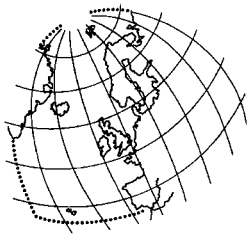
Hazardous Substances Series

Whole Effluent Assessment



OSPAR Commission

2005

The Convention for the Protection of the Marine Environment of the North-East Atlantic (the "OSPAR Convention") was opened for signature at the Ministerial Meeting of the former Oslo and Paris Commissions in Paris on 22 September 1992. The Convention entered into force on 25 March 1998. It has been ratified by Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, Netherlands, Norway, Portugal, Sweden, Switzerland and the United Kingdom and approved by the European Community and Spain.

La Convention pour la protection du milieu marin de l'Atlantique du Nord-Est, dite Convention OSPAR, a été ouverte à la signature à la réunion ministérielle des anciennes Commissions d'Oslo et de Paris, à Paris le 22 septembre 1992. La Convention est entrée en vigueur le 25 mars 1998. La Convention a été ratifiée par l'Allemagne, la Belgique, le Danemark, la Finlande, la France, l'Irlande, l'Islande, le Luxembourg, la Norvège, les Pays-Bas, le Portugal, le Royaume-Uni de Grande Bretagne et d'Irlande du Nord, la Suède et la Suisse et approuvée par la Communauté européenne et l'Espagne.

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Justification note:
Whole Effluent Assessment (WEA) within OSPAR

contents

1 Background on this note

This note sets out how Whole Effluent Assessment (WEA) supports the objectives of the OSPAR Hazardous Substances Strategy. WEA can be characterized as follows:

- WEA consists of a variety of (biological) tests to the determine persistence, bioaccumulation and toxicity (PBT-criteria). These are the same criteria that are used within OSPAR's Hazardous Substances Strategy. The difference is that WEA tests are applied to the *entire effluent sample* instead of to the individual substances.
- This means that not only the effects of the known substances, but also those of unknown substances are measured. WEA allows identification of adverse effects that result from substances that cannot be chemically identified or substances that have been identified but have not yet been assessed on PBT. Furthermore, WEA measures the result synergistic (combined) effects of constituent chemicals.
- This is particularly useful additional information for complex effluents that contain a large variety of unidentified substances. WEA can be used by OSPAR as a complement to the substance–based approach through supporting the identification of adverse effects of complex industrial effluents.
- The tests methods are derived from the methods used to assess PBT for individual substances. For toxicity the tests are sufficiently developed and can be utilized. For persistence and bioaccumulation further work needs to be done to make the available tests more widely applicable and easy to interpret for WEA purposes (see 4.3).

2 OSPAR Hazardous Substances Strategy: positioning of Whole Effluent Assessment

2.1 Outline of OSPAR's Hazardous Substances Strategy

OSPAR's Hazardous Substances Strategy is explained in detail in the box below [3]. In brief the Strategy can be described as follows:

- The objective is to reach (very) low levels of hazardous substances in the marine environment,
- The strategy is to continuously reduce discharges, emissions and losses of hazardous substances,
- The hazard characteristics of substances is assessed with PBT criteria,
- Single hazardous substances are selected and prioritised with the DYNAMEC system (assessment of PBT) followed by a survey on the background of the substances (sources, volumes, pathways, etc.),
- The relevant measures are taken, resulting in lower concentrations in the surface water system and thus in the marine environment.

2.2 Some background on the OSPAR Hazardous Substances Strategy [3]

I. What is the objective of this strategy?

As set out in the Hazardous Substances Strategy, the objective of the Commission with regard to hazardous substances is to prevent pollution of the maritime area by continuously reducing discharges, emissions and losses of hazardous substances with the ultimate aim of achieving concentrations in the marine environment near background values for naturally occurring substances and close to zero for man-made synthetic substances. The Commission will implement this strategy progressively by making every endeavour to move towards the target of the cessation of discharges, emissions and losses of hazardous substances by the year 2020.

II. What does the Commission do to achieve the objective of the Hazardous Substances Strategy?

a. selection and prioritisation of substances (or groups of substances) on the basis of PBT criteria agreed by OSPAR (or other intrinsic properties that lead to an equivalent level of concern) followed by ranking of substances taking account of exposure data;

b. for substances identified by OSPAR for priority action, the establishment of a background document describing (i) an identification of all sources of the substance and its pathways to the

marine environment, (ii) monitoring data, a quantification of sources and an assessment of problems, (iii) the desired reduction, (iv) an identification of possible measures and (v) the choice for action/measures. As a tool for assessing risks of potential hazardous substances in the marine environment, OSPAR has adopted the common EU/OSPAR approach on risk assessment for the marine environment (Section 4 of Chapter 3 in Part II of the EU Technical Guidance Document);

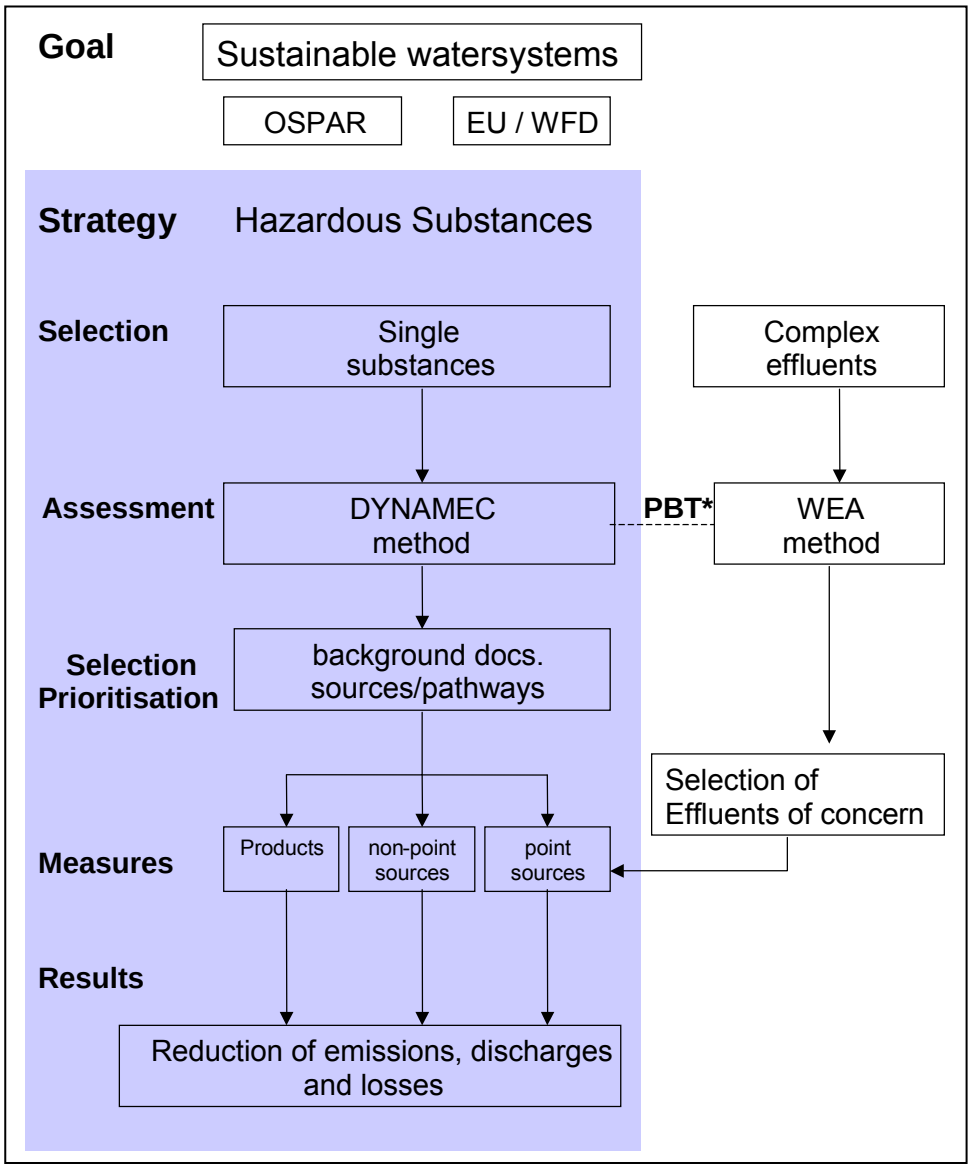
In the Bremen Statement of the ministerial meeting of the OSPAR Commission the role and position of OSPAR with respect to the other international organisations was clearly identified. For the subject of hazardous substances in particularly the European Community was encouraged to take full account of the need to protect the marine environment and therefore to take account of OSPAR's commitments to move towards the cessation of emission, discharges and losses of hazardous substances (2020 target).

- OSPAR shall ensure and will therefore focus its work on continuing to identify hazardous substances that cause concern for the marine environment;
- monitoring and assessing the range of measures adopted by various authorities in order to check if they are adequate for achieving the OSPAR 2020 target;
- where a shortfall is identified, taking action to make it good.

III. What do Contracting Parties do to achieve the objectives?

First of all, the Contracting Parties make a collective input to contribute to the work of the Commission as described in the previous paragraph. Additionally, they have the obligation to implement the OSPAR measures which they have adopted (some Contracting Parties have reservations on some measures). On top of that nearly all Contracting Parties are bound by the relevant EU policies and regulations, i.e. IPPC Directive, Water Framework Directive and the EC Chemicals Policy (see also paragraph 3). It is expected that the future programmes and measures on the WFD priority (hazardous) substances will also have large consequences for the regulation by competent authorities in Member States concerning hazardous substances. The European Commission intends to make optimal use of existing directives for implementing this WFD policy, but there is also the intention to produce WFD daughter directives where necessary.

Figure 1: Outline of OSPAR's Hazardous Substances Strategy with relation to the position of WEA.



* a number of Contracting Parties have pointed out that the DYNAMEC PBT assessment and the WEA PBT assessment are not comparable.

2.3 WEA as a complementary tool

The substance approach covers a broad field: prioritisation of substances, followed by measures with regard to both products, non-point sources as well as point sources (see Figure 1).

WEA can be used as a complementary tool to the substance-based approach in order to reach the objectives. The working field of WEA is smaller and reflects only the point sources that have a complex nature (see Figure 2). WEA can address the adverse effects of effluents which cannot be explained through information on PBT of individual substances alone. It is generally recognised that in complex samples, only a small fraction of (around 20%) the substances present can be identified. When adverse effects (PBT) are measured in the samples of effluents, sediments and surface waters, only a part of these effects can be related to the PBT properties of identified substances. Failing to address the effects of those substances that cannot be identified can mean that a (large) part of the adverse effects from effluents, is neglected.

The substance approach has shown to be a successful policy instrument (OSPAR Strategy, EC Dangerous Substances Directive): it has led to reductions in the concentrations of hazardous substances in surface waters. However it is generally recognised that the substance approach shows certain limitations, especially

when the medium to be examined has a complex composition of numerous substances (effluent, surface water, sediment):

1) Only a limited number of substances that are presently produced and used (around 50 000) can be analysed or *identified*. Hence, we are only looking at a small number of the total number of potentially harmful substances;

2) Experimental *PBT data* are available for only for a limited number of the substances that have been identified. Producing experimental data is a time consuming process.

> It is generally recognised and frequently shown that in complex samples, only a small fraction of (around 20%) the substances present can be identified. This is consistent with the observation that when adverse effects (PBT) are measured in the samples of effluents, sediments and surface waters, only a part of these effects can be explained using information on PBT of identified substances. This means that un-identified substances can be responsible for a (large) part of the adverse effects from effluents, surface waters or sediments.

These limitations give rise to interest in the development and implementation of tests that can be applied to entire environmental samples, like effluents, surface water or sediments. One might refer to these as 'sample' approaches. These test-systems offer a "short cut" in working towards the objectives for the protection of the marine environment, without the need to identify every constituent of a complex effluent and to determine the potential harm for each individual constituent.

WEA must be seen as a safety net (other than the formal DYNAMEC safety net) for the substance-by-substance approach and does not replace existing approaches with regard to the reduction of releases of hazardous substances. WEA is one of the tools which will help to make an assessment of impact. WEA will make it possible to check point sources for their overall potential to cause adverse effects. In this way, it can provide important additional information to fulfil the objectives of OSPAR's Hazardous Substances Strategy.

The OSPAR Convention, 1992, justifies the application of WEA in the sense that: "Contracting Parties agree to take all possible steps to prevent and eliminate pollution and to take the necessary measures to protect the maritime area against adverse effects".

2.4 Added value depending on type of effluent

Application of WEA is regarded as having added value where the substance oriented approach can not guarantee an adequate assessment. Figure 2 indicates the field of application where WEA has an added value; that is focussing on effluents with a complex composition.

The substance oriented approach is efficient for assessing the input side and product side from an industrial site (see Figure 2). We have a good knowledge of substances in raw materials, feedstock, auxiliary chemicals and products. In cases where PBT data are not available, they can be obtained by testing with the identified substances. In many cases the substance oriented approach is also adequate in assessing the effluent quality. When the processes result in 'simple' waste water with a predictable chemical composition, chemicals assessment may provide sufficient information to estimate the environmental hazard of the effluent.

However, for complex effluents where e.g. side-products are formed that end-up in the effluent, the composition of the wastewater is less predictable and many unknown or unidentifiable substances may be present. In these situations the basic chemical analyses provide an incomplete insight, since it covers a limited part of the substances in the effluent. This is the current situation in the chemical industry (sector). Usually an extensive chemical analysis is carried out, resulting in the identification of more substances. However, identification will not be complete and PBT data on the identified substances will still have to be obtained.

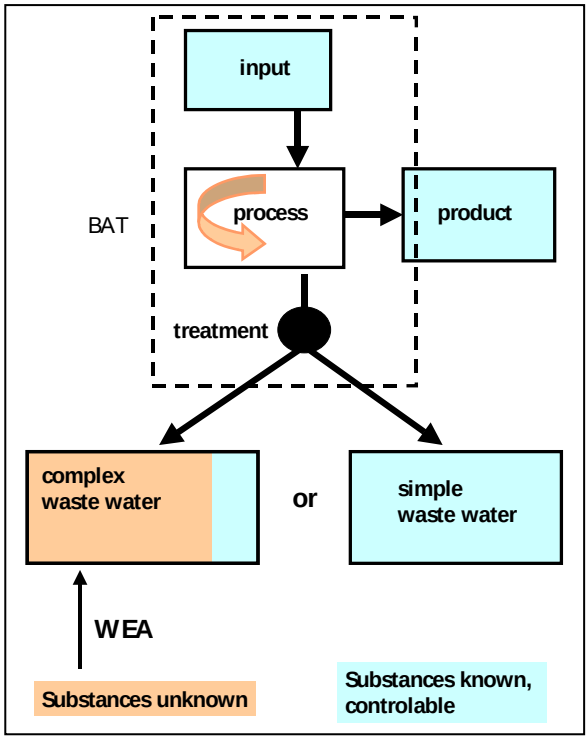
With WEA a short cut can be made since the effects of all the substances in the effluent can be measured. Therefore, WEA could be used in identifying effluents to be given priority and could thus contribute to 'flagging-up' effluents of concern. The added value has been shown within the practical study programme carried out in 2003 (see the text block below).

Conclusion from the practical study programme 2003

The added value of WEA was evaluated by comparing WEA results with permit requirements and with an extended chemical specific approach. It was concluded that in many effluents, testing toxicity has an added value, since toxicity was found that could not be explained with the (extended) chemical specific approach. The added value is most apparent in effluents that have a complex constitution.

Figure 2: Useful application fields for WEA

An industrial process is schematically divided into 1) the input of chemicals that enter the process, 2) the process, 3) the products and 4) the wastewater. The blue coloured boxes represent the field where the substance oriented approach is efficient, while the light brown coloured boxes indicate the field where the WEA approach is applicable.



It should be recognised that WEA should always include chemical analyses. Some of the physical-chemical parameters (e.g. sum and group parameters on organic load like TOC, DOC, COD AOX, nutrients, pH, conductivity) are even necessary for interpretation of the WEA results (salt effect, pH, ammonia-toxicity).

3 Whole Effluent Assessment within the EU policy

3.1 Water Framework Directive

Within the Water Framework Directive (WFD) targets are described for 'good chemical status' and 'good ecological status'. For the chemical status, the selection of priority substances, elaboration of discharge controls and quality standards are based on the same intrinsic properties of hazardous substances (PBT) as within OSPAR's strategy. For substances that are being classified as priority hazardous substances, cessation targets will be set. This means that WEA also has the potential of being able to contribute to the WFD targets for priority substances.

As to the ecological status, applying WEA on effluents may help to understand or maybe to predict the effects on the ecological characteristics of the aquatic environment.

3.2 IPPC

The IPPC Directive addresses large industrial activities with a special focus on transboundary pollutants. For discharges to water the list of dangerous substances of Council Directive 76/464/EEC is an annex to the IPPC Directive. It is expected that in the near future the IPPC directive will also contribute to discharge and emission controls for priority substances under the WFD.

One of the main elements of the IPPC Directive is the exchange of information on Best Available Techniques with the publication of so called BAT Reference documents (BREF). The performance of BAT can also be expressed as the absence or reduction of negative effects in effluents measured by means of WEA parameters.

So far, information on WEA has been incorporated in 3 (out of 12) BREF documents i.e. on Large Volume Organic Chemicals, Cooling Systems and Waste Water and Waste Gas Treatment and is proposed in the draft BREF on Economics and Cross Media issues. [refs pm].

Since the complexity of substances in effluents is increasing and the methods for assessing the effects are improving at the same time, it seems obvious that WEA parameters will be used regularly for the benchmarking of BAT performance. Also, biological testing of waste water is one of the innovative developments in water pollution control and meets a growing interest from industry.

4 Whole Effluent Assessment: outlook and further work

4.1 Prerequisites

Implementation of WEA in the future should of course serve the objectives of OSPAR's Hazardous Substances Strategy, as was described under section 1 (see information block). The following aspects should be kept in mind:

a. the WEA tool should assess effluents on the basis of PBT criteria agreed by OSPAR (or other intrinsic properties that lead to an equivalent level of concern). An equivalent level of concern may include endocrine disrupting effects or genotoxic effects of industrial effluents;

b. the marine environment should be of primary concern in the discussion on the design of a WEA tool within OSPAR making good use of the various WEA instruments that have been successfully implemented in the practices of discharge control;

c. the WEA tool should flag up effluents that show hazardous effects (of substances) that can cause concern for the marine environment and OSPAR should help to develop effective programmes to handle those hazardous effects or encourage various bodies and organisations to take appropriate further action.

Furthermore, some additional criteria can be defined based on previous discussions within the Hazardous Substances Committee in 2002 [4]:

a. WEA should be a robust, reliable and cost-effective method to guarantee success in applying this methodology in addition to the substance-by-substance approach;

b. Application of WEA should be subsidiary and flexible;

c. The WEA tool for achieving the objective of the OSPAR Hazardous Substances Strategy should be made operational in a “learning by doing” approach.

4.2 State of the art March 2004

In the year 2003 a practical study programme was conducted by the IEG-WEA, followed by an OSPAR workshop in September. Both the practical study programme and the discussions within the workshop are reported elsewhere [6].

- *Robustness*

  WEA is based on the same assessment criteria (PBT) and consists of a combination of techniques in order to reveal (potential) effects: toxicity (acute and chronic), genotoxicity, potential for bioaccumulation and persistency. The tests required to determine PBT in the effluent samples, are derived from the protocols that are available from the assessment of single substances.

  For toxicity methods protocols are available, and only little further effort is required to make ISO protocols. Some more attention should be given to methods used for chronic toxicity tests.

  As for bioaccumulation, two different tests were applied, that require further comparison as well as discussions on validation to bioaccumulation within organisms.

The persistency test data gave rise to more discussion, both on the type of testing as well as on the interpretation of the results.

- *Cost-effectiveness*

  The cost-effectiveness of WEA was estimated on a preliminary basis and compared to the costs of an extended substance oriented approach. It was concluded that the costs are of the same magnitude while the value for money with WEA is larger since the effects of all known and unknown substances are assessed. Of course costs for WEA might decrease when testing becomes daily practice and developments lead to simpler WEA test protocols. Furthermore, detailed chemical analysis of some specific substances may be extremely expensive.

- *Work to be done on WEA*

  It was concluded and recommended that WEA should include a tool box with different tests, so that a tailor-made choice of these tests should be possible. The tool box should be accompanied by a guidance, in which the way to chose and interpret test results and make management decisions on further action is explained.

In February 2004 an informal IEG meeting was organised on the kind invitation of the ECETOC working group of CEFIC. During this workshop the proposal for a multi annual working programme, that had been outlined during SPDS 2003, was further developed [8].

In the coming years work of the IEG-WEA should continue in two directions. The first one (in depth) would address specific items with regard to test developments, protocols as well as the guidance (flow charts). The second direction (in breadth) would be a practical line, where a test programme could be conducted in order to obtain more data and to increase the uptake from participating parties. The work from 2003 has shown that this concept of 'learning by doing' is effective.

5 References

1. Explanatory note SPDS 2002
2. Compatibility note SPDS 2003
3. Pm. Official reference of Strategy OSPAR
4. Summary Record HSC 02/11/1, Annex 15; Summary Record HSC 02/10/1, Annex 11
5. References of 3 BREFs from IPPC where WEA is mentioned
6. Summary Record WEA Workshop September; SPDS 03/13/7, Annex 2: WEA 03/6/1-E
7. Report on the practical study programme 2003
8. Proposal for a multi annual work programme HSC 2004

OSPAR practical study programme 2003 on Whole Effluent Assessment (WEA)

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Summary

Introduction

The OSPAR Point and Diffuse Sources working group set-up an intersessional expert group (IEG-WEA) in 1999 to examine the value of a whole effluent assessment approach in helping to achieve the OSPAR objectives for protection of the marine environment. To date the group has discussed possible ways of using persistence (P), liability to bioaccumulate (B) and toxicity (T) data for whole effluents within the OSPAR remit and has produced reviews of suitable methods. For WEA persistence is defined as the persistence of toxicity and/or liability to bioaccumulate after a period of biodegradation. The applied persistence tests are adjusted versions of tests that are validated for use on single substances.

The IEG-WEA conducted a practical study programme in 2003, in order to test these methods to assess P, B and T on a limited number of effluents around Europe.

The goal of the practical study programme was to assess whether WEA can have an added value with regard to the task of identifying effluents of concern to the marine environment. Furthermore, the programme should assess whether the WEA methods are robust and reliable and evaluate the cost-effectiveness of WEA. Seven Contracting Parties participated in the programme: Ireland, Belgium, United Kingdom, Portugal, Germany, Sweden and the Netherlands. The programme was designed in cooperation with all participating countries, while the coordination was in the hands of the Netherlands. This evaluation report has been written in cooperation with all participating parties, while a draft report was discussed on an OSPAR workshop in September 2003, where the total IEG group was invited. The OSPAR workshop was meant to evaluate the results of the practical study programme and to make conclusions on feasibility, added value and required future work on WEA within OSPAR.

Highlights of the results

Within the relatively short period of a few months, all tests were conducted and reported. In general toxicity and liability to bioaccumulate were found to decrease when effluents were treated more intensively. No relationship between a sector and the range of toxicity or liability to bioaccumulate could be found. This probably demonstrates that the variability within a sector in terms of processes and water treatment is greater than the apparent uniformity might suggest.

Toxicity and liability to bioaccumulate were sometimes found to be persistent with respect to biodegradation: that is a period of incubation did hardly decrease the degree of toxicity and liability to bioaccumulate. However, some additional discussions on the testing and the interpretation of persistence are required.

The *added value* of WEA was evaluated by comparing WEA results with permit requirements and with an extended chemical specific approach. It was concluded that in many effluents, testing toxicity has an added value, since toxicity was found that could not be explained with the (extended) chemical specific approach. The added value is most apparent in effluents that have a complex nature. On an added value of WEA testing of liability to bioaccumulate and persistence tests no firm conclusions can be drawn at this stage for that applied tests methods require further validation. This validation is part of the work programme 2004 – 2006.

The *robustness* has only been evaluated in a preliminary way, since the programme was too small to make statistical analysis on e.g. the reliability. The applied tests for toxicity and liability bioaccumulate were shown to be able to discern effluent samples containing high and low toxicity and liability to bioaccumulate.

For most of the applied toxicity methods ISO protocols are available, for others making ISO protocols seems feasible. Maybe some more attention should be given to methods used for chronic toxicity tests. For toxicity tests with fish, a discussion came up with regard to the added value they have in relation to other toxicity tests. Here the ethical aspects that apply to vertebrate animals play a role. As for liability to bioaccumulate, two different tests were applied, that will need further comparison as well as discussions on validation to bioaccumulation within organisms. The persistence tests gave reason for more discussions: on the type of testing as well as on the way to interpret the results.

The *cost-effectiveness* of WEA was estimated on a preliminary basis and compared to the costs of an extended chemical specific approach. It was concluded that the costs are of the same magnitude (around 3500 Euro per effluent), while the value for money with WEA is larger since the effects of all known and unknown substances are assessed. Of course costs for WEA might decrease when testing becomes daily practice and when a tailor-made choice will result in a smaller WEA test. Furthermore, chemical analysis of

some specific substances may be extremely expensive. It is dependent on the way of application within a Contracting Party whether costs for WEA should only be partly or fully added up to costs for the chemical specific approach.

General conclusions and recommendations for future work

WEA can have an added value for (complex) effluents with regard to flagging up effluents of concern to OSPAR. In the future WEA should consist of a toolbox of different tests. Depending on situation and effluent, a tailor-made choice of these tests should be possible. The toolbox should be accompanied by a guidance flow chart, in which the way to chose and interpret tests is explained. WEA tests for toxicity are available, but for liability to bioaccumulate and persistence, some further work is required. More data would be useful to make better evaluations on robustness and costs. In the coming years the work of the IEG-WEA should continue in two directions. Firstly the particular items that have been noted above should be addressed. This includes tests for liability to bioaccumulate and persistence, as well as drawing up several guidance flow charts. Secondly a new monitoring programme should be conducted, in order to obtain more data and to increase the uptake from participating parties.

1. Introduction

1.1 Background on whole effluent assessment (WEA)

1.1.1 WEA in general

The chemical specific approach has been, and still is, a successful policy instrument (OSPAR Strategy, EC Dangerous Substances Directive). Nevertheless it is generally recognised that this approach has some shortcomings.

Results from chemical analysis of samples of wastewater, surface water or sediments, have indicated that only a limited number of the substances that may be present in those samples, can be analysed, identified and quantified.

This is one of the main reasons for (ongoing and growing interest) in the development and implementation of biological tests that can be applied to entire environmental samples, like effluents, surface water or sediments. These biological tests have already been performed and have shown that the measured adverse effects can only partly be explained by the substances identified by analytical methods. This means that a large fraction of the adverse biological effects in (waste) water and sediments is caused by “unknown” substances or by combinations of substances.

Whole Effluent Assessment (WEA) can be defined as the assessment of effluents by using a range of biological methods in order to reveal (potential) effects, based on an assessment of persistence, bioaccumulation and toxicity (PBT-criteria). Since the entire effluent sample is tested, WEA increases the understanding of the combined effects of all known and unknown substances within effluents, especially in complex mixtures.

1.1.2 OSPAR and the position of WEA

Relation between OSPAR's Hazardous Substances Strategy and WEA

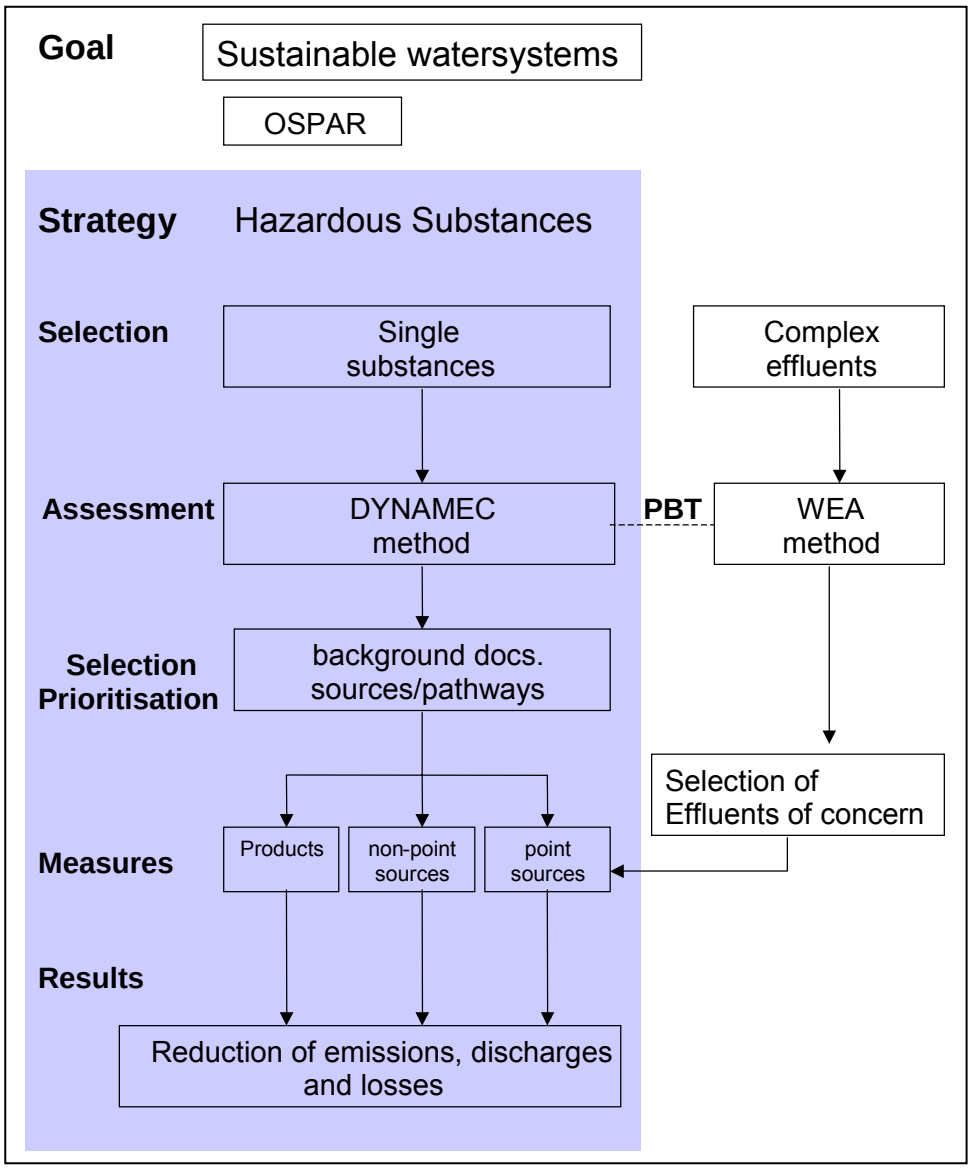
The relation between WEA and OSPAR's Hazardous Substances Strategy is depicted in Figure 1.1.

