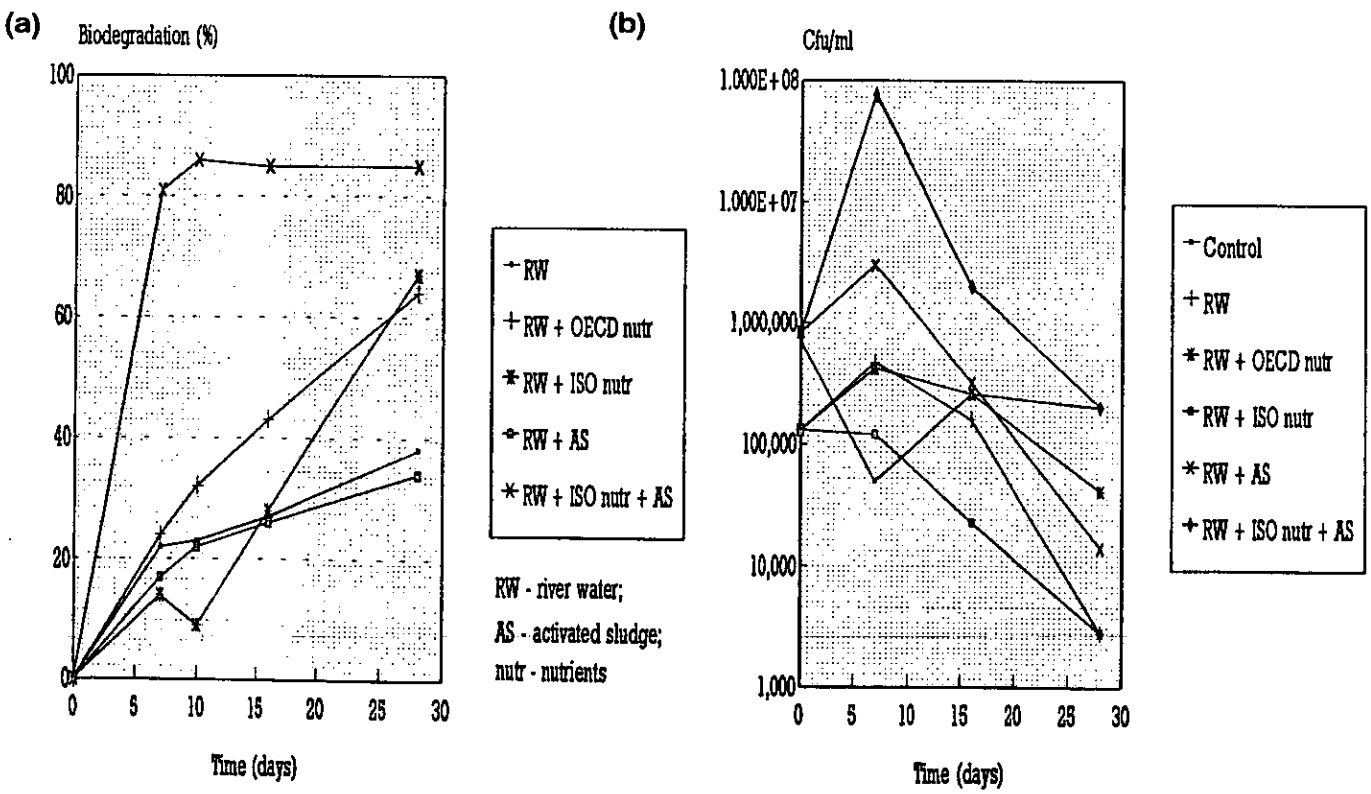


FIGURE 16: Biodegradation of river water in the DOC reduction test measuring DOC (a) and microorganism numbers (b) (Experiment 2)

TABLE 40: Biodegradability of river water (DOC reduction test)

Sample	Concentration (mg/ℓ or cfu/mℓ) after degradation period (days):																		
	0					7					10	16					28		
	DOC	TOC	COD	COD uf	Mic	DOC	TOC	COD	COD uf	Mic	DOC	DOC	TOC	COD	COD uf	Mic	DOC	COD	Mic
Control	<0.5	<0.5	0	0	$7.0x10^6$	0.5	0.7	11	47	$5.0x10^4$	0.8	0.9	0.5	9	23	$2.6x10^6$	1.3	16	$2.0x10^5$
River water	8.8	8.9	3	16	$1.3x10^6$	7.4	7.7	12	11	$4.7x10^6$	7.6	7.3	6.4	11	10	$1.6x10^6$	6.8	11	$2.8x10^3$
River water plus OECD nutrients	8.8	8.9	3	16	$1.3x10^6$	7.2	7.3	12	16	$4.2x10^6$	6.8	5.9	5.3	16	17	$2.6x10^6$	4.5	9	$4.1x10^4$
River water plus ISO nutrients	8.8	8.9	3	16	$1.3x10^6$	8.1	7.5	14	14	$1.2x10^6$	8.8	7.2	6.2	10	18	$2.3x10^4$	4.2	10	$2.8x10^3$
River water plus activated sludge	8.8	8.9	3	16	$8.3x10^6$	7.8	7.1	14	54	$3.0x10^6$	7.7	7.4	6.4	14	26	$3.2x10^6$	7.1	11	$1.4x10^4$
River water plus ISO nutrients and activated sludge	8.8	8.9	3	16	$8.3x10^6$	2.2	2.1	26	64	$7.6x10^7$	2.0	2.2	1.9	18	64	$2.0x10^6$	2.6	24	$2.0x10^6$
River water plus aniline	36.9	35.5	132	159	$1.3x10^6$	16.9	16.7	43	57	$4.1x10^6$	9.9	7.8	7.2	22	28	$1.3x10^7$	5.1	14	$2.0x10^5$
River water plus aniline plus ISO nutrients and activated sludge	36.9	35.5	132	159	$8.3x10^6$	8.3	7.7	31	126	$4.7x10^6$	6.0	2.8	2.8	44	57	$3.1x10^6$	2.9	24	$3.7x10^5$
River water plus $HgCl_2$	8.8	8.9	3	16	$1.3x10^6$	-	-	-	-	-	-	-	-	-	-	-	7.4	10	-

uf Unfiltered sample (COD was always determined on filtered samples, except in this study where COD was measured on both filtered and unfiltered sample)
Mic Microorganisms
- Not determined

The river water was not toxic, as illustrated by the DOC, TOC and COD analyses (biodegradation >40% after 7 days and microbial numbers increased) (Table 40). The DOC and COD concentrations of river water remained fairly constant when treated with $HgCl_2$, indicating an absence of abiotic degradation (Table 40).

Paper mill effluent was studied by testing the effluent alone, effluent plus media (OECD and ISO), effluent with activated sludge inoculum, and effluent with inoculum and medium (ISO). Toxicity was evaluated by adding aniline to the effluent in the absence of inoculum and medium, and with inoculum and medium. $HgCl_2$ was added to effluent to evaluate abiotic degradation. Biodegradation was measured in terms of DOC, TOC, COD (filtered) and microorganisms. The initial DOC, TOC and COD concentrations were 38.7, 42.0 and 117 mg/ℓ, respectively (Table 41). Biodegradation results are presented in Figure 17. The results obtained for DOC and TOC were similar (Figures 17a and 17b). The best biodegradation was found with effluent plus inoculum and nutrients (approximately 60% after 14 days), closely followed by effluent with ISO nutrients (approximately 60% after 14 to 21 days). This was followed by effluent with OECD medium (approximately 40% after 14 days). The biodegradation in effluent alone and effluent with inoculum ranged between 20 and 30% after 14 days. The trend was the same as that of river water. COD analysis (Figure 17c) showed the best biodegradation in the presence of inoculum and nutrients (50% after 21 days), followed by effluent with OECD medium (41% after 21 days), and effluent with activated sludge inoculum (37% after 14 days). The lowest biodegradation was obtained when effluent was tested alone and with ISO medium (approximately 30%). None of the results met the required levels to suggest ready biodegradability (80% level for the ISO test and 70% for the OECD test). Organic chemical concentrations at the end of the experiment were high (DOC and TOC: 18-32 mg/ℓ; COD: 62-84 mg/ℓ). In most instances the % degradation decreased at the end of the experiment. Some degree of precipitation was observed in the samples where media was added, which could have interfered with the results. Microbial numbers showed an increase after 7 days and a decrease after 14 days (Figure 17d).

In case of DOC and TOC analysis the biodegradation of effluent plus aniline was <40% in 7 days suggesting toxicity. However, when medium and nutrients were added, the biodegradation exceeded the required level, indicating that the effluent was not toxic. COD results showed an absence of toxicity (Table 41). The results in Table 41 show that no abiotic degradation occurred.

An additional experiment was carried out with mixtures of the effluent (DOC: 52.3 mg/ℓ) and Apies river water (DOC: 7.4 mg/ℓ) without the addition of inoculum or medium. The results are presented in Figure 18. The river water alone showed a biodegradation of 47% after 23 days and the effluent 30% after 10 days (similar to results of previous experiments). A 50:50% mixture of the effluent and river water reached 30% biodegradation after 23 days. The 25:75% effluent-river water mixture showed only 23% biodegradation after 23 days. The DOC in the effluent was 39 mg/ℓ at the end of the test and that of the river water 4.3 mg/ℓ. The results show low biodegradation of the effluent upon dilution.

4.3.1.2 CO_2 production test

The test was slightly modified to test water and effluent. Purging was carried out for 24 h with empty reaction flasks. On day 0 the 0.0125 M $Ba(OH)_2$ was added to the absorber flasks while purging continued. All the test solutions were prepared in separate containers. The Milli-Q water and test samples were added to containers at the required volumes. This was followed by the addition of nutrients and inoculum. Containers were then swirled for mixing.

TABLE 41: Biodegradability of effluent (DOC reduction test)

Sample	Concentration (mg/ℓ or cfu/mℓ) after degradation period (days):																	
	0				7				14				21			28		
	DOC	TOC	COD	Mic	DOC	TOC	COD	Mic	DOC	TOC	COD	Mic	DOC	TOC	COD	DOC	TOC	COD
Control	<0.5	<0.5	10.0	$2.6x10^4$	0.6	<0.5	0	$6.3x10^4$	<0.5	<0.5	15.0	$2.3x10^4$	<0.5	<0.5	0	<0.5	<0.5	1.0
Effluent	38.7	42.0	117	$5.5x10^5$	39.3	33.8	91.0	$5.0x10^6$	31.1	30.2	94.0	$2.7x10^4$	29.9	30.5	81.0	29.8	29.4	74.0
Effluent plus OECD nutrients	38.7	42.0	117	$5.5x10^5$	31.7	35.6	74.0	$5.8x10^6$	23.8	25.8	83.0	$2.5x10^6$	23.1	25.7	63.0	23.7	24.8	64.0
Effluent plus ISO nutrients	38.7	42.0	117	$5.5x10^5$	20.5	27.1	78.0	$1.5x10^6$	16.7	18.6	94.0	$6.5x10^5$	13.1	17.7	79.0	18.0	18.8	84.0
Effluent plus activated sludge	38.7	42.0	117	$5.7x10^5$	34.8	40.6	78.0	$6.7x10^5$	28.2	28.0	82.0	$1.4x10^4$	28.7	28.6	71.0	32.1	29.6	72.0
Effluent plus ISO nutrients and activated sludge	38.7	42.0	117	$5.7x10^5$	17.5	22.4	87.0	$7.5x10^5$	14.1	17.0	79.0	$4.3x10^5$	14.6	16.1	54.0	17.9	18.1	62.0
Effluent plus aniline	68.5	68.0	267	$5.3x10^5$	54.8	55.1	142	$3.4x10^6$	29.9	28.7	88.0	$6.4x10^5$	27.6	30.6	57.0	25.0	23.9	62.0
Effluent plus aniline plus ISO nutrients and activated sludge	68.5	68.0	267	$5.3x10^5$	20.6	20.6	80.0	$1.0x10^7$	14.6	16.5	69.0	$7.3x10^5$	12.8	16.8	41.0	13.6	14.6	50.0
Effluent plus $HgCl_2$	38.7	42.0	117	$5.5x10^5$	-	-	-	-	-	-	-	-	-	-	-	40.0	42.9	111