The OSPAR's Hazardous Substances Strategy is quoted in the box below and can briefly be described as follows:

- The objective is to reach (very) low levels of hazardous substances in the marine environment,
- The strategy is to continuously reduce discharges, emissions and losses of hazardous substances,
- The hazard characteristics of substances is assessed with PBT criteria,
- Single hazardous substances are selected and prioritised with the DYNAMEC system (assessment of PBT) followed by a survey on the background of the substances (sources, volumes, pathways, etc.),
- The relevant measures are taken, resulting in lower concentrations in the surface water system and thus in the marine environment.

OSPAR’s objective with regard to hazardous substances is to prevent pollution of the maritime area by continuously reducing discharges, emissions and losses of hazardous substances, with the ultimate aim of achieving concentrations in the marine environment near background values of naturally occurring substances and close to zero for man-made synthetic substances. In achieving this objective OSPAR selects and prioritises substances on the basis of criteria for Persistence, Liability to Bioaccumulate and Toxicity (P, B and T); criteria that reflect the intrinsic hazardous properties of substances.

Figure 1.1: Outline of OSPAR's Hazardous Substances Strategy with relation to the position of WEA



The substance approach has shown to be a successful policy instrument (OSPAR Strategy, EC Dangerous Substances Directive): it has led to reductions in the concentrations of hazardous substances in surface waters.

However it is generally recognised that the substance approach shows certain limitations:

1) Only a limited number of substances that are presently produced and used (around 50 000) can be analysed or *identified*. Hence, we are only looking at a small number of the total number of potentially harmful substances;

2) Experimental *PBT data* are available for only for a limited number of the substances that have been identified. Producing experimental data is a time consuming process.

WEA could form a short cut to these limitations, since it consists of a variety of (biological) tests to determine the same criteria that are used within OSPAR's Hazardous Substances Strategy (PBT-criteria), but now applied to the *entire effluent sample* instead of to the individual substances. This means that not only the effects of the known substances, but also those of unknown substances are measured.

In 1997 it was concluded that biotests and other effect tests are very valuable additional instruments for assessing waste water on an OSPAR workshop regarding 'ecotoxicological evaluation of waste water' In 1999 the OSPAR Point and Diffuse Sources working group set up an intersessional expert group (IEG) to examine the value of whole effluent assessment (WEA) in support of the implementation of the OSPAR hazardous substances strategy.

The OSPAR Convention, 1992, justifies the application of WEA in the sense that: "Contracting Parties agree to take all possible steps to prevent and eliminate pollution and to take the necessary measures to protect the maritime area against adverse effects".

WEA must be seen as a safety net for the substance-by-substance approach and does not replace existing approaches with regard to the reduction of releases of hazardous substances. WEA is one of the tools which will help to make an assessment of impact. WEA will make it possible to check point sources for their overall potential to cause adverse effects. In this way, it can provide important additional information to fulfil the objectives of OSPAR's Hazardous Substances Strategy.

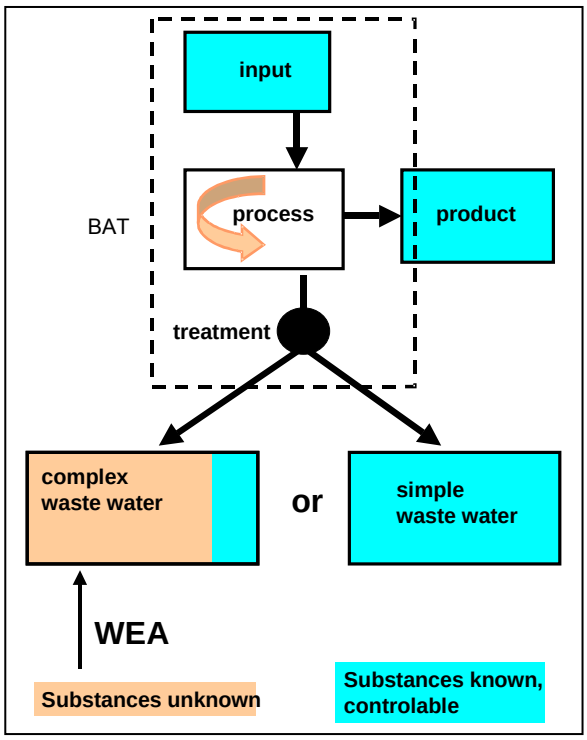
Added value only for complex effluents

Application of WEA is regarded as having added value where the substance oriented approach can not guarantee an adequate assessment. This is not the case for all types of effluents. Figure 1.2 indicates the field of application where WEA has an added value, that is focussing on effluents with a complex composition.

The substance oriented approach is efficient for assessing the input side and product side from an industrial plant (see Figure 1.2). There is sufficient knowledge of substances in raw materials, feedstock, auxiliary chemicals and products. In cases where PBT data are not available, they can be obtained by testing with the identified substances. In many cases the substance oriented approach is also adequate in assessing the quality of the waste water. When the processes result in 'simple' waste water with a predictable chemical composition, chemicals assessment may provide sufficient information to estimate the environmental hazard of the effluent.

Figure 1.2: Useful application fields for WEA

An industrial process is schematically divided into 1) the input of chemicals that enter the process, 2) the process, 3) the products and 4) the wastewater. The blue coloured boxes represent the field where the substance oriented approach is efficient, while the light brown coloured boxes indicate the field where the WEA approach is applicable.



However, for complex effluents where e.g. side-products are formed that end-up in the effluent, the composition of the wastewater is less predictable and many unknown or unidentifiable substances may be present. In these situations the basic chemical analyses provide an incomplete insight, since it covers a limited part of the substances in the effluent. This is the current situation in the chemical industry (sector). Usually an extensive chemical analysis is carried out, resulting in the identification of more substances. However, identification will not be complete and PBT data on the identified substances will still have to be obtained.

In these cases, where the methodology is sufficiently robust, WEA can have an added value. It should be recognised that WEA should always include chemical analyses. Some of the physical-chemical parameters (e.g. sum and group parameters on organic load like TOC, DOC, COD AOX, nutrients, pH, conductivity) are even necessary for interpretation of the WEA results (salt effect, pH, ammonia-toxicity).

Prerequisites for OSPAR put forward at the OSPAR workshop on WEA

Implementation of WEA in the future should of course serve the objectives of OSPAR's Hazardous Substances Strategy. The following aspects should be kept in mind:

a. the WEA tool should assess effluents on the basis of PBT criteria agreed by OSPAR (or other intrinsic properties that lead to an equivalent level of concern). An equivalent level of concern may include endocrine disrupting effects or genotoxic effects of industrial effluents;

b. the marine environment should be of primary concern in the discussion on the design of a WEA tool within OSPAR making good use of the various WEA instruments that have been successfully implemented in the practices of discharge control;

c. the WEA tool should flag up effluents that show hazardous effects (of substances) that can cause concern for the marine environment and OSPAR should help to develop effective programmes to handle those hazardous effects or encourage various bodies and organisations to take appropriate further action.

Furthermore, some additional criteria can be defined based on previous discussions within the Hazardous Substances Committee in 2002:

WEA should be a robust, reliable and cost-effective method to guarantee success in applying this methodology in addition to the substance-by-substance approach;

Application of WEA should be subsidiary and flexible;

The WEA tool for achieving the objective of the OSPAR Hazardous Substances Strategy should be made operational in a "learning by doing" approach.

1.2 OSPAR practical study programme on WEA

1.2.1 Motivation and objectives

Motivation

In 1999 the OSPAR Point and Diffuse Sources working group set-up an intersessional expert group on WEA (IEG-WEA) to examine the added value of WEA in helping to achieve the OSPAR objectives for the protection of the marine environment. The group has carried out an inventory with regard to the application or interest in WEA within the OSPAR member states. The inventory showed that interest was present in many countries and that some countries were already applying WEA in one form or the other within their licences or as part of the licensing procedure. Hence the group has discussed possible ways of using WEA within the OSPAR framework. These discussions where focussing on the added value and the cost-effectiveness of WEA and the reliability of tests. In order to make some steps forwards in these discussions, seven Contracting Parties agreed to participate in the practical study programme of 2003.

Objectives

- The primary goal of the practical study programme was to demonstrate that WEA has an added value when it comes to the task of identifying effluents of concern to the marine environment. More specifically, to show how WEA can be used to identify priorities for action; to trigger further site specific investigations and chart progress in reducing whole effluent persistence, liability to bioaccumulate and toxicity. This was to be achieved by showing that in a substantial number of effluents WEA can detect potential harmful effects, which cannot be measured using a chemical specific approach alone.
- The second goal was to demonstrate the robustness and reliability of WEA methods for persistence, liability to bioaccumulate and toxicity assessment in the hands of OSPAR Contracting Parties ensuring that the data generated by these methods are "fit for purpose".
- The third objective was to evaluate the costs effectiveness of WEA.

The following step would be to convince Contracting Parties not participating in the demonstration programme of the added value of WEA, so that they would participate in the follow-up. This follow up would be an OSPAR monitoring programme (in 2004 and later), where more countries could participate and more effluents could be assessed with WEA.

1.2.2 Programme design

In September 2002 seven Contracting Parties participated in the programme: Ireland, Belgium, United Kingdom, Portugal, Germany, Sweden and the Netherlands. The programme was designed in cooperation with all participating countries, while the overall coordination was in the hands of the Netherlands.

Each of the seven participating countries selected and tested around 2 effluents. The effluents should preferably be BAT-regulated. Since the programme was designed for research objectives only, the names and locations of the industries concerned will not be made public. CEFIC facilitated the selection of effluents. All effluents were assessed both with WEA measurements as well as with the chemical specific approach.

As much as possible comparable WEA tests would be applied, to improve comparison. As for toxicity, all effluents were at least assessed with toxicity to bacteria, algae and crustaceans. It was agreed that the WEA tests should produce high quality data. When possible all results should be accompanied by statistical reliability criteria, such as 95% confidence intervals.

The chemical specific approach was based on the effluent measurements regulated by the permit and the results of an extended chemical analysis (GC/MS screening). The effluent characteristics were compared with current permit limit values. For substances identified with the extended chemical analysis (GC/MS screening), a query on existing PBT data will be performed.

The costs of an effluent assessment were described and evaluated. The basis for this evaluation were the current prices for WEA tests and extended chemical analysis.

1.2.3 Function and status of this report

The practical study programme has been planned, executed and reported in draft within a period of one year, from September 2002 to September 2003. This is a relatively short period when taking into account the number of Contracting Parties and the extent of the programme; about 200 WEA tests were performed. The results and conclusions in the draft report were examined and discussed by the Contracting Parties during the OSPAR WEA workshop on 24 and 25 September in Utrecht, the Netherlands. During this workshop also a consensus was found with respect to the conclusions that could be drawn. In the present report the remarks made during the workshop as well as the amended conclusions are incorporated.

2. Methods

2.1 Selection of effluents

In order to elicit the added value of WEA it was preferred to select complex effluents: effluents from industries producing fine chemicals, pharmaceuticals, textiles and pulp and paper. When available, historical data on the toxicity, bioaccumulation and persistence of effluents were used to select the effluents. Each contracting party selected a number of effluents.

In total 17 effluents were selected. Table 2.1 summarises the final selection of these effluents and provides information on the type of industry, treatment, discharge, permit requirements and compliance. The selected effluents originated from and 3 hospitals and three types of industry, 6 pharmaceutical, 4 chemical and 4 textile. Eight effluents received biological treatment prior to discharge, two received physical/chemical treatment and seven were not treated at all. Four of latter ones were also indirect effluents, meaning that they would still pass some sort of treatment, e.g. in a municipal waste water treatment plant, before being discharged. The remaining three are discharged to the receiving water system without further treatment. For one effluent, UK-1, only a part of the effluent has received some biological treatment. In this study the effluent is regarded as not biologically treated

The effluents were sampled and tested for persistence, liability to bioaccumulate and toxicity in the period of May to August 2003. Samples for liability to bioaccumulate and extended chemistry were stored at –20ºC in plastic or glass (NL) containers. The containers were transported to the Netherlands by express mail. SPME and GC-MS screenings were carried out during the period of July to September of 2003. Test results and data on effluent treatment, permit requirements and compliance were collected during the months July to September 2003.

Table 2.1. Effluents selected for the OSPAR practical study programme on WEA with information on treatment, discharge, permit required measurements and compliance

Code	Type	Treatment	[1)]	Discharge	Measurements required by permit	Compliance
NL-1	Chemical	Active sludge	B	Direct	Flow, COD, N-Kjeldahl, N-total, P-total, suspended solids, EOX, bisphenol-A, Mono-aromatic carbohydrates, copper, zinc, methylene-chloride, volatile phenoles, chlorine (free)	Compliant
NL-2	Pharma	Active sludge	B	Direct	Flow, BOD, COD, chloride, bromide, sulphate, copper, zinc, phosphate, aluminium, EOX, iodide, N-Kjeldahl, nitrate, suspended solids	Compliant
UK-1	Chemical	Partly biological treatment + settlement	-*	Direct	Ammonia, pH, oxidised N, orthophosphate, cadmium	Compliant
UK-2	Chemical	None	-	Direct	Ammonia, pH, suspended solids, arsenic, benzene, BOD, cadmium, chloroform, chromium, copper, cyanide, lead, 1,2-DCE, mercury, nickel, nitrate, nitrite, nonylphenol, oxidized N, phenol, toluene, trichloroethene, m-p xylene, o-xylene, total xylenes, zinc	Occasional non-compliance
P-1	Textile	Active sludge, colour removal	B	Indirect	BOD, CQO, SST, conductivity, pH, B, As, Pb, cyanide(total), Cu, Cr, Fe, Ni, Se, Zn, metals (total), hydrocarbons (total), phenols, NH4-N, surfactants	Not compliant
P-2	Pharma	Stripping, pH adj, aeration, decantation, final pH adj.	P/C	Indirect	Flow, BOD, COD, phenols, suspended solids, total, total cyanide, sulphite, surfactants. N-Kjeldahl, P-total, cadmium, copper, nickel, lead, zinc, iron, chloride, NH4-N	Not compliant
D-1	Textile	pH-adj.	P/C	Indirect	COD, TOC, BOD/COD, NH4-N, N-Kj, conductivity, AOX, copper, chromium, sulphite, coloration	Compliant
D-2	Pharma	Active sludge, denitrification	B	Direct	TOC, AOX, NO_3-N, NH_4-N, N-Kj, conductivity, PO_4-P, EDTA, NTA, DTPA	Compliant
D-3	Pharma	Active sludge, denitrification	B	Direct	TOC, NO_3-N, NH_4-N, N-Kj, AOX, conductivity, PO_4-P, chloride	Not compliant
D-4	Textile	Active sludge + Active carbon + Bio- filter	B	Direct	COD, TOC, AOX, Vanadium, SO_3, benzene, toluene, xylene and other aromatic solvents	Compliant
BE-1	Hospital	None	-	Indirect	pH, flow, temp., POX, detergents, suspended solids, BOD, COD	-
BE-2	Hospital	None	-	Indirect	Not specified	-
BE-3	Hospital	None	-	Indirect	pH, flow, temp., POX, detergents, suspended solids, BOD, COD, NO3-N, NO2-N, N-Kjeldahl, P, 13 metals, Cl, F, phenols, sulphate, AOX, EOX, CN, VOX	-
SE-1	Chemical	Aerated basin + chem. precip.	B	Direct	Flow, BOD, COD, N-tot. suspended solids, boron, fluoride, toxicity as TEF (Q x 100/EC50, 15min Microtox)	Compliant
IR-1	Pharma	Biological treatment	B	Direct	< 10 TU's required for permit (except algal tox)	Compliant
IR-2	Pharma	Unknown	- **	Indirect	< 5 TU's required for permit	Not compliant
IR-3	Textile	None	-	Direct	< 10 TU's required for permit	Not compliant

[1)] B = Biological P/C = Physical/Chemical - = None

* Only a small part of the UK-1 effluent was biologically treated

** *T*he IR-2 effluent is a mix of process wastes & factory sewage, untreated as it is, then discharged to a municipal WWTP.

2.2 Selection of tests

2.2.1 Toxicity

General background on toxicity tests

Toxicity tests or bioassays are carried out in order to assess the direct adverse effects on living organisms or cells. In bioassays test organisms or cells are exposed to the test substance in a concentrations range, or, as in the case of effluents, to a dilution series of the effluent. A dose-effect curve as illustrated in Figure 2.1 can than be obtained by evaluating the adverse effects, such as mortality, mobility or reproduction, at the different exposure levels after a certain period of exposure. From this dose-effect curve the concentration where 50% effect occurs, the EC50 is estimated. The EC50 is mostly requested in acute toxicity studies. In the case of lethality or inhibition as the measured effect the EC50 is usually given as respectively the LC50 to IC50. Bioassays can also be used to obtain a No Observed Effect Concentration (NOEC), the highest tested concentration at which no statistically significant adverse effects are observed. The NOEC is often requested from chronic toxicity studies.

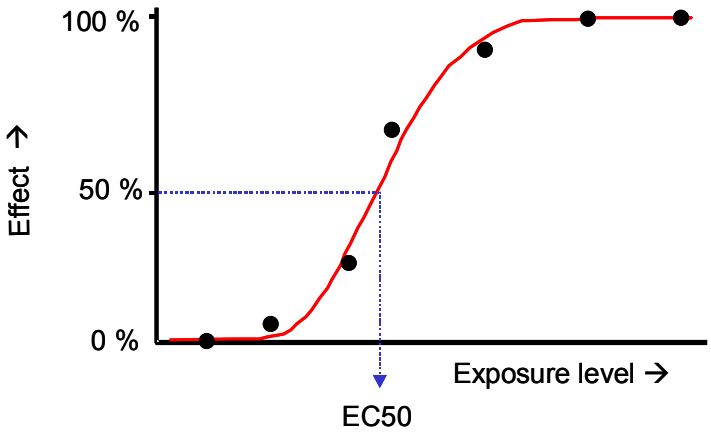


Figure 2.1: Dose-effect curve obtained from bioassays

The present study all results of all the toxicity tests are presented in terms of Toxic Units (TU). The TU can be seen as the effective effluent dilution factor and is calculated as 100/EC50 (%) or 100/NOEC (%), depending on the type of test.

When no toxicity could be measured at the highest concentration tested this results in a low number of Toxic Units accompanied by the '<' sign in the figure. When the EC50 or NOEC is below the lowest tested concentration the calculated number of toxic units is accompanied by a '>' sign in the figure.

Selection of toxicity test

For the practical study programme it was agreed that all Contracting Parties would apply the tests they were most familiar with. An overview of the selected tests is given in Table 2.2. More details for the toxicity tests with species names, test endpoints and the protocols applied can be found in section 7.2.

All effluents were at least tested with bacteria, algae and crustaceans and in many cases also a fish toxicity test was performed. Some Contracting Parties omitted the fish testing, thus recognising the ethical concerns over toxicity testing using vertebrates. In some cases more than one species from a certain taxonomic group was tested or the same species were tested both for acute and chronic toxicity. For a small number of effluents toxicity was also assessed with species from other taxonomic groups (bivalves, water plants).

Some Contracting Parties additionally assessed their effluents for genotoxicity and/or endocrine disruption endpoints. The methods used for genotoxicity and endocrine disruption were in line with the recommendations of the OSPAR IEG reviews "Survey on Genotoxicity test Methods for the Evaluation of Wastewater within whole effluent assessment" by Gartiser and Schnurstein and "OSPAR background document on the use of effect related methods to assess and monitor wastewater discharges – testing of endocrine disruption" by Knacker.

Originally it was intended to apply the chronic Microtox tests, a relatively new and potentially sensitive, short-term chronic toxicity test, to all effluents. However, due to technical failure this could eventually not be achieved.

2.2.2 Liability to Bioaccumulate

Due to the many technical difficulties associated with measuring the bioaccumulation of substances directly in living organisms, it is generally accepted that the liability of substances to bioaccumulate in organisms may be estimated from their lipophilic properties: their affinity for fatty tissue. Whether a substance will actually accumulate in the organisms fatty tissue will, among other factors, greatly depend on the organisms ability to metabolise and excrete the substance.

In the present study two methods for measuring the liability to bioaccumulate were applied, the Solid Phase Micro Extraction (SPME) method and the LPE-EGOM method, a liquid-liquid extraction method. Both methods are in line with the findings of the OSPAR IEG review "Persistence and bioaccumulation – methods in use or under development in whole effluent assessment" prepared by Åke Undén (Sweden) and the reader is referred to this document for more background information.

Various Contracting Parties measured the liability to bioaccumulate in their effluent samples using the SPME method. Next to this it was also agreed that all effluents would be tested in the Netherlands with the SPME method. The protocol used for the SPME method is given in section 7.2.2.

Briefly, the SPME method is a method by which the total amount of Potentially Bioaccumulating Substances (PBS) is measured in 'biomimetic' fibres of polydimethylsiloxane (PDMS). Due to the resemblance with animal fat, the total amount of substances in the fibre reflects the affinity of the measured substances to accumulate in animal fatty tissue. The substances absorbed by the fibre are quantified by making use of an external standard, 2,3-dimethylnaphthalene, and expressed as mM of PBS in the fibre. Note that the dimensions here are amounts per litre of fibre. This is essentially different from the dimensions in methods using liquid/liquid extraction, where the dimensions are amounts per litre of sample. PBS analyses were carried out in two laboratories in the Netherlands, the RIZA and RIVO. For comparison three samples were measured by both laboratories.

2.2.3 Persistence

For single substances the persistence is measured by measuring the concentrations of substances before and after a period of incubation in test for biodegradation. Since in WEA we are primarily interested in the effluent as a whole, persistence in WEA is rather interpreted as the persistence of toxicity and/or liability to bioaccumulate. For an extensive discussion on this issue the reader is referred to the OSPAR IEG review on "Persistence and bioaccumulation – methods in use or under development in whole effluent assessment" prepared by Ake Unden (Sweden).

In WEA the same tests are used as for individual substances, but instead of measuring the concentration of substances it is the liability to bioaccumulate and the toxicity that are measured before and after the incubation. For some Contracting Parties, recalcitrant matter measured as TOC, for instance, is also a reason for concern (see section 3).

In the practical study programme, the persistence of toxicity and liability to bioaccumulate was measured in 9 effluents. Two methods for the incubations were used; the 'modified DOC-die-away' test (OECD301E & ISO 7827) and the 'Zahn-Wellens' test (OECD 302 B & ISO 9888). The DOC-die-away test simulates biodegradation in a natural environment and therefore measures the 'readily biodegradability'. This test was used for the 8 direct effluents in the programme. The Zahn-Wellens test simulates biodegradation in a sewage treatment plant, where conditions are better for biodegradation. Therefore the results show the 'inherent biodegradability' that might occur under optimal conditions. This test was used only for effluent D-1, since this effluent would undergo further treatment.

The reduction of Dissolved Organic Carbon (DOC) in samples can serve as a measure of the biodegradation of substances during the incubation.

2.2.4 Overview of tests

Table 2.2 gives an overview of the test performed with each effluent, both with the unaltered effluents and with the effluents after a biodegradation step. In section 7.2 of Appendix 7 detailed information on individual tests and protocols is provided.

Table 2.2. Overview of the tests carried out with each effluent before and, when applicable, after a 'ready' or 'inherent' biodegradation test.

Code	Liability to Bioaccumulate	Toxicity								
		Bacteria	Algea	Crustaceans		Fish		Other	Genotox	ED[5]
				acute	chronic	acute	chronic			
NL-1	X	X	X	X	X		X		X	
after 'ready' test	X	X		X						
NL-2	X	X	X	X	X		X		X	
after 'ready' test	X				X					
UK-1	X[1]	X	X	X	X	-		X[3]		
after 'ready' test	X		X		X			X[3]		
UK-2	X[1]	X	X	X	X			X[3]		
after 'ready' test	X				X			X[3]		
P-1	X[1]	X	X	X	X					
P-2	X[1]	X	X	X	X					
D-1	X[1]	X	X	X					X[2]	X
after 'inherent' test biodeg.	X[1]	X	X	X					X[2]	X
D-2	X[1]	X	X	X		X	X		X[2]	X
after 'ready' test	X[1]	X	X	X					X[2]	X
D-3	X[1]	X	X	X		X	X		X[2]	X
after 'ready' test	X[1]	X	X	X					X[2]	X
D-4	X[1]	X	X	X		X	X		X[2]	X
after 'ready' test	X[1]	X	X	X					X[2]	X
BE-1	X	X	X	X		X			X	X
BE-2	X	X	X	X		X			X	X
BE-3	X	X	X	X		X			X	X
SE-1	X[1]	X	X	X		X	X			
after 'ready' test	X[1]	X	X	X		X	X			
IR-1	X	X	X	X[2]		X[2]		X[4]		
IR-2	X	X	X	X		X		X[4]		
IR-3	X	X	X	X		X[2]		X[4]		

[1]: Measured in two laboratories,

[2]: Measured with more than one species or method

[3]: Tests with oyster larvae (Bivalva: *Crassostrea gigas*)

[4]: Test with duckweed (*Lemna minor*)

[5]: Endocrine disruption

2.3 Extended chemistry (GC-MS screening)

Samples of all effluents were sent to RIZA for SPME measurements. Next to these measurements the effluent samples were also screened for the presence of organic substances. For this purpose the samples were extracted using a liquid-liquid extraction with dichloromethane (DCM). The organic substances in the extract were identified using the state-of-the-art method in analytical chemistry, i.e. gas-chromatography with mass-spectrometry (GC-MS) together with the peak identification according to the latest version of the AMDIS software and the NIST98 library on mass-spectra.

2.4 Evaluation of WEA

2.4.1 Added value

The added value of WEA as compared to the chemical specific approach in identifying effluents of concern, was evaluated by comparing the outcome of the WEA assessment with that of a an extended chemical specific assessment for toxicity.

The chemical specific approach primarily comprised of evaluating effluent chemistry against permit requirements. Therefore, the Contracting Parties were asked to evaluate their own effluents and give their personal view on the added value of the WEA measurements. They were asked to compare the WEA results with historical knowledge of the effluents and with the basic effluent chemistry and permit requirements.

There were large differences between the effluents in terms of the measurements required by the permit and the effluent quality criteria in the permits. Therefore, an evaluation on the basis of the extended effluent chemistry seemed more appropriate. For that purpose a query on toxicity data was performed, using the EPA AQUIRE database. The goal of this query was to see if observed toxicity in the effluents could be explained on the basis of substances identified in the effluents. WEA is considered to have an added value when permit requirements are met, but toxicity is observed that can not be explained on basis of the effluents chemistry. Furthermore, WEA may also have an added value when both the effluent chemistry and WEA indicate a low concern. In this case WEA functions as a check or safety net, for excluding the presence of unidentified toxic substances.

2.4.2 Robustness (and size of toolbox)

If WEA is to become an additional instrument in assessing effluents, the methods to assess P, B and T should be robust. The robustness of test methods for application should be evaluated on the basis off:

1) variations within a test
2) the inter-laboratory and
3) intra-laboratory variation and
4) its ability to effectively discriminate between samples.

The variation within a certain test is evaluated by simultaneously testing either a number of repetitions (replicates) or a number of concentrations as in a concentration range. The latter is usually applied in toxicity tests in order to reliably derive an effect concentration like the LC50 (the concentration that is lethal to 50% of the tested population) from the test results. By using replicates and/or a concentration range the test data are traditionally used to calculate the reliability or confidence of a test result.

An evaluation of the inter- and intra-laboratory variation requires a totally different approach. Usually the inter-laboratory variation is checked by testing one or more chemicals or well-defined mixtures a number of times. Preferably this should be done at different times and by different persons. A similar approach is used to evaluate the intra-laboratory variation but instead of the repetitions of tests within one laboratory, the tests will be repeated in a number of laboratories. The practical study programme was not designed to evaluate either the inter- or intra-laboratory variation of any of the applied test methods. Moreover, the focus in the practical study programme was to evaluate as many effluents as possible with as many tests as possible. Therefore, the inter- or intra-laboratory variation of applied methods is not discussed further in this report.

The fourth and perhaps most important criterion for evaluating the robustness of a certain method is a test's ability to effectively discriminate between samples. For instance, when the potential range of test results is small relative to the test variability, including inter-and intra- laboratory variation, it will be difficult to draw meaningful conclusions. The opposite is also true. Despite a relatively large test variation and inter and intra laboratory variations, the test results can be meaningful when the range of test results is large enough. In the evaluation of the test applied in the practical study programme this criterion will be discussed as appropriate.

2.4.3 Cost-effectiveness

In order to assess the costs for the application of WEA, the costs for the different WEA tests were requested from each participating country. With some assumptions the costs for application of WEA were estimated and compared with the costs of an extended chemical specific approach.

The cost assessment is for only one complete P,B,T measurement for only one effluent. Reason for this is that once an effluent has been assessed for the first time, the cost effectiveness of the use of WEA tests and chemical analysis should be evaluated for each effluent individually. For instance, the closeness of the examination should be related to the representativeness of the samples. For highly variable sample compositions low cost screenings or limit tests repeated in a short interval may provide more information (time series).

3. Results

In total 17 effluents were used in the practical study programme (see Table 2.1). The results for toxicity, liability to bioaccumulate and persistence are presented and discussed in separate paragraphs. A summary of these data can be found in Appendix 7.3.1. For 7 effluents toxicity and liability to bioaccumulate were determined a second time, after a period of biodegradation. The results of these tests are discussed in the paragraph on persistence only.

The measurements Endocrine Disruption are not discussed in this report. They are however included in Section 7.3.1 of Appendix 7.

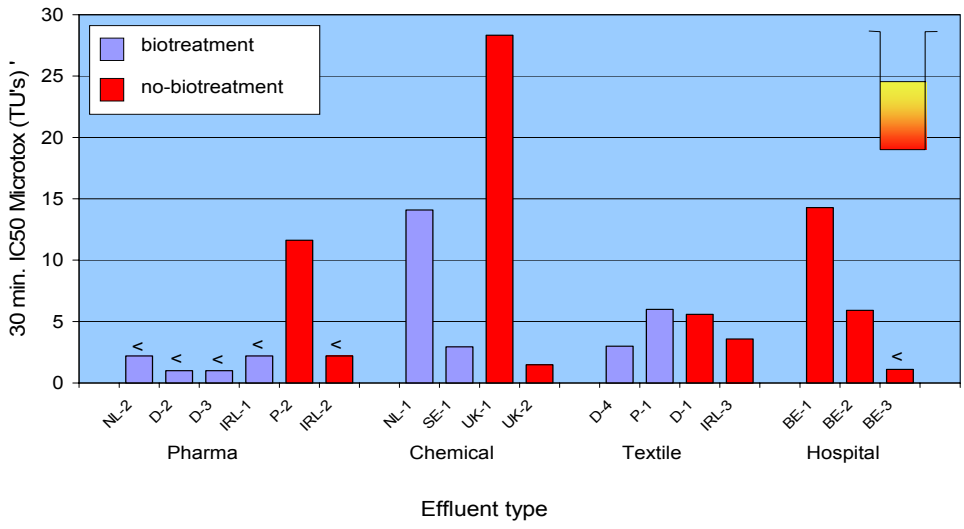
3.1 Toxicity

3.1.1 Microtox

All 17 effluent samples were tested for toxicity to bacteria with the acute Microtox test. The values reported are the 30 min IC50 (the 50% Inhibition Concentration). The results of these measurements are expressed as Toxic Units (TUs) and presented in Figure 3.1.

Eleven out of 17 effluents showed toxicity in the Microtox test, with TUs ranging from 1,5 for UK-2 to 28 for UK-1. As can be seen from the figure the effluents not receiving any biological treatment tend to be the most toxic in the Microtox test. One exception to this seems the NL-1 effluent, which, despite biological treatment is still quite toxic.

Fig 3.1 The toxicity in 17 effluent samples using the Microtox test

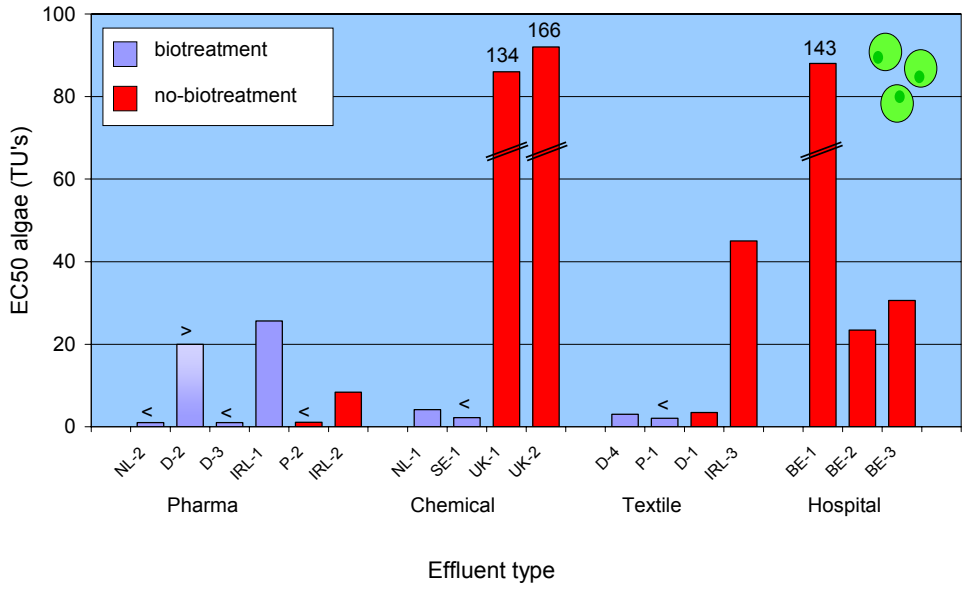


Algae

Two species of algae were used. NL, P, BE, D and SE used only *Pseudokirchniella subcapitata* (previously *Selenastrum capricornutum*), a freshwater species, and the UK used only the marine species *Skeletonema costatum.* Ireland used both species. Due to the limited data it was not possible to distinguish between the species in this evaluation. The EC50 of the algal tests are expressed as the toxic units in Figure 3.2.

Two out of 8 biologically treated effluents showed moderate toxicity for the algae. Eight out of 9 not-biologically treated effluents were moderately to very toxic to algae. Very high TU values were found for the Chemical effluents UK-1 and UK-2 and the Hospital effluent BE-1. As can be seen in the figure, algal toxicity can be found for all types of effluents.

Fig 3.2 The toxicity in 17 effluent samples using algal tests



3.1.2 Crustaceans

Various species of crustaceans were tested and both acute and chronic effects were measured (see section 7.3 for details on species, endpoints and protocols). The results of the acute tests are presented in Figure 3.3. The values are expressed as toxic units. The species are not distinguished in this figure.

The biologically treated effluents showed no more than moderate toxicity to the crustaceans. This was not the case for the effluents without biotreatment. Seven out of 9 effluents showed toxicity and of these, very high TU values were found for Pharma effluent IRL-2 and Chemical effluent UK-1. The species used for testing these effluents were respectively the freshwater species *Daphnia magna* and the marine species *Tisbe battagliai* For the Irish textile effluent IRL-3, both *Daphnia* and *Tisbe were* used in an acute test. *Tisbe* proved to be twice as sensitive in this case.

3.1.3 Fish

Six effluents receiving bio-treatment and five without bio-treatment effluents were tested for fish toxicity. Acute tests were performed for the Belgian and Irish effluents. The species used were the freshwater species *Oncorhynchus mykiss* (rainbow trout), and the marine fish species *Scophthalmus maximus (turbot)*. The Netherlands, Germany and Sweden used (sub-) chronic fish tests with the zebrafish (*Danio rerio*). The protocols applied by the Netherlands and Germany were developed for ethical reasons, to replace the acute fish test with juvenile and adult fish and to replace the standard early life stage tests. In the figures all the fish test toxicity data are combined. The acute EC50's and sub-chronic NOEC's are expressed as TU's and presented in Figure 3.4.

Fig 3.3 The acute toxicity in 17 effluent samples using crustaceans

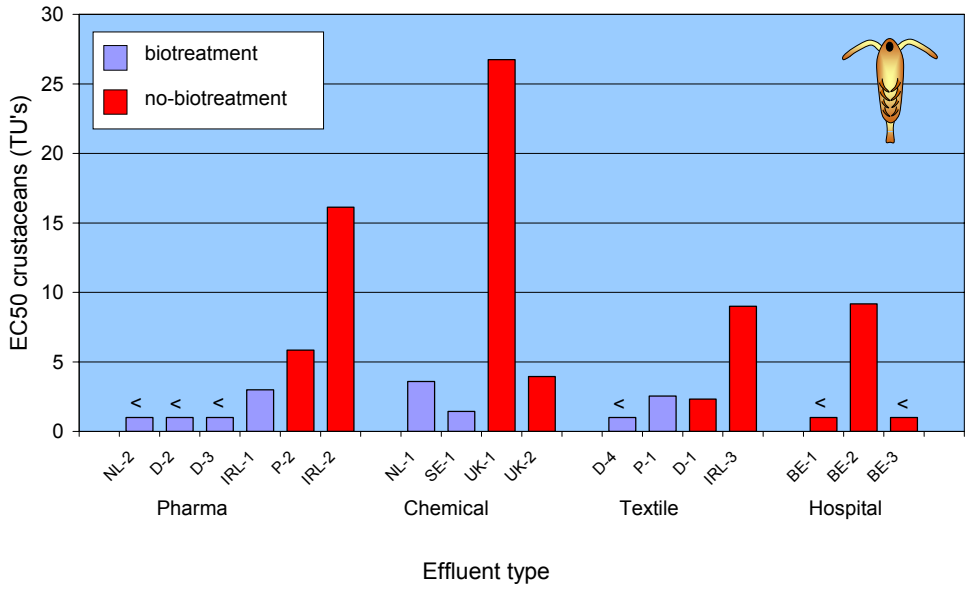
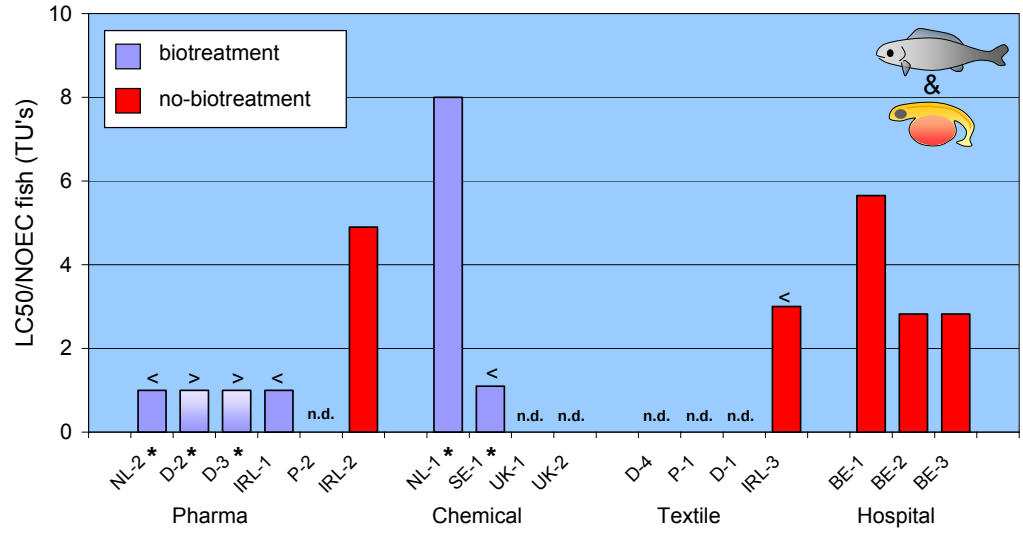


Fig 3.4 The toxicity in 17 effluent samples in acute and chronic fish tests



***** : (sub-)chronic toxicity

Four out of five effluents without bio-treatment showed moderate toxicity to rainbow trout. Adverse effects are observed in the fish tests carried out with the undiluted Pharma effluents D-2 and D-3. Together with the toxicity observed for Chemical effluent NL-1 it may thus be concluded that three out of the six biologically treated effluents are toxic to fish.

3.1.4 Other taxa

In addition to the tests with the Microtox, algae, crustaceans and fish, species from other taxa were used by the UK and Ireland. Data can be found in Appendix 7.3.1. The UK applied a chronic test with oyster larvae (*Crassostrea gigas*) whereas Ireland applied an acute test with duckweed (*Lemna minor*), an aquatic plant. Essentially these tests are comparable to the other tests in this programme, i.e. effluents are tested in a dilution range in order to arrive at an EC50 or a NOEC. Due to the limited data the results of these tests are not discussed separately in this report. Nevertheless, the results are incorporated in the discussion about overall results of the practical study project (section 4.3).

3.1.5 Genotoxicity

Test for genotoxicity were carried out for nine effluents, notably the Dutch, German and Belgian effluents. The results of these tests can be found in section 7.3.1 of Appendix 7.

Genotoxicity (or genotoxic activity) is expressed as an induction factor relative to the background level of induction. Induction factors below 1,5 are considered insignificant or comparable with the background.

Although only a limited number of effluents, from very few sectors were tested for genotoxicity the results show a marked difference between those effluents that were biologically treated and those that were not. More explicitly, all the untreated effluents showed genotoxic activity, against none of the biologically treated effluents. The four effluents that were genotoxic were those of the hospitals and one of the textile industry. Earlier studies in Germany and Belgium have shown that the genotoxicity of these effluents can be attributed to the presence of antibiotics and textile dyes (Gartiser, personal communication).

3.2 Liability to bioaccumulate

3.2.1 SPME GC-FID method

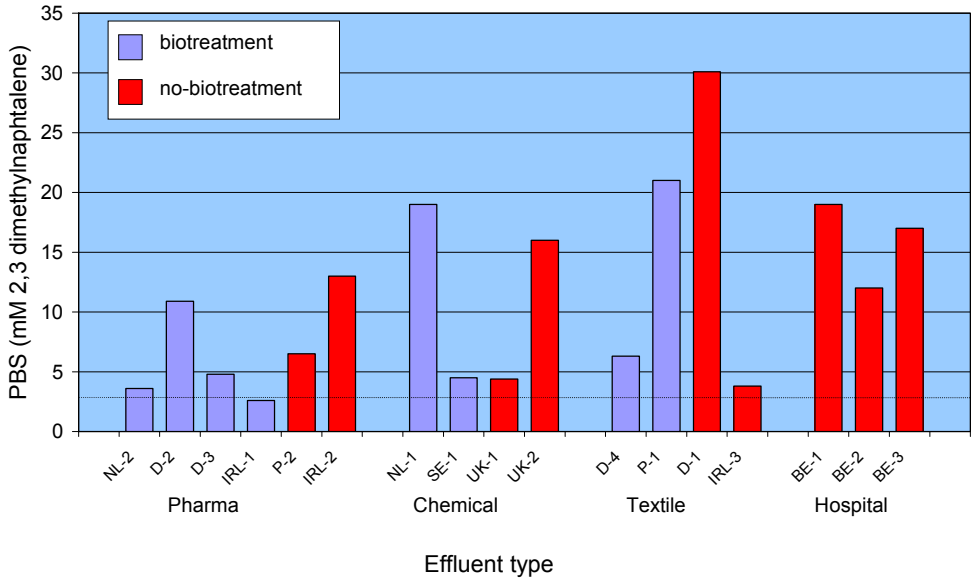
All 17 effluents were analysed for liability to bioaccumulate using the biomimetic SPME method. The amounts of Potentially Bioaccumulating Substances (PBS) resulting from this method are presented in Figure 3.5.

PBS levels in tap water from empty plastic bottles were 2,4 ± 0,6 mM (n=3). For practical reasons it was therefore assumed that PBS levels below 3 mM should be regarded as background levels. Compared to this background level a maximum ten-fold increase in PBS can be seen for Textile effluent D-1.

Due to the sorption to the active sludge particles, a biological treatment is expected to eliminate lipophilic substances quite efficiently. However, from Figure 3.5 it can be seen that there is not a clear distinction between the concentrations of PBS and the type of treatment, biological versus no biological treatment. High and low levels of PBS can be found for both types of treatment. The most obvious explanation for this is that our distinction between treatment types is too much of a simplification. Apparently there are other, more important factors that complicate the interpretation of these results.

The Swedish effluent was additionally tested with the so-called LPE(EGOM) liquid extraction method (see section 7.2 of Appendix 7 for method and section 7.3.1 of Appendix 7 for results). Since the SPME PBS values relate to the fibre concentration rather than the sample concentration as in the LPE(EGOM) method, a direct comparison was not possible. However, the SE-1 data indicate better correlation between the SPME PBS values and the unseparated extract (EGOM) values rather than with the PBS values (after separation). This is unexpected and merits further study. An extended comparison is recommended to take place in the working programme of 2004.

Fig. 3.5 Potentially Bioaccumulating Substances (PBS) in 17 effluent samples using the SPME fibre method.

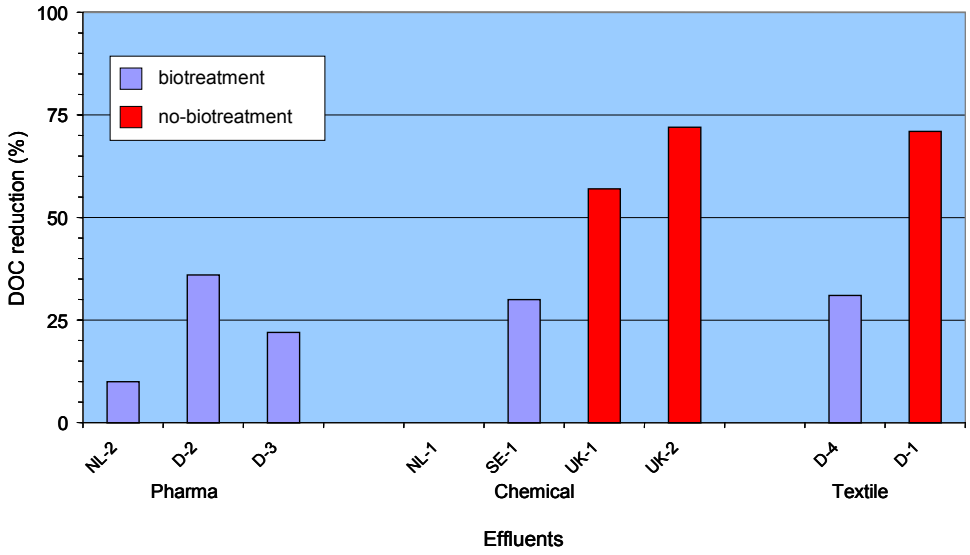


3.3 Persistence

Nine effluents underwent a persistence test. The rationale behind these tests is that during a period of incubation, toxic and/or bioaccumulating pollutants in the effluent are potentially eliminated or formed. For eight effluents this incubation was in a modified 'DOC die-away' test system. The method used is carried out in line with international agreed guidelines for measuring the 'Ready-Biodegradability' of individual substances. For one effluent (D-1) persistence was evaluated using a modified 'Zahn-Wellens' test, a test commonly performed to measure the 'Inherent-biodegradability' of individual substances. Instead of persistence the authors prefer to refer to this test as a test for 'Treatability' rather than persistence. To the authors best knowledge this is the first study in which persistence in terms of toxicity and liability to bioaccumulate has been the subject of an international programme on effluent screening.

A generic way to measure the reduction of organic pollutants is through measuring the reduction of Dissolved Organic Carbon (DOC). The results of these measurements are given in Figure 3.6. From this figure it can be seen that DOC reduction is between 10 and 32% for the effluents that already received biological treatment. As expected DOC reduction was much higher, up to 72%, for the effluents that not received any form of biological treatment yet. Note here that at low DOC levels, calculating DOC reduction may become unreliable due to fluctuating background values (Gartiser, personal communication).

Fig 3.6 Persistency of 8 efffluents from the OSPAR Demonstration Programme



As expected the highest DOC reductions were found for the effluents that had not received any biological treatment; UK-1, UK-2 and D-1. In the case of D-1 it concerns an indirect effluent, i.e. an effluent that will receive further treatment in a domestic or municipal wastewater treatment plant. Additional elimination of biodegradable constituents can thus be expected. For UK-1 and UK-2 this is not the case. Both effluents are discharged directly into the receiving water and it may thus be anticipated that a relatively large portion of the organic matter will remain in the aquatic environment for a long period of time.

Both before and after the period of incubation in the persistence test the effluents were tested for liability to bioaccumulate and toxicity. The results of these measurements are discussed in the following paragraphs.

3.3.1 Persistence of Toxicity

Various toxicity tests were applied to measure toxicity before and after biodegradation. The results of these tests are presented in Figure 3.7 to Figure 3.9. The overall picture appears to be that toxicity is somewhat reduced during the persistence test. However, strong and significant reductions in toxicity were only seen for the effluents that had not received any biological treatment. This finding is in line with the observations on DOC reduction. Here also the highest reductions in DOC are observed for those effluents that had not been biologically treated. One exception to this general picture is Pharma effluent D-2. Although biologically treated, most of its toxicity to algae (20 TU's) was eliminated in the persistence test. This observation could not be explained with the reduction of DOC for this effluent.

The tests with the textile effluent D-1 shows that the genotoxicity is reduced through biodegradation. This is also true for the endocrine activity as seen in the Pharma effluent D-3 (data not shown, see section 7.3).

Fig 3.7 Toxicity in Microtox test before and after biodegradation test.

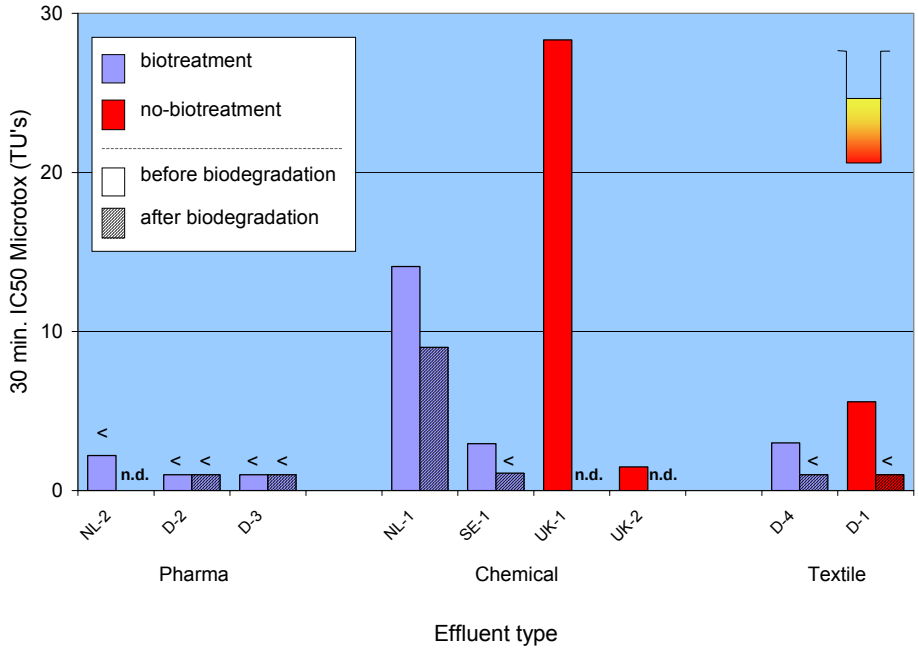


Fig 3.8 Toxicity for algae test before and after biodegradation test.

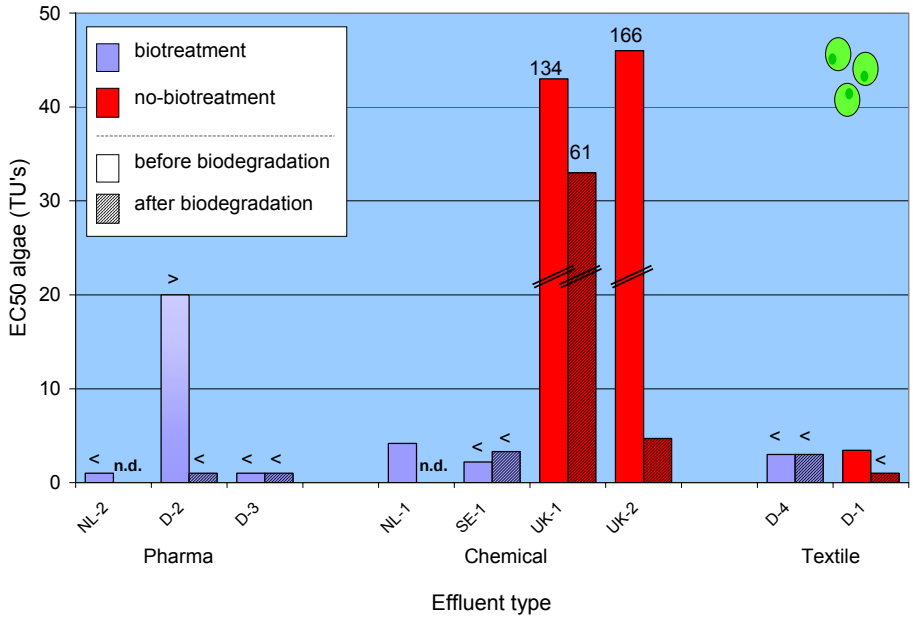
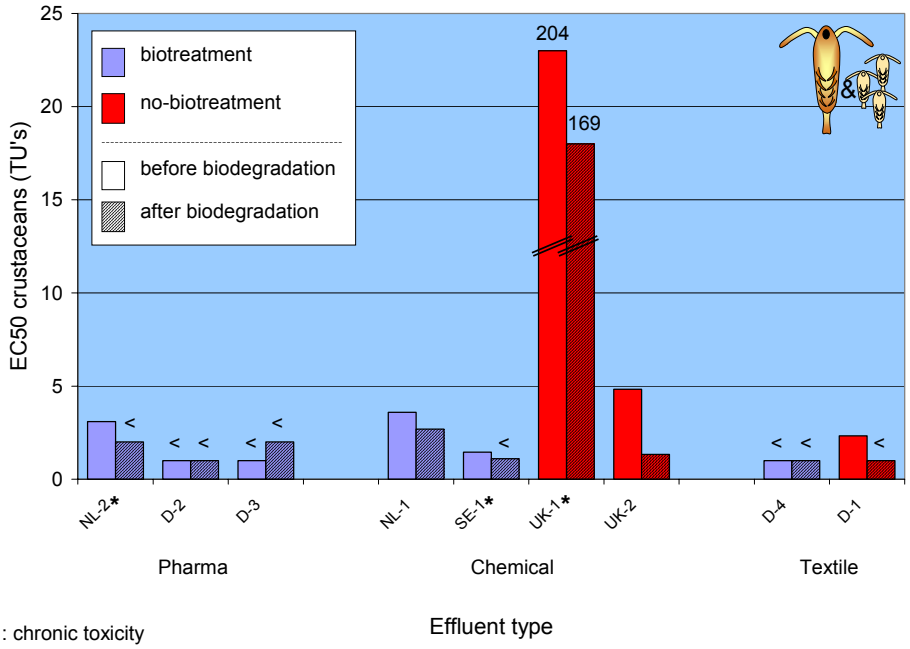


Fig. 3.9 Toxicity for crustacean before and after biodegradation test.

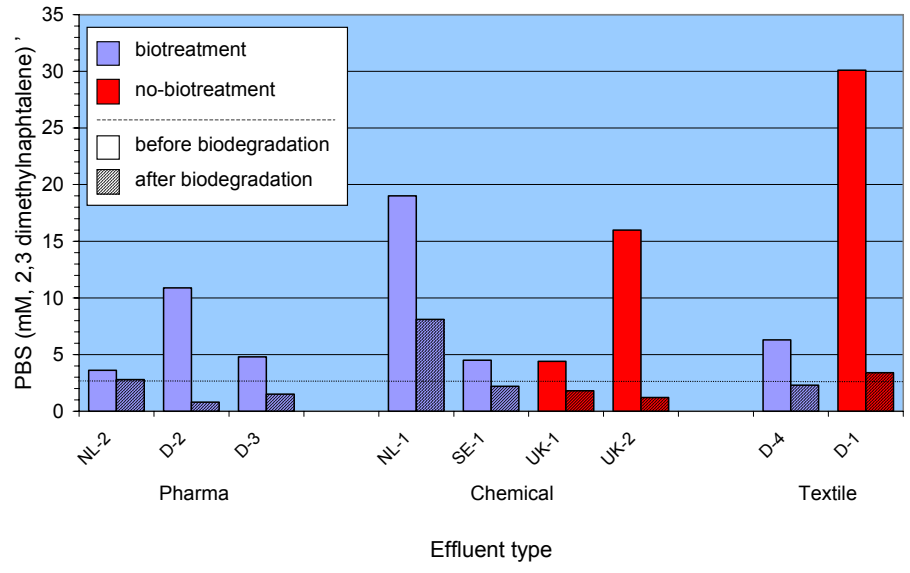


* : chronic toxicity

3.3.2 Persistence of Liability to bioaccumulate

The total amount of PBS was measured using the SPME method both before and after incubation in the persistence test. The results of these measurements are presented in Figure 3.10.

Fig 3.10 Liability to Bioaccumulate before and after biodegradation test.



After biodegradation the amount of PBS was lower for all effluents. The strongest reductions are seen for Pharma effluent D-2, Chemical effluent UK-2 and Textile effluent D-1. For the latter two this should not be surprising since these were the effluents that had not received any biological treatment. For the biologically treated effluent D-2 it is however surprising. The strong PBS reduction for this effluent is in line with the strong reduction of the toxicity to algae but not with the DOC removal, which was only 36% here. However, initial DOC levels were already relatively low, 5,9 mg/l and in the same range as the blank values of the inoculum. Thus is can be concluded that in this specific case DOC removal is not a good indicator of the removal of constituents with toxic or bioaccumulating properties.

When comparing the data on DOC reduction and those on PBS reduction is appears that PBS reduction is more efficient than DOC reduction. A possible explanation for this could be that the relatively large, lipophilic, molecules that accumulate on the SPME fibres are broken down into smaller, less lipophilic fragments. This process is not easily captured by measuring DOC reduction but it will be by PBS measurements since this method is selective for lipophilic substances. However, here again it should be noted that calculating DOC reduction may become unreliable at low initial DOC levels.

With regard to the Swedish method, data shown in section 7.3, the content of PBS in sample SE-1 was reduced only moderately, when determined as mg C/ml with the liquid extraction method. Hence, further biotreatment of this wastewater does not help significantly although the DOC reduction was about 30%. As already said above this apparent disagreement between the two methods (SPME and LPE) will be studied further in the next cycle.

3.4 GC-MS screening

Next to the SPME-PBS measurements the effluent samples were also screened for the presence of organic substances. For this purpose the samples were extracted using a liquid-liquid extraction with dichloromethane (DCM). The organic substances in the extract were identified using the state-of-the-art method in the area of analytical chemistry, i.e. gas-chromatography with mass-spectrometry (GC-MS) together with the peak identification according to the latest version of the AMDIS software and the NIST98 library on mass-spectra.

The results of the GC-MS screenings are summarised in lists with names of substances, CAS numbers, retention times, together with concentrations and quality indexes (model fits). Here it should be noted that the delivery of these results is accompanied by some important remarks from the chemists carrying out the analysis. These remarks were:

1) It is unknown how many substances are left unidentified.

2) For many identified substances, identification is unsure.

3) The concentrations of the identified substances have a large uncertainty. The reason for this is that the MS responses of the substances are often unknown (they may differ more than a factor of 10) and the variation of the internal standards is relatively high, approximately 26%. The given concentrations should preferably not be used in a quantitative way.

Because of these uncertainties the lists of potentially identified substances reported in this study only contains those that were reliably identified (>80% confidence according to reverse-fit method). Peaks smaller than 5 times the background signal (approximately 10,000 counts) were not included. The peaks of substances identified with more than 80% confidence were all manually checked for errors.

One of the Dutch laboratories was asked to evaluate their results with respect to the percentage of identified substances and the total amount of organic load the identified substances represent. The results are summarised in Table 3.1. The percentage of successfully identified substances varied between 2 and 53%. The percentage of explained total peak area varied between 9 and 67%. A large deal of the total peak area could be explained for the effluent samples of UK-2, after biodegradation, and BE-2. Nevertheless, for the majority of the effluents most substances present in the sample remained unidentified.

Table 3.1: Estimated percentages of successfully identified substances and explained peak areas in the OSPAR samples, using the GC-MS screening method.

Effluent code	Estimate of total number of substances	Total of identified substances[1]	% substances identified	% total peak area explained[2]
UK 1	350	7	2	4
UK 1 (after biodegradation)	70	37	53	15
UK 2	350	62	18	42
UK 2 (after biodegradation)	100	17	17	67
NL-1 (after biodegradation)	200	14	7	18
P-1	525	48	9	9
P-2	100	10	10	25
BE-1	300	36	12	19
BE-2	425	76	18	62
BE-3	325	35	11	12

[1]: only substances identified with >80% confidence

[2]: peak area explained by the identified substances

4. Evaluation

The primary objective of this practical study programme was to evaluate the added value of the WEA approach as compared to an extended chemical specific approach. Another objective was to compare the two approaches in terms of costs. In the following paragraphs these issues will be discussed separately, but before doing so it is essential to make a few remarks.

For a comparison between the WEA and the chemical specific approach, both in terms of added value and in terms of costs, it is important to keep in mind the crucial differences between the two approaches.

WEA is a tool that can be especially useful in the assessment of complex effluents. The major benefit to be gained from WEA is that the environmental hazards in terms of P, B and T of all substances present in the effluent can be measured in one go per variable. The output of the WEA approach is therefore measured values for P, B and T of the whole effluent.

Unlike the WEA approach, the chemical specific approach is a two-step process. The first step is the identification and quantification of substances in the effluent. The second step is to estimate P, B and T for these substances on the basis of existing PBT data. The output of the chemical specific approach is a PBT estimate for the successfully identified and quantified substances in the effluent. The above reasoning is illustrated in Figure 4.1.