Mic Microorganisms
- Not determined

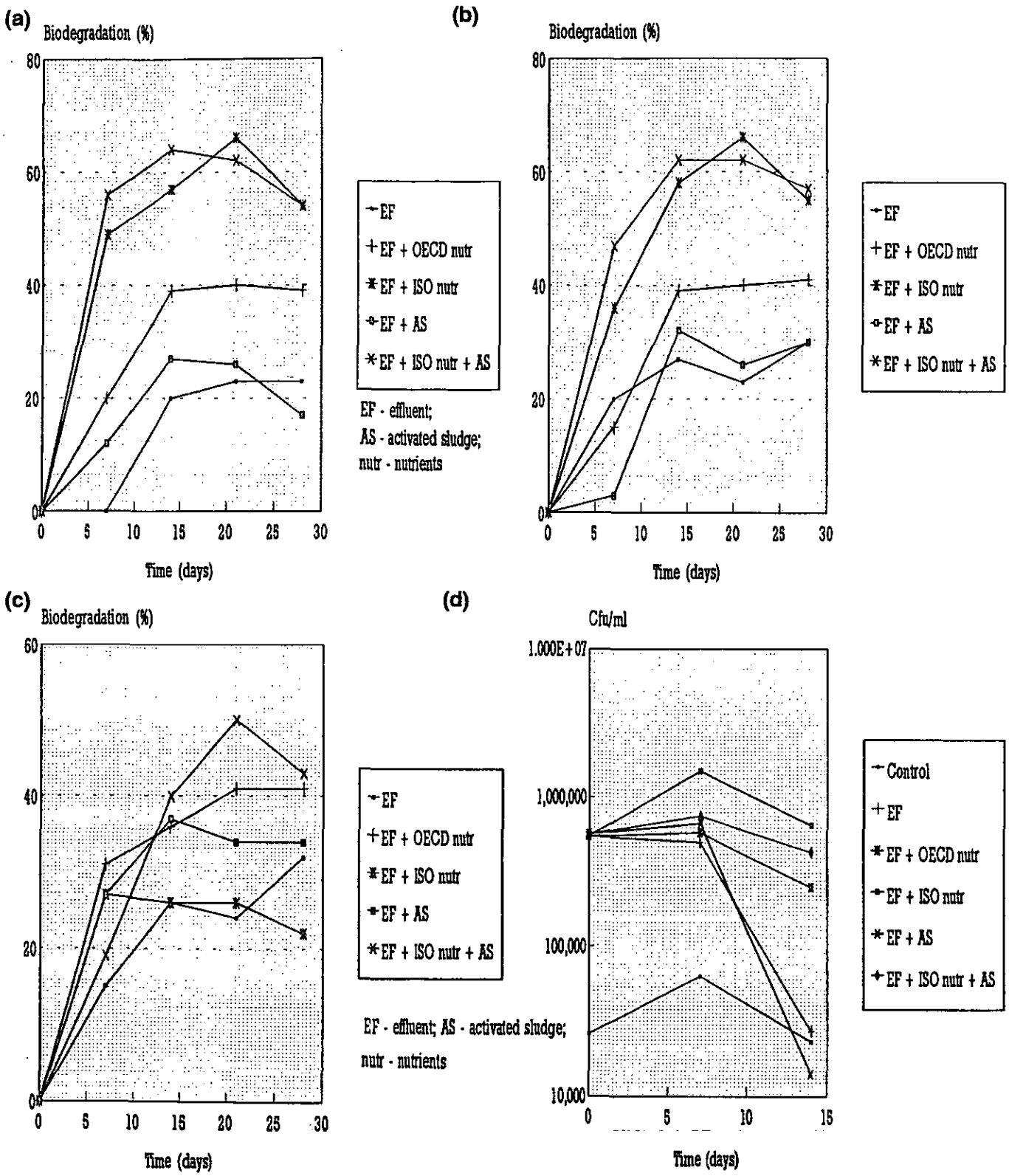
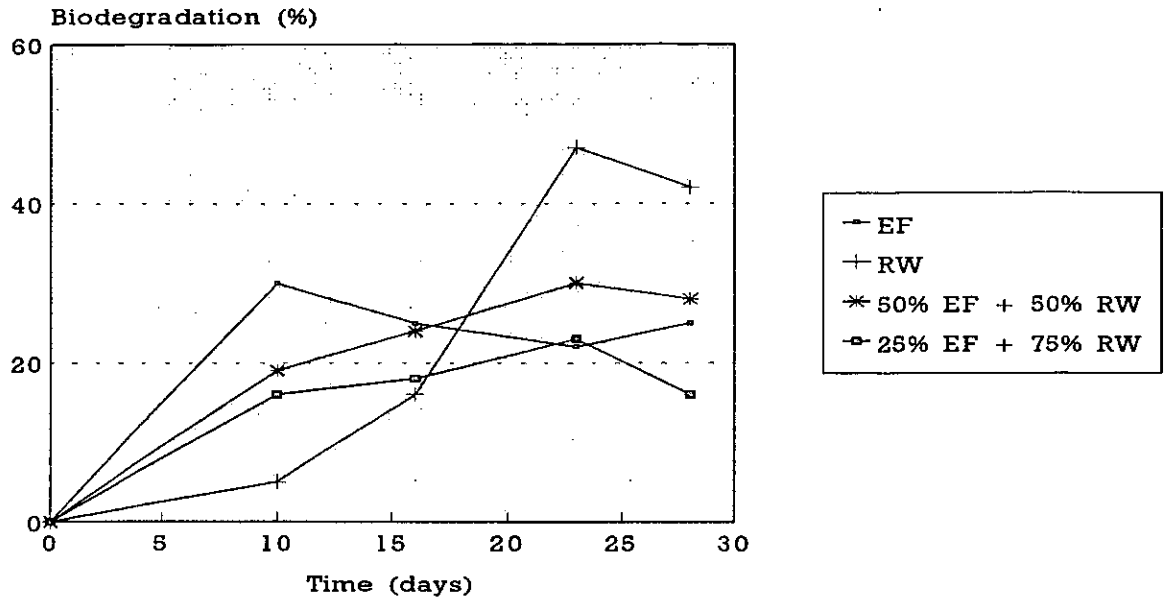


FIGURE 17: Biodegradation of paper mill effluent in terms of DOC reduction test measuring DOC (a), TOC (b), COD (c) and microorganism numbers



EF - effluent; RW - river water

FIGURE 18: Biodegradation of effluent and river water mixtures in the DOC reduction test

The three absorber flasks were then sealed off to avoid CO_2 entry. While purging continued different solutions were added rapidly to reaction flasks. The absorber flasks were then reconnected to reaction flasks. Samples were immediately taken for DOC analysis. All the reaction flasks were acidified (1 mℓ concentrated HCl/ℓ) at the end of an experiment to decompose the carbonates and bicarbonates in test solutions and to drive off the carbon dioxide, while aeration continued for a further 24 h. CO_2 analyses were then carried out on the remaining absorbers. Final DOC samples were taken before acid addition.

The results of an experiment on Apies river water are shown in Table 42 and Figure 19a. River water was tested alone, with nutrients, with activated sludge inoculum and with inoculum and medium. 100 mℓ of $Ba(OH)_2$ was used in absorber flasks and the additional scrubbers were omitted. In general, the differences between duplicate test and control results were small. The total CO_2 produced in control flasks was 97.3 mg/ℓ when the calculated value of 50 mg/ℓ was used and 61.9 mg/ℓ when the actual value (47.5 mg/ℓ) was used. The best results were obtained for river water plus inoculum and nutrients (359% on day 28). This was followed by the river water plus nutrients (240% on day 28) and river water plus inoculum (175% on day 28). The river water alone also showed biodegradation and reached 127% on day 28. The results were in agreement with those obtained with the DOC reduction test (4.3.1.1). The very high % biodegradability makes interpretation in terms of ready biodegradability difficult. The results were very much depended on the TCO_2 value, which in this case only was 27.2 (DOC of river water x 3.67 = 7.4 x 3.67). The addition of acid on day 28 resulted in a large amount of CO_2 produced, indicating that carbonates and bicarbonates were present in the reaction flasks. The increase was particularly high when medium and inoculum were added. Biodegradation was also established by conducting DOC, TOC, COD and microbial analyses on the samples. TOC and COD results fluctuated and could not be applied for calculation. The results obtained with DOC and microbial analyses are presented in Figures 19b and 19c.

TABLE 42: Biodegradation of river water during different experimental conditions[a] (CO_2 production test)

Sample	% Biodegradation at time (days):												
	2	3		4	7	10	11	14	21	28	29		
River water	24	31	39	42	55	65	71	83	102	127	170	190	191
River water plus nutrients	150	169	182	184	193	206	211	222	235	240	383	407	414
River water plus inoculum	49	68	82	83	104	117	124	141	161	174	346	446	465
River water plus nutrients and inoculum	156	207	232	234	267	301	310	326	345	359	506	539	548

[a] Calculated according to paragraph 3.3.2

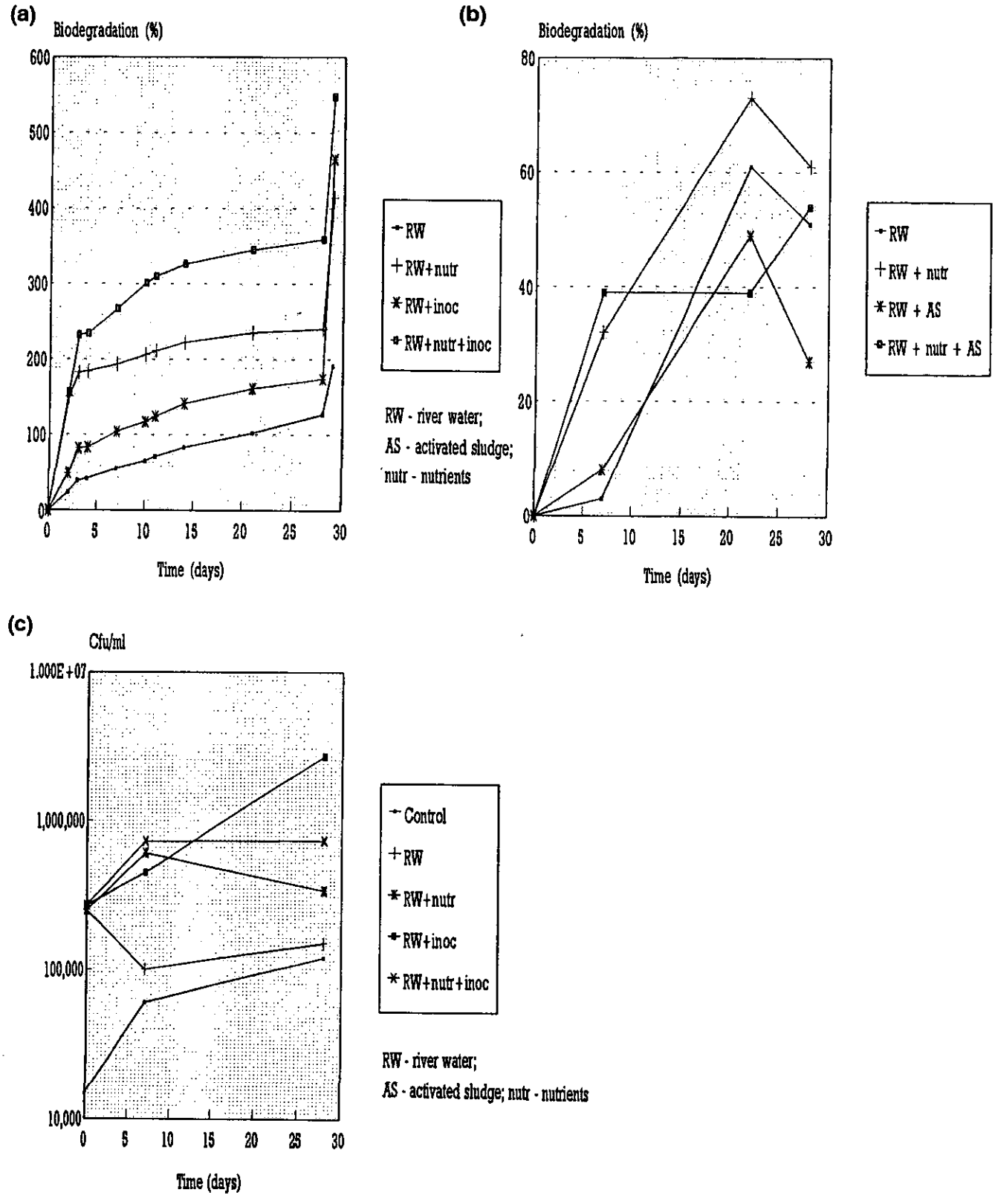


FIGURE 19: Biodegradation of river water in the CO_2 production test measuring CO_2 (a), DOC (b) and microorganism numbers (c)

The maximum biodegradation in terms of DOC was obtained with river water plus nutrients. This was followed by river water alone and river water with nutrients and inoculum. The river water with inoculum showed the lowest biodegradation. These results differed from those obtained with CO_2 production (Table 42 and Figure 19a) and DOC reduction (4.3.1.1), and were erratic. Microbial numbers showed a decrease or increase after time (erratic) (Figure 19c).