The WEA and chemical specific approach have similar drawbacks when it comes to optimal sampling, the conservation and storage of samples and laboratory quality assurance. Nevertheless, the chemical specific approach has two more drawbacks that the WEA approach does not have. Namely, the number of substances that can be reliably identified and quantified is limited (see section 3.4) and for those who are quantified, data on P,B and T are often not available (see also Figure 4.1). Furthermore, in the chemical specific approach the bioavailability and mixture toxicity are not taken into account. The consequence of this is that the reliability of the PBT estimate will always be a matter of dispute and it follows that the costs of the chemical specific approach very much depends on the effort that is put in arriving at a reliable PBT estimate. Comparing the costs of the two approaches is thus not only a comparison between prices, but it should be a judgement of the ‘value for money’.

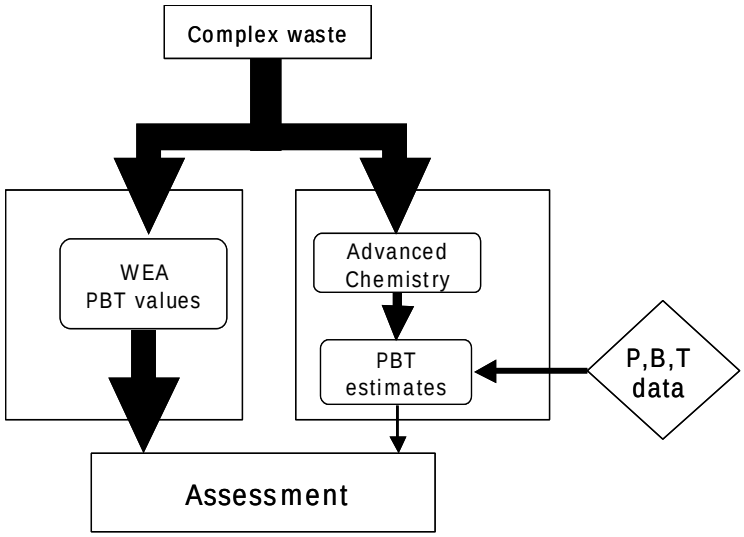


Fig 4.1: WEA and chemical specific approach; scheme 'value for money' evaluation. The thickness of arrows indicates the number of substances that are included in the assessment.

4.1 Added value

In this paragraph the results from the WEA assessments are compared with assessments on the basis of the chemical composition of the effluents. For an assessment according to the chemical specific approach the compliance to the permits are checked. Furthermore additional analyses (GC-MS) have been carried out. For the successfully identified substances an inventory is made of existing data on toxicity. On the basis of this inventory the extended chemical assessment is carried out.

Table 4.1 summarizes the findings concerning the added value of the WEA measurements. An overview of the GC-MS results and toxicity data for the most relevant substances is given in section 7.3 of Appendix 7.

Most of the effluents contain a wide variety of known and unknown substances which cannot sufficiently be characterised in terms of their chemical profile. Even for the chemicals identified there is insufficient information on their environmental hazard and / or effects. In this context WEA could provide further insight in these environmental hazards / effects from the effluent as a whole. This would provide an additional benefit compared to chemical monitoring and offers a safety net to identify effluents that need further assessment or control.

From Table 4.1 it is obvious that added value from WEA in relation to the chemical specific approach, is shown for most effluents. This was the case in effluents where the identified substances could not or only partly explain the toxicity that was measured with WEA. In the following paragraphs more details are presented.

Table 4.1. Overview of the added value of WEA for the different effluents

Effluent	Added value WEA
NL-1	The toxicity could not be explained by the chemical composition. Toxicity did not decrease after biodegradation.
NL-2	The low toxicity and liability to bioaccumulate was confirmed by effluent chemistry.
UK-1	The toxicity in this effluent appeared to be persistent. This was in line with DOC removal in the biodegradation test. However, the toxicity observed before and after the degradation test could not be explained by the chemical composition.
UK-2	The toxicity and liability to bioaccumulate in this effluent disappeared almost entirely after the biodegradation test. This was in line with the fact that this effluent was characterised as Readily Biodegradable. WEA may be used to demonstrate potential over-regulation in the case of this effluent
P-1	The observed (high) toxicity could partly be explained by the chemical analysis performed
P-2	For this effluent permit requirements were exceeded. Therefore it was difficult to demonstrate the added value of WEA
D-1	WEA testing is part of the permit.
D-2	WEA testing is part of the permit.
D-3	WEA testing is part of the permit.
D-4	WEA testing is part of the permit.
BE-1	The relative high toxicity in these effluents could not be explained by the toxicity data of the identified compounds
BE-2	The relative high toxicity in these effluents could not be explained by the toxicity data of the identified compounds
BE-3	The relative high toxicity in these effluents could not be explained by the toxicity data of the identified compounds
SE-1	The observed toxicity and liability to bioaccumulate (SPME & EGOM) were lower after the biodegradation test, which was partly in agreement with a DOC decrease (-30%). However, these effects could not be explained by the (basic) chemical analysis performed .
IR-1	No chemical permit requirements. WEA testing is part of the permit.
IR-2	No chemical permit requirements. WEA testing is part of the permit.
IR-3	No chemical permit requirements. WEA testing is part of the permit.

4.1.1 Dutch effluents

Effluent NL-1, chemical plant with biological treatment

The toxicity measured in this effluent is substantial for bacteria, crustaceans and fish. Liability to bioaccumulate and toxicity for algae were relatively low. The chemicals regulated in the permit did not exceed the permits limit values. Although not required for the permit, a number of priority substances were also analysed. An overview of the measured substances is given in Table 4.2. Most of the measured priority substances were under the limit of detection. Although toxicity data were found on some of the substances, the concentrations in the effluent were much too low to explain the effects observed (see interlude below).

Additional GC-MS analyses showed a large number of substances present in the effluent, most of them disappeared after the biodegradation test. However, toxicity did not decrease after the biodegradation test. The toxicity data for these substances could not explain the observed effects. This means that WEA clearly has an added value for this effluent.

Interlude

The LC50 value for phenol for D. magna is 12 mg/l, which is 3 orders of magnitude higher than the concentration found for the sum of phenols in this effluent.

For dichloromethane, 23 data for fish, 17 data for invertebrates and 6 data for algae were evaluated using the quality criteria recommended by EU authorities. Both acute and chronic toxicity studies were taken into account and assessment factors were used to define a final Predicted No Effect Concentration (PNEC) value of 830 µg/l, which is much higher than the concentration found in this effluent.

The LC50 value for zinc for D. magna is 350 µg/l. The LC50 value for this effluent for D. magna was 19 %, which resembles a concentration of zinc of 19 µg/l (0,05 TU).

Acute toxicity levels for Bisphenol A have been measured in a variety of aquatic organisms, including freshwater and saltwater algae, invertebrates and fish. LC50 values range from 1000 to 20,000 µg/L The No-Observed-Effect-Concentration (NOEC) of Bisphenol A in a 21-day chronic reproduction test in Daphnia was 3160 µg/L. Effect concentrations at the 10% level (EC10) were determined for both freshwater and marine algae to be 1360 to 1680 µg/L and 400 to 690 µg/L, respectively. All these concentrations are much higher than the concentrations found in this effluent (20 µg/l).

Table 4.2. Chemicals measured in effluent NL-1

				Parameter	**value**	**unit**
			limit	1,1,1-Trichloroethane	<0,1	µg/l
BisFenol_A	20	µg/l	1140	1,1,2-Trichloroethane	<0,5	µg/l
BOD	139	mg/l	170	1,1-Dichloroethane	<1	µg/l
Cu	<13	µg/l	100	1,2-dichloroethane	<5	µg/l
dichloromethane	6	µg/l	114	1,2-Xylene	<0,01	mg/l
flow	4876	m3/d	8800	2,6 DiMethylphenol	<10	µg/l
EOX	<0,1	mg/l	0,06	2-methylphenol	<10	µg/l
KjN	1,8	mg/l	11,4	Arsenic	2,5	µg/l
P	0,9	mg/l	23	Benzene	<0,01	mg/l
Zn	100	µg/l	850	Cd	<4	µg/l
Sum of _NO3 +NO2	4,7	mg/l	23	Cl	43000	mg/l
Sum of phenols	20	µg/l	114	Cr	<8	µg/l
				Cum	<0,01	mg/l
				DiChloroBromoMethane	0,1	µg/l
				ethylbenzene	<0,01	mg/l
				Phenol	<10	µg/l
				Hg	<0,1	µg/l
				NO2	<0,1	mg/l
				NO3	4,7	mg/l
				Ni	<24	µg/l
				Pb	<20	µg/l
				Styrene	<0,01	mg/l
				tetrachloroethene	<0,1	µg/l
				tetrachloromethane	<0,1	µg/l
				tribroommethane	<0,1	µg/l
				trichloroethene	<0,1	µg/l
				trichloromethane	3,3	µg/l
				Toluene	<0,01	mg/l
				Sum of_1,3 and 1,4 Xylene	<0,02	mg/l

Effluent NL-2, pharmaceutical plant, after biological treatment

Relatively little toxicity and a low liability to bioaccumulate were measured. Therefore there was no urgent need for additional substance research. The chemicals regulated in the permit did not exceed the limit values listed in this permit (table 4.3). Besides that, a number of heavy metals were measured. The effluent easily met the limits from the licence.

Table 4.3. Chemicals measured in effluent NL-2

Parameter	value	unit	Permit	Parameter	value	unit
Cl	46	mg/l	3300	Ni	11	ug/l
BZV5a	6	mg/l	15	Pb	<10	ug/l
Br	1,5	mg/l	333	Hg	<0,1	ug/l
CZV	119	mg/l	150	As	<1	ug/l
Debiet	2517	m3/d	4500	Cr	<4	ug/l
Cu	60	ug/l	300	Cd	<2	ug/l
SO4	98	mg/l	1111			
ZS	<10	mg/l	30			
Zn	55	ug/l	180			
P	1,1	mg/l	11,1			
KjN	8,3	mg/l	50			

4.1.2 Belgian effluents

Belgium selected three medical institutions, discharging their wastewaters in the sewage system for treatment in a wastewater treatment plant. Wastewater from hospitals can contain a wide range of dangerous substances e.g. antibiotics, disinfectants, antiseptics, radiodiagnostics and hormones. As a consequence, the discharge of this wastewater can lead to different effects in the receiving waters or sewage systems. The untreated wastewaters were therefore tested for (1) effects of acute toxicity, (2) potential for hormone disruption and (3) genotoxicity.

All effluents displayed an acute toxicity signal. This signal was strongest with algae as a testing organism. The effluents also showed positive results for effects of endocrine disruption and genotoxicity.

The same testing is in the pipeline for the treated effluents (after wastewater treatment plant) but results are not yet available. This part of the study will give an idea of the persistence of the toxicity and as a consequence of the treatability of hospital effluents in a municipal waste water system.

A GC-MS analysis yielded no useful information concerning the relevant substances for the three observed effects. A wide range of substances was observed but toxicity of only a small number of substances could be identified according to the literature. These data could not explain the high toxicity values found in the tests. This illustrates the difficulty for complex mixtures to pinpoint toxicity to specific substances and to regulate all those substances and possible by-product by means of a permit.

An additional TIE study on the effluents with high algae toxicity demonstrated a significant reduction of toxicity after solid phase extraction. A sequence of C-18 (for nonpolar compounds) and EN (for more polar compounds) showed presence of both groups of compounds that appeared to be responsible for acute toxicity in the algae test. Phase II of TIE with identification by GC-MS & HPLC has not yet been performed.

4.1.3 German effluents

In Germany WEA is already incorporated in the permit requirements for some wastewater sectors, such as the pharmaceutical/chemical industry, which in a way also puts the added value of WEA into the permits. In other sectors, such as the textile industry, the application of WEA depends from local authorities. The following description gives some background information about the effluents tested:

Effluent D-1, Textile finishing, indirectly discharged

The wastewater permits for such effluent follow principally Annex 38 of the wastewater ordinance, not including WEA methods (see effluent D-4). Some parameters like COD, BOD and nutrients do not apply for indirectly discharged effluents. Local authorities require the performance of the Zahn-Wellens test as an additional parameter. Formerly a BOD_5/COD value of 0,4 was applied. A COD-elimination of 70-80% in the Zahn-Wellens test after 7 days is assumed as sufficient for a treatment in the municipal wastewater plant.

The COD- and DOC-elimination of the sample analysed were around 70%. While the moderate Daphnia and algae toxicity was completely removed after the Zahn-Wellens-test, that of the *Vibrio fisheri* assay remained stable at a relatively high toxicity (EC50 47% and LID = 24). This is probably due to the colouration of the sample, which interferes with the luminescence measurement. Both the Ames (TA 98+S9) and the umu assay (-S9) showed clear genotoxicity. The effect in the umu assay, although reduced, was also detectable after the Zahn-Wellens test. A slight effect was also measured in the yeast assay for endocrine effects. The potential bioaccumulating substances as measured via SPME analysis revealed the highest value measured in the practical study program at all. After the Zahn-Wellens test only 1/8 of the amount was detected, which was comparable to that observed with textile effluent D-4. The colouration of the sample was eliminated by 47% (yellow), 42% (red) and 58% (blue), considering the dilution factor in the Zahn-Wellens test.

The results show clearly the added value of WEA for a hazard assessment of the effluent. A TIE approach should be considered in order to identify the sources of genotoxicity. The origin of the "recalcitrant" COD/DOC should also be addressed by calculating the COD load contribution of the different textile chemicals.

Chemical analysis revealed the following parameters (mean values of 6 years if not indicated otherwise):

COD	mg/L	925 (this sample)	Copper-ion	mg/l	0,035
TOC	mg/L	279 (this sample)	Chromium-ion	mg/L	0,01
BOD/COD	mg/L	0,3	Sulphite	mg/L	<18,5
NH_4-N	Mg/L	7,25	Colouration, spectral absorption coefficient		
Kjeldahl-N mg/L	Mg/L	16,3	436 nm (yellow)	m^{-1}	39,4 (this sample)
conductivity	MS/m	311	525 nm (red)	m^{-1}	16,5 (this sample)
AOX	Mg/L	0,65	620 nm (blue)	m^{-1}	10,4 (this sample)

The COD and colouration of the sample at 435 nm, 535 nm and 620 nm were within the reference values measured before, thus the sample analysed can be assumed to be representative.

Effluent D-2, Pharmaceutical plant, directly discharged

The pharmaceutical plant produces especially cytotoxic and non-cytotoxic parenteral drugs such as cytostatics. The wastewater of an associated company specialised in the scaling up of pharmaceuticals is treated in the same treatment plant.

The results of chemical analysis and ecotoxicity tests showed no evidence of relevant contaminants. Only the algae test was somewhat elevated, but in several tests performed with the sample a low reproducibility of algae results was observed and in comparative measurements in another laboratory no toxicity at all was determined. After the biological treatment no algae toxicity was measured. In the past a higher algal toxicity (LID 32-128) was measured and has been attributed to emissions of the solvent n-heptane. After the rearrangement of vacuum pumps in the respective plant, background values of LID 1-4 were observed, while the emission permit value is LID 16.

The concentration of potential bioaccumulating substances was significantly reduced after the biological treatment but no comparative values are available for an assessment of those data.

Non-biodegradable mother liquors (criteria >90% elimination in the Zahn-Wellens test) are collected and disposed of via waste incineration in order to improve the DOC-Elimination of the wastewater treatment plant.

The results of chemical analysis performed with the sample were

TOC	mg/L	5,4	Conductivity	mS/m	139
AOX	mg/L	0,056	PO_4-P (total)	mg/L	0,6
NO_3-N	mg/L	10,0	EDTA	mg/L	0,022
NH_4-N	mg/L	<0,05	NTA	mg/L	<0,0005
Kjeldahl-N mg/L	mg/L	10,0	DTPA	mg/L	<0,0001

The effluent complies with the requirements for wastewater from the chemical/pharmaceutical industry, which is regulated in Annex 22 of the wastewater ordinance. Here, among others, the following parameters are given:

Requirements of total effluent at the pipe			**Requirements before mixture of different effluents**		
COD	mg/L	75 (or >90% elimination)	AOX	mg/L	0,3-8 mg/l depending on production
Phosphorus total	mg/L	2	Copper	mg/L	0,1-0,5
NH4-N+NO3-N+ NO2-N	mg/L	20	Chromium	mg/L	0,05-0,5
Fish toxicity	LID	2	Mercury	mg/L	0,001-0,05
Daphnia toxicity	LID	8	Cadmium	mg/L	0,005-0,2
Algae toxicity	LID	16	Nickel	mg/L	0,05-0,5
Bacteria toxicity	LID	32	Lead	mg/L	0,05-0,5
Genotoxicity umu	LID	3 (no genotoxicity)	Zinc	mg/L	0,2-2
			Purgeable halogenated hydrocarbons	mg/L	10
			TOD-load of different wastewater parts must only be mixed if the elimination in the Zahn-Wellens test is >80%		

The added value of WEA has been proven in the past, where higher algae toxicity data have been measured and the sources have been found and eliminated.

Effluent D-3, Chemical/pharmaceutical plant, directly discharged

The pharmaceutical plant produces intermediates for pharmaceuticals and the food processing industry such as caffeine. The effluent complies with the wastewater permits according to Annex 22 of the wastewater ordinance (see sample D-2). There was no substantial ecotoxicity or genotoxicity detected in the sample. The yeast assay revealed clear endocrine effects, which were removed after the treatment in the DOC, die away assay. An appraisal of that effect at the moment is not possible because comparative data are lacking. Historical data from 1996 on show higher algae toxicity in some samples (n=42, LIF_{max}=256, mean LID=23). Nevertheless the median of the algae toxicity was LID=1, indicating, that most samples show no toxicity.

The added value of WEA has been proven in the past, where algae toxicity had been detected and was effectively reduced.

The results of chemical analysis performed with the sample were

TOC	mg/L	24,7	AOX	mg/L	0,081
NO3-N	mg/L	22,7	conductivity	mS/m	1109
NH4-N	mg/L	<0,08	PO4-P (total)	mg/L	0,7
Kjeldahl-N mg/L	mg/L	28,7	chloride	mg/L	2695

Comparative data of effluent chemical analysis the last 2,5 years were in the range of 29 mg/L TOC, 72 m/L CSB, 72 µg/L AOX, 0,7 m/L PO_4-P, 0,7 mg/L NH_4-N and 21,2 mg/L NO_3-N (median, n~50). The sample analysed can therefore be assumed to be representative.

Effluent D-4, Textile finishing, directly discharged

The requirements of Annex 38 are clearly met by the sample analysed. WEA methods are required by local authorities as additionally supervisory parameters. The Practical study program revealed a moderate ecotoxicity: (LID 1 (Daphnia) to LID 12 (bacteria)). While the *Vibrio fisheri* effect was completely removed after the DOC die away test, the algae effect was stable at LID 3, probably due to the colouration of the sample. No genotoxicity or endocrine effects were determined. The SPME analysis of the original sample must be repeated. After the DOC die away test the SPME revealed values comparable to that observed with textile effluent A after the Zahn-Wellens test. The colouration of the sample was eliminated by 18% (yellow), 17% (red) and 25% (blue), considering the dilution factor in the DOC die away test.

The results of chemical analysis performed with the sample were:

COD	mg/L	103	Benzene, toluene, xylene and other aromatic solvents	mg/L	< 0,5
TOC	mg/L	33,1			
AOX	mg/L	0,084	Colouration, spectral absorption coefficient		
Vanadium	mg/l	< 0,01	436 nm (yellow)	m^{-1}	5,1
SO_3	mg/L	<0,5	525 nm (red)	m^{-1}	2,3
			620 nm (blue)	m^{-1}	1,2

According to Annex 38 of the wastewater ordinance the following requirements are needed for effluents discharged directly to surface water:

Requirements of total effluent at the pipe			**Requirements before mixture of different effluents**		
COD	mg/L	160	AOX	mg/L	0,5
BOD	mg/L	25	Sulphide	mg/L	0,5
Phosphorus total	mg/L	2	Copper	mg/L	0,5
NH4-N	mg/L	10	Chromium total	mg/L	1
NH4-N+NO3-N+ NO2-N	mg/L	20	Nickel	mg/L	0,5
Sulphite	mg/L	1	Zinc	mg/L	2
Fish toxicity	LID	2	Tin	mg/L	2
Colouration spectral absorption coefficient			**Further demands**		
436 nm (yellow)	m^{-1}	7	No products with Cr(VI), As, Hg, APEO, EDTA, DTPA		
525 nm (red)	m^{-1}	5	Only use of degradable sizes, complexing agents and tensides (Zahn-Wellens test >80%)		
620 nm (blue)	m^{-1}	3	Exception: phosphoric, polyacrylic and maleic acids		

Historically waste water data of the textile finishing plant from 1987 to 2003 (n ~ 30-196) revealed the following parameters (median values):

COD	mg/L	134	Copper	mg/L	0,01
TOC	mg/L	39	Chromium	mg/L	0,01
BOD/COD		0,16	Mercury	mg/L	0,02
PO4-P	mg/L	0,4	Cadmium	mg/L	0,04
NH4-N	mg/L	2,5	Nickel	mg/L	0,01
NO3-N	mg/L	0,7	Lead	mg/L	0,01
NO2-N	mg/L	0,39	Zinc	mg/L	0,03
PO4-P	mg/L	0,40	Iron	mg/L	1,05
AOX	mg/L	0,04	Chloride-ion	mg/L	587,5
Fish toxicity	LID	2	Sulphate	mg/L	614

The sample analysed was therefore representative.

4.1.4 Portuguese effluents

Effluent P-1, Textile, Indirect discharge

This effluent showed to be acutely toxic to bacteria and chronically toxic to crustaceans. Measured liability to bioaccumulate was high. The chemicals that are regulated in the permit did not exceed the limit values listed in this permit (Table 4.4) except for Boron. Some parameters were under the detection limit. GC-MS analyses displayed several siloxane-derivates to be present. Some of these derivates showed high toxicity towards aquatic organisms, which could partly explain the observed effects. WEA has no strong added value for this effluent.

Table 4.4. Chemicals measured in effluent P-1

Parameter	unit	value	Permit limit	Limits for direct discharge
pH		7,8		5,5-9,0
CBO5	mg/l	66	500	100
CQO	mg/l	371	2000	250
SST	mg/l	70	1000	60
Conductivity	µS/cm	2540	3000	
B	mg/l	**3,0**	1,0	
As	mg/l	0,01	0,05	1,0
Pb	mg/l	<0,02	0,05	1,0
Total cyanide	mg/l	<0,0025	1,0	0,5
Cu	mg/l	0,18	1,0	1,0
Cr (total)	mg/l	<0,11	2,0	2,0
Fe (total)	mg/l	0,37	2,5	2,0
Ni (total)	mg/l	<0,09	2,0	2,0
Se (total)	mg/l	<0,0044	0,05	
Zn (total)	mg/l	0,21	5,0	
Metals (total)	mg/l	<0,99	10	
Total hydrocarbons	mg/l	<10	50	
Phenols	mg/l	<0,015	40	0,5
Ammonium Nitrogen	mg/l	1,6	100	10
Surfactants	mg/l	0,056	50	2,0

Effluent P-2, Pharmachem, Indirect discharge

The effluent was toxic to bacteria and crustaceans, both in acute and chronic tests. The level of PBS determined was under 10. For this effluent, the added value of WEA could not be confirmed, because the limits of the permit are exceeded in several chemical parameters (CBO5, CQO, Pb, Fe, Hg, total cyanide). The GC-MS analysis of this effluent showed a small number of compounds in relative high concentrations. Toxicity of only three compounds could be identified, but could not explain the effects found in the effluent.

Table 4.5. Chemicals measured in effluent P-2

Parameter	unit	value	Permit limit	Limits for direct discharge
pH		8,8		6,0-9,0
CBO5	mg/l	**1900**	500	40
CQO	mg/l	**4200**	1000	150
COT	mg/l	1100		
SST	mg/l	90	1000	60
As	mg/l	<0,03	0,05	1,0
Pb	mg/l	**<0,2**	0,05	1,0
Cu	mg/l	0,04	1,0	1,0
Cr (total)	mg/l	<0,1	2,0	2,0
Fe (total)	mg/l	**3,5**	2,5	2,0
Ni (total)	mg/l	1,0	2,0	2,0
Zn (total)	mg/l	0,51	5,0	
Cd	mg/l	<0,03		0,2
Hg	µg/l	**<5**	0,05	0,05
Total cyanide	mg/l	**<10**	1,0	0,5
Fluoride	mg/l	20		
Chloride	mg/l	3300	1500	
Sulphide	mg/l	<0,4	2,0	1,0
Phenols	mg/l	25	40	0,5
Ammonium Nitrogen	mg/l	35	100	10
Nitrate	mg/l	<0,5	80	50
Nitrite	mg/l	<0,2	10	
P (total)	mg/l	1,0	20	10

4.1.5 UK effluents

UK1 – chemical plant with some biological treatment and some settling

The acute toxicity measured in this effluent is substantial for bacteria, algae and crustaceans compared to the other effluents. However it does not appear to have appreciable bioaccumulating effects. The biodegradation test suggested that the content of the effluent is not readily biodegradable, and this is supported by the post-biodegradation toxicity data, which shows significant toxicity remaining to algae and crustaceans.

There are not many chemical based compliance criteria for this effluent, but the effluent is compliant with those that exist. The highest recorded values for the compliance criteria for samples taken in the period February 2001 – February 2003 are shown in Table 4.6.

Table 4.6. Chemical values measured in UK-1

Cadmium	50ug/l
Ammonia	1020mg/l
Oxidised N	1,8mg/l
Orthophosphate	161mg/l

Literature values have been found for Cadmium (0,06mg/l 48 hour *Daphnia magna*), Ammonia (UK EQS 150ug/l) and Nitrate (approx 1300mg/l 96 hour range of fish species). No data could be found for orthophosphate.

These data suggest that the primary cause of toxicity could be ammonia. However, the Practical study Programme has shown that the toxicity is not biodegradable to any great extent, implying that the toxicity is not due to ammonia alone. The GC-MS analysis showed a large number of compounds, in relative high concentrations that could be related to the processes of the plant. A number of these compounds were still present after biodegradation. However, no toxicity data could be found for these compounds. This means that WEA has added value in terms of knowledge of the effluent characteristics (i.e. not biodegradable and not bioaccumulative) that cannot be derived from the current chemical specific approach. This gives insight into the fate of the toxicity, and information on its causes that is not immediately apparent from the chemical measurements alone.

UK2 – Suite of chemical plants with some providing limited treatment

The acute toxicity measured in the effluent is high for algae in comparison with the other effluents, but relatively low for bacteria and crustaceans. The biodegradation test suggests that the effluent is readily biodegradable, and indeed that toxicity to algae and crustaceans is considerably reduced following biodegradation. The SPME fibre method suggested that substances within the effluent had the liability to bioaccumulate, but again this potential was considerably reduced following biodegradation. These results were confirmed by the GC-MS analyses, which showed a considerable amount of substances (~60), which disappeared for the greater part after the biodegradation test. For most of these compounds no toxicity data could be found.

In this case, the PBT approach within WEA added value in that toxicity data alone would suggest that the effluent is of concern to the environment. However the biodegradation studies and the SPME work, showed that although the effluent was toxic and liable to bioaccumulate, it was also readily biodegradable and that the chemicals causing the toxicity and liability to bioaccumulate appeared to be in the biodegradable fraction. However there is a learning point of note here, and that is that the toxicity was not fully removed, either for the algae, crustacean, or for the alternative invertebrate used (*Crassostrea gigas*). It was suspected that some of the toxic effects shown in the post biodegradation toxicity tests were due to the biodegradation media added to the effluent, as there was some toxicity shown in the media controls.

4.1.6 Sweden

Bacteria, algae and crustacea indicate a wastewater of intermediate acute toxicity. Further treatment as in the degradation test reduces this effect somewhat for bacteria and crustacea, but there is still a remaining acute effect. The apparent toxicity increases in the algae test, but it may be suspected that the laboratory was not aware of the salt effects. The fish embryo/larvae test shows no toxic influence. Overall, there is cause to evaluate effects in the recipient waters.

The respiration test showed that the sample did not inhibit the inoculum activity for the degradation test. The 28 d degradation test resulted in a further 30 % DOC reduction, thus demonstrating that the wastewater treatment is not optimal. Hence, although three-stage as built now, there is room for improvement if the recipient conditions so require.

Table 4.7. Degradation test with SE-1

Inhibition of respiration	Value	Degradation test	Value
ISO 8192, method A	inhibition at 80%: -7,2 %	EN ISO 7827:1995	DOC elimination after 28 d: 30 %

The EGOM value, 3,2 mg/l is high enough to justify a full PBS study according to the Swedish practice. And a PBS value of 0,75 mg C/l, which is found to be substantially (about 90 %) persistent is also at a level that may cause further treatment studies. Still, it should be recognised that this effluent has improved considerably in a decade, with nearly ten times as much potentially bioaccumulating material being released prior to the present treatment was built.

Table 4.8. Chemical analyses, sample SE-1

Analysis	Method	Values		
		Before degradation	After degradation	
BOD-7	SS028143 electr.	6,6	< 3,0	mg/l
COD-Cr	Hach	120	93	“
TOC (NPOC)	SS028199	37	31	“
DOC (NPOC)	“	28		“
Suspended solids	SSEN872	24		”
PH	SS028122 mod	5,9	6,7	
Conductivity	SSEN27888	149	195	mS/m
Nitrogen, total	SS028131 FIA	1,6	4,6	mg/l
Phosphorous, total	SS028127	0,42	122	“
AOX	SSEN1485	0,60	0,26	“
EOX	-	0,43	0,024	“
Nitrogen, total/filtered GF/A	-	0,89		
Phosphorous, total/filtered GF/A	-	0,35		

AOX is high, but well under the provisional limit that would motivate further study. The high EOX is a suspected artefact, although there are so far no explanations for it.

Permit compliance

Conditions for discharges to water and compliance 2002:

The discharge of P must be lower than 1 kg/d as a monthly average. The condition is complied with. (The value in the present study corresponds to about 0,4 kg/d).

The discharge of pollutants, quantified as COD must be lower than 720 kg /d as a yearly average and 850 kg/d as a monthly average. The present performance is much lower, i.e. the condition is complied with. (The value in the present study corresponds to about 100 kg/d).

The release of toxic substances, calculated as TEF (effluent flow per d times 100/EC50 (Microtox, 15 min) should be lower than 10 700 m^3/d. The condition is complied with. (The value in the present study corresponds to about TEF 2000 m^3/d). This is one of rather few cases in Sweden where the licensing authority has set a toxicity condition.

There are also conditions prescribing what type of treatment should be used, etc, which are also complied with.

On the basis of the present study, there may be reason to check the situation in the recipient waters. This is done by the regional Water Protection Union, and the data may well be available although it has not been possible to append them to this study. The company complies with the permit conditions, mostly with considerable margin.

On the basis of the production processes, a substance-by-substance might provide additional information, but only with respect to known reactants and products. WEA provides essential information with respect to compliance and recipient load.

4.1.7 Ireland

In Ireland WEA is also already incorporated in the permit requirements. Some exceedings of the permit limits were found.

IRL 1 is a treated pharmaceutical effluent discharged to the marine environment via local authority sewers along with several other pharmachem effluents. It is in compliance with permit for all species with the constant exception of *Skeletonema*.

IRL 2 is a pharmaceutical & healthcare company. The limit of 5 T.u's applies to a discharge which after in-house treatment enters the local authority WWTP & is then discharged to river. Respirometry trials deem this waste non-toxic to the biological treatment plant.

The company would be responsible for approx. 20% of the load to the WWTP.

Here, *Daphnia* & *Pseudokirchneriella* are over the limit.

IRL 3 is a small textile company with no effluent treatment of any kind discharging to an estuarine receiving system. Huge dilution is available to a small, intermittent discharge licensed by local authority (limit 10 T.u's) The results are good (i.e.: low when compared to historical range).

The toxicity for algal species is not in compliance with the permit limits.

4.2 Robustness

As was explained in section 2.4.2, the results of the practical study programme only allowed for an evaluation of the robustness of WEA in terms of test variation and discriminating power. In the following two paragraph these two issues will be discussed separately.

4.2.1 Test variation

In analytical chemistry the test variation or the reliability of one specific measurement is usually determined through the repetition of the complete procedure from sample to result. These repetitions are then used to calculate average values and corresponding standard deviations.

In toxicity tests this is done in a very different manner. The test results, EC or LC50's or NOEC and LOEC's, are calculated on the basis of a set of data derived from a test with multiple repetitions and with multiple exposure concentrations and sometimes even with multiple observation times. It is evident that such a complex data set requires a tailor-made statistical approach. For instance, data on the exposure concentrations are usually log transformed for further calculations.

Although usually only one number (like the EC50) is reported, the dataset contains much more useful information than only this number. For instance, both the shape of the dose-response curve and the rate at which effects come about are very meaningful parameters in a toxicological sense. The EC50 is directly derived from the dose-response curve and as a consequent the reliability of the EC50 depends on the statistical fit of the curve (see also section 2.3.1). Based on the statistical parameters for the curve fitting procedure the upper and lower 95% confidence limits are also calculated.

The reliability of the liability to bioaccumulate and toxicity methods applied in the practical study programme are summarized in Table 4.9. Data on the variation of the persistence test were not available in this study. For comparison purposes the 95% confidence toxicity data are converted to standard deviations by making use of a well known statistical rule of the thumb. This rule is that the 95% confidence limits are roughly equal to the mean plus/minus the standard deviation times two.

Table 4.9. Relative Standard Deviations (RSD), in percent for the measurements on liability to bioaccumulate (PBS) and toxicity (EC and LC50).

	Median RSD (%)	Minimum RSD (%)	Maximum RSD (%)	Number Of tests
Liability to bioaccumulate PBS	20	3	73	26
Toxicity[1]				
Bacteria	12	4	21	11
Algae	6	1	17	10
Crustaceans acute	9	7	25	10
Chronic[2]	13	6	22	4
Fish acute	17	17	17	3

[1]: The relative standard deviations for the toxicity tests are calculated from the statistical confidence interval lower and upper limits as: RSD = 100% x 0,5 x ($^{\text{upper limit}}/_{\text{lower limit}}$-1) / ($^{\text{upper limit}}/_{\text{lower limit}}$+1).

[2]: The NOEC's for chronic tests have no confidence interval. Instead the next lower and higher test concentrations are used instead.

For the PBS measurements the numbers in Table 4.9 show us that at this moment the median and maximum relative standard deviations are relatively high, respectively 20% and 73%. This implies a large uncertainty relative to the total range of PBS values, from 3 for the background to 30 for effluent D-1.

For a small number of samples the PBS method was also applied by some other laboratories participating in the practical study programme. However, since for most of these laboratories it was their first time they used the method, the data should not be considered valid for an intra-lab comparison. Moreover, the data in Table 4.9 and the experience from practical study programme have shown that the SPME method and protocol needs some improvements before it can be applied on a routine basis. Improving the SPME method could be part of a follow up on the practical study programme.

The data for the relative standard deviations of EC and LC50's derived form the toxicity tests show that in general they are low, all less than 25% and sometimes even very low, down to 1%. This is in accordance with the state of development of these tests: both test designs as well as statistical analysis procedures have reached the limits of perfection over the last decades. On the whole it can be concluded that toxicity test variability is relatively small and EC and LC50's can be determined with a high level of certainty.

In cases where standard deviations are relatively high special attention should be paid to the complex nature of the substance or mixture under investigation. Especially at higher effluent exposure concentrations the test results may be affected by so-called confounding factors like the pH, conductivity, hardness, ammonia, etc. These factors may obscure the toxic effects that have our primary interest. Identifying the causes of toxic effects will require a thorough understanding of the composition of the effluent and the presence of potentially confounding factors. For instance, UK testing used marine species. Salinity adjustment of post biodegradation samples using hyper-saline brine solution was required before toxicity testing (as the biodegradations were carried out at low effluent concentrations in freshwater media). This may have introduced confounding factors. This issue needs further attention in the future.

4.2.2 Discriminating power

As stated in section 2.4.2 the robustness of a specific test is very much dependent on its ability to discriminate between samples. Therefore the range of response is very important. Figure 4.1 gives an overview of the results of the toxicity tests in the practical study programme (genotoxicity and endocrine disruption are not included).

On the whole the most important taxa involved in this practical study programme (bacteria, algae, crustaceans and fish) displayed a wide variety of toxicity in the selected effluents, ranging from <1 to 143 Toxic Units. None of the tests was too sensitive, in a sense that in every effluent an effect is observed; or not sensitive enough, in a sense that hardly any effects are observed in the range of effluents tested. Moreover, none of the test species appeared to be "the most sensitive species", each of them having their own added value.

A very large variation in sensitivity between different taxa was observed, with in many cases, the algae as the most sensitive species. A good second place is reserved for the chronic crustacean tests. Interestingly, in the most extreme case of variation, Pharma effluent P-2, there is a factor more that 100 between the most and the least sensitive species, the crustacean (chronic) and alga respectively. This example clearly illustrates the need to use more than one taxum when evaluating an effluent's toxicity.

On the other hand, in a number of effluents there is only very little variation in sensitivity of the tests. Using a set of tests therefore provides useful information on the species sensitivity distribution. This is especially interesting, when there is a need to extrapolate the results to other species as might be the case in risk assessment.

As to the added value of the fish tests, some questions may arise. When looking at Figure 4.1 two conclusions catch the eye. The first one is that for fish the range of toxicity (the difference between the least and the most toxic effluent) was only 5,8 Toxic Units. This seems very low as compared to the other tests and the discriminating power of the fish tests is therefore limited. The second conclusion is that in none of the cases, the fish test was the only or the most sensitive. This, in combination with the costs and the ethical discussions, may lead to the conclusion to omit this test. On the other hand, fish tests may be more representative than other animals. In Sweden, for instance, fish testing, including histology, liver functions and blood functions have at present a major role in the evaluation of pulp and paper effluent treatment. Moreover, the amount of data in the practical study programme is too little to make a final conclusion. It is recommended to address this issue in a follow-up programme.

When looking at the data it can be seen that for all three hospitals, algae were the most sensitive as compared to other test organisms. Such information may be very useful when deciding on what species to use in a first screening programme for this type of effluents. It opens up ways to focus and prioritize efforts for environmental improvements.

For the liability to bioaccumulate measurements with the SPME method the discriminating power can be derived from Figure 3.5. PBS measurements ranges from 3, the assumed background level, to 30 for effluent D-1. This range is relatively small, especially when compared to the sometimes very high standard deviation of the measurements, up to 73% (see Table 4.8). Thus, in the further developed of this method it is recommended to minimize test variation and to get a clearer understanding of the true background levels of PBS. Since this method is not fully developed it cannot yet be considered 'robust'.

4.3 Cost-effectiveness

In this paragraph the costs for a WEA PBT value and a chemical specific PBT estimate will be compared on the basis of estimates. The comparison is made for one P, B, T assessment for one effluent.

Costs for WEA PBT values

In order to make an estimate of the costs, an inventory was made of the costs of a base set of full scale toxicity tests in the countries of the Contracting Parties. The base set consisted of the Microtox test, an algal test and an acute crustacean test. A summary of costs is given in the Table 4.10. For simplicity reasons chronic tests with crustaceans, and tests with fish or taxa other than the base set are left out of this comparison. The same account for tests for genotoxicity and endocrine disruption.

Table 4.10. Estimates of prices for base set of WEA toxicity tests in Euros.

	NL	BE	UK	Ire	P	Se	D
Microtox	140	110	200	200	200	300	360
Algae	680	735	600	1200	350	1100	1290
Crustaceans	370	420	600	900	200	900	950
Total	**1190**	**1265**	**1400**	**2300**	**750**	**2300**	**2600**

The total price of the base sets varies between € 750 in Portugal to € 2600 in Germany. Here it must be noted that Germany normally uses adapted protocols that greatly reduce the costs of regular testing. It is not fully clear whether all the prices mentioned above are inclusive of project management and reporting. For Sweden reporting is included so possibly the differences between the Contracting Parties is somewhat

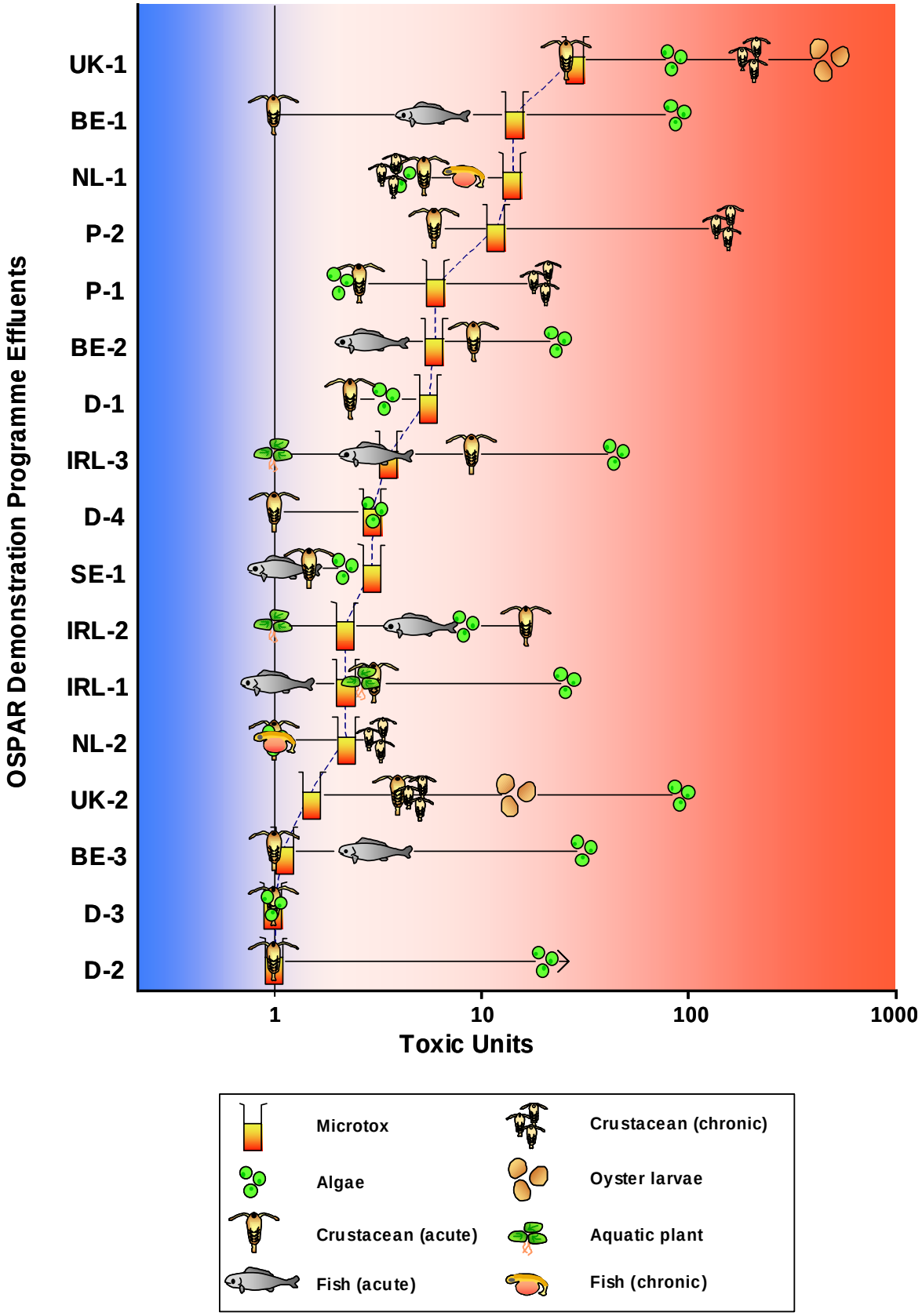
overestimated. Based on the figures the overall price of a base set (project management and reporting included) for one effluent sample is expected to be somewhere between 1200 and 2600 Euros. For this evaluation € 1750 has been used.

When more than one sample is assessed the prices go down. It may be expected for every five effluent samples the overall price doubles (€ 3500 for five samples), provided that the samples can be measured simultaneously. Per effluent sample a base set would then be € 700 including project management and reporting.

The costs of liability to bioaccumulate test with the PBS method are about € 1000 for one effluent sample in the Netherlands. Because of a larger efficiency the price for five samples the overall price will hardy increase and is estimated at approximately € 250 per sample. In Sweden the price for two samples is estimated at € 2600. It should however be noted that this method is a low-volume application performed by only few laboratories in Sweden. It can be expected that if this method becomes a routine analysis, prices will significantly drop in the future.

The costs for one biodegradation test in the Netherlands are estimated at € 3000 (DOC, measurements included). These costs were relatively high since we tested only one effluent and the majority of the costs were accounted for by the positive control with aniline. For five effluents tested simultaneously we estimate the costs of the persistence step to be approximately € 1600 per sample. In Germany the costs for a 28 day Zahn-Wellens and DOC Die Away Test are slightly lower, 1000 and 1200 Euros respectively. Measuring persistence could imply that toxicity and liability to bioaccumulate will be measured both before and after biodegradation. For this assessment we assumed that the complete base set is measured again after the biodegradation test.

Fig 4.1 Overview of test results for the toxicity tests



The overall costs of a WEA PBT assessment is given in Table 4.11.

Table 4.11. Overall costs of WEA PBT assessment

Test type	Costs per sample (€)	
	One sample	Five samples
T (full base set)	1750	700
B (using the PBS method)	1000	250
P biodegradation	3000	1600
T after biodegradation (full base set)	1750	700
B after biodegradation	1000	250
Total	**8500**	**3500**

Costs for the chemical specific PBT estimate

Estimation of the PBT values through the chemical specific approach is a two-step approach: chemical identification and quantification of substances, followed by looking up data on P, B and T and estimate P, B and T from these. Some examples can be found in section 4.1.

In the practical study programme the chemical analysis was performed with a GC-MS screening. The costs for a GC-MS screening depend very much on the amount of effort put into the identification and quantification of the substances in the sample. We have no information about the cost for one effluent sample but from our experience we estimated the costs for a routine GC-MS screening to be € 1000 per sample when more than at least 10 samples are measured simultaneously.

An attempt was made to estimate the toxicity of an effluent by calculating toxic units on the basis of the concentrations of the identified substances. This was done for several effluents, which took about one day (€ 800) per effluent on retrieving and interpreting toxicity data. The results were disappointing since for the majority of substances no toxicity data were available. The overall result of this exercise was that despite the investment of 'expert' time it was impossible to adequately estimate toxicity from the chemical composition of the effluent. Even when some results were obtained, great uncertainty remained about the value of it.

No attempt was made to retrieve data for liability to bioaccumulate or persistence, but considering the lack of databases on measured values for B and P it may be assumed that an attempt to do so would have been very hard to accomplish. For this evaluation the costs for such an attempt are estimated to be comparable to those for estimating toxicity.

Comparison of costs

The results of the cost evaluation are summarized in Table 4.12. Since we have no information on the costs for one single GC-MS analysis the comparison is made for 10 effluent samples being assessed simultaneously.

The costs for both types of PBT assessment are comparable; around € 3500 per sample. In the OSPAR workshop of September 2003 it was recognised that these costs were comparable, although based on rough calculations and some assumptions. Furthermore, the costs for WEA testing will lower when biological testing is carried out on a routine basis. Also, it was mentioned that for some specific compounds, chemical analysis is extremely expensive. For each effluent or case a specific cost-evaluation should be made.

Table 4.12. Estimated costs of PBT assessment with WEA and chemical specific approach, in Euros per effluent. (Based on 10 effluent samples assessed simultaneously).

	WEA PBT values	Chemical specific PBT estimates
GC-MS screening	-	1000
Toxicity	700	800
Liability to bioaccumulate	250	800
Persistence (incl. T and B after biodegradation)	2550	800
Total	**3500**	**3400**

Although the costs for both the assessments are comparable, one should keep in mind that the amount and kind of information obtained from the two approaches is totally different. With WEA all effects of all substances present in the effluent are assessed. With the chemical specific approach not all substances are identified and quantified and for those substances that can be identified the combined PBT value is an estimate and not directly measured. In other words, the value for money with WEA is larger than with chemical specific approach.

5. Conclusions and recommendations

5.1 Conclusions

Many complex effluents that contain a wide variety of known and unknown substances, cannot not be characterised sufficiently in terms of their chemical profile. And what is more, for the chemicals identified there is insufficient information on their environmental hazard and/or effects. In this context WEA could provide further insight in the understanding of the environmental effects from the effluent as a whole. This would provide an additional benefit compared to chemical monitoring and offers a safety net for the identification of effluents that need further assessment or control. WEA has the potential to become a technical instrument on a holistic level to support achievement of the objective of the OSPAR Hazardous Substances Strategy, which could also facilitate the use of such methods in a wider European framework.

In the present study the *added value* of WEA was evaluated by comparing WEA results with permit requirements and with an extended chemical specific approach. It was concluded that in many effluents, testing toxicity has an added value, since toxicity was found that could not be explained with the (extended) chemical specific approach.

The added value from WEA in relation to the chemical specific approach, has been shown within the practical study programme for the majority of effluents. The added value was most apparent in those cases where the substances identified in effluents could not, or only partly explain the toxicity that was measured with WEA.

The *cost-effectiveness* of WEA was estimated on a preliminary basis and compared to the costs of an extended chemical specific approach. It was concluded that the costs are of the same magnitude (around 3500 Euro per effluent), while the value for money with WEA is larger since the effects of all known and unknown substances are assessed. Of course costs for WEA might decrease when testing becomes daily practice and when a tailor-made choice will result in a smaller WEA test. Furthermore, chemical analysis of some specific substances may be extremely expensive. It is dependent on the way of application within a Contracting Party whether costs for WEA should only be partly or fully added up to costs for the chemical specific approach.

The methods within WEA are derived from internationally agreed protocols for measuring the persistence and toxicity of individual substances. For effluents these protocols have often been adapted. For most toxicity tests protocols are available or need (minor) adjustments. Looking at the confidence intervals, variation between replicates within one test appeared to be relative small. With regard to toxicity tests with fish, an analysis of costs, the added value of including this trophic level and the ethical discussion versus the ecological relevance on working with vertebrate organisms should be made. For liability to bioaccumulate and persistence more detailed investigations are necessary.

In general toxicity and liability to bioaccumulate were found to decrease when effluents were treated more intensively. No relationship between a sector and the range of toxicity or liability to bioaccumulate could be found. This probably demonstrates that the variability within a sector in terms of processes and water treatment is greater than the apparent uniformity might suggest.

Toxicity and liability to bioaccumulate were sometimes found to be persistent with respect to biodegradation: that is a period of incubation did hardly decrease the degree of toxicity and liability to bioaccumulate. However, some additional discussions on the testing and the interpretation of persistence are required.

The methods for liability to bioaccumulate of the Swedish solvent extraction (LPE&EGOM) method and the solid-phase micro extraction (SPME) method, showed differences that to a large extent could be explained. The LPE method has been used for more than a decade, and its first part – EGOM – is an efficient screening test. The results of the newly applied SPME method look promising as a screening tool to assess potential bioaccumulative substances. Nevertheless, the tests applied for liability to bioaccumulate will need further comparison as well as discussions on validation to bioaccumulation within organisms.

For persistence the question was raised whether the test results showed persistence or treatability, as the latter might be related to the type of effluent and test to be used. There is a need to pay particular attention to test conditions as concentrations in the test medium can significantly influence the measured biodegradation. It was widely acknowledged that the combined use of biodegradation and toxicity or biodegradation and liability to bioaccumulate potential provides a solution to indicate recalcitrant toxicity or liability to bioaccumulate potential.

The practical study programme has generated, within a relatively short period, a large quantity of data that could be used to highlight differences in toxicity, liability to bioaccumulate and persistence in effluents. This indicates that methods are not only available, but also robust enough to be applied and result in sufficient differentiation between effluents.

The ‘learning by doing’ concept of the practical study programme was found to be effective.

The WEA instrument that could be applied in the future, should consist of two parts: a tool box and a guidance flow chart.

The "tool box" should contain a set of adequate methods that can be used in a flexible way, relevant for the specific circumstances of the effluents concerned (tailor-made approach) The toolbox should include tests that can measure the parameters that have a specific relevance for OSPAR, e.g. (acute and) chronic toxicity, persistence, liability to bioaccumulate and, if possible, genotoxicity and endocrine disruption.

The guidance flow chart should enable us to understand what criteria are used to make management decisions. The flow chart should consist of objective and measurable criteria and decision points, and might help environmental managers to become familiar with the tool. The flow chart should also indicate either a parallel or triggered approach with regard to P, B and T.

5.2 Recommendations

The overall recommendation is to continue the work on WEA within the OSPAR IEG group, establishing a working programme for several years and with a two-legged approach.

The first leg should result in a guidance flow chart and a toolbox. Here specific items that need further investigations (including liability to bioaccumulate, persistence, fish tests) and discussion (flow chart) should be addressed.

The second leg should consist of a follow up of the practical study programme, a monitoring programme, where – following the concept of ‘learning by doing’ – data are gathered and experience is gained. This programme will not be a simple ‘redoing’ or extension of the practical study programme, but will have a focused approach. In this line, the new monitoring programme could be used to test the toolbox and the flowchart, and to explore the advantages of using artificial effluents (in addition to the real effluents) in order to increase understanding of results. Finally, more participants could be involved.

For the near future it is suggested to further work on standardization and validation of the SPME method for liability to bioaccumulate, to carry out a ring test and prepare proposals for a protocol. This should not exclude the use of other appropriate methods that yield equivalent results. A good comparison with e.g. the Swedish method and the SPME method should be made.

Further evaluation of the use of the fish tests (added value, limitations for ethical reasons and high costs) should be made, including various forms now in practice, such as the fish egg/larvae test, and other modifications.

For the near future it is also suggested to further elaborate the role of, and methods for, measuring persistence in the context of WEA.

6 References

Leonards, P., A. Kruijt, P. Wezenbeek (2003). Determination of potential bioaccumulating substances using GC-FID/MS and screening van organic substances in effluents (in Dutch). RIVO-report C053/03

Aquasense (2003). Practical study Programme Whole Effluent Assessment (WEA) 2003 (in Dutch). Aquasense report 1998.

VITO (2003) WEA-OSPAR Practical study Programme. Vito report 9936451

Hynning, P-Å (1996) Separation, identification and quantification of components of industrial effluents with bioconcentration potential. Wat. Res. 30 1103-1108.

7. Appendices

7.1 WEA proposal for practical study programme

OSPAR Intersessional Expert Group on Whole Effluent Assessment
Proposal for a Whole Effluent Assessment Demonstration Programme

1. Background

The OSPAR Point and Diffuse Sources working group set-up an intersessional expert group (IEG-WEA) in 1999 to examine the value of a whole effluent assessment approach in helping to achieve the OSPAR objectives for protection of the marine environment. The group has to date discussed possible ways of using persistence (P), bioaccumulation (B) and toxicity (T) data for whole effluents within the OSPAR remit and has produced reviews of suitable methods[1]. The IEG-WEA is now proposing to trial these methods to assess P, B and T on a limited number of effluents around Europe in a Whole Effluent Assessment demonstration programme. The primary goal of the programme is to convince Contracting Parties with limited experience of WEA and its test methods of the added value of the approach. The secondary goals are to collect data on the robustness and reliability of the methods and a preliminary assessment of the PBT-loads of effluents. This document provides a project proposal for this demonstration programme. The demonstration programme should be seen in a broader context of the progress of the IEG. A proposal for an OSPAR workshop is submitted alongside this project proposal. It is intended to present the results of the demonstration programme at this OSPAR workshop.

2. Objectives

To convince Contracting Parties of the usefulness of WEA by demonstrating that a WEA approach can "add value" to the task of identifying effluents of concern to the marine environment. This will be achieved by demonstrating that harmful effects can be measured using the whole effluent approach that can not be measured using a chemical by chemical approach and by demonstrating that the cost of a whole effluent approach can be significantly lower than that of the chemical by chemical approach for complex effluents.
To demonstrate how a whole effluent approach could be used to identify effluents of concern to the marine environment and to initiate action to reduce this concern. More specifically to show how the approach can be used to identify priorities for action; to trigger further site specific investigations and to chart progress in reducing whole effluent persistence, bioaccumulation and toxicity.

To demonstrate the robustness and reliability of whole effluent persistence, bioaccumulation and toxicity assessment methods in the hands of OSPAR Contracting Parties ensuring that the data generated by these methods is "fit for purpose".

To convince Contracting Parties not participating in the Demonstration Programme that they should participate in the follow-up OSPAR monitoring programme.

3. Contracting Parties expressing an interest in participating in the Programme

Letters of intent will be submitted by to PDS by the following Contracting Parties: -

Belgium
Germany
The Netherlands
Sweden
The United Kingdom

CEFIC representing the European chemical industry have in principle offered support to the programme.

[1] For more info on the goals and work of the Intersessional Expert group see the Explanatory Note [ref]

4. Methodology

Selection of effluents

All types of effluents will be considered for use in the study, but ideally the effluents will be those regulated by IPPC e.g. organic chemicals (fine chemicals); pharmaceuticals; textiles; pulp and paper. Each contracting party will select at least two effluents for testing in the demonstration programme. CEFIC will work with all Contracting Parties where appropriate to help facilitate the selection of effluents. The names and locations of the industries concerned will not be made public knowledge. Ideally industries will volunteer to be included in the programme.

Whole Effluent Assessment Methods

In order to obtain data of the highest comparability persistence, bioaccumulation and toxicity of each effluent should be assessed at the same time in the same effluent sample. In practice, to speed the process of data capture, bioaccumulation and toxicity data will be obtained and only where this is significant a second assessment will be made of all three parameters on a second sample. To ensure data comparability, bioaccumulation and toxicity must be assessed both before and after the assessment of persistence.

For persistence and bioaccumulation, each contracting party will select the assessment methods to be used in line with the findings of the OSPAR IEG review “Persistence and bioaccumulation – methods in use or under development in whole effluent assessment” prepared by Ake Unden (Sweden) – see attachment for recommended methods.

As for persistence and bioaccumulation, each contracting party will select the toxicity assessment methods to be used. Measures of both acute and chronic toxicity are required. Genotoxicity and endocrine disruption endpoints can also be measured. Fish testing may be omitted recognising the concerns over toxicity testing using vertebrates – see attachment for recommended methods.

For Genotoxicity, methods should be selected in line with the recommendations of the findings of the OSPAR IEG review “Survey on Genotoxicity test Methods for the Evaluation of Wastewater within whole effluent assessment” prepared by Stefan Gartiser and Andreas Schnerstein (Germany) - see attachment for recommended methods.

For Endocrine Disruption, methods should be selected in line with the recommendations of the findings of the OSPAR IEG review “OSPAR background document on the use of effect related methods to assess and monitor wastewater discharges – testing of endocrine disruption” prepared by Thomas Knacker (Germany).

There is some benefit to be gained from the selection of similar methods and so all Contracting Parties will be asked to put forward their preferred method selections for a final group discussion prior to the start of any practical work.

All data must be generated by a laboratory that operates a quality system (e.g. GLP).

Supporting Chemical Specific Information

As one of the key objectives of the programme is to demonstrate that a whole effluent assessment approach can add value to the current chemical by chemical approach for complex effluents, it is important to collate the current chemical data being collected for each effluent i.e. current chemical data; details of any licence conditions and the results of compliance assessment.

Supporting Process Information

It is also important to gather information on the processes and abatement in place at each site - in particular are process operations typical, what level of effluent treatment is in operation and whether or not BAT is applied on site. Participating Contracting Parties may choose to measure, persistence, bioaccumulation and toxicity not only downstream but also upstream of any effluent treatment plants so that the effectiveness of treatment and BAT with respect to these parameters can be evaluated.

Cost of sampling and testing

All costs associated with this programme will be paid by either the participating Contracting Parties or the industries themselves.

Timetable

It is anticipated that the programme will start later this year and be completed by the end of August 2003.

OSPAR workshop

In a separate proposal (see Annex 5 of Progress report) it is proposed to organise an OSPAR workshop in September 03 on the outcome of the demonstration programme. For details see the terms of reference in that proposal.

Attachment

For persistence – recommended methods

- Zahn-Wellens test (OECD 302B) – where effluent is discharged to a biological treatment plant.

- Die-away test or shake flask test (OECD 301) – where effluent is discharged direct to a river, estuary or marine water.

For bioaccumulation – recommended method

- SPME procedure – a test protocol on the use of this method can be obtained from Gert-Jan de Maagd (The Netherlands).

For acute toxicity – recommended methods

-Fish acute method (ISO/OECD) or Fish egg test – freshwater or marine
-Daphnia acute (ISO/OECD) – freshwater; or Tisbe acute - marine

For chronic toxicity – recommended methods

-Algal growth (ISO/OECD) – freshwater or marine
-Daphnia chronic (ISO/OECD) – freshwater; or Tisbe chronic – marine
-Fish Partial life-cycle and/or reproduction

For genotoxicity – recommended methods

-Bacterial mutagenicity tests – Ames, umuC-test & SOS-chromo
-Eucaryotic cells – micronucleus or Comet assay with permanent cell lines or suitable organisms

For endocrine disruption - recommended methods

E-Screen test (based on human breast cancer cell line MCF-7
Yeast Assay
Fish Partial life-cycle and or reproduction cited above may pick up ED effects

7.2 WEA methods

7.2.1 Toxicity tests

In Table 7.2 an overview is given of the tests used for toxicity, including the species, the protocols and the end points.