The first experiment on paper mill effluent included aniline as reference chemical (40 mg/ℓ). In addition, 40 mg/ℓ of stearic acid was tested to establish whether or not the results obtained during experiments 1 to 4 on individual chemicals (4.2.2) could be improved. (Aniline was added to the separate container during test solution preparation while stearic acid was added directly to the sample after introduction into the reaction flasks). Mixtures of the chemical compounds and paper mill effluent were tested to examine possible toxicity. The mixed samples received inoculum (activated sludge) but no medium. The effluent was tested alone and with inoculum. Medium was not added because of the possibility of precipitation. The additional scrubber devices containing soda lime and $CaCl_2$ were placed between the compressed air valves and NaOH solutions to establish whether the CO_2 produced in control tests could be reduced (4.2.2.1). Two hundred millilitres of $Ba(OH)_2$ was used in absorber flasks. The study was carried out over 29 days because readings could not be taken on day 28.

On a number of sampling occasions duplicate control CO_2 values showed larger differences (as high as 6 mg/ℓ CO_2) than in previous experiments. Large differences were also more frequently obtained between test sample duplicates, particularly with effluent samples (as high as 30 mg/ℓ CO_2). These differences could be ascribed to possible differences in air flow during aeration or because clogging took place (stopping air flow). The effluent contained particulate matter which could also have contributed to different biodegradation rates. According to the ISO method the CO_2 production in the control should be insignificant and not exceed 70 mg/ℓ (usually 30 to 40 mg/ℓ) at the end of the test. However, in this study the CO_2 produced when the actual value (97 mℓ HCl) was considered was 89.8 mg. When the calculated value was used (100 mℓ HCl), the CO_2 produced was 126.1 mg. Both values were much higher than the specified value.

The % biodegradation of the effluent, chemical compounds, and mixtures of chemicals and effluent is shown in Table 43. The TCO_2 used to calculate the results of aniline and stearic acid was 146.8 (3.2.2). The TCO_2 of the effluent was 160.6 (DOC of effluent x 3.67 = 43.8 x 3.67) and that of the effluent plus chemical compounds was 307.27 (3.2.2). The results obtained with the chemical compounds show a lag period of <5 days. The maximum biodegradation at day 30 was 78% for aniline and 60% for stearic acid. The results obtained with aniline as reference chemical (degradation ≥50% within 14 days) show that the inoculum was suitable for use. The biodegradation of stearic acid was <50% in 14 days, verifying the results of experiments 1 to 4 (4.2.2) that the chemical is difficult to biodegrade and is not suitable as reference chemical.

The effluent, with and without inoculum, showed a rapid biodegradation throughout the incubation period. 50% degradation was reached after 14 days, indicating ready biodegradability (Table 43 and Figure 20a). The maximum biodegradation reached at day 29 was 77% in case of effluent without inoculum and 113% in case of effluent with inoculum.

The results of mixtures of the effluent and chemical compounds (Table 43) showed that the effluent was not toxic (% degradation >25% in 29 days). Results show that there was little

TABLE 43: Biodegradation of effluent and mixtures of effluent and chemical compounds[a] (CO_2 production test)

Sample	% Biodegradation at time (days):										
	1	2	5	8	12	15	21	26	29	30	30
Aniline	-2	-1	11	43	62	72	76	77	76	78	78
Stearic acid	1	4	14	24	33	38	41	46	52	58	60
Effluent without inoculum and nutrients	14	20	36	45	56	65	74	75	77	104	125
Effluent with inoculum but without nutrients	18	30	47	61	78	82	92	107	113	155	166
Effluent plus aniline, with inoculum but without nutrients	7	11	19	34	46	53	61	67	69	93	100
Effluent plus stearic acid with inoculum but without nutrients	9	14	27	38	50	54	60	62	63	90	100

[a] Calculated according to paragraph 3.3.2

increase in the total amount of CO_2 produced after acidification of aniline and stearic acid, indicating that very little carbonates and bicarbonates were present in reaction flasks. On the other hand, large amounts of CO_2 were released in flasks containing effluent and mixtures of effluent and chemicals, indicating the presence of carbonates and bicarbonates.

Table 44 shows the biodegradation in terms of DOC. In general, control values were <0.5 mg/ℓ. The control results of days 23 and 26 were very high and could have been due to an experimental error. With a few exceptions, duplicate test results were in agreement. A large difference was observed for the mixtures of effluent and aniline on days 0, 23 and 26. The DOC values of the two effluent samples differed by about 10 mg/ℓ on day 0. The reason for this is not clear.

Aniline showed 100% degradation within 7 days (Table 44). The biodegradation of the effluent samples increased until day 13 (between 35 and 44%). On the next two sampling dates the DOC values increased above those initially measured (Table 44 and Figure 20b) and the biodegradation values decreased. On day 29 the biodegradation values were between 23 and 38%. The effluent-aniline mixture showed similar results (Table 44). The reason why the DOC values were extremely high on days 23 and 26 is not clear. It is possible that organic material was released from particulate matter, which could previously not pass through the membrane filter. It is also likely that errors were made with the analysis. Additional parameters were included in other studies on effluent and water to enhance interpretation of results (*e.g.* microbial numbers, TOC and COD). The maximum biodegradation value obtained for the effluent was 44%, which implies that the effluent is not biodegradable. The results obtained for the mixture indicate that the effluent was not toxic (approximately 50% in 7 days).

The results of a second CO_2 production test on paper mill effluent is shown in Table 45 and Figure 21a. The effluent was tested alone, with nutrients, with inoculum and with inoculum and nutrients. 100 mℓ of $Ba(OH)_2$ was used in absorber flasks and the additional scrubbers were omitted. In general, differences between duplicate control and test values were small. The total CO_2 produced in control flasks was 97.3 mg/ℓ when the calculated value of 50 mg/ℓ was used and 61.9 mg/ℓ when the actual value (47.5 mg/ℓ) was used. The TCO_2 value used to calculate % biodegradation was 191.9 (DOC value x 3.67 = 52.3 x 3.67).

The highest biodegradation was obtained when nutrients and inoculum were added to the effluent (106% after 28 days). This was followed by the effluent containing nutrients (94% after 28 days). The results obtained for the effluent alone and effluent with inoculum were similar (60-70% after 28 days). The results were in agreement with those obtained with the DOC reduction test (4.3.1.1). The % biodegradation was >50% within 14 days (ISO test), suggesting ready biodegradability for all samples. The addition of acid on day 28 resulted in an increase in CO_2 produced, indicating that carbonates and bicarbonates were present in the reaction flasks. Biodegradation was also established by conducting DOC (Figure 21b), TOC (Figure 22a), COD (Figure 22b) and microbial (Figure 22c) analyses on the samples. DOC, TOC and COD results were similar, showing that the highest biodegradability was obtained with effluent plus nutrients and inoculum (approximately 50%). This was followed by effluent with nutrients, effluent with inoculum and effluent alone. These results were in agreement with those obtained in DOC reduction tests and the CO_2 production test. However, the requirement for ready biodegradability was not met. Microbial numbers (Figure 22c) of the control test showed an increase after time. Microbial numbers of test solutions decreased after 7 days and increased thereafter.

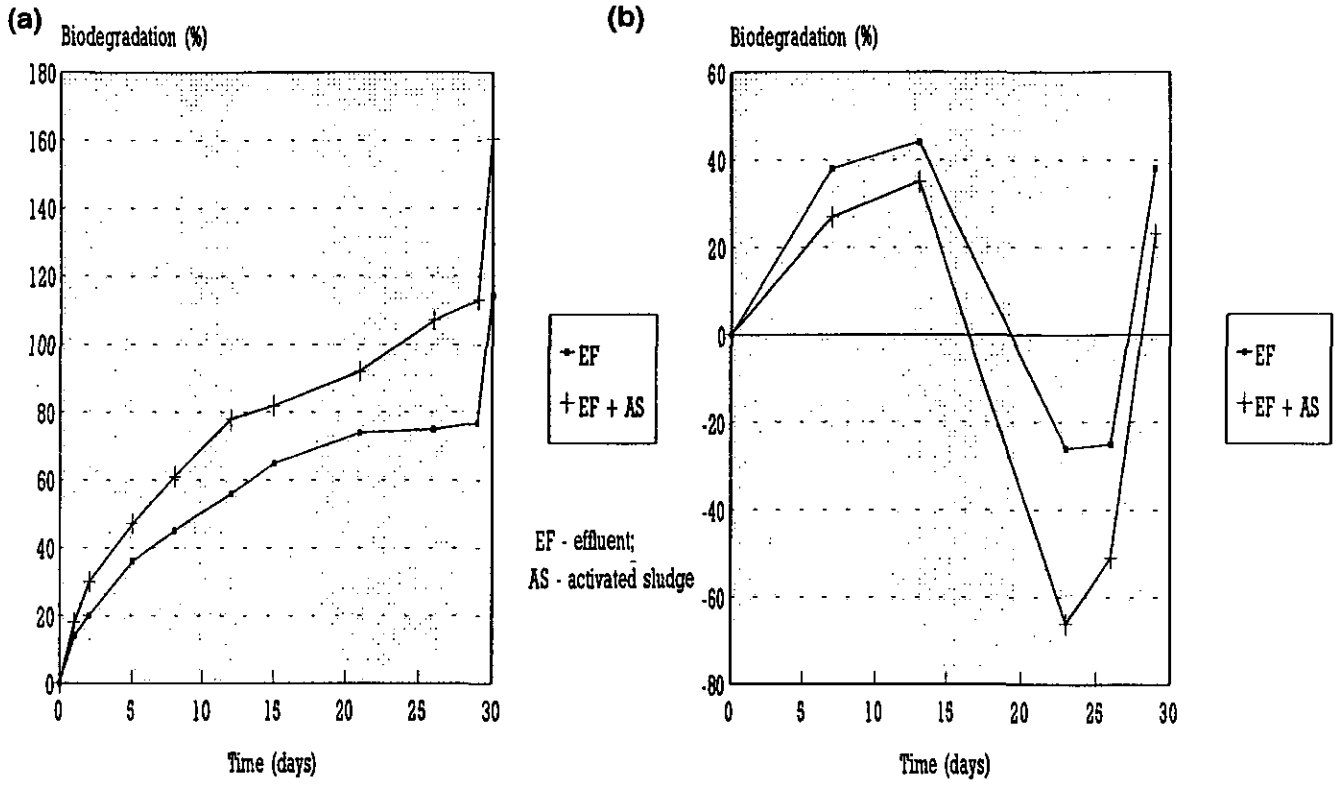


FIGURE 20: Biodegradation of paper mill effluent in the CO_2 production test measuring CO_2 (a) and DOC (b) (Experiment 1)

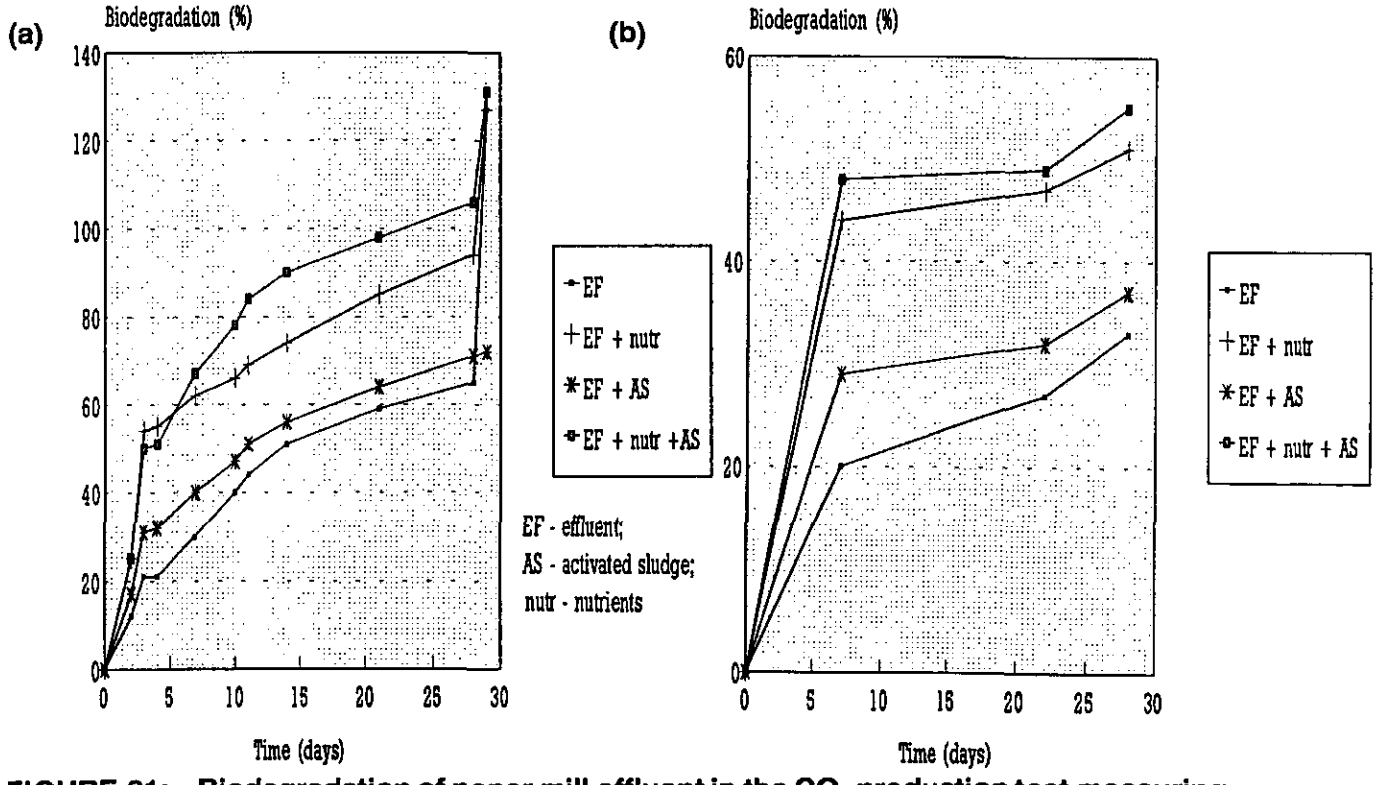


FIGURE 21: Biodegradation of paper mill effluent in the CO_2 production test measuring CO_2 (a) and DOC (b) (Experiment 2)

TABLE 44: Biodegradation of effluent and mixtures of effluent and aniline[a] (DOC reduction)

Sample	% Biodegradaton after time (days):					
	0	7	13	23	26	29
Aniline	0	100	97	-	100	100
Effluent without inoculum and nutrients	0	38	44	+26	+25	38
Effluent with inoculum but without nutrients	0	27	35	+66	+51	23
Effluent plus aniline, with inoculum but without nutrients	0	47	57	1	6	47

[a] Calculated according paragraph 3.3.1

TABLE 45: Biodegradation of effluent using different experimental conditions[a] (CO_2 production test)

Sample	% Biodegradation at time (days):											
	2	3		4	7	10	11	14	21	28	29	
Effluent	12	18	21	21	30	40	44	51	59	65	89	115	127
Effluent plus *nutrients*	25	46	54	55	62	66	69	74	85	94	118	125	127
Effluent plus inoculum	17	26	31	32	40	47	51	56	64	71	71	71	72
Effluent plus nutrients and inoculum	25	45	50	51	67	78	84	90	98	106	124	129	131

[a] Calculated according to paragraph 3.3.2

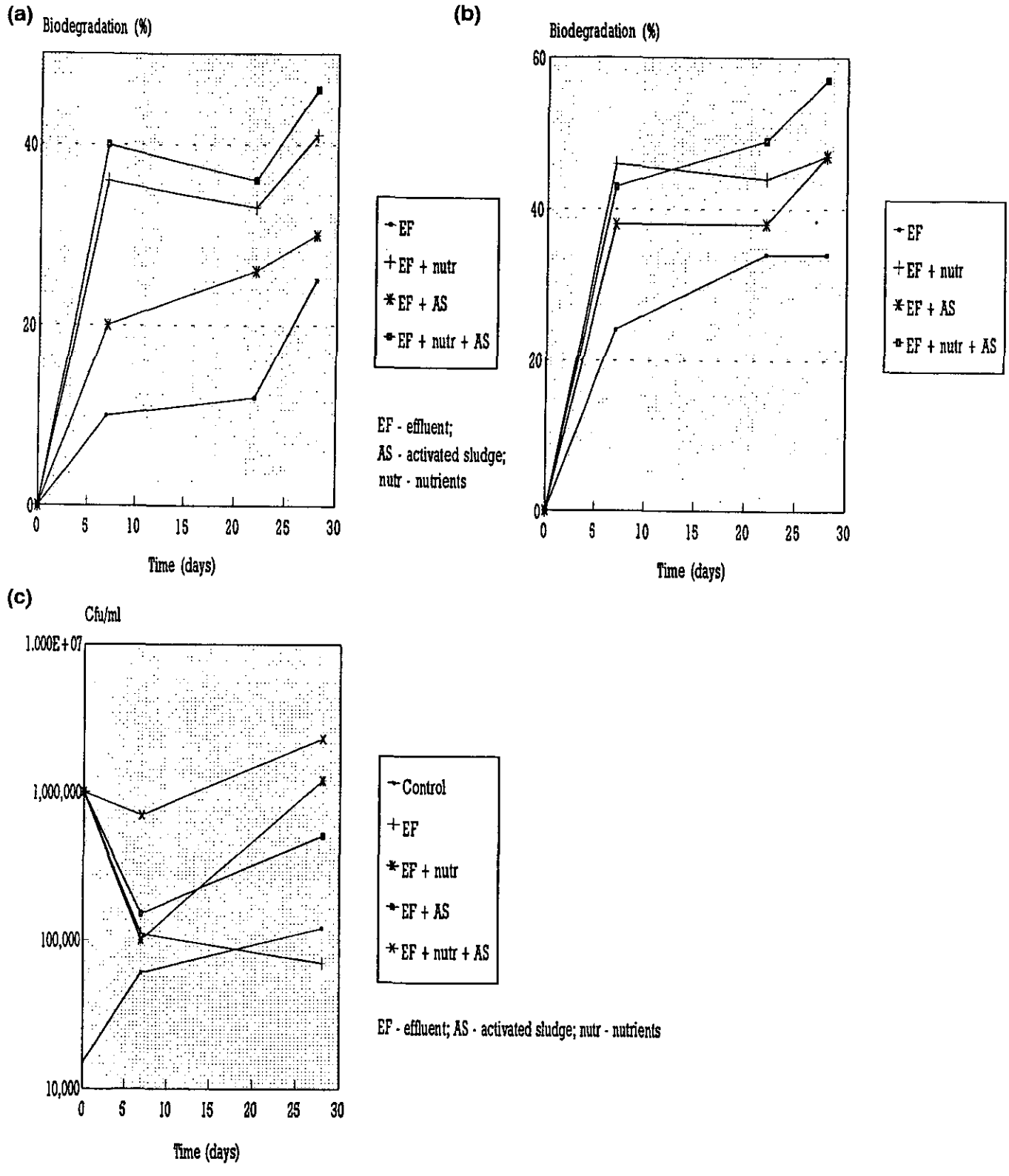


FIGURE 22: Biodegradation of paper mill effluent in the CO_2 production test measuring TOC (a), COD (b) and microorganism numbers

4.3.1.3 O_2 depletion test

In the first experiment on river water the water was tested alone and with inoculum and medium. The oxygen concentration of the water was <5 mg/ℓ. One set of tests was carried out on controls and water without aeration and another on controls and river water continuously aerated until use. Samples were prepared as described in the method (3.1.3). River water alone was transferred directly into test bottles from sample containers. $HgCl_2$ was added to one bottle of each series of tests to examine changes under sterile conditions.

Controls and blank controls showed considerable variation after different incubation periods. On day 10 there were large differences between blank control duplicates. The values obtained on this date for controls and blank controls were also low as compared to that on other dates. Temperature has a large influence on oxygen content. It is possible that these variations occurred because of temperature changes, as the study was carried out in the winter months. The oxygen level in the control was about the same after 14 days as in the beginning of the test. The blank control was about the same when samples were not aerated before use and higher (0.8 mg/ℓ) when continuous aeration was used. The oxygen in test bottles was depleted by day 7.

Although the control values did not meet the requirements of the OECD (1981a) test, results were calculated to evaluate the method. The % biodegradation was calculated by using the formula:

$$\% \text{ Biodegradability} = \frac{O_2 \text{ depletion (mg/ℓ)/measured DOC (mg/ℓ)}}{\text{measured COD (mg/ℓ)}}$$

The DOC and COD of the river water was 6.1 mg/ℓ and 19.5 mg/ℓ, respectively. Table 46 shows that the maximum degradation reached for all samples was 5% after 7 days. This value indicates that the organic concentration was too high and oxygen was completely depleted, preventing further degradation to take place.

The second test on Apies river water was carried out on the sample directly (without inoculum or medium, aerated) and on dilutions thereof (receiving inoculum and medium). Dilutions were prepared with Milli-Q water. A single series of bottles were prepared. Control and blank control results were much lower than the results obtained in previous studies (5.0 to 6.0). Oxygen content in the control bottles varied by 0.4 mg/ℓ during the 28 day period while that of the blank control increased with 1.0 mg/ℓ. Within 4 days the oxygen in the bottles containing the undiluted river was depleted (≤0.4 mg/ℓ). The 10 and 20% water contained residual oxygen at the end of the 28 day period (2.4 and 3.7 mg/ℓ, respectively). The DOC and COD values used in the calculation of % biodegradation were 7.0 and 31 mg/ℓ, respectively. The biodegradation is shown in Table 47 and Figure 23. The maximum level of biodegradation reached by the undiluted river water was 3%, indicating that the organic concentration was too high for the test (7.0x31=217 mg/ℓ C). The 20% sample showed a maximum degradation of 42%, which was reached after 15 days. The organic content of this preparation was also high (1.4x6.2=8.68 mg/ℓ C). The 10% sample showed 101% biodegradation after 15 days. The organic content of this dilution was within the required 2.0 mg/ℓ C concentration (0.7x3.1=2.17 mg/ℓ C). If the sample was biodegradable the residual oxygen content in the 20% river water should have been very low because of the high organic concentration in the sample. The fact that the 20% sample reached a peak after 15 days while there was still enough oxygen left for biodegradation suggests that the organic chemicals in the water were not biodegradable, as was found with the DOC reduction test.

TABLE 46: Biodegradation of river water[a] (Oxygen depletion test) (Experiment 1)

Experimental condition	Sample	% Biodegradation after time (days):				
		0	3	7	10	14
No aeration directly before use	River water without inoculum and nutrients	1	2	5	4	5
	River water with inoculum and nutrients	1	2	5	4	5
Continuous aeration before use	River water without inoculum and nutrients	1	3	5	5	5
	River water with inoculum and nutrients	1	2	5	5	5

[a] Calculated according to paragraph 3.3.3

TABLE 47: Biodegradation of river water[a] (Oxygen depletion test) (Experiment 2)

Sample	% Biodegradation after time (days):				
	0	4	7	15	28
River water without inoculum and nutrients, continuously aerated	0	3	3	3	3
20% River water with inoculum and nutrients	+2	15	27	42	40
10% River water with inoculum and nutrients	37	37	23	101	101

[a] Calculated according to paragraph 3.3.3

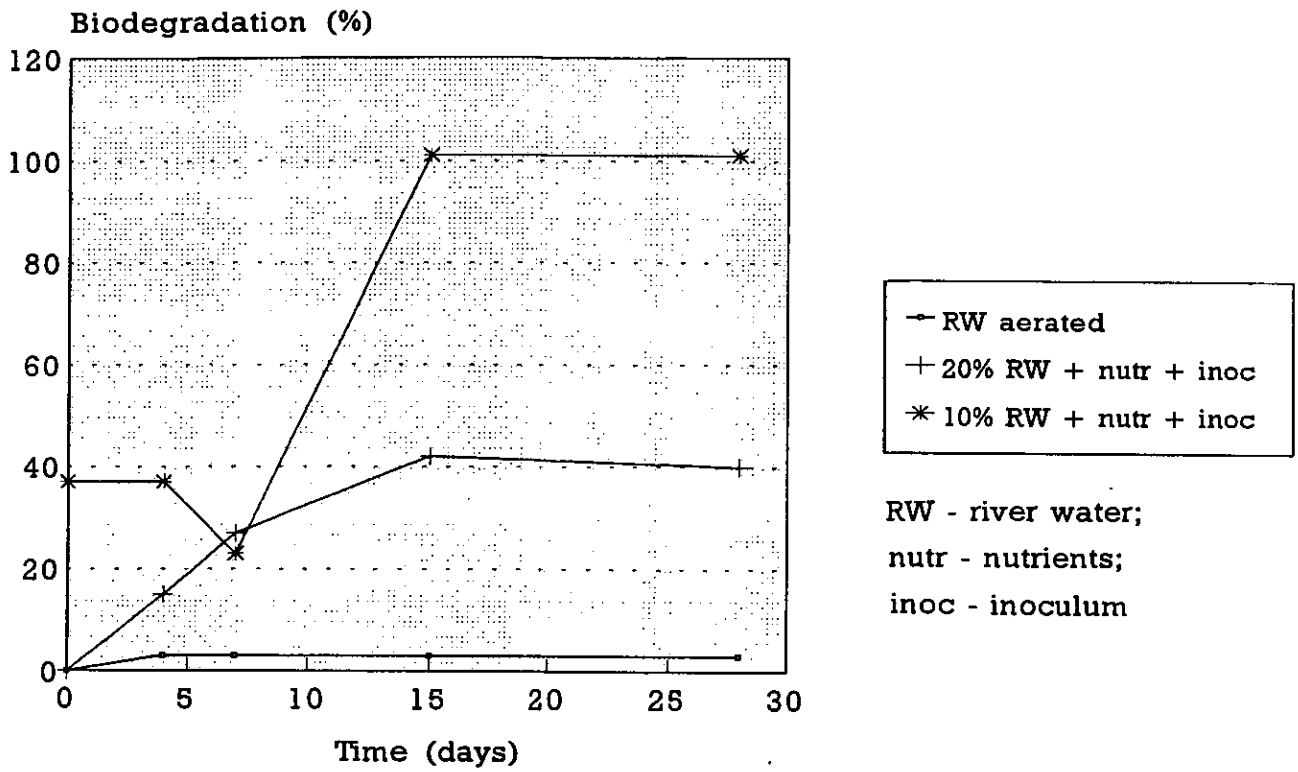


FIGURE 23: Biodegradation of river water in the oxygen depletion test (Experiment 2)