Table 7.2. Overview of the species, test endpoints[1] and test protocols used by the Contracting Parties in the 2003 OSPAR practical study programme

	TAXA						
	Bacteria	Algae	Crustacean		Fish		Other taxa
			Acute	Chronic	Acute	Chronic	
Du	*V. fisheri* 30 min EC50 *ISO 11348-2*	*R. subcapitata* 72h EC50 *DIN 38412-33*	*D. magna* 48h IC50 *DIN 38412-30*	-	-	*D. rerio* 2d NOEC *DIN 38415-6*	-
Se	*V. fisheri* 30 min EC50 *ISO 11348-3*	*R. subcapitata* 72h EC10 *ISO 8692*	*N. spinipes* 96h EC50 *SS 02 81 06*	-	-	*D. rerio* 14d NOEC *SS 02 81 93*	-
Ir	*V. fisheri* 30 min EC50 *ISO 11348-3*	*R. subcapitata* 72h IC50 *ISO 8692* *S. costatum* 72h IC50 *ISO 10253*	*D. magna* 48h EC50 *ISO 6341* *T. battagliai* 48h LC50 *ISO 14669* *C. crangon* 96h LC50 *MAFF-BEG/030* *T. brevicornis* 48h LC50 ICES #28, 2001	-	*O. mykiss* 96h LC50 *OECD 203* *S. maximus* 96h LC50 *OECD 203*	-	Aquatic plant *L. minor* 7d IC50 *OECD, 1998*
P	*V. fisheri* 30 min EC50 *AZUR-Microtox*	*R. subcapitata* 72h EC50 *ISO 8692*	*D. magna* 48h EC50 *ISO 6341*	*D.magna* 21d NOEC *OECD 211*	-	-	-
NL	*V. fisheri* 30 min EC50 *AZUR-Microtox*	*R. subcapitata* 72h EC50 *ISO 8692*	*D. magna* 48h EC50 *OECD 202*	*D.magna* 16d NOEC *OECD 211 (mod)*		*D. rerio* 8d NOEC *OECD 212 (mod)*	-
Be	*V. fisheri* 30 min EC50 *AZUR-Microtox*	*R. subcapitata* 72h EC50 *OECD 201*	*D. magna* 48h EC50 *OECD 202*	-	*O. mykiss* 96h LC50 *OECD 203*	-	-
UK	*V. fisheri* 30 min EC50 *AZUR-Microtox*	*S. costatum* 72h EC50 *ISO 10253*	*T. battagliai* 48h EC50 *ISO 14669*	*T. battagliai* 16d EC50 *in house method*	-		Bivalve *C. gigas* 24h EC50 *ICES method 11*

[1]: EC50, LC50 and IC50 are the concentrations at which respectively 50 percent effect, lethality and inhibition are observed. The NOEC is the no observed effect concentration, i.e. the highest tested concentration without a significant adverse effect.

Nitocra spinipes method

The test animal of *Nitocra spinipes* is obtained by sexual fertilization. The breeding animal is held in 100 ml glass vessels in natural brackish water taken from the Swedish east coast at Studsvik south of Stockholm. The animal is held in accordance with the standard method (SS028106) Adult animals at the age of 3-4 weeks are used in the test.
The sample was osmotically adjusted to the same level as the natural brackish water (salinity 6,3).

A dilution series of 5 concentrations was selected after preliminary tests. Each test concentration is held in 5 glass centrifuge tubes, with 5 animals in 10 ml solution. Conductivity, pH and dissolved oxygen concentration is measured before and after the test period. The measurements before test is done in the original sample. pH and dissolved oxygen concentration was within the approved interval (pH 7,7-8,3 and O_2 > 70 % saturation).

The light was dim for 12 h a day. The temperature was in the interval 20,5 ^{o}C+/- 0,5.

The evaluation of toxicity was done in a computer based probit-evaluation program(Probit 2.3 from Swedish Environmental Protection Agency). NOEC is estimated by Mann-Whitney U-test.

Danio rerio method

The method used was SS 02 81 93 “Determination of embryo-larval toxicity to freshwater fish-semistatic method”.

Fertilised eggs (with an age of 2-4 hours) of zebrafish (*Danio rerio*) were incubated in a concentration gradient of a test sample. New test solutions were made daily and eggs (embryos) and larvae were transported to new petri dishes containing new test solutions.

40 newly fertilised eggs were transported to the control- and test concentration petri dishes (Day 0). After 24 h, dead eggs were removed and the number of eggs were reduced to 20 per petri dish. The control group contained two replicates while the test concentrations contained one replicate each. During the experimental period the number of dead and hatched eggs were observed as well the number of surviving larvae.

The control group was exposed to dilution water with an oxygen saturation of about 100% and a pH of 7,5±0,2. The dilution water is specified in SS 02 81 93 and described below in “control article”. The experiments were carried out in petri dishes with 50 ml of test solution/dish. The petri dishes were incubated at 26 $\pm$1 °C in normal laboratory light with a light:darkness relation of 12h:12 h.

Temperature, pH and oxygen saturation were measured daily in newly made test solutions and dilution water. The same parameters were measured in the controls as well as in the lowest and highest test concentrations in the old (24 h) solutions.

7.2.2 Liability to bioaccumulate tests

Protocol for determination of Potentially Bioaccumulating Substances (PBS) using the LPE method

Approximately 1 L sample was extracted twice with 50 ml cyclohexane. pH was adjusted to 10 and extraction was repeated twice with 50 ml cyclohexane. All extractions were performed in separatory funnels on a shaker for at least 2h. The four extracts were combined. The volume was reduced by rotary evaporation and finally by a gentle flow of nitrogen to dryness in a weighed vial. The residue was redissolved and an aliquot was injected on a gas chromatograph (HP5890A) with a capillary column (DB5 30m x 0,32 mm, 0,25 µm film, J&W Scientific) and flame ionisation detector. The temperature of the column was programmed from 40°C to 320°C. The area under the chromatogram in the range corresponding to the normal alcanes n-C_{10} to n-C_{40} was integrated. Quantification was made using eicosane (n-$C_{20}H_{42}$) as a standard. The result is referred to as EGOM (extractable gas chromatographic organic material).

The extract was dissolved in methanol and fractionated with HPLC (Consta Metric 4100) on a C18 column (HyPurity C18 5µ, 250 x 4,6 mm, Thermo Hypersil) that was eluted with a gradient from 30% phosphate buffer pH 2,5 in methanol to 100% methanol. The system was calibrated using compounds with known logP_{OW} so that fractions logP_{OW} < 3, 3-5 and >5 could be collected. The fractions were concentrated, diluted with 0,5M HCl and extracted with cyclohexane. These extracts were injected on the gas chromatograph as described above. The procedure is in accordance with Hynning (1996).

Protocol for determination of Potentially Bioaccumulating Substances (PBS) using the SPME method

1. MATERIALS

1.1 PDMS fibre and fibre holder

PDMS fibres (Supelco, Bellafonte, CA, USA) have a length of 1 cm and an internal diameter of 55 μm. On the fibre a coating of 100 μm polydimethylsiloxane (PDMS) is placed. The volume of the PDMS phase is 0,621 μl.

To prevent damaging of the fibre and the coating during analysis a fibre holder, which can be obtained from the manufacturer is indispensable.

CONDITIONING AND CLEANING OF NEW FIBRES
Before the fibre can be used, it has to be conditioned by placing it in an injection chamber for 2 hours at 260°C. Afterwards the quality of the cleaned fibre needs to be evaluated, see paragraph 2 below.

1.2 External standard

As an external standard for quantification of the samples 2,3-dimethylnaphtalene (40 mg/l ethyl acetate) is used.

1.3 Test bottles

Extractions have to be performed in a 250 ml flat bottom round bottle with a screw-cap with a PTFE seal. The bottles have to be filled with sample to the rim in order to prevent head space. A hollow needle (or a piece of GC column) is stuck through the septum to remove the high pressure, which develops during stirring.

1.4 Stirring

During extraction the sample has to be mixed with a 2 cm glass magnetic stirrer at a minimal speed of 500 rpm.

1.5 GC-column and GC-liner

A 10 meter column has to be used (J&W, Folsom, CA, USA, DB-1, length 10 m, ID 0,25 mm, film thickness 0,1 μm) and an insert liner for SPME (8 mm liner, Supelco, Bellafonte, CA, USA).

2. CHECKING THE FIBRE QUALITY

2.1 Maximal allowed molar concentration of cleaned fibres

In order to determine the maximal allowed molar concentration of the cleaned fibres, the molar response of the cleaned fibres has to be determined according to the protocol. The C_{fibre} concentration may be 0,05 ±0,06 mM.

2.2 Adsorption capacity fibres

Before testing the adsorption capacity of the fibres a solution of 50 µl 1-octanol in 1 litre of demineralised water (solution A) is made. Next 60 ml of solution A is added to 3000 ml of demineralised water (solution B). The C_{fibre} concentration of solution B is determined. As a quality criterion for the adsorption capacity of a fibre the next range is determined: 0,025 to 0,049 mM.

3. GC-FID CIRCUMSTANCES

Before analysis the suitable injection depth for the fibres has to be determined, in order to fit the fibre in the middle of the GC liner. Fibres have to be desorbed splitless during the complete GC run at a temperature of 250 °C. A relative short temperature program is used, starting at 40 °C during 2 min and elevated to 290 °C at 30 °C/min.

4. EXTERNAL STANDARD

Triplicate samples from 1µl from a 2,3-dimethylnaphtalene solution (40 mg/l in ethylacetate) are splitless injected using the above-mentioned GC-temperature program and a normal GC-liner.

The external standard has to be measured in triplicate both at the beginning as the end of a series of samples, in which fibres are analysed. The standard deviation of both triplicates has to be smaller than 5%, and no significant difference is detected ($p < 0,05$) between both triplicates.

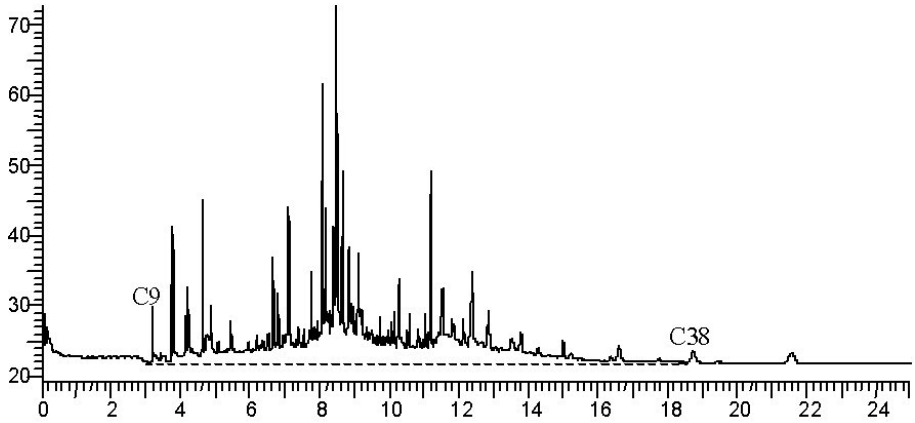


Figure 1: Example of an integration of a chromatogram from an effluent sample.

5. EXECUTION OF SPME-EXTRACTION OF WATER SAMPLES

Sample in round bottom bottle. No headspace. Bottle is kept in the dark or wrapped in tinfoil.

Fibre with fibre holder is brought in solution through the seal. The fibre should be placed in the middle of the solution.

During the extraction period, which lasts for 24 hours, keep stirring.

Remove fibre and fibre holder after 24 hours from bottle, dry gently with a tissue en inject directly on GC-FID with special GC SPME-liner.

6. CALCULATIONS

De average response of all measurements of the external standard (2,3-dimethylnaphftalene) is used to determine the molar response; peak area responding to 1 mole of the reference substance. When using an FID, the molar response of an unknown substance may be set equal to the molar response of the standard.

By using the molar response of the external standard and the PDMS volume on the fibre (0,621 µl) de peak area belonging to an SPME-fibre can be recalculated to the molar concentration in the fibre (C_{fibre} in mM).

C_{fibre} = peak area/ (molar response external standard [µl] * 0,621 µl)

7.3 Results

7.3.1 Toxicity and Liability to bioaccumulate

Effluent	Liability to Bioaccumulate			Toxicity (% wastewater)								
Code: type	SPME RIZA/RIVO	Other labs	LPE & (EGOM)	Bact	Algae	Crustaceans Acute	Chronic	Fish Acute	Chronic	Other [3]	Gentox[4]	Endocrine Disruption
	mM[1]	mM[1]	mg C/L	EC50	EC50	EC50	NOEC/EC50	EC50	NOEC	NOEC	IF	IF
NL-1: Chemical	19			7,1 (6,1-8,5)	24	25,0 (13-38)	25		12,5		<1,5 / <1,5	
post degradation	8,1			11 (8-15)		35 (25-50)						
NL-2: Pharma	3,6			>45	>98	>100	32		>100		<1,5 / <1,5	
post degradation	2,8						>50					
UK-1: Chemical	4,4			3,53 (3,07-4,06)	0,75 (0,73-0,76)	3,74 (3,07-4,06)	0,496 (0,39-0,55)			0,21 (0,20-0,23)		
post degradation	1,8				1,64 (1,28-1,83)		0,47 (0,40-0,51			0,69 (0,65-0,73)		
UK-2: Chemical	16	4,05		67,4 (53,9-89,0)	0,60 (0,42-0,70)	25,3 (21,6-29,6)	21,65 (13,3-28,0)			5,3 (5,0-5,7)		
post degradation	1,2	1,14			21,4 (18,3-23,0)		38,9 (24,7-62,8)			10,7 (10,4-11,0)		
P-1: Textile	21	7,6		16,7 (15,3-18,3)	48,9	39,2 (31,2-49,4)	5,1					
P-2: Pharma	6,5	6,8		8,6 (5,5-13,6)	>90	17,1 (14,6-19,1)	0,7					
D-1: Textile	30,1	84		17,9	29	43					2,24 / 3,2	Neg. cytotox.
post degradation	3,4	10,7		46,9	>100	> 100			<100		<1,5 / 1,9	1,7
D-2: Pharma	10,9	10,6		>100	<5	> 100			<100		<1,5 / <1,5	neg.
post degradation	0,8	1,8		>100	>100	> 100			100		<1,5 / <1,5	neg.
D-3: Pharma	4,8	4,9		>100	>100	> 100			<100		<1,5 / <1,5	1,9
post degradation	1,5	2,9		>100	>100	>50			<100		<1,5 / <1,5	neg.
D-4: Textile	6,3	22,0		33,4	>33	>100					<1,5 / <1,5	neg.
post degradation	2,3	9		>100	>33	>100					<1,5 / <1,5	neg.
BE-1: Hospital	19	21,4		7 (5,3-9,2)	0,7 (0,62-0,79)	>100		17,7(12,5-25)			15,9 / 2,2	148,3@ / 602,3&
BE-2: Hospital	12	24,3		16,9 (13,3-21,5)	4,27(3,93-4,64)	10,9 (9,4-12,5)		35,4 (25-50)			2,97 / 0,98#	75,5@ / 975,0&
BE-3: Hospital	17	15		>91	3,3 (3,0-3,6)	>100		35,4 (25-50)			4,28 / 1,0#	95,6@ / 1012,4&
SE-1: Chemical	4,5		0,75&(3,2)	34 (31-38)	>46	69 (58-84)			>90			
post degradation	2,2		0,68&(1,7)	>90	>30	>90			>90			
IRL-1: Pharma	2,6			>45	3,9 (3,4-4,3)	>56 />56 />33 31,2 (24,9-50)		>100		37 (27,6-51,7)		
IRL-2: Pharma	13			10 (8-13)	11,9 (9,4-14,9)	6,2		20,4		>100		
IRL-3: Textile	3,8			28 (24,6-30,5)	2,2 (1,6-3,2) / 5 (2,3-6,1)	20,8 (17,2-24,4) / 11,1 (9,1-13,5)		>33 / >33		>100		

[1]: mM on fibre, not in the original concentration in the effluent

[2]: Measured with more than one type of test

[3]: Toxicity also measured with oyster lavae (C. gigas) in the UK and with aquatic plant (*L. minor*) in Ireland.

[4]: Genotoxicity is measured by NL en BE with the Umu-C assay either without or with S9 addition. D used the Ames and Umu-C test, both without additions.

#: negative, IF< 1,5 is considered not to be genotoxic for environmental samples.

@: calculated estrogenic activity as ng/l E2 equivalent (estradiol)

&: calculated androgenic activity as ng/l DHT equivalent (dihydroxytestosterone)

7.3.2 Additional chemistry

In this section the results on the additional chemistry and toxicity data for most relevant substances are presented per effluent.

NL-1

Name	Cas-nr.	Conc. (µg/l)	Toxicity (µg/l)			
			Fish		crustacean	algae
			chronic	acute	acute	
triisobutylphosphate	126-71-6	2,91	no data found			
xylenol (vic-m-)	576-26-1	2,84			11200	
? bis(2-methoxyethyl) phthalate	117-82-8	1,87	no data found			
diisobutyl phthalate	84-69-5	1,81	no data found			
??? cyclopentasiloxane, decamethyl-	541-02-6	1,63	no data found			
? bis(2-ethylhexyl) phthalate	117-81-7	1,38	62	139000	2000	960
? phenol, di-(tertbutyl)-	no CAS132	1,08				
acetophenon, 4-isopropyl-	645-13-6	0,93				
? phenol, p-tert-butyl-	98-54-4	0,83				
??? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	0,73				
??? octadecanoic acid, butyl ester	123-95-5	0,63				
? BHT (butylated hydroxytoluene, antioxidant, Ionol)	128-37-0	0,62				
phenol, 2-(1-methylethyl)-	88-69-7	0,55				
kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,47				
phthalic acid, diethyl ester	84-66-2	0,27				
isoquinoline	119-65-3	0,25				
hexanoic acid, 2-ethyl-	149-57-5	0,25				
??? tetradecane	629-59-4	0,21				
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,21				
diphenyl sulfone	127-63-9	0,20				
??? isooctanol	26952-21-6	0,19				
phenol, 4-(1-methylethyl)-	99-89-8	0,18				
? di-2-ethylhexyladipate	103-23-1	0,17				
acetophenone	98-86-2	0,12				

UK-2

		Conc.	Toxicity (µg/l)			
		µg/l	Fish	Fish	Crustacean	
CAS	Name		chronic	acute	acute	algae
98-95-3	Benzene, nitro-	43	-	100000	30000	-
112-92-5	1-Octadecanol	28	no toxicity data found			
526-73-8	Benzene, 1,2,3-trimethyl-	26	no toxicity data found			
2136-72-3	Ethanol, 2-(octadecyloxy)-	18	no toxicity data found			
117-81-7	Bis(2-ethylhexyl) phthalate	18	62	139000	2000	960
108-95-2	Phenol	16	100	8600	11000	76000
84-69-5	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	15	150	900	1000	190
88-75-5	Phenol, 2-nitro	14			210	
112-30-1	1-Decanol	11	420	2400	7300	2100
818-81-5	1-Octanol, 2-methyl-	9,3				
36653-82-4	1-Hexadecanol	8,4	no toxicity data found			

57-11-4	Octadecanoic acid	8,4	no toxicity data found			
57-10-3	n-Hexadecanoic acid	8,0	no toxicity data found			
10522-26-6	2-Methyl-1-undecanol	7,6	no toxicity data found			
616-03-5	2,4-Imidazolidinedione, 5-methyl-	7,3	no toxicity data found			
84-74-2	Dibutyl phthalate	6,6	10	350	3900	400
85-68-7	Benzyl butyl phthalate	4,7	140	630	2200	190
467-84-5	Phenadoxone	3,9	no toxicity data found			
872-50-4	2-Pyrrolidinone, 1-methyl-	3,7	no toxicity data found			
10522-26-6	2-Methyl-1-undecanol	3,4	no toxicity data found			
91-20-3	Naphthalene	3,4	450	120	5700	33000
4884-24-6	[1,1'-Bicyclopentyl]-2-one	3,4				
10522-26-6	1-methyl-1-undecanol	3,2				
2416-20-8	Hexadecenoic acid, Z-11-	3,0				
934-80-5	Benzene, 4-ethyl-1,2-dimethyl-	2,9				
101-84-8	Diphenyl ether	2,6				
13643-96-4	2H-Thiopyran-3-carboxaldehyde, 5,6-dihydro-2,6-dimethyl-	2,4				
95-48-7	Phenol, 2-methyl-	2,4				
25265-71-8	Dipropylene glycol	2,4				
54446-78-5	Ethanol, 1-(2-butoxyethoxy)-	2,2				
629-76-5	1-Pentadecanol	2,2				
100-51-6	Benzyl Alcohol	2,0		10000	55000	2600000
622-96-8	Benzene, 1-ethyl-4-methyl-	1,8				
22428-87-1	1,4-Dioxaspiro[4.5]decan-8-ol	1,8				
935-51-3	1,4-Dioxaspiro[4.5]decane, 8-methyl-	1,7				
544-63-8	Tetradecanoic acid	1,7				
20600-49-1	Benzothiazole, 2-(o-aminophenyl)-4-methyl-	1,7				
827-54-3	Naphthalene, 2-ethenyl-	1,6	no toxicity data found			
584-02-1	3-Pentanol	1,6	no toxicity data found			
2425-77-6	1-Decanol, 2-hexyl-	1,6	no toxicity data found			
930-68-7	2-Cyclohexen-1-one	1,5				
112-42-5	1-Undecanol	1,4				
19780-33-7	2-Ethyl-1-dodecanol	1,4				
3622-84-2	Benzenesulfonamide, N-butyl-	1,4				
120-89-8	2,4,5-Trioxoimidazolidine	1,3				
611-14-3	Benzene, 1-ethyl-2-methyl-	1,3	no toxicity data found			
140-66-9	Phenol, 4-(1,1,3,3-tetramethylbutyl)-	1,2	40000		90000	
620-14-4	Benzene, 1-ethyl-3-methyl-	1,2				
103-84-4	Acetamide, N-phenyl-	1,1				
645-66-9	Lauric anhydride	0,9	no toxicity data found			
4536-30-5	Ethanol, 2-(dodecyloxy)-	0,8				
585-34-2	Phenol, m-tert-butyl-	0,8	no toxicity data found			
EPA-222513	Tetramethyl diphosphan-oxide-sulfide	0,7				
87-41-2	1(3H)-Isobenzofuranone	0,7				
646-06-0	1,3-Dioxolane	0,7				
496-11-7	Indane	0,6	no toxicity data found			
766-97-2	Benzene, 1-ethynyl-4-methyl-	0,6				
123-63-7	Paraldehyde	0,6				

UK-1

CAS	Name	Concentration (µg/l)	Toxicity Fish chronic	Fish acute	Crustacean acute	Algea
100-54-9	3-Pyridinecarbonitrile	626	no toxicity data found			
23996-53-4	3-Imidazol-1-ylpropanenitrile	259	no toxicity data found			
1633-44-9	Pyridine-3,4-dicarbonitrile	182	no toxicity data found			
27090-63-7	1,6-Hexanediamine, N,N,N',N'-tetrabutyl-	128	no toxicity data found			
4177-16-6	Pyrazine, ethenyl-	123	no toxicity data found			
23996-53-4	3-Imidazol-1-ylpropanenitrile	88	no toxicity data found			
EPA-191171	4-Aminonicotinonitrile	77	no toxicity data found			
626-17-5	1,3-Benzenedicarbonitrile	56	no toxicity data found			
EPA-72017	2,3,4-Trimethoxymandelic acid, di-TMS	30	no toxicity data found			
1820-80-0	3-Aminopyrazole	30	no toxicity data found			
68-94-0	6H-Purin-6-one, 1,7-dihydro-	25	no toxicity data found			
98-92-0	Niacinamide	22	no toxicity data found			
544-13-8	Pentanedinitrile	19	no toxicity data found			
288-88-0	1H-1,2,4-Triazole	19	no toxicity data found			
108-50-9	Pyrazine, 2,6-dimethyl-	16	no toxicity data found			
10570-40-8	4H-1,2,4-Triazole, 4-methyl-	13	no toxicity data found			
17851-53-5	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	12	no toxicity data found			
117-81-7	Bis(2-ethylhexyl) phthalate	12	62	139000	2000	960
113124-09-7	3-Pyridinecarbonitrile, 5,6-dimethyl-	12	no toxicity data found			
626-17-5	1,3-Benzenedicarbonitrile	12	no toxicity data found			
253-66-7	Cinnoline	12	no toxicity data found			
271-29-4	1H-Pyrrolo(2,3-c)pyridine	10	no toxicity data found			
56145-23-4	2-Pyrrolidinecarboxylic acid, 1,2-dimethyl-5-oxo-, methyl ester	10	no toxicity data found			
7126-38-7	1H-Pyrrole-3-carbonitrile	8,2				
10347-14-5	Benzene-1,2,4-tricarbonitrile	7,5				
40160-23-4	Imidazole. 5-[2-(aminocarbonyl)ethyl]-	7,5				
62249-52-9	Bicyclo[2,2.2]oct-5-ene-2,3-dicarbonitrile	6,8				
84-74-2	Dibutyl phthalate	6,2	_	1480	2990	400
4458-33-7	Ethyl di-N-butylamine	5,3				
16411-13-5	Butanedinitrile, 2,3-dimethyl-	5,2				
504-29-0	2-Pyridinamine	4,6				
761-65-9	Formamide, N,N-dibutyl-	4,3				
931-54-4	Benzene, isocyano-	3,8				
27798-57-8	Benzoic acid, 2,6-bis(trimethylsiloxy)-, methyl ester	3,8				

P2

CAS	Name	Conc. ug/l	Toxicity Fish chronic	Fish acute	crustacean acute	crustacean chronic	Algae
44898-60-4	2-Propen-1-amine, N,N-bis(1-methylethyl)-	273		no toxicity data found			
28900-91-6	Formylmethylenetriphenylphosphorane	226	no toxicity data found				
111-96-6	Ethane, 1,1'-oxybis[2-methoxy-	115			340000	28000	
58-08-2	Caffeine	65		805000	47000		47000
828-94-4	5-Methoxy-2,3-dimethylindole	54			no toxicity data found		
516-95-0	Epicholestanol	54			no toxicity data found		
117-81-7	Bis(2-ethylhexyl) phthalate	30	62	139000	2000		960
57-10-3	n-Hexadecanoic acid	29			no toxicity data found		
519-73-3	Triphenylmethane	21			no toxicity data found		
76-84-6	Benzenemethanol, à,à-diphenyl-	20			no toxicity data found		

P1

CAS	Name	Conc. ug/l	Fish chronic	Fish acute	Crustacean acute	Crustacean chronic	algae
EPA-196185	1-Octadecanamine, N-(1-methoxyethyl)-N-methyl-	23					
541-02-6	Cyclopentasiloxane, decamethyl-	12	no toxicity data found				
112-79-8	9-Octadecenoic acid, (E)-	9,3	no toxicity data found				
85-69-8	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	8,1		>190	56		>320
57-10-3	n-Hexadecanoic acid	7,9	no toxicity data found				
2130-62-3	Phenol, 2,4,6-tricyclohexyl-	7,4	no toxicity data found				
540-97-6	Cyclohexasiloxane, dodecamethyl-	7,1	7	28	40		33
54446-78-5	Ethanol, 1-(2-butoxyethoxy)-	6,0	no toxicity data found				
541-01-5	Heptasiloxane, hexadecamethyl-	4,8	no toxicity data found				
84-74-2	Dibutyl phthalate	4,0	100	350	3900		400
n/a	1-Triacontanol	3,9	no toxicity data found				
556-67-2	Cyclotetrasiloxane, octamethyl-	3,8	280	360		1,7	260
107-41-5	Hexylene Glycol	3,7		9500000	3200000		
630-03-5	Nonacosane	3,4	no toxicity data found				
57-11-4	Octadecanoic acid	3,3	no toxicity data found				
55759-91-6	2-Propenal, 3-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-	3,2	no toxicity data found				
630-02-4	Octacosane	2,9	no toxicity data found				
100-51-6	Benzyl Alcohol	2,7			26000		
107-50-6	Cycloheptasiloxane, tetradecamethyl-	1,9	no toxicity data found				
111-90-0	Ethanol, 2-(2-ethoxyethoxy)-	1,8		134000000	3340000		
111-96-6	Ethane, 1,1'-oxybis[2-methoxy-	1,6	no toxicity data found				
556-68-3	Cyclooctasiloxane, hexadecamethyl-	1,5	no toxicity data found				
119-65-3	Isoquinoline	1,1					
629-96-9	1-Eicosanol	1,0					
120714-42-3	Benzoic acid, 2-acetyl-3-methoxy-	1,0					
111-77-3	Ethanol, 2-(2-methoxyethoxy)-	1,0					
544-63-8	Tetradecanoic acid	1,0					
5746-58-7	Tetradecanoic acid, 12-methyl-, (S)-	0,9					
EPA-143498	6,10,14-Trimethyl-pentadecan-2-ol	0,9					
100-54-9	3-Pyridinecarbonitrile	0,9					
140-29-4	Benzyl nitrile	0,9					
2719-62-2	Benzene, (1-pentylheptyl)-	0,8					
EPA-146517	Tetracosamethyl-cyclododecasiloxane	0,8					

556-71-8	Cyclononasiloxane, octadecamethyl-	0,7	no toxicity data found
18772-36-6	Cyclodecasiloxane, eicosamethyl-	0,7	no toxicity data found
EPA-146517	Tetracosamethyl-cyclododecasiloxane	0,7	
2719-64-4	Benzene, (1-propylnonyl)-	0,7	
EPA-197209	Benzeneethanamine, á-hydroxy-à-methyl-N-octadecyl-	0,6	
19780-33-7	2-Ethyl-1-dodecanol	0,6	
108-95-2	Phenol	0,6	
2398-66-5	Benzene, (1-methylnonadecyl)-	0,5	
2234-75-5	Cyclohexane, 1,2,4-trimethyl-	0,4	

BE-1

			Toxicity (µg/l)			
CAS	Name	Conc. ug/l	Fish acute	crustacean acute	crustacean chronic	algae
117-81-7	Bis(2-ethylhexyl) phthalate	343	139000	2000	77	960
112-79-8	9-Octadecenoic acid, (E)-	273	no toxicity data found			
57-10-3	n-Hexadecanoic acid	129	11000			
58-08-2	Caffeine	97	805000	47000		47000
59-50-7	Pheno, 4-chloro-3-methyl	95	1000	19000	13000	
112-18-5	1-Dodecanamine, N,N-dimethyl-	77	no toxicity data found			
111-76-2	Ethanol, 2-butoxy-	75		1815000		
85-68-7	Benzyl butyl phthalate	73	820	1000	260	100
516-95-0	Epicholestanol	62	no toxicity data found			
84-69-5	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	31	no toxicity data found			
106-44-5	Phenol, 4-methyl-	30		1400	1000	
78-51-3	Ethanol, 2-butoxy-, phosphate (3:1)	21	no toxicity data found			
137-58-6	Lidocaine	21	no toxicity data found			
84-74-2	Dibutyl phthalate	20	1480	2990	500	400
120-32-1	Clorophene	16	720	590		
645-66-9	Lauric anhydride	16	no toxicity data found			
122-99-6	Ethanol, 2-phenoxy-	13		52000	3800	69500
84-66-2	Diethyl Phthalate	13	12000			84100
3622-84-2	Benzenesulfonamide, N-butyl-	12				
111-90-0	Ethanol, 2-(2-ethoxyethoxy)-	11				
EPA-129243	N,N-Dimethyltetradecanamine	10				
67-64-1	Acetone	10				
102-18-1	1,2-Ethanediamine, N,N'-dimethyl-N,N'-bis(phenylmethyl)-	10				
104-76-7	1-Hexanol, 2-ethyl-	9,3				
36653-82-4	1-Hexadecanol	8,8				
2416-20-8	Hexadecenoic acid, Z-11-	8,2				
1120-36-1	1-Tetradecene	7,9				
60-12-8	Phenylethyl Alcohol	6,9				
108-95-2	Phenol	5,1				
54446-78-5	Ethanol, 1-(2-butoxyethoxy)-	5,0	no toxicity data found			
18829-55-5	2-Heptenal, (E)-	4,6				
3913-81-3	2-Decenal, (E)-	4,3				
55619-05-1	1,3,5-Cycloheptatriene, 3-chloro-	4,1				
56143-21-6	Benzeneacetic acid, à-methoxy-, methyl ester, (ñ)-	4,0				
112-70-9	1-Tridecanol	4,0				