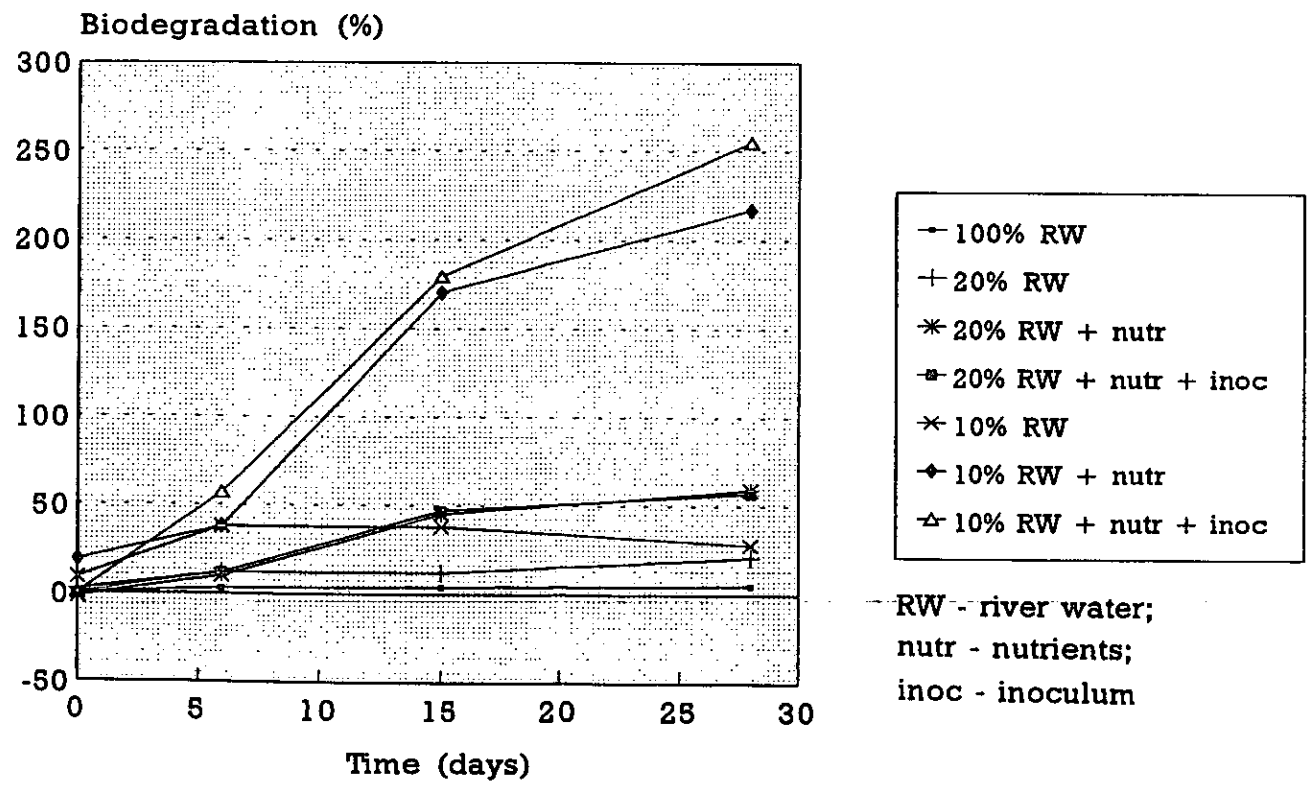


FIGURE 24: Biodegradation of river water in the oxygen depletion test (Experiment 3)

The oxygen concentrations of effluent sterilized with $HgCl_2$ are shown in Table 48. A decrease of between 0.1 and 0.7 mg/ℓ oxygen was noticed in control and test results. The decrease was such that abiotic degradation was not suspected. In one instance there was an increase of 0.6 mg/ℓ in the oxygen content.

TABLE 48: Degradation of river water in the presence of $HgCl_2$

Experimental condition	Sample	Oxygen concentration (mg/ℓ) at time (days):	
		0	14
No aeration directly before use	Control	6.7	6.4
	Blank control	6.6	6.5
	River water without inoculum and nutrients	5.5	5.2
	River water with inoculum and nutrients	6.1	5.4
Continuous aeration before use	Control	6.5	6.4
	Blank control	6.0	5.5
	River water without inoculum and nutrients	5.5	6.1
	River water with inoculum and nutrients	5.7	5.5

The third experiment was carried out on river water and dilutions thereof (Table 49). Here the variation between duplicate results were small and control and blank controls met the requirements for the bottle test. Percentage biodegradation was calculated by using DOC and COD values of 8.8 and 12 mg/ℓ, respectively. The residual oxygen in the 100% river water sample at the end of 28 days was 1.2 mg/ℓ. The residual oxygen concentration in the other test flasks were ≥3.8 mg/ℓ. The maximum level of biodegradation reached by the undiluted river water (Table 49 and Figure 24) was 5%, indicating that the test concentration was too high (8.8x12=105.6 mg/ℓ C). The 20% river water (1.76x2.4=4.22 mg/ℓ C) showed the best biodegradability when nutrients and nutrients and inoculum were added (59 and 57%, respectively). Only 21% biodegradation was reached with the 20% river water alone. The 10% river water alone showed a degradation of 28%. This value increased to 217 and 255% respectively when nutrients, and nutrients and inoculum were added. The organic content in the 10% preparation was below the stated 2.0 mg/ℓ C (0.88x1.2=1.06 mg/ℓ C), explaining the high biodegradation values. The fact that the 20% sample (nutrients and nutrients and inoculum) almost reached a peak after 15 days while there was still enough oxygen left in the sample for biodegradation suggests that the organic chemicals in the water were not biodegradable.

In the first experiment on paper mill effluent various dilutions were tested (Table 50). Dilutions were prepared with Milli-Q water. The samples received nutrients and inoculum. Aniline was included as reference chemical (1.5 mg/ℓ). $HgCl_2$ was added to one bottle of each series of tests to examine changes under sterile conditions. The initial oxygen concentrations in the 5 and 10% effluent samples were low compared to that of the controls (5.5-5.7 mg/ℓ as compared to 6.9-7.1 mg/ℓ). In general, there were considerable differences between duplicate

TABLE 49: Biodegradation of river water[a] (Oxygen depletion test) (Experiment 3)

Sample	% Biodegradation after time (days):			
	0	6	15	28
100% River water	0	3	4	5
20% River water	2	12	12	21
20% River water plus nutrients	-2	10	45	59
20% River water plus nutrients and inoculum	0	12	47	57
10% River water	9	38	38	28
10% River water plus nutrients	19	38	170	217
10% River water plus nutrients and inoculum	0	57	179	255

[a] Calculated according to paragraph 3.3.3

TABLE 50: Biodegradation of effluent[a] (Oxygen depletion test) (Experiment 1)

Sample	% Biodegradation after time (days):			
	0	5	15	29
10% Effluent	2	0	4	10
5% Effluent	12	5	6	29
2% Effluent	+10	7	33	23
Aniline, 1.5 mg/ℓ C	+14	31	64	76

[a] Calculated according to paragraph 3.3.3

control and test results (as much as >1.0 mg/ℓ). The blank control values did not differ by more than 0.3 mg/ℓ. The oxygen was depleted in the 10% effluent sample by day 29. The 2 and 5% samples had residual oxygen concentrations of 6.4 and 3.7 mg/ℓ at the end of the test period. The % biodegradation was calculated using DOC and COD values of 43.8 and 164 mg/ℓ, respectively. The biodegradation is shown in Table 50 and Figure 25. The maximum level of biodegradation reached by the 10% effluent was 10% (day 29), indicating that the organic concentration was too high (4.38x16.4=71.83 mgℓ C). The 5% effluent showed a maximum degradation of 29%. The organic concentration of this dilution was also high (2.19x8.2=17.96 mg/ℓ C). The concentration of the 2% sample was acceptable for calculation (0.88x3.28=2.89 mg/ℓ C), but a maximum biodegradation of only 33% was reached. The fact that there was a residual oxygen level >3.0 mg/ℓ in both the 2 and 5% samples, while the % biodegradation was low, indicate that the effluent was not biodegradable. Similar results were found when DOC was measured in the CO_2 production test (4.3.1.2).

Aniline was biodegradable (≥60% within the 10 days after the lag period) (Table 50), indicating that the inoculum was acceptable. A residual oxygen concentration of 4.25 mg/ℓ was present at the end of the study. The maximum biodegradation was 76%, and was reached by day 29.

The oxygen concentrations of effluent sterilized with $HgCl_2$ are shown in Table 51. Some of the samples showed similar results at the beginning and end of the test while others showed large increases. This was noticed for the control (6.9 to 8.8 mg/ℓ), 10% effluent (5.7 to 8.5 mg/ℓ) and 5% effluent (5.5 to 6.1 mg/ℓ). It is possible that the high values obtained for the control and 10% effluent were due to experimental error.

TABLE 51: Degradation of effluent in the presence of $HgCl_2$

Sample	Oxygen concentration (mg/ℓ) at time (days):	
	0	29
Control	6.9	8.8
Blank control	7.1	7.0
10% Effluent	5.7	8.5
5% Effluent	5.5	6.1
2% Effluent	7.2	7.0
Aniline, 1.5 mg/ℓ C	7.4	7.6

The results of a second study on effluent are shown in Table 52. Dilutions of 10, 5 and 2% were once again tested. Samples were tested alone, with nutrients, and with nutrients and inoculum. The differences between duplicate results were small. In general, control and blank control values were within the requirements of the test. The oxygen in the 10% samples was depleted (0.1 mg/ℓ) at day 28. The 2 and 5% samples had residual oxygen concentrations of ≥3.5 and ≥1.5 mg/ℓ, respectively at the end of the test period. The % biodegradation was calculated using DOC and COD values of 38.7 and 117 mg/ℓ, respectively. The % biodegradation is shown in Table 52 and Figure 26. The maximum level of biodegradation reached by the 10% effluent (with and without nutrients and inoculum) was 14% (day 28),

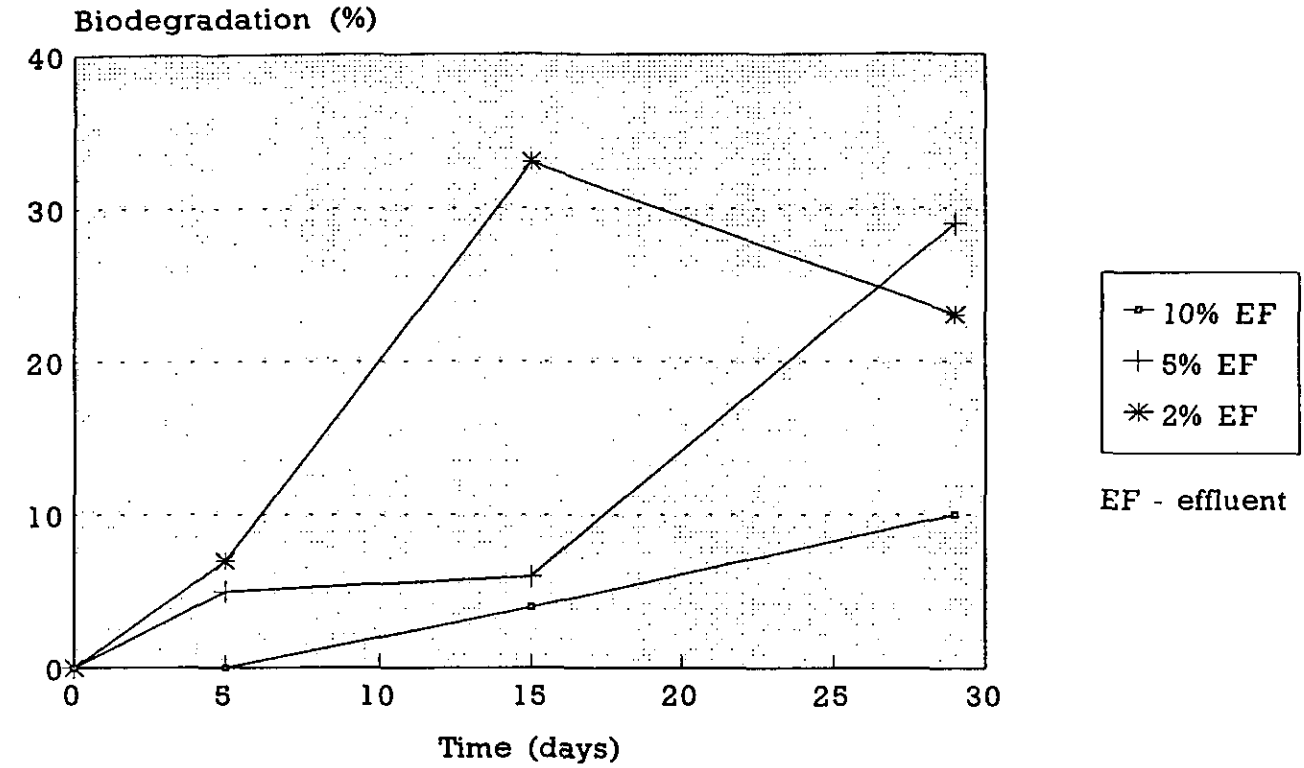


FIGURE 25: Biodegradation of paper mill effluent in the oxygen depletion test (Experiment 1)

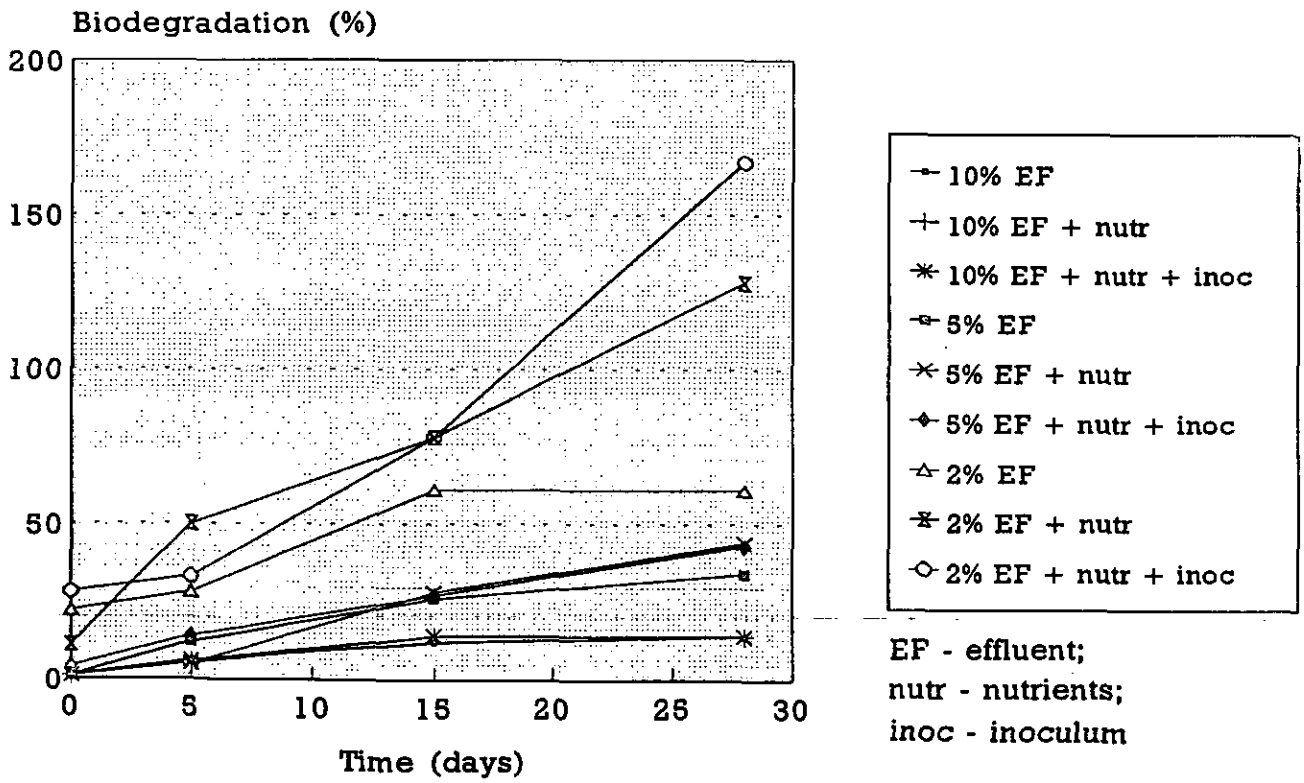


FIGURE 26: Biodegradation of paper mill effluent in the oxygen depletion test (Experiment 2)

TABLE 52: Biodegradation of effluent[a] (Oxygen depletion test) (Experiment 2)

Sample	% Biodegradation after time (days):			
	0	5	15	28
10% Effluent	1	6	12	14
10% Effluent plus nutrients	1	5	14	14
10% Effluent plus nutrients and inoculum	1	6	14	14
5% Effluent	1	12	26	34
5% Effluent plus nutrients	1	5	28	44
5% Effluent plus nutrients and inoculum	4	14	27	43
2% Effluent	22	28	61	61
2% Effluent plus nutrients	11	50	78	128
2% Effluent plus nutrients and inoculum	28	33	78	167

[a] Calculated according to paragraph 3.3.3

indicating that the test concentration was too high (3.87x11.7=45.28 mg/ℓ). The 5% effluent showed a maximum degradation of approximately 45% with nutrients and nutrients and inoculum. The biodegradation in the 5% effluent without nutrients and inoculum was lower at 34%. The organic content of the 5% preparation was above the required 2.0 mg/ℓ C for the test (1.94x5.85=11.34 mg/ℓ). The 2% effluent showed a biodegradation of 61% in the absence of nutrients or inoculum. This level was reached after 15 days. In the presence of nutrients the biodegradation was 128% and with nutrients and inoculum 167%. The organic content of the 2% sample was in close proximity of the 2.0 mg/ℓ C required for the test (0.77x2.34=1.8 mg/ℓ C). The results obtained with the 2% effluent suggest that the effluent was ready biodegradable (>60% within 14 days).

4.3.2 Final tests

The samples used in final tests and their DOC and COD concentrations are presented in Table 53. The Willards effluent became turbid very quickly indicating microorganism growth which expectedly interfered with the DOC analysis. The DOC concentration reported for this effluent was too high compared to the COD value. The river and Iscor samples were collected during a very rainy period which resulted in a lower organic load than anticipated. The river/stream and Iscor effluent samples contained sand and other particles. Willards effluent contained suspended food particles (potato) and had a milky colour. The cutting oil (colourless) and pesticide (yellow-green) effluents were clear.

Because of low DOC concentrations the river/stream water and Iscor effluent were tested directly (100%) in the DOC reduction and CO_2 production tests. Ten-fold dilutions of the Willards and cutting oil effluent were used (measured DOC: 20.0 and 111 mg/ℓ, respectively). Because of the high organic content, the pesticide effluent was diluted 200-times (0.5%) (measured DOC: 49.3 mg/ℓ).

Only selected samples were evaluated in the oxygen depletion test because all the samples could not be accommodated. Dilutions were tested to ensure a suitable TOD value for calculation. The Moreleta stream water and Iscor effluent were tested at 16.7 and 20%, and the pesticide effluent at 0.013 and 0.017%.

Final tests were conducted in the presence of inoculum and nutrients, because in general, the best biodegradation was achieved under these experimental conditions (4.3.1).

TABLE 53: Water and effluent used in final tests

Determinand (mg/ℓ)	Apies river water	Moreleta stream water	Iscor effluent	Willards effluent	Cutting oil effluent	Pesticide effluent
DOC	14.4	7.8	9.2	1352	956	10203
COD	10	21	20	873	4010	23700

4.3.2.1 DOC reduction test

Biodegradation was measured in terms of DOC, COD and microbial numbers. TOC concentrations generally corresponded with DOC values. However, sometimes these concentrations were considerably lower than the DOC values, especially when the organic

TABLE 54: Biodegradation of different waters and effluent[a] (DOC reduction test)

Sample	% Biodegradation after time (days):			
	0	7	22	28
100% Apies river water	0	57	60	59
100% Moreleta stream water	0	54	42	44
100% Iscor effluent	0	52	57	59
10% Willards effluent	0	91	92	93
10% Cutting oil effluent	0	72	92	92
0.5% Pesticide effluent	0	88	95	97
100% Apies river water plus 40 mg/ℓ aniline	0	80	90	88
100% Moreleta stream water plus 40 mg/ℓ aniline	0	75	86	87
100% Iscor effluent plus 40 mg/ℓ aniline	0	85	91	89
10% Willards effluent plus 40 mg/ℓ aniline	0	90	96	95
10% Cutting oil effluent plus 40 mg/ℓ aniline	0	80	92	93
0.5% Pesticide effluent plus 40 mg/ℓ aniline	0	93	95	96
40 mg/ℓ aniline	0	89	97	97

[a] Calculated according to paragraph 3.3.1

content was low. Because these samples were unfiltered, it is expected that microbial degradation took place if samples were standing at room temperature (analytical room) for a few hours before analysis. TOC was, therefore, omitted from the final tests.

The biodegradation of the different samples when using DOC analysis is shown in Table 54 and Figure 27a. A final biodegradation of between 50 and 60% was reached within 7 days for the river/stream water and Iscor effluent. According to the validity criteria given for the ISO test, these samples were not ready biodegradable. The other effluents showed biodegradation of >90% at between 7 and 22 days, indicating ready biodegradability. In general, DOC concentrations were reduced to between 2.0 and 4.0 mg/ℓ at day 28. None of the samples were toxic (Table 54), as mixtures of aniline and the samples showed biodegradation >40% after 7 days. The test with aniline showed that the activated sludge microorganisms were active. The COD values were erratic, especially for controls and samples with low organic content. This determinand was, therefore, only applicable to samples with high organic composition. The results obtained for Willards, cutting oil and pesticide effluent are shown in Figure 27b. Willards showed a 100% biodegradation within 7 days, indicating ready biodegradability. The cutting oil effluent was close to the 80% biodegradation specified for ready biodegradability (76% after 7 days), with a maximum of 78% after 28 days. The pesticide effluent only reached 52% biodegradability (after 7 days), and from these results did not appear to be ready biodegradable. Microbial numbers (Figure 27c) decreased in the control and samples with low organic content during the first 18 days, and then increased. In case of the Willards, cutting oil and pesticide effluent increases were noticed until day 28. In general, measurement of microorganisms, especially in samples with low organic carbon, was not successful, because results were erratic. Table 55 provide information on abiotic biodegradation. Only the Willards effluent showed abiotic degradation. It is possible that the $HgCl_2$ concentration was not sufficiently high to kill the large amount of microorganisms active in this sample.

TABLE 55: Abiotic degradation of different waters and effluent (DOC and COD reduction)

Sample	Concentration (mg/ℓ) after degradation period (days):			
	0		28	
	DOC	COD	DOC	COD
100% Apies river water	8.6	22	8.4	15
100% Moreleta stream water	8.3	27	8.3	19
100% Iscor effluent	8.0	27	8.3	31
10% Willards effluent	20	79	6.7	24
10% Cutting oil effluent	111	424	152	516
0.5% Pesticide effluent	49.3	131	74.2	171

4.3.2.2 CO_2 production test

Because the DOC reduction test was run in parallel with this test, only CO_2 production was measured. The results are presented in Table 56 and Figure 28a. In all instances the biodegradation was above the required 50% level for ready biodegradability by day 14. All