BE-2

		Toxicity (µg/l)				
CAS	Name	Conc. µg/l	Fish acute	crustacean acute	crustacean chronic	algae
112-18-5	1-Dodecanamine, N,N-dimethyl-	586	no toxicity data found			
100-44-7	Benzyl chloride	465	4000			
4169-04-4	1-Propanol, 2-phenoxy-	459	no toxicity data found			
EPA-129243	N,N-Dimethyltetradecanamine	399				
3878-46-4	Carbonic acid, ethyl phenyl ester	296	no toxicity data found			
n/a	N-Methyl-N-benzyltetradecanamine	260				
122-99-6	Ethanol, 2-phenoxy-	260	no toxicity data found			
6180-61-6	1-Propanol, 3-phenoxy-	242	no toxicity data found			
EPA-129243	N,N-Dimethyltetradecanamine	210				
117-81-7	Bis(2-ethylhexyl) phthalate	187	>100	>160	77	>100
57-10-3	n-Hexadecanoic acid	143	no toxicity data found			
57-11-4	Octadecanoic acid	136	no toxicity data found			
98-55-5	3-Cyclohexene-1-methanol, à,à4-trimethyl-	120	no toxicity data found			
EPA-129242	N-Methyl-N-benzyltetradecanamine	118				
112-79-8	9-Octadecenoic acid, (E)-	116	no toxicity data found			
112-53-8	1-Dodecanol	90	no toxicity data found			
6180-61-6	1-Propanol, 3-phenoxy-	85	no toxicity data found			
58-08-2	Caffeine	68	805000	47000		47000
80-97-7	Cholestanol	65	no toxicity data found			
111-76-2	Ethanol, 2-butoxy-	60		1815000		
36653-82-4	1-Hexadecanol	58	no toxicity data found			
84-69-5	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	55	no toxicity data found			
EPA-210384	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol	53				
104-76-7	1-Hexanol, 2-ethyl-	37	32000			
544-63-8	Tetradecanoic acid	32	no toxicity data found			
645-66-9	Lauric anhydride	32	no toxicity data found			
111-02-4	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl	29				
84-74-2	Dibutyl phthalate	28	1480	2990	500	400
4536-30-5	Ethanol, 2-(dodecyloxy)-	24				
84-66-2	Diethyl Phthalate	24	12000			84100
3622-84-2	Benzenesulfonamide, N-butyl-	23				
103-83-3	Benzenemethanamine, N,N-dimethyl-	21				
112-92-5	1-Octadecanol	17				
78-51-3	Ethanol, 2-butoxy-, phosphate (3:1)	17				
120-51-4	Benzyl Benzoate	15				
138-87-4	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-	15				
2136-70-1	Ethanol, 2-(tetradecyloxy)-	15				
EPA-124558	Cholestadiene	12				
464-45-9	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-	11				
60-12-8	Phenylethyl Alcohol	11				
586-82-3	3-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-	10				
EPA-128086	D,à-Tocopherol	10				
58422-92-7	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	10				
1164-16-5	N-Benzyloxycarbonyl-L-tyrosine	8,3				
17696-61-6	Isobutyl p-hydroxybenzoate	7,9				
94-13-3	Propylparaben	7,7				
85-68-7	Benzyl butyl phthalate	7,5	820	1000	260	100

94-26-8	Butylparaben	7,5
629-76-5	1-Pentadecanol	6,4
112-34-5	Ethanol, 2-(2-butoxyethoxy)-	6,3
58422-92-7	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	6,1
7299-40-3	cis-á-Terpineol	6,0
470-67-7	7-Oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-	5,9
646-31-1	Tetracosane	5,7
2566-97-4	9,12-Octadecadienoic acid, methyl ester, (E,E)-	5,7
18769-46-5	Cholestan-3-ol, (3à)-	5,6
17696-61-6	Isobutyl p-hydroxybenzoate	5,3
58422-92-7	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	5,2
119-61-9	Benzophenone	5,0
120-47-8	Ethylparaben	5,0
99-76-3	Methylparaben	4,9
112-70-9	1-Tridecanol	4,7
1085-12-7	Benzoic acid, 4-hydroxy-, n-heptyl ester	4,7
n/a	Benzaldehyde, 3,4-dibenzyloxy-	4,7
1632-73-1	Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-	4,4
3777-70-6	Furan, 2-hexyl-	4,1
17312-55-9	Decane, 3,8-dimethyl-	3,9
111-87-5	1-Octanol	3,8
62108-16-1	1H-Indole, 2,3-dihydro-4-methyl-	3,8
621-87-4	2-Propanone, 1-phenoxy-	3,6
41977-45-1	Bicyclo[4.1.0]heptane, 7-pentyl-	3,5
24863-70-5	1-Cyclopentene-1-acetic acid, 3-oxo-2-pentyl-, methyl ester	3,5
33351-43-8	Isoquinoline, 1-butyl-3,4-dihydro-	3,5
115-96-8	Tri(2-chloroethyl) phosphate	3,5

BE-3

CAS	Name	Conc. µg/l	Toxicity (µg/l) Fish chronic	Fish acute	crustacean acute	crustacean chronic	algae
112-79-8	9-Octadecenoic acid, (E)-	239		14000			
117-81-7	Bis(2-ethylhexyl) phthalate	198	62	139000	2000		960
57-10-3	n-Hexadecanoic acid	194	no toxicity data found				
122-99-6	Ethanol, 2-phenoxy-	128			52000	3800	69500
57-11-4	Octadecanoic acid	115	no toxicity data found				
111-76-2	Ethanol, 2-butoxy-	107			1815000		
58-08-2	Caffeine	105			160000		
80-97-7	Cholestanol	80	no toxicity data found				
EPA-210384	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol	61					
544-63-8	Tetradecanoic acid	52	no toxicity data found				
102-69-2	1-Propanamine, N,N-dipropyl-	30	no toxicity data found				
17851-53-5	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	26	no toxicity data found				
20324-32-7	2-Propanol, 1-(2-methoxy-1-methylethoxy)-	20	no toxicity data found				
645-66-9	Lauric anhydride	19	no toxicity data found				
54446-78-5	Ethanol, 1-(2-butoxyethoxy)-	19	no toxicity data found				
106-44-5	Phenol, 4-methyl-	18					
112-18-5	1-Dodecanamine, N,N-dimethyl-	14					
103-23-1	Hexanedioic acid, bis(2-ethylhexyl) ester	13					
1002-84-2	Pentadecanoic acid	13					
10482-56-1	3-Cyclohexene-1-methanol, à,à,4-trimethyl-, (S)-	13					
102-18-1	1,2-Ethanediamine, N,N'-dimethyl-N,N'-bis(phenylmethyl)-	13					
84-74-2	Dibutyl phthalate	12		1480	2990	500	400
108-95-2	Phenol	10					
78-51-3	Ethanol, 2-butoxy-, phosphate (3:1)	9,2					
36653-82-4	1-Hexadecanol	7,5					
2363-88-4	2,4-Decadienal	6,8					
3913-81-3	2-Decenal, (E)-	6,5					
104-68-7	Ethanol, 2-(2-phenoxyethoxy)-	5,9					
112-53-8	1-Dodecanol	5,4					
18829-55-5	2-Heptenal, (E)-	5,4					
3622-84-2	Benzenesulfonamide, N-butyl-	5,1					
868-77-9	2-Propenoic acid, 2-methyl-, 2-hydroxyethyl ester	5,0					
104-68-7	Ethanol, 2-(2-phenoxyethoxy)-	4,9					
84-66-2	Diethyl Phthalate	4,8		12000			84100

D-1

Name	CAS-nr.	Concentration (µg/l)
?? ethanol, 2-(2-butoxyethoxy)-	112-34-5	12,32
m-pyrol	872-50-4	11,31
9,10-anthracenedione	84-65-1	4,47
ethanol, 2-(2-ethoxyethoxy)-	111-90-0	4,28
??? n-tricosane (C23)	638-67-5	2,23
??? benzene, 1-methoxy-4-nitro-	100-17-4	1,55
??? docosane (C22)	629-97-0	0,90
toluidine (o-), 5-chloro-	95-79-4	0,76

??? hexadecanoic acid (palmitic acid)	57-10-3	0,74
diisobutyl phthalate	84-69-5	0,61
??? cyclopentasiloxane, decamethyl-	541-02-6	0,59
??? heptadecane	629-78-7	0,51
??? bis(2-methoxyethyl) phthalate	117-82-8	0,48
?? dibutyl phthalate	84-74-2	0,45
??? stearineacid (octadecanoic acid)	57-11-4	0,42
? bis(2-ethylhexyl) phthalate	117-81-7	0,39
??? heneicosane (C21)	629-94-7	0,35
??? octadecane (C18)	593-45-3	0,29
??? hexadecane	544-76-3	0,25
??? phytane (hexadecane, 2,6,10,14-tetramethyl-)	638-36-8	0,24
??? eicosane (C20)	112-95-8	0,24
dimethyl phthalate	131-11-3	0,22
2-propanol, 1-butoxy-	5131-66-8	0,21
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,20
triisobutylphosphate	126-71-6	0,19
ethanol, 2-butoxy-	111-76-2	0,14
phthalic acid, diethyl ester	84-66-2	0,13
benzene, 1-chloro-2-nitro-	88-73-3	0,13
??? pentadecane	629-62-9	0,12
2-propanol, 1-butoxy-	5131-66-8	0,12
hexanoic acid, 2-ethyl-	149-57-5	0,12
isoquinoline	119-65-3	0,10
ethanol, 2-butoxy-	111-76-2	0,10

D-2

Name	CAS-nr.	Conc. (µg/l)	Toxicity (µg/l)			
			Fish chronic	Fish acute	crustacean acute	algae
diisobutyl phthalate	84-69-5	2,658	No data found			
? bis(2-methoxyethyl) phthalate	117-82-8	2,549	No data found			
??? cyclopentasiloxane, decamethyl-	541-02-6	1,760	No data found			
? bis(2-ethylhexyl) phthalate	117-81-7	1,360	62	139000	2000	960
triisobutylphosphate	126-71-6	1,329	No data found			
hexanoic acid, 2-ethyl-	149-57-5	0,903				
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,691	No data found			
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,412				
9,10-anthracenedione	84-65-1	0,387				
toluene, a-chloro-	100-44-7	0,384				
??? squalene (all trans) (2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamet	111-02-4 / 7683	0,377				
??? undecene-1	821-95-4	0,369				
limonene	138-86-3	0,348				
??? stearineacid (octadecanoic acid)	57-11-4	0,341				
pyrrolidinedion(2,5), 1-methyl-	1121-07-9	0,306				
??? tetradecane	629-59-4	0,279				
??? tetradecane	629-59-4	0,271				
toluidine (o-), 5-chloro-	95-79-4	0,264				
??? diaziridine, 1,2-dipropyl-	6794-92-9	0,258				
phthalic acid, diethyl ester	84-66-2	0,253				
??? oxirane, [(2-propenyloxy)methyl]-	106-92-3	0,188				

??? cyclopentanon	no CAS109	0,175
??? hexadecanoic acid (palmitic acid)	57-10-3	0,149
? heptanoic acid	111-14-8	0,137
??? di-2-ethylhexyladipate	103-23-1	0,127
?? tetradecane, 1-chloro-	2425-54-9	0,110
toluene, 3-chloro-	108-41-8	0,101
??? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	0,098
??? o-hydroxybiphenyl	90-43-7	0,093
??? eicosene	3452-07-1	0,093
??? catechol, 4-tert-butyl-	98-29-3	0,093
??? triethyleneglycol diacetate	111-21-7	0,092
??? sulphur (S8)	10544-50-0	0,088
benzenamine, n,n-diethyl-	91-66-7	0,085
??? pentadecane	629-62-9	0,079
??? decanol-1	112-30-1	0,076
??? octadecanoic acid, butyl ester	123-95-5	0,074
??? n-octacosane (C28)	63-02-4	0,074
??? nonanoic acid	112-05-0	0,073
acetophenone	98-86-2	0,073
??? hexane, 2-methyl-	591-76-4	0,068
benzenesulfonamide, N-butyl-	3622-84-2	0,062
??? dioxolane-1,3, 2-(2-propenyl)-	38653-49-5	0,059
? heptane, 2,2,4,6,6-pentamethyl-	13475-82-6	0,058
??? decene-1	872-05-9	0,056
??? oxirane, (2-ethylhexyl)oxy methyl-	2461-15-6	0,056
??? hexanol-1, 5-methyl-	627-98-5	0,052
??? pentane, 3,3-dimethyl-	562-49-2	0,051
??? C9-alcohol	28473-21-4	0,051
??? benzene, 1-methoxy-4-nitro-	100-17-4	0,050

DU-3

		Toxicity				
Name	CAS-nr.	Conc. (µg/l)	Fish chronic	Fish acute	crustacean acute	algae acute
? bis(2-methoxyethyl) phthalate	117-82-8	1,740	no data found			
??? cyclopentasiloxane, decamethyl-	541-02-6	1,492	No data found			
diisobutyl phthalate	84-69-5	1,401	No data found			
??? octadecanoic acid, butyl ester	123-95-5	1,025	No data found			
triisobutylphosphate	126-71-6	1,000	No data found			
hexanoic acid, 2-ethyl-	149-57-5	0,719	No data found			
? bis(2-ethylhexyl) phthalate	117-81-7	0,694	62	139000	2000	960
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,376	No data found			
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,235				
??? undecene-1	821-95-4	0,216				
??? tetradecane	629-59-4	0,161				
??? squalene (all trans) (2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamet	111-02-4 / 7683	0,099				
??? cyclopentanon	no CAS109	0,081				
?? di-2-ethylhexyladipate	103-23-1	0,079				
heptanoic acid	111-14-8	0,078				
phthalic acid, diethyl ester	84-66-2	0,071				
??? tetradecene-5	41446-66-6	0,068				

acetophenone	98-86-2	0,057
??? pentene-1, 3-methyl-	760-20-3	0,047
??? decanol-1	112-30-1	0,047
BHT (butylated hydroxytoluene, antioxidant, Ionol)	128-37-0	0,046
limonene	138-86-3	0,045
??? eicosene	3452-07-1	0,044
??? decene-1	872-05-9	0,044
??? pentene, 2,4-dimethyl-	2213-32-3	0,041
?? heptane, 2,2,4,6,6-pentamethyl-	13475-82-6	0,039
??? pyrrolidine, 2,5-dion-3-ethyl-1,3-dimethyl-	13861-99-9	0,037
? hexane, 2,3,5-trimethyl-	1069-53-0	0,037
?? tetradecane, 1-chloro-	2425-54-9	0,036
?? pentadecane	629-62-9	0,036
??? C9-alcohol	28473-21-4	0,036
??? 1-hexanol	111-27-3	0,036
nonane	111-84-2	0,034
nonanal	124-19-6	0,032
alpha-Methylstyrene	98-83-9	0,026
??? hexane, 2-methyl-	591-76-4	0,025
??? butane-2,3-diol	513-85-9	0,025
tributyl phosphate	126-73-8	0,022
??? pentane, 1-chloro-	543-59-9	0,022
? pyrrolidinedion(2,5), 1-methyl-	1121-07-9	0,020
??? nonanol-1	143-08-8	0,020
??? acetic acid, octadecyl ester	822-23-1	0,019
??? pentane, 3,3-dimethyl-	562-49-2	0,019
heptane, 2,3-dimethyl-	3074-71-3	0,018
? n-octacosane (C28)	63-02-4	0,018
hexane, 2,3,3-trimethyl-	16747-28-7	0,018
? hexanon-2	591-78-6	0,018
?? nonanoic acid	112-05-0	0,017
benzothiazole	95-16-9	0,016
butane, 2-methoxy-2-methyl-	62016-49-3	0,016
acetic acid, 1-methylethyl ester (isopropylacetate)	108-21-4	0,016
??? cyclohexanol	108-93-0	0,015
tridecane	629-50-5	0,014
?? triethyleneglycol diacetate	111-21-7	0,014
??? decanal	112-31-2	0,013
??? dodecane	112-40-3	0,013
styrene	100-42-5	0,012
??? propylacetate	109-60-4	0,011
??? cyclohexane, 1,1,3-trimethyl-	3073-66-3	0,011
phthalic acid, benzyl butyl ester	85-68-7	0,010
tris(2-chloroisopropyl)phosphate (Fyrol-PCF)	13674-84-5	0,010
??? p/m-xylene (1,4-dimethyl-benzene/1,3-dimethyl-benzene)	106-42-3/108-38	0,009
??? phenol, 2,4,6-tri-tert-butyl- ?	732-26-3	0,009
??? octanal	124-13-0	0,009
dimethyl phthalate	131-11-3	0,009
??? palmitoleic acid, methyl ester	1120-25-8	0,008
??? cyclohexane, 1,1,2-trimethyl-	7094-26-0	0,008
octane	111-65-9	0,008

hexadecane	544-76-3	0,008
??? hexadecanoic acid (palmitic acid)	57-10-3	0,007
??? n-nonacosane (C29)	630-03-5	0,007
cyclohexane, 1,2,3-trimethyl-	1678-81-5	0,007
benzene, 1,2,4-trimethyl-	95-63-6	0,007
?? n-hexacosane (C26)	630-01-3	0,006
? octane, 3,6-dimethyl-	15869-94-0	0,006
n-heptacosane (C27)	593-49-7	0,006
stearineacid (octadecanoic acid)	57-11-4	0,006
? juvabione	17904-27-7	0,006
docosane (C22)	629-97-0	0,006
octadecane (C18)	593-45-3	0,006
cyclohexane, 1,3,5-trimethyl-	1839-63-0	0,005
?? phenanthrene	85-01-8	0,005
??? hexane, 2,2-dimethyl-	590-73-8	0,005
? phenol, di-(tertbutyl)-	no CAS132	0,005
benzenesulfonamide, N-butyl-	3622-84-2	0,005
?? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	0,005

DU-4

Name	CAS-nr.	Conc. (µg/l)	Toxicity (µg/l)			
			Fish chronic	Fish acute	crustacea acute	algae
??? cyclopentasiloxane, decamethyl-	541-02-6	2,051	No data found			
? bis(2-methoxyethyl) phthalate	117-82-8	1,496	No data found			
diisobutyl phthalate	84-69-5	1,309	No data found			
triisobutylphosphate	126-71-6	1,130	No data found			
? bis(2-ethylhexyl) phthalate	117-81-7	0,897	62	139000	2000	960
hexanoic acid, 2-ethyl-	149-57-5	0,769				
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,567	No data found			
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,339				
??? undecene-1	821-95-4	0,239				
??? squalene (all trans) (2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamet	111-02-4 / 7683	0,181				
??? tetradecane	629-59-4	0,174				
phthalic acid, diethyl ester	84-66-2	0,153				
? heptanoic acid	111-14-8	0,080				
??? decanol-1	112-30-1	0,075				
??? cyclopentanon	no CAS109	0,073				
??? cyclopentane, isobutyl-	3788-32-7	0,064				
?? di-2-ethylhexyladipate	103-23-1	0,061				
??? triethyleneglycol diacetate	111-21-7	0,057				
acetophenone	98-86-2	0,057				
??? eicosene	3452-07-1	0,054				
? nonanal	124-19-6	0,050				
??? decene-1	872-05-9	0,047				
?? pentadecane	629-62-9	0,043				
??? C9-alcohol	28473-21-4	0,042				
? tetradecane, 1-chloro-	2425-54-9	0,041				
??? nonanoic acid	112-05-0	0,040				
??? C9-alcohol	28473-21-4	0,040				
??? heptane, 2,2,4,6,6-pentamethyl-	13475-82-6	0,038				

benzothiazole	95-16-9	0,034
??? hexadecanoic acid (palmitic acid)	57-10-3	0,033
??? cyclopropane, 1-heptyl-2-methyl-	74663-91-5	0,030
limonene	138-86-3	0,029
??? nonanol-1	143-08-8	0,027
??? n-hexacosane (C26)	630-01-3	0,026
alpha-Methylstyrene	98-83-9	0,025
??? decanal	112-31-2	0,024
??? hexane, 2-methyl-	591-76-4	0,023
??? pentane, 3,3-dimethyl-	562-49-2	0,022
??? octaneacid (caprylacid)	124-07-2	0,019
? heptane, 2,3-dimethyl-	3074-71-3	0,018
??? dioxolane-1,3, 2-(2-propenyl)-	38653-49-5	0,018
??? pentane, 1-chloro-	543-59-9	0,018
??? hexane, 2,3,5-trimethyl-	1069-53-0	0,017
??? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	0,016
? triacetin	102-76-1	0,016
tributyl phosphate	126-73-8	0,016
formamide, N,N-dibutyl-	761-65-9	0,015
?? tridecane	629-50-5	0,015
BHT (butylated hydroxytoluene, antioxidant, Ionol)	128-37-0	0,015
??? hexanol-1, 5-methyl-	627-98-5	0,015
surfynol 104 (2,4,7,9-tetramethyl-5-decyne-4,7-diol)	126-86-3	0,015
phthalic acid, benzyl butyl ester	85-68-7	0,014
butane, 2-methoxy-2-methyl-	62016-49-3	0,014
acetic acid, 1-methylethyl ester (isopropylacetate)	108-21-4	0,013
??? dodecane	112-40-3	0,013
styrene	100-42-5	0,013
??? isoalkane (C21)	no CAS165	0,012
tris(2-chloroisopropyl)phosphate (Fyrol-PCF)	13674-84-5	0,012
??? 1-hexanol	111-27-3	0,012
?? stearineacid (octadecanoic acid)	57-11-4	0,011
??? phenol, di-(tertbutyl)-	no CAS132	0,010
? n-nonacosane (C29)	630-03-5	0,010
??? heptanon-2, 1-ethoxy-	51149-70-3	0,010
? dimethyl phthalate	131-11-3	0,010
??? p/m-xylene (1,4-dimethyl-benzene/1,3-dimethyl-benzene)	106-42-3/108-38	0,009
furan, tetrahydro-2,5-dimethoxy-	696-59-3	0,008
? acetophenon, 4-(1-hydroxy-1-methylethyl)-	54549-72-3	0,008
??? octanal	124-13-0	0,008
n-heptacosane (C27)	593-49-7	0,008
? benzenesulfonamide, N-butyl-	3622-84-2	0,007
benzene, 1,2,4-trimethyl-	95-63-6	0,007
? ethyl citrate	77-93-0	0,007
??? pentanon-2, 5-methoxy-	17429-04-8	0,007
? isopropyl myristate	110-27-0	0,007
??? ethanol, 2-butoxy-	111-76-2	0,007
? azobenzene	103-33-3	0,006
??? octadecanoic acid, butyl ester	123-95-5	0,006
?? octadecane (C18)	593-45-3	0,006
?? methyldihydrojasmonate	24851-98-7	0,006

??? naphthalene, 2-ethyl-	939-27-5	0,006
hexadecane	544-76-3	0,006
benzene, propyl-	103-65-1	0,005
??? cyclohexane, dodecyl-	54105-66-7	0,005

IR-1

Name	Cas-nr.	Concentration (µg/L)
??? cyclopentasiloxane, decamethyl-	541-02-6	2,81
diisobutyl phthalate	84-69-5	2,19
? bis(2-methoxyethyl) phthalate	117-82-8	2,07
triisobutylphosphate	126-71-6	2,05
??? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	1,20
? bis(2-ethylhexyl) phthalate	117-81-7	1,18
??? octadecanoic acid, butyl ester	123-95-5	1,01
hexanoic acid, 2-ethyl-	149-57-5	0,88
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,74
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,42
limonene	138-86-3	0,29
??? undecene-1	821-95-4	0,25
??? tetradecane	629-59-4	0,24
? BHT (butylated hydroxytoluene, antioxidant, Ionol)	128-37-0	0,21
phthalic acid, diethyl ester	84-66-2	0,18
pyrrolidinedion(2,5), 1-methyl-	1121-07-9	0,16
??? 1-hexanol	111-27-3	0,14
??? diaziridine, 1,2-dipropyl-	6794-92-9	0,14
??? cyclopentane, isobutyl-	3788-32-7	0,14
??? m-pyrol	872-50-4	0,14
tributyl phosphate	126-73-8	0,14
??? stearineacid (octadecanoic acid)	57-11-4	0,13
? heptanoic acid	111-14-8	0,13
nonanal	124-19-6	0,13
??? cyclopentene, 3-methyl-	1120-62-3	0,11
??? oxirane, [(2-propenyloxy)methyl]-	106-92-3	0,11

IR-2

Name	Cas-nr.	Concentration (µg/l)
??? hexadecanoic acid (palmitic acid)	57-10-3	4,19
? bis(2-methoxyethyl) phthalate	117-82-8	2,60
diisobutyl phthalate	84-69-5	2,16
??? stearineacid (octadecanoic acid)	57-11-4	2,10
? bis(2-ethylhexyl) phthalate	117-81-7	1,67
??? cyclopentasiloxane, decamethyl-	541-02-6	1,06
triisobutylphosphate	126-71-6	0,94
hexanoic acid, 2-ethyl-	149-57-5	0,76
??? n-dodecanoic acid (n-lauric acid)	143-07-7	0,64
??? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	0,61
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,60
??? octadecanoic acid, butyl ester	123-95-5	0,53
toluene, a-chloro-	100-44-7	0,39
caffeine	58-08-2	0,39
phthalic acid, diethyl ester	84-66-2	0,29
? heptanoic acid	111-14-8	0,28
?? octaneacid (caprylacid)	124-07-2	0,21
cresol (m-) (phenol, 3-methyl-)	108-39-4	0,19
??? dioxolane-1,3, 2-(2-propenyl)-	38653-49-5	0,14

dimethyl phthalate	131-11-3	0,14
??? tetradecane	629-59-4	0,14
??? triethyleneglycol diacetate	111-21-7	0,13
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,13
pyridine, 2,4,6-trimethyl-	108-75-8	0,13
??? nonanoic acid	112-05-0	0,13
??? ethanol, 2-(2-butoxyethoxy)-, acetate	124-17-4	0,13
??? di-2-ethylhexyladipate	103-23-1	0,12
??? undecene-1	821-95-4	0,11

IR-3

Name	CAS-nr	Concentration (µg/l)
bis(2-ethylhexyl) phthalate	117-81-7	6,379
triisobutylphosphate	126-71-6	3,574
diisobutyl phthalate	84-69-5	2,732
??? hexadecanoic acid (palmitic acid)	57-10-3	2,704
??? n-dodecanoic acid (n-lauric acid)	143-07-7	2,699
?? bis(2-methoxyethyl) phthalate	117-82-8	2,575
benzothiazole	95-16-9	2,146
??? cyclopentasiloxane, decamethyl-	541-02-6	2,028
1-Hexanol, 2-ethyl-	104-76-7	1,986
?? benzothiazole, 2-(methylthio)-	615-22-5	1,444
??? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	1,034
hexanoic acid, 2-ethyl-	149-57-5	1,011
??? octadecanoic acid, butyl ester	123-95-5	0,970
??? stearineacid (octadecanoic acid)	57-11-4	0,735
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,658
??? n-nonacosane (C29)	630-03-5	0,450
??? tetradecane	629-59-4	0,385
??? n-heptacosane (C27)	593-49-7	0,372
??? benzenemethanol, alpha,alpha-dimethyl-	617-94-7	0,326
??? benzene, azido	622-37-7	0,286
??? n-hexacosane (C26)	630-01-3	0,239
??? isooctanol	26952-21-6	0,229
? heptanoic acid	111-14-8	0,222
aniline	62-53-3	0,182
??? propofol	2078-54-8	0,173
? n-triacontane (C30)	638-68-6	0,170
??? pentadecane	629-62-9	0,165
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,160
caprolactam	105-60-2	0,160
??? di-2-ethylhexyladipate	103-23-1	0,152
indole	120-72-9	0,150
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,124
??? heptadecane	629-78-7	0,112
phthalic acid, diethyl ester	84-66-2	0,108
??? eicosene	3452-07-1	0,106
??? decene-1	872-05-9	0,101

SE

Name	CAS-NR.	Concentration (µg/L)
??? cyclopentasiloxane, decamethyl-	541-02-6	5,19
triisobutylphosphate	126-71-6	2,71
diisobutyl phthalate	84-69-5	1,64

? bis(2-methoxyethyl) phthalate	117-82-8	1,35
hexanoic acid, 2-ethyl-	149-57-5	0,81
??? ethane, 1,2-dibromo-	106-93-4	0,74
??? octadecanoic acid, butyl ester	123-95-5	0,72
??? butylpalmitate (hexadecanoic acid, butyl ester)	111-06-8	0,71
? bis(2-ethylhexyl) phthalate	117-81-7	0,69
?? benzene, 1,4-dimethoxy-	150-78-7	0,68
??? cyclotetrasiloxane, octamethyl-	556-67-2	0,63
? kodaflex txib (2,2,4-trimethyl-1,3-pentanediol diisobutyrate)	6846-50-0	0,60
2,4,8,10-tetraoxaspiro[5.5]undecane	126-54-5	0,40
?? tetradecane	629-59-4	0,21
??? thiophene, 2-methyl-5-propyl-	33933-73-2	0,21
??? undecene-1	821-95-4	0,18
??? squalene (all trans) (2,6,10,14,18,22-tetracosahexaene , 2,6,10,15,19,23-hexamet	111-02-4 / 7683	0,17
? heptanoic acid	111-14-8	0,16
? tributyl phosphate	126-73-8	0,15
? di-2-ethylhexyladipate	103-23-1	0,15
??? octane, 6-ethyl-2-methyl-	62016-19-7	0,15
??? furan, tetrahydro-2,2-dimethyl-5-secbutyl-	33978-70-0	0,14
??? 4-methoxyphenol	150-76-5	0,13
??? nonane, 2,3-dimethyl-	2884-06-2	0,13
?? cyclopentene, 3-methyl-	1120-62-3	0,10
??? nonanoic acid	112-05-0	0,08
phthalic acid, diethyl ester	84-66-2	0,08
??? 1-hexanol	111-27-3	0,07
??? cyclohexanone	108-94-1	0,07
??? cyclopentane, isobutyl-	3788-32-7	0,07
??? octanal	124-13-0	0,07
??? eicosene	3452-07-1	0,07
??? BHT (butylated hydroxytoluene, antioxidant, Ionol)	128-37-0	0,07
acetophenone	98-86-2	0,06
??? decanol-1	112-30-1	0,06
benzene, 1-methyl-4-isopropyl- (p-cymene)	99-87-6	0,06
? bicyclo[310]hexane, 4-methylene-1-isopropyl	3387-41-5	0,06
??? 3,5-xylenol	108-68-9	0,06
??? tetradecane, 1-chloro-	2425-54-9	0,06
??? methylacetate, (4-methylphenyl)sulfonyl-,	50397-64-3	0,06
??? hexanol-1, 5-methyl-	627-98-5	0,05
??? octaneacid (caprylacid)	124-07-2	0,05
??? C9-alcohol	28473-21-4	0,05
?? phenol, 2,6-dimethoxy-	91-10-1	0,05
??? pentadecane	629-62-9	0,05
?? heptanon-2, 1-ethoxy-	51149-70-3	0,05
? benzene, 1,2,3-trimethyl-	526-73-8	0,05
limonene	138-86-3	0,05
??? 2,5-dimethyl-1,4-dioxane	15176-21-3	0,04
??? acetic acid, octadecyl ester	822-23-1	0,04
??? hexadecanoic acid (palmitic acid)	57-10-3	0,04
??? decene-1	872-05-9	0,04
? benzene, 1,2-dimethoxy-4-(1-propenyl)-	93-16-3	0,04
??? n-triacontane (C30)	638-68-6	0,04
??? b-citronellol	106-22-9	0,03
?? dioxolane-1,3, 2-(2-propenyl)-	38653-49-5	0,03
? surfynol 104 (2,4,7,9-tetramethyl-5-decyne-4,7-diol)	126-86-3	0,03

carene-3	13466-78-9	0,03
naphthalene, 1-methyl-	90-12-0	0,03
??? styrene, p-ethyl-	no CAS230	0,03
??? methanesulfanilide	1197-22-4	0,03
?? phthalic acid, benzyl butyl ester	85-68-7	0,02
quinoline, 2,7-dimethyl-	93-37-8	0,02
?? quinoline, 2,4-dimethyl-	1198-37-4	0,02
??? decanal	112-31-2	0,02
??? catechol, 4-tert-butyl-	98-29-3	0,02
tris(2-chloroisopropyl)phosphate (Fyrol-PCF)	13674-84-5	0,02
??? benzaldehyde, 4-hydroxy-3-methoxy-	121-33-5	0,02
??? benzofuranon-2(3H), 3-methyl-	32267-71-3	0,02
??? diphenyl methyl fosfine oxide	2129-89-7	0,02
? stearineacid (octadecanoic acid)	57-11-4	0,02
??? phenol, 2-(1,1-dimethylethyl)-	88-18-6	0,02
??? decanol-1	112-30-1	0,02
??? diphenylmethane	101-81-5	0,02
??? cyclohexaneamine, N-butylidene-	1197-52-0	0,02
?? n-octacosane (C28)	63-02-4	0,02
??? salicylaldehyde (of isomeer)	90-02-8	0,01
isopropyl myristate	110-27-0	0,01
??? ethyl citrate	77-93-0	0,01
?? n-hexacosane (C26)	630-01-3	0,01
??? n-heptacosane (C27)	593-49-7	0,01
styrene	100-42-5	0,01
aceton, 1-phenyl-	103-79-7	0,01
??? thiophene, 3-acetyl-	1468-83-3	0,01

Degradability and liability to bioaccumulate – Methods used in whole effluent assessment –

Report from the WEA IEG under OSPARCOM SPDS, February 2004

Contents

Summary and conclusions

1. In addition to single substances the Hazardous Substances strategy recognises that for many industries the complexity of their effluents means that it is more realistic to deal with the substances present as a single entity rather than attempting to measure them individually. A methodology for such an evaluation will include, in particular, biological test methods for the determination of persistence or biodegradability, acute and chronic ecotoxicity, genotoxicity/mutagenicity, and endocrine disruption as well as chemical simulation or surrogate methods for the evaluation of potential bioaccumulating substances.