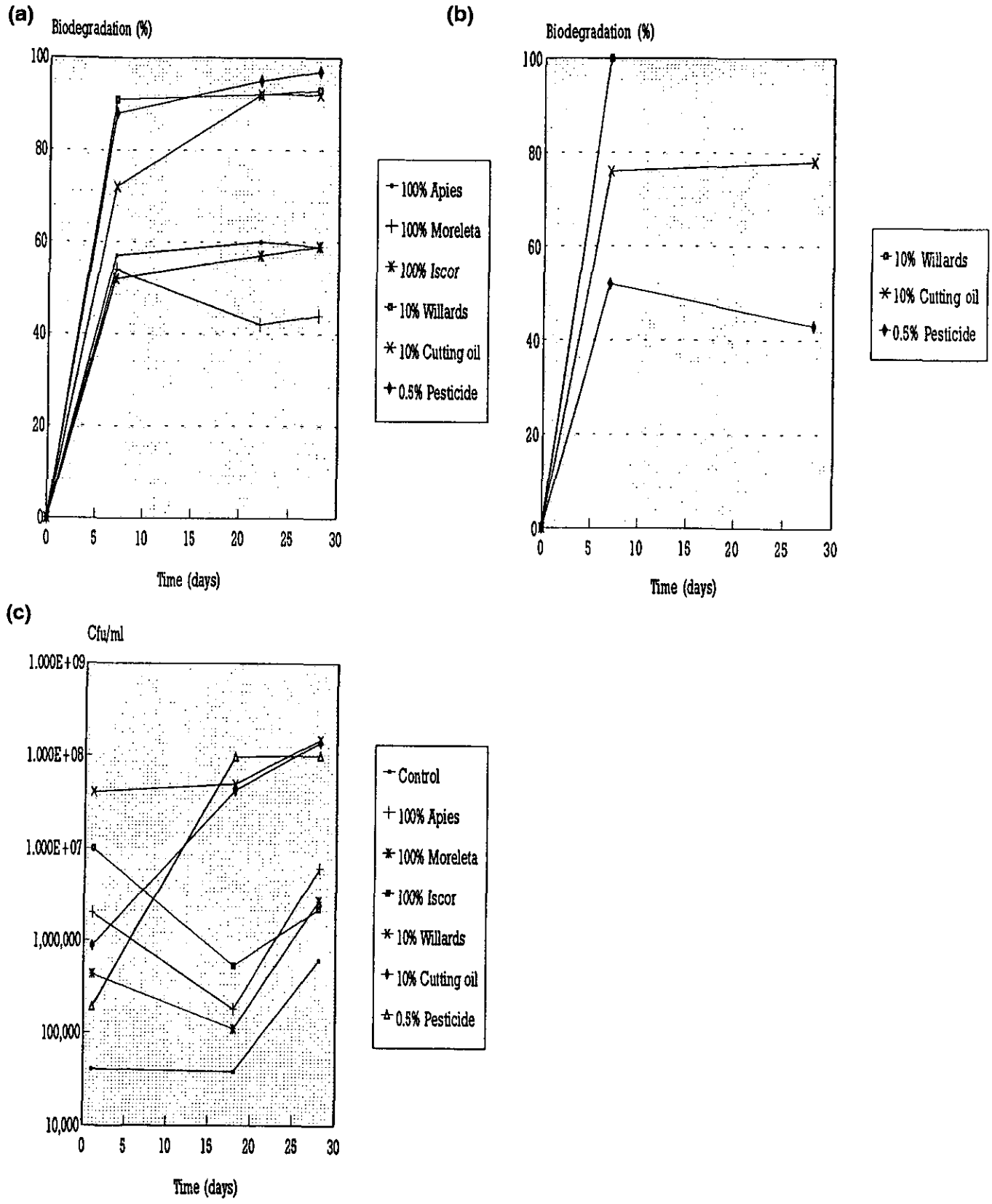


FIGURE 27: Biodegradation of different waters and effluent in the DOC reduction test measuring DOC (a), COD (b) and microorganism numbers (c)

TABLE 56: Biodegradation of different waters and effluent[a] (CO_2 production test)

Sample	% Biodegradation at time (days):												
	1	4		6	8	11	13	20	22	28	29		
100% Apies river water	68	147	177	194	200	213	224	232	232	245	265	275	279
100% Moreleta stream water	65	138	162	179	193	201	214	215	217	241	261	261	269
100% Iscor effluent	77	173	190	212	229	244	237	245	249	257	255	255	261
10% Willards effluent	20	74	83	107	121	127	138	146	147	152	153	153	156
10% Cutting oil effluent	0	11	23	34	46	57	68	78	82	85	88	89	90
0.5% Pesticide effluent	2	26	48	61	70	72	76	80	84	86	86	86	87

[a] Calculated according to paragraph 3.3.2

TABLE 57: Biodegradation of different waters and effluent[a] (Oxygen depletion test)

Sample	% Biodegradation after time (days):			
	0	5	14	28
20% Moreleta stream water	-2	17	40	35
16.7% Moreleta stream water	-2	26	35	66
20% Iscor effluent	7	13	71	84
16.7% Iscor effluent	6	28	94	90
0.017% Pesticide effluent	2	27	36	25
0.013% Pesticide effluent	-2	19	33	19

[a] Calculated according to paragraph 3.3.3

the samples, except the cutting oil effluent, showed a rapid biodegradation (>50% within first 4 days). High final biodegradation values were obtained for the river/stream water and Willards effluent at day 28. The low TCO_2 values used in the calculation contributed to the high values. Very little CO_2 was released upon acidification of the effluent samples, indicating an absence of carbonates and bicarbonates in reaction flasks. The results indicated that Apies river and Moreleta stream water contained carbonates and bicarbonates. The CO_2 production in the control exceeded the specified 70 mg/ℓ (calculated value: 115 mg/ℓ; actual value: 74 mg/ℓ).

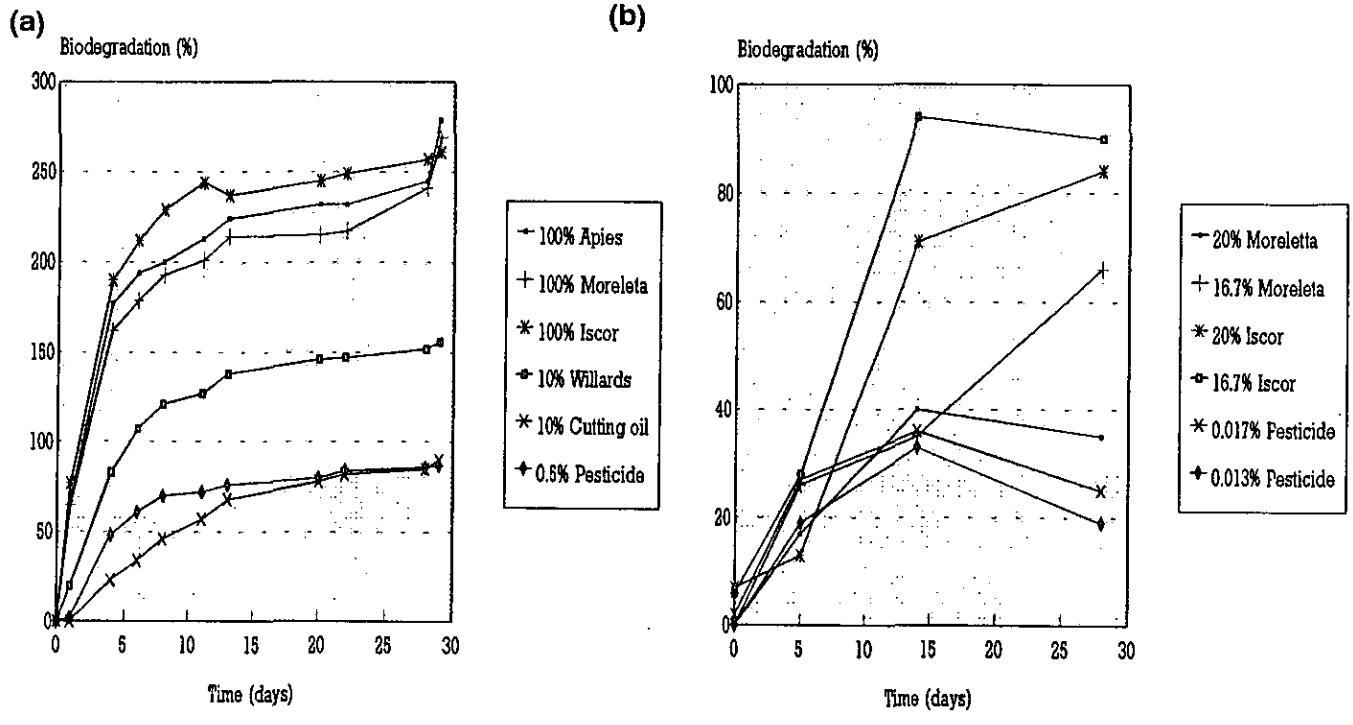


FIGURE 28: Biodegradation of water and effluent in the CO_2 production (a) and oxygen depletion (b) tests

4.3.2.3 O_2 depletion test

The DOC and COD concentrations used for the calculation of the TOD are given in Table 53. Only the 20% Iscor sample showed complete oxygen depletion by day 28, indicating that the organic content was too high. The residual oxygen in the 16.7% Iscor sample was 1.8 mg/ℓ and that in the other samples between 3.4 and 5.6 mg/ℓ. The results obtained with the oxygen depletion test are shown in Table 57 and Figure 28b. A good biodegradability was obtained with the Iscor effluent, meeting the required limit of 60% within 14 days after the lag phase. The Moreleta steam water and pesticide effluent did not appear to be biodegradable, particularly because high residual oxygen levels were present at the end of the test. The control and blank control results met the validity requirements for the test.

4.4 Summary of biodegradability results

4.4.1 Biodegradability of chemical compounds

The results of the different biodegradability tests on chemical substances are shown Table 58. Aniline and lauryl sulphate was found to be ready biodegradable in all the tests. Diethylene glycol was difficult to biodegrade, and showed ready biodegradability only in the DOC reduction test when activated sludge (ISO test) and a low test concentration was used. Stearic acid was ready biodegradable in the oxygen depletion test, but not in the CO_2 reduction test.

TABLE 58: Biodegradability of chemical compounds

Organic compound	Concentration (mg/ℓ C)	DOC reduction test (ISO)	DOC reduction test (OECD)	CO_2 production test	O_2 depletion test
		Ready biodegradable (number tests positive/number tested)			
Aniline	40	3/3	3/3	3/3	not tested
	10	3/3	3/3	not tested	0/1[1]
	2	not tested	not tested	not tested	3/3
Lauryl sulphate	40	3/3	0/3	3/3	not tested
	10	3/3	3/3	not tested	0/1[1]
	2	not tested	not tested	not tested	3/3
Diethylene glycol	40	0/3	0/3	0/3	not tested
	10	3/3	0/3	not tested	0/1[a]
	2	not tested	not tested	not tested	0/3
Stearic acid	40	not tested	not tested	0/3	not tested
	10	not tested	not tested	not tested	0/1[1]
	2	not tested	not tested	not tested	2/3

[a] Organic content too high, causing total oxygen depletion within a few days

4.4.2 Biodegradability of water and effluent

Table 59 shows that the CO_2 production test was positive (ready biodegradable) for all the samples (biodegradation: 85 to 359%). The DOC reduction test showed ready biodegradability for one of the Apies river water 1 samples and Willards, cutting oil and pesticide effluents. The DOC reduction evaluation during the CO_2 production test tested negative for the Apies river water. The remaining DOC at the end of the 28 day biodegradation period ranged from 2.6 in case of river water to 34 mg/ℓ for paper mill effluent. The oxygen depletion test only showed ready biodegradability for one paper mill effluent sample and the Iscor sample.

TABLE 59: Biodegradability of water and effluent[a]

Sample	DOC reduction test[b]		COD production test			Oxygen depletion test[c]	
	+/-	DOC (mg/ℓ) remaining	+/-	DOC reduction[b] +/-	DOC (mg/l) remaining	+/-	Residual oxygen (mg/ℓ)
Apies 1	- (72%)	2.8	not tested	not tested	not tested	- (42%)	2.4
Apies 1	+ (86%)	2.6	+ (359%)	- (73%)	3.6	- (57%)	4.1
Apies 2	- (60%)	3.3	+ (245%)	not tested	not tested	not tested	not tested
Moreletta	- (54%)	4.4	+ (241%)	not tested	not tested	- (66%)	3.4
Paper mill	not tested	not tested	+ (113%)	- (35%)	34	- (29%)	4.7
Paper mill	- (64%)	18.1	+ (106%)	- (55%)	27	+ (167%)	3.5
Iscor	- (59%)	3.1	+ (257%)	not tested	not tested	+ (90%)	1.8
Willards	+ (93%)	1.4	+ (152%)	not tested	not tested	not tested	not tested
Cutting oil	+ (92%)	9.2	+ (85%)	not tested	not tested	not tested	not tested
Pesticide	+ (97%)	1.4	+ (86%)	not tested	not tested	- (25%)	4.7

[a] Tests carried out in presence of nutrients and inoculum
[b] Highest biodegradation during the 28 day period
[c] Best result obtained at a certain dilution
\+ Biodegradable
\- Not biodegradable

5. CONCLUSIONS AND RECOMMENDATIONS

The evaluation of the three types of ready biodegradability tests showed that all the tests have a viable role to play in assessing the ultimate biodegradability of chemical products in local water laboratories. The appropriate method will depend on the chemical to be tested. For example, soluble compounds can be tested in all the tests. The CO_2 production and oxygen depletion tests are more suitable for poorly soluble chemicals. When low concentrations of chemicals need to be tested, for example when test compounds are toxic at the normal levels of 10 and 40 mg/ℓ, the oxygen depletion test will be the most applicable.

The DOC reduction test was found to be the most simple and straightforward of the tests with reference to preparation, maintenance and data analysis. The test was reliable and reproducible, showing little variation between duplicate tests and produced similar biodegradability results during repetitive evaluations.

The DOC reduction test using river water as inoculum (OECD, 1981b) was more sensitive, and therefore more stringent, than the test using activated sludge (ISO, 1984). However, activated sludge was found to be a more suitable source for inoculation as the concentration (SS) was determined directly before use, while in the case of river water a standard volume based on previous analysis of the water was used. It is possible to determine the microbial numbers in river water on the day of use if alternative counting techniques are used. The use of activated sludge resulted in a microbial concentration in test solutions similar to that found in our surface waters (10^3 to 10^4/mℓ), providing a more realistic test than when river water is used for inoculation.

The CO_2 production test has great potential for chemical product testing, especially when used in combination with DOC reduction analysis (two tests in one). Some problems were, however, experienced. According to the requirements of the standard method too much CO_2 was produced by the controls and the variation between duplicates was sometimes large. It is recommended to use the actual titrated value for $Ba(OH)_2$ (mℓ) instead of the calculated value to reduce the CO_2 produced by controls to below the required 70 mg/ℓ.

The preparation of the O_2 depletion test was found to be more tedious than that of the other tests in order to ensure that the dissolved oxygen concentration remains constant during sample distribution. Problems have been experienced in that the oxygen depletion in some of the controls and blank controls were larger than specified in the OECD test of 1981. However, according to the requirement of the revised method of 1992, all the control results were within the specified limit (oxygen depletion: $\leq$1.5 mg/ℓ).

Aeration was found to be a suitable substitute for stirring and shaking of solutions in the DOC reduction test. This type of agitation will also be more cost effective for routine use.

Differences in light intensity in the laboratory did not appear to influence the results of the DOC reduction test. The same should be true for the CO_2 production test. Algae can, however, influence the results in the O_2 depletion test (low concentrations). It is thus, recommended to follow the standard test instructions using complete darkness during incubation in the O_2 depletion test and diffused light for the other two tests.

DOC reduction was enhanced at high temperatures and slowed down at low temperatures. It is, therefore, important to standardize the temperature for testing. A temperature of between 20 and 25°C, as used in standard tests, seems to be acceptable. It should, however, be

kept in mind that slower breakdown can be expected in the natural environment during the winter period.

Additional scrubbers containing soda lime, placed in front of the normal NaOH and $Ba(OH)_2$ scrubbers, did not reduce the amount of CO_2 produced by control flasks in the CO_2 production test, indicating that the high CO_2 produced by controls was not due to inadequate removal of CO_2 from the air. Clogging occurred in additional scrubbers, particularly in high humidity, rendering such devices inadequate and dangerous because pressure can build up in flasks.

Studies using 100 mℓ and 200 mℓ $Ba(OH)_2$ in end absorber flasks in the CO_2 production test resulted in similar absorption of CO_2 in control flasks, indicating that the use of larger volumes of $Ba(OH)_2$ in end absorbers did not increase CO_2 concentrations in control tests. Furthermore, larger volumes of $Ba(OH)_2$ did not result in a better absorption of CO_2 in test flasks, indicating that the contact time between bubbles and 100 mℓ $Ba(OH)_2$ in Erlenmeyer flasks was sufficient. Stirring in addition to aeration resulted in an increased rate of biodegradation but did not improve the large variation sometimes noticed between duplicate tests (both CO_2 production and DOC reduction).

Bubble formation in flasks in the oxygen depletion test did not appear to contribute to the large oxygen depletion in controls and blank controls. Similar results were obtained when flasks were standing upright and inverted with stoppers in Milli-Q water.

As in standard biodegradability tests, it will be necessary to include, in addition to controls and tests, a test on a reference chemical, an abiotic test and a test to examine possible toxicity, in order to understand and verify results. Although not examined in our study, a test to examine adsorption (DOC reduction tests) could also be included. Such a test is prepared in the same way as the abiotic test, but inoculum is also added (OECD, 1992). When complex compounds are tested it is recommended that a toxicity test is carried out before the biodegradation test to avoid toxicity. Oxygen uptake tests with activated sludge or bacterial growth inhibition tests (Slabbert, 1994) were found to be useful for this purpose. The OECD guideline (1992) also provides information on toxicity testing.

In order to examine abiotic degradation, a sufficiently high $HgCl_2$ concentration should be used ($\geq$10 mg/ℓ) to avoid microbial growth.

Aniline and lauryl sulphate proved to be useful reference chemicals. If chemical products which are expected to be non-biodegradable are tested it could be useful to include a chemical like diethylene glycol which is difficult to biodegrade as example.

In our study the non-soluble stearic acid was directly (weighed off on microscope slide) added to test flasks (CO_2 production test) or dissolved in an organic solvent (oxygen depletion test). More information on the preparation of poorly soluble organic compounds can be obtained from an ISO guidance document (ISO, 1992).

It is recommended that the standard methods as outlined in ISO and OECD protocols, with certain modifications, are used for local application (Table 60).

Biodegradation studies on water and effluent showed that the best biodegradation was achieved when medium and inoculum, as specified in the standard tests, were added to test solutions. When Apies river water and paper mill effluent were tested, precipitation occurred

in the DOC reduction and CO_2 production tests, which could have interfered with the interpretation of results. Dilution in the oxygen depletion test is such that precipitation will not occur.

TABLE 60: Recommended biodegradability test methods

DOC reduction test		
Standard procedure (ISO, 1984)	**Modification**	**Explanation**
1. 250 mℓ sample	1. 1 ℓ sample	1. Sufficient sample for frequent DOC analysis
2. Activated sludge/secondary effluent/surface water inoculum	2. Activated sludge inoculum	2. Final microbial numbers similar to that found in surface waters - more realistic test
3. Use membrane filter of 0.2 µm porosity to filter samples for DOC analysis	3. Use 0.5 µm SLCR millipore filters to prepare samples for DOC analysis	3. Does not remove or add carbon to sample
4. Stirring for aeration and mixing	4. Aeration to mix	4. As effective as stirring/shaking and more cost effective
CO_2 production test		
Standard procedure (ISO, 1990)	**Modification**	**Explanation**
1. Use Drechsel bottles as end absorbers	1. Use 250 mℓ Erlenmeyer flasks with tighly sealed stoppers as end absorbers	1. More cost effective and work relatively well
2. Stirring for aeration and mixing	2. Aeration to mix (stirring for poorly soluble substances)	2. As effective as stirring/shaking and more cost effective
3. Activated sludge/secondary effluent/surface water inoculum	3. Activated sludge inoculum	3. Final microbial numbers similar to that found in surface waters - more realistic test
Oxygen depletion test		
Standard procedure (OECD, 1992)	**Modification**	**Explanation**
1. Use 0.05 to 5 mℓ/ℓ activated sludge for inoculation	1. Instead of inoculating 0.05 mℓ/ℓ activated sludge effluent directly, first make a dilution and add a larger volume	1. More accurate
2. Place flasks upside down in water bath	2. Flasks placed upright in a constant temperature cupboard	2. As effective and more cost effective (does not need expensive equipment)
3. Maximum oxygen concentration: approximately 9 mg/ℓ	3. Maximum oxygen concentration: approximately 7 mg/ℓ	3. Saturation

The evaluation of additional parameters such as TOC, COD and microbial analysis in the DOC reduction and CO_2 production tests during studies on water and effluents showed that TOC results were, in general, in agreement with DOC results. In some instances TOC levels were lower than DOC concentrations, indicating biodegradation (no filtration) before analysis. DOC, therefore, proved to be a more reliable determinand than TOC. In general, COD values

showed large fluctuations, particularly for control solutions and when organic concentrations were low, indicating that this determinand is not an accurate measure. However, when samples of high organic content is evaluated, this determinand could supply useful additional information. Microorganism numbers showed large fluctuations and no specific pattern of growth, therefore this parameter is not appropriate to use.

Compared to tests on individual chemicals high biodegradation values (much larger than 100%) were in some instances obtained for the CO_2 production and O_2 depletion tests. Measured DOC and COD values were used to calculate the TCO_2 and TOD, which are in turn used to calculate biodegradability. It is known that the COD is often not as high as the TOD as some chemicals are poorly oxidized in the CO_2 test, resulting in falsely high values for biodegradation (OECD, 1992).

The results obtained for water and effluent with the different biodegradability tests were generally not in agreement. The CO_2 production showed ready biodegradability for all the water and effluent samples while the DOC reduction test and oxygen depletion tests showed positive results in a few instances. The biodegradation in the DOC reduction test was particularly low when river waters were tested, where reduction from approximately 8 to 3 mg/ℓ C occurred.

The study indicated that methods developed for the ready biodegradability testing of chemical substances are not so applicable to environmental samples. Further development is required to establish applicable protocols for biodegradability testing of complex samples.

When the standard tests are applied to environmental samples, the following should be considered: The DOC reduction test should be used with medium and inoculum. Dilutions should be made for the oxygen depletion test to have a TOD of approximately 2.0 mg/ℓ (using measured DOC and COD for calculation). If there is a large residual oxygen concentration in the test flasks at the end of the test while the calculation show a relative amount of organic carbon to be degraded, the test is considered to be negative (not ready biodegradable).

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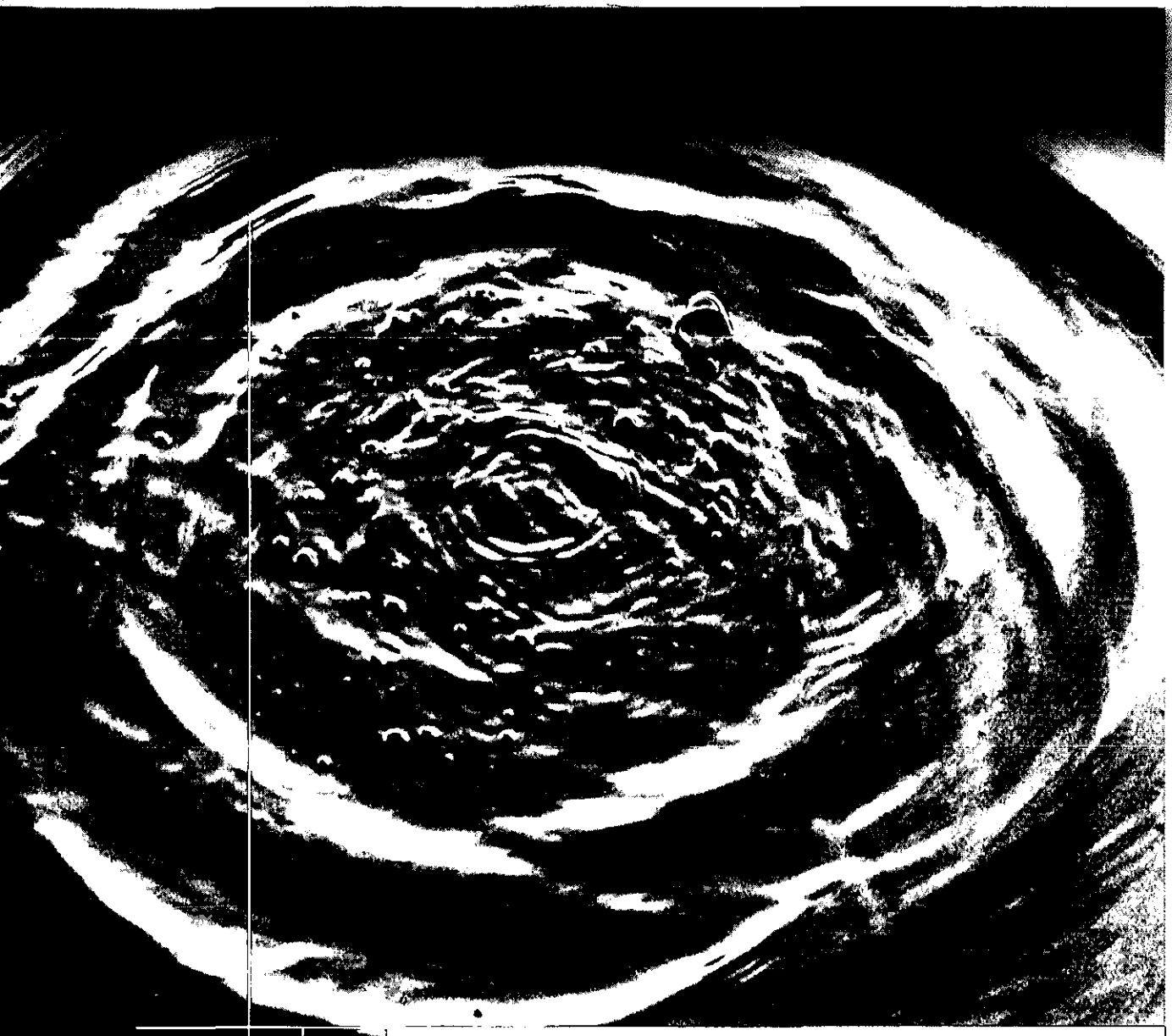
ARCHIVING

Only the final results are presented in this report. The raw and calculated data will be stored at Building 21, CSIR in written and electronic format.



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