2. A number of methods defined and validated for single substances have been adapted and are being used by some Contracting Parties for an assessment of the whole effluent content of persistent and potentially bio-accumulating substances.

3. Making a definition of persistence or degradability in the context of whole effluents is complex. For whole effluent assessment a working definition of persistence at present is taken as the persistence of toxicity and/or liability to bioaccumulate after a period of degradation.

4. Some of these methods, in particular those for biodegradability assessment, are used routinely by several laboratories, but there are differences between those Contracting Parties regarding under what circumstances they are used. Thus there is also experience in interpreting the results and assessing whether an effluent constitutes a potential problem in terms of showing hazardous characteristics. Such an assessment often requires both biological and chemical tests and always has a considerable degree of uncertainty as a consequence of the complex character of the effluent.

5. The main concern with persistent matter is when it is also potentially bioaccumulating. There are also Contracting Parties where toxicity in the persistent matter is the main concern, or where persistence alone is seen as an indication that entails further study of the wastewater. Although the use of these whole effluent assessment methods is supported by several Contracting Parties, there is as yet no common recommended set of methods.

6. The OSPAR practical study programme carried out in 2003 provided further information on the stage of development of the various available methods and showed the Contracting Parties the utility and the limitations of this approach.

7. In the opinion of the IEG, the proper choice of method for degradability (as a more appropriate term than persistence) assessment depends on the further destination of the wastewater:

   a) Wastewater discharged to a biological treatment plant should be tested with a method using a high inoculum concentration, such as the Zahn-Wellens test (ISO 9888/OECD 302B).

   b) Wastewater directly discharged to a natural recipient should be tested with a method using a low inoculum concentration, such as methods based on the ISO/OECD dissolved organic carbon die-away methods (ISO 7827/OECD 301A), or headspace CO_2 evolution test in closed vessels (ISO 14593/OECD 310 draft).

8. The methods for determination of potential bioaccumulation based on liquid extraction and chromatographic separation for the estimation of the octanol-water partition coefficient have provided useful information, and are in regular use by some Contracting Parties. The SPME method is an example of the more recent adsorbent based techniques. Although it does not account for elimination through the metabolism it seems to be closer to the natural situation and utilises fewer steps. Nevertheless it is still in the development stage.

1 Introduction

The OSPAR strategy with regard to hazardous substances aims at the prevention of pollution of the maritime area by continuous reduction of discharges, emissions and losses of hazardous substances, thereby moving towards the target of their cessation by 2020. The key element of the strategy is a dynamic selection and prioritisation mechanism and the development of criteria and methods that may be used for identification. Hazardous substances are defined as substances, which are persistent (P), liable to bioaccumulate (B) and toxic (T) and as substances, which do not meet all of the mentioned criteria, but give rise to an equivalent level of concern. In addition to a single substance approach as addressed in this strategy, it is recognised that the complexity of many industrial effluents makes it more realistic to analyse the substances present in those effluents as an entity rather than attempting to analyse them individually. A methodology for such an evaluation will include in particular biological test methods for the determination of persistence, acute and chronic ecotoxicity, genotoxicity/mutagenicity, and endocrine disruption as well as chemical simulation or surrogate methods for the evaluation of "Potential Bioaccumulating Substances", PBS. Such methods have been integrated in various national concepts for the evaluation of wastewater discharges ("Whole Effluent Assessment", WEA).

Whereas several of the Contracting Parties prescribe toxicity testing on a regular basis, bioaccumulation or degradability testing is performed with less regularity although methods are available or under development. This paper tries to give an outline of methods in use for wastewater characterisation, and to discuss to what extent they are relevant for OSPAR's goals. Further, important development work that is going on in some Contracting Parties is presented.

It is evident from the OSPAR background document concerning the elaboration of programmes and measures relating to Whole Effluent Assessment (OSPAR Commission 2000), as well as from the recent BREF on wastewater and waste gas treatment (EC 2002) that there is not a common way of applying WEA techniques or utilising the measurements as criteria in permitting among the Contracting Parties. In some Contracting Parties toxicity is considered to be the important variable, in others persistence is the variable of concern, especially in combination with bioaccumulation, whereas toxicity is not regarded to be quite as important. There is agreement that PBT measurements on whole effluent provides the added value of accounting for interactive effects that may be present in a complex water.

There are two major ways in which to apply WEA in the permitting of industrial facilities. Some Contracting Parties primarily set toxicity limits when defining the conditions of the permit. In these cases the tests will be performed with some regularity as specified in the permit. And there are Contracting Parties that in a permit prescribe a set of WEA tests, which are used to check whether the wastewater treatment is adequate to ascertain that negligible harm is done to the environment, as far as is detectable in a set of PBT tests. Some examples of the latter approach are presented in Annex 1 to this report. It is not the intention in this document to advocate one approach over the other. It should be stressed, however, that in order to assess whether an effluent could be environmentally hazardous not only acute toxicity should be tested, and that WEA is more than merely a PBT analysis. It has been stated that "learning by doing" in developing and applying these methods should not have legal consequences, but the extent to which these measurements are utilised in the national law application clearly varies.

Further, some Contracting Parties consider the determination of any remaining toxicity (after a degradation test) to be the most appropriate measurement of persistence, whereas in others chemical analysis is considered to be the correct method, the combination with toxicity being part of the WEA strategy as a whole. Apart from these general comments we will try to limit the discussion to effluent applications, i.e. recipient water tests, if such are feasible with these techniques, will not be considered.

To satisfy both operators and authorities the WEA methodology should have at least the following characteristics.

a. They should be scientifically valid, i.e. validated tests suitable for complex effluents, and with a clear test strategy.

b. They should also be robust, thus making it possible to set up in many laboratories for a stable performance.

c. They should be cost-effective, as simple and as rapid as is commensurate with the requirements above. There should be low-cost methods available for routine application.

d. The variables monitored and the endpoints chosen should predict real environmental effects.

e. Methodologies should be tiered so as to reduce time and expenditure while providing adequate information of the studied effluent.

Some laboratory standard tests should preferably be harmonised in Europe, whereas tests intended for recipient waters cannot be standardised due to wide differences in temperature, salinity and fauna in different regions. Because of this it also makes sense to have laboratory tests that use the local fauna, when possible.

1.1 Definitions (with some practical interpretations)

Primary degradation is an alteration in the chemical structure of a substance, resulting in the loss of a specific property of that substance (Blok & Balk 1994).

In this context it is important to recognise that toxicity may increase when breakdown products are formed, that is the product or products can be more toxic than the parent chemical. It should also be noted that in a complex sample, with several unknown substances present, toxicity would be an inadequate measure of degradation.

Ultimate biodegradation is the level of degradation achieved when the test compound is totally utilised by microorganisms resulting in the production of carbon dioxide, water, mineral salts and microbial biomass (Blok & Balk 1994).

Ready biodegradation is an arbitrary but widely accepted (EC 1967) classification of chemicals, which have passed certain tests for ultimate biodegradability (EC 1992, Annex C.4 A-F; OECD 1992a), whereas *Inherent biodegradation* is a classification of chemicals for which there is unequivocal evidence of biodegradation (primary or ultimate) in any test thereof (Blok & Balk 1994; EC 1988; OECD 1992b).

Although both classes of test may be carried out using microorganisms without previous exposure to the test substance (Beek et al. 2001), it is perhaps more common to use an adapted inoculum for the inherent tests. Still, the ready tests are more stringent and it is assumed that compounds passing these tests will rapidly and completely biodegrade in aquatic environments under aerobic conditions. According to the Technical Guidance Document (EC 2003), in tests for inherent biodegradability the test conditions are designed to be more favourable to the microorganisms in that the ratio of substance to cells is lower than in the ready tests and there is no requirement for the (bio)degradation to follow a time pattern as in the ready tests. Further, a pre-exposure of the inoculum resulting in pre-adaptation of the microorganisms may be allowed. The time permitted for the study is normally limited to 28 days, but it may be continued for much longer; six months have been suggested as the maximum duration for the inherent test. Because of the strongly favourable conditions for biodegradation that are present in these tests the results of the inherent tests should be extrapolated with great caution. The typical complex wastewater will contain both “ready” and “inherent” substances, and thus this distinction is of less significance in the present context.

Persistence is the inverse of degradability. Apart from biodegradation, hydrolysis and photolysis may also contribute to the observed degradation. Further, adsorption and evaporation can erroneously contribute to the apparent degradation. The test set-up should involve abiotic or sterile controls for the evaluation and compensation of these factors (OSPAR Commission 2000).

Persistence is often defined as the half-life for a compound, for instance >50 d in the DYNAMEC mechanism (see section 2.2) but for the typical complex wastewater a simpler concept is preferable. The determination of half-life should include an assessment of possible metabolites with PBT characteristics (EU 1996).

Most OECD tests for ready biodegradation of a substance require that in order to pass the test 60 or 70 % should be degraded within ten days after that the degradation has started in a test (within a “10 day window”), that is after the lag phase. According to Danish experience 60 to 70 % represents almost full degradation, that is a much smaller amount than 30 to 40 % of unknown and potentially stable matter is likely to remain. One approach that has been used is to narrow down the pass margin such that less than 20 % of the original amount of organic matter, determined as dissolved organic carbon, DOC, is allowed to remain for it to pass as degradable (SEPA 1997). This measurement problem is recognised as a key problem in P assessment in complex water samples and is further discussed in the text below (sections 1.2, 2.1, 2.3.2, 2.6 Sweden). The information should be regarded as semi-quantitative, although this has been suggested to invalidate the approach, it is as close as you can get with an unknown and complex waste water. The practical study programme run in 2003 [ref] showed that specific chemical analyses is not an alternative for such a matrix.

Bioconcentration is the process by which a compound is absorbed from water through gills or epithelial tissues and is concentrated in the body. BCF, the bioconcentration factor, is the ratio between the concentrations in the organism and the surrounding water. BCF can be assessed with the OECD 305 A - E

fish bioaccumulation test, there has also been established correlations between BCF and $K_{ow,}$ the partition coefficient for octanol/water.

Bioaccumulation is the process by which a compound is taken up by an aquatic organism, both from water and through food. It may be estimated by K_{ow} (as potentially bioaccumulating substances).

Biomagnification denotes the process by which the concentration of a compound increases in different organisms, occupying successively higher trophic levels (Murty 1986).

For single substances log $K_{ow} \geq 4$ or BCF ≥ 500 have been set to be cut-off values for the initial selection procedure of the OSPAR DYNAMEC mechanism (see 2.2), in line with a harmonised system for the classification of chemicals, which are hazardous to the aquatic environment (OECD 2001). In earlier work with complex waters log $K_{ow} \geq 4$ or ≥ 3 have been used (SEPA 1997). The lower limit has also been used by Germany and Norway, see section 5.

1.2 Methods in use

It is clear from the background document concerning WEA (OSPAR Commission 2000), that the methodology developed by ISO and OECD is or may be applied by several Contracting Parties. The methods listed in Table 1 are the standard methods that have been modified by the Contracting Parties in the table, and that are used for WEA application after proper validation in a complex sample environment. In general the methods are modified to function in a complex sample environment. It should be stressed that these are screening methods that need to be properly validated to allow interpretation in the whole effluent application.

Table 1. Standard methods that are used in modified form in WEA applications

Method	Biodegradation	Bioaccumulation
Denmark	OECD 301 E	OECD 117
Finland	ISO 14593	
Germany	EN ISO 9888, OECD 303A	
The Netherlands	OECD 301 E	
Norway	ISO 7827 (OECD 301 A) & OECD 301 F	OECD 107
Sweden	ISO 7827	OECD 107 & 117
United Kingdom	OECD 301 E & F	OECD 107 & 117

Beek et al. (2001) state that tests based on (so-called) summary parameters (the commonly used environmental analytical variables BOD, COD, DOC, CO_2 production, etc.) are only applicable to single substances, because a decrease of the amount of the different compounds cannot be differentiated and metabolites formed cannot be quantified. Thus it cannot be distinguished whether the observed partial degradation results from the complete degradation of one constituent or from several substances undergoing only partial degradation. Since a full chemical analysis of such a sample can be impossible, this may still be the best information obtainable in practical terms. Modifications of these methods have been used in several Contracting Parties, and have provided useful results. Note moreover, that the original Zahn & Wellens (1974) paper addresses both single substances and effluents. More information of the methods is given in subsequent sections.

1.3 Cost aspects

23. It is essential that the cost for testing and analyses is not seen as too high for the information it can provide. Examples of prices charged in 2002 in Finland, Germany and Sweden are given in Table 2. One of the laboratories states that the price will among other things be influenced by whether the laboratory can provide both the biological tests and chemical analyses, or as is often the case, part of the testing is subcontracted.

Table 2. Cost per sample in euros 2002

Method	Single sample	2 – 4 samples	5 – 9 samples
301 A (DOC die-away)	1200[1]	1030	950
301 F (BOD 28 days)	420 - 900		
EN ISO 9888 Zahn-Wellens[2]	600	350 - 500	300 - 450
PBS	1560[3]	1420	1300
EGOM (section 4.2)	(300 -)[4] 450	370	310

[1] ISO 14592 is estimated to be somewhat lower in cost than the DOC tests

[2] 28 d tests approximately twice this cost

[3] HPLC separation and analysis somewhat higher

[4] Lower for small sample size

2 Persistence – methods for biodegradability testing

2.1 Design objectives

The appropriate design of the biodegradation test depends on the destination of the wastewater to be tested. Two main test types may be distinguished (Beek *et al.* 2001):

- Tests with low inoculum concentration and comparatively high initial substrate concentration (tests for ready biodegradation). These tests try to simulate conditions that are found in surface waters although in general both organism density and substrate concentration are much lower in the surface water.
- Tests with high inoculum concentration and comparatively low initial substrate concentration (tests for inherent biodegradation). The conditions in an industrial wastewater treatment plant may be of this nature. It is difficult to extrapolate to degradation rates in the environment from a positive inherent test. Conversely a negative inherent test can normally be interpreted as an indication of persistence of the test substance in the environment.

Standardised procedures are available in the ISO guidelines and OECD 301 test series for the ready tests, the OECD 302 test series for the inherent tests. Test variables for ultimate biodegradation are oxygen consumption and CO_2 evolution. Other variables such as DOC elimination are also used, but strictly speaking a reduction in DOC can be interpreted as biodegradation only when degradation follows a typical growth curve with lag, acceleration and stagnation phases. Else adsorption may be an essential part and the results should be referred to as elimination, in the sense removal from the wastewater (OSPAR Commission 2000). It may be noted that if the adsorbing phase follows the treated wastewater to the recipient it is not eliminated from the environment, but merely transferred from one compartment to another.

The design of the ready biodegradation tests assumes relatively low test substance concentrations of 10 to 50 mg TOC/l compared to test substance concentrations in inherent tests. Focussing on readily degrading substances these tests do not favour biodegradation, since the ratio of test material to microorganisms is high. Although described as biodegradation it will also contain hydrolytic and photolytic degradation, depending on the test design. On the other hand the test design for wastewater indirectly discharged via municipal treatment plants should correspond to the conditions in those plants, which are high COD/TOC and inoculum concentrations with activated sludge as inoculum. The commonly used Zahn-Wellens (ISO 9888) test belongs to that category. These inherent tests thus favour the microbial action, but there may also be some scope for other lytic mechanisms, in addition to biodegradation.

The persistence of a substance reflects not only the potential for long-term exposure of organisms but also the potential for the substance to reach the aquatic environment and to be transported to remote areas. Effluents will often contain a complex combination of substances, some of which are easily degraded in the environment (that is non-persistent) and some of which are more or less stable (Johnson & Watts 2001).

It is incorrect to discuss the "persistence of effluents". Only compounds in the effluent or a property that is an indirect effect of these compounds (such as toxicity) can be persistent. The quantity of such compounds may

be estimated by measuring remaining (recalcitrant) COD or DOC, for instance, as already said above. One of the major problems with determining the persistence of mixtures is the presence of rapidly degradable (non-persistent) compounds together with persistent ones. A low concentration of a highly persistent chemical, in the presence of much higher concentrations of more easily degradable substances (such as an organic solvent), is likely to give a result that suggests that the effluent contents are not persistent, even though it may contain highly persistent material. This dilemma led Beek and co-authors (2001) to the conclusion that tests using such measurements that do not differentiate between compounds that are easily degraded and compounds that are not are unsuitable for the testing of complex samples. This is the central dilemma with these tests, and we shall come back to it later in this document.

The biodegradability of wastewater samples is also commonly estimated by determining biochemical oxygen demand over 5 or 7 days (BOD). The BOD is compared to the chemical oxygen demand (COD), and a BOD/COD ratio of more than 0,5 is classified as an indication of biodegradability (Beek *et al.* 2001). An elevated BOD in directly discharged wastewater indicates that it is not given treatment commensurate with BAT (best available technique).

There is one further design consideration, which must be mentioned. Although the degradability of the sample content may be important, the qualities of the stabilised sample, that is after a degradation test, is often what has to be determined when an operator has to qualify for a permit. It can therefore be required that the degradation setup satisfies the following conditions (note the Norwegian description below, where instead separate tests are performed):

- Adequate volumes of stabilised sample should result from the experiment.
- The inoculum concentration should be chosen such that influence on subsequent tests and analyses, in particular that of potential bioaccumulation, is minimised.
- The medium concentration that is used in the degradation vessel should be chosen such that it does not influence sensitive organisms used in subsequent toxicity testing. This has been shown to be important for algae growth tests.

2.2 Relationship to the DYNAMEC mechanism

Since 1998 work has been undertaken within the OSPAR Commission to establish a dynamic selection and prioritisation mechanism for hazardous substances (DYNAMEC). In this mechanism for individual chemicals, the persistence criterion allows the use of different types of available information on the biodegradability of a substance and three different levels of information are defined:

- Level 3: Experimental data on persistence in the marine environment
- Level 2: Experimental data from Ready or Inherent Biodegradability Tests. Note the requirements listed in 2.4.3 for the application of inherent biodegradability tests.
- Level 1: Data from biodegradation estimation models

The DYNAMEC mechanism advocates, where possible, the use of Level 3 data from simulation test systems such as ISO 14592 (OECD 309) surface water biodegradation simulation test that determine the half-life under relevant environmental conditions. Data from these tests provide the most environmentally realistic information given the nature of the test conditions.

The mechanism also recognises that neither the standard ready biodegradability test nor the standard inherent biodegradability tests are ideal for the assessment of the persistence of a substance, particularly for the marine environment. However, since these methods often provide the only available data on substances of concern, their use is indispensable for the application of the DYNAMEC mechanism.

The review of the methods below to begin with considers tests designed to assess the degradability of individual substances, which measure either changes in the concentration of that substance or a surrogate measurement such as DOC or TOC. Since these methods are standardised, they are a good background for a description of adaptations required for complex water samples. It should be recognised that for such samples, where the nature of the components is unknown, an alternative method may be to assess changes in toxicity, at least if toxicity is the main concern. This provides important information in situations where, for example, substances may be degraded to more or less toxic metabolites even though the level of DOC or TOC does not change. As already said above, the changes in toxicity will not necessarily reflect the degradation of compounds in the sample. For comparison, an adaptation of a single substance method to an effluent application is described after that in 2.3.1.

2.3 Tests for directly discharged wastewater

Hence, although our focus in this document is the characterisation of wastewater, we have chosen to list the pure substance methods, since they are more often referred to, although in actual application to an effluent they are suitably modified. Annex 2 summarises the test conditions of OECD Guidelines (OECD 1995) and ISO Standards (as defined in ISO/TR 15462, ISO 1997) that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium. The ISO Standards (with the corresponding OECD Guidelines) are:

- ISO 7827: Dissolved Organic Carbon (DOC) Die Away Test (301A, 301E)
- ISO 9439: Carbon Dioxide (CO_2) Evolution Test (Modified Sturm Test) (301B)
- ISO 10707: Closed Bottle Test (301D) (ISO 10/94)
- ISO 9408: Manometric Respirometry Test (301F)
- ISO 14593: CO_2 headspace test (OECD 311 draft)

In all these tests a solution, or suspension, of the test substances in a mineral medium is inoculated and incubated under aerobic conditions in the dark or diffuse light over a 28-day period.

Test substances, which are soluble in water to at least 100 mg/l, can be assessed by all methods, provided they are non-volatile and non-adsorbing. The test inoculum may be derived from a variety of sources such as surface water, sewage treatment works effluents, which is not chlorinated, activated sludge or a mixture of these. For the DOC Die-Away, CO_2 Evolution and Manometric Respirometry methods, if activated sludge is used it should be taken from a treatment plant or laboratory scale unit receiving predominantly domestic sewage. Inocula from other sources, usually yielding lower cell densities, have been found to result in a greater scatter of results. For the Modified Screening and Closed Bottle methods, a more dilute inoculum without sludge flocs is needed and the preferred source is a secondary effluent from a domestic wastewater treatment plant or laboratory-scale unit (Johnson & Watts 2001).

2.3.1 DOC die-away test (ISO 7827)

One version of this test, which is one of the most commonly used, can be described as follows. The test chemical is added as the sole source of carbon at 10 to 40 mg/l to a mineral salts medium, which is buffered at pH 7,4. The medium is inoculated to 10^4 – 10^6 cells/ml in duplicates with controls containing only inoculated medium but no test substance. Performance is checked with a reference chemical such as aniline, benzoate or acetate. The flasks are incubated in the dark at 22±2º. Samples are withdrawn throughout the 28-day incubation period with a frequency that allows an adequate degradation curve of % DOC removal to be drawn.

The lag time is defined as the time from inoculation until the removal reaches 10 % of the start concentration. The degradation time is defined as the time from the end of the lag phase until when 90 % of the maximum level of degradation has been reached. The pass criterion is 70 % DOC reduction, which should be reached within 10 days after the end of the lag phase (OECD 1995).

One modification for wastewater samples of ISO 7827 is as follows. The test period is 28 days or until the reduction in DOC is less than 10 % within 4 days. For some wastewaters with a large fraction of persistent or only inherently biodegradable organic substances it may be necessary to extend the degradation period to for example 40 or 80 days. The complex character of the wastewater may give rise to interpretation problems that might be partly met by supplementary analysis of DOC on days 0, 4, 7, 14, 21, 25, 28 and, if the test is extended, days 35, 40 and on the final day. BOD is also measured on days 0, 4, 7, 14, 28 and on the final day and COD and TOC on day 0 and on the final day (SEPA 1997).

Based on knowledge from tests of inhibitory effects on activated sludge the wastewater sample is diluted so as to facilitate the degradation process. The recommended concentration of wastewater (sample) in the degradation test corresponds to the EC20 value for inhibition of respiration of the activated sludge fraction used as inoculum. It is important not to dilute the sample more than necessary, because it is advantageous to carry out the further testing on the stabilised sample on as concentrated a sample as possible. The amount of DOC must not be less than 10 mg/l at the beginning of the test.

Primary settled water from a municipal sewage plant is used as inoculum (1 ml per litre), and the test medium is prepared as described in ISO 7827. If algal growth tests are to be performed, the medium concentration should be modified. The total volume of the degradation test should be at least 15 l, more if fish tests are part of the subsequent programme.

The pass criterion is 80 % DOC reduction within the test period. If potential bioaccumulating substances is to be determined in the stabilised sample, it is essential not use a heavy inoculum, which will introduce artefacts in that analysis.

The main reason for choosing a uniform inoculum has been to obtain comparable results, another that the test could be conducted at any of several laboratories. As a consequence, it is not certain that the degradation process resembled that to which local conditions in the recipient give rise. In one project it was recommended that the recipient conditions should be taken into account when selecting inoculum material in connection with investigations of persistent characteristics of wastewater. These conclusions are further elaborated in section 2.6, Sweden.

2.3.2 Biodegradability in seawater

In the ISO 16221 standard (ISO 2001), five biodegradation tests, based on the conventional Closed Bottle test, Two-phase Closed Bottle Test, Carbon Dioxide Evolution Test, CO_2-ISO Headspace Test and DOC-Die-away Test, have been adopted for screening biodegradability in the marine environment. Natural or artificial seawater is used and incubation period is prolonged to 60 d while temperature is fixed within the range of 15°C to 25°C. The feasibility of the methods has been demonstrated in an OSPARCOM ring test.

OECD 306, which is a variant of the modified OECD Screening Test (OECD 301E), is available for assessing biodegradability of individual substances in seawater. Method 306 (which corresponds to ISO Standards 7827 and 10707) can be carried out as either a shake flask or closed bottle method and the only microorganisms added are colony-forming heterotrophic bacteria in the test seawater to which the test substance is added. The test guideline states that "the results from this test are **not** designed to be taken as indicators of ready biodegradability but are to be used specifically for obtaining information about the biodegradability of chemicals in marine environments". If toxic effects are expected or possible it is advisable to include an inhibition experiment in the test design.

The characteristics of this test method are also summarised in Annex 2. In the test, failure to satisfy the test criteria (namely >70% DOC removal or >60% reduction in Theoretical Oxygen Demand (ThOD)) does not preclude the potential for biodegradability of the substance of interest in the marine environment, but rather indicates that further study is needed (Johnson & Watts 2002). Obviously, a thorough simulation of degradation in seawater should account for photolysis and hydrolysis as well.

ISO 14592 (OECD 309) is a surface water biodegradation simulation test that determines half-life under relevant environmental conditions. In the ISO 14593 CO_2 headspace test it is to some extent possible to test samples containing volatiles and non-soluble fractions (Battersby 1997). It can be applied to seawater cases (Ingeslev & Nyholm 2000, Ahtiainen et al. 2003).

2.4 Treatability of wastewater in municipal wastewater treatment plants

2.4.1 Significance of indirectly discharged industrial wastewater

The biological treatment of industrial wastewater in municipal treatment plants is one of the most frequently applied treatment processes. In Germany about half of the total industrial wastewater flow is discharged to public sewers. This corresponds to roughly one third of the total municipal wastewater flow and COD/BOD-loads treated in around 4 000 activated sludge plants in Germany. In some industrial sectors such as the textile finishing industry more than 90% of the wastewater is treated together with domestic wastewater (Killer *et al*. 1993). In the United Kingdom about 80% of the trade effluent is discharged indirectly via sewers (UK Department for the Environment, Food & Rural Affairs 1998).

Thus the (bio)degradability of indirectly discharged industrial wastewater in municipal treatment plants plays an important role. Considering the biological conditions of treatment plants two different approaches have been applied, to assess treatability in treatment plants based on both high inoculum concentrations of activated sludge and COD/DOC-measurement. The first approach uses flow-through laboratory activated sludge plants, the second the Zahn-Wellens test.

2.4.2 Laboratory activated sludge plants

Flow-through laboratory activated sludge plants according to EN ISO 11733 and OECD 303A have been used to simulate the fate of wastewater in municipal treatment plants, but time and cost prevent their broad application. The wastewater is continuously dosed into the activated sludge vessel, and nutrients and/or a synthetic sewage matrix may be added to enhance simulation characteristics. A control unit is only fed with the synthetic sewage. Both units may be coupled by interchanging a defined volume of activated sludge once a day. DOC is measured in the effluent, and the daily DOC-elimination is calculated after correcting for the material transfer due to the inoculation procedure. The test has occasionally been used to assess the elimination of effluents in sewage treatment plants (Gartiser *et al*. 1996) but more often the focus is the optimisation of process parameters of real treatment works. Further extensions of the test method with an additional anoxic vessel for denitrification processes are under development.

2.4.3 Zahn-Wellens test

A second tool used to assess the biological treatability of wastewater is a simple batch test with activated sludge, which is used to determine the removal of organic ingredients by biodegradation and adsorption (=bioelimination) (the so-called "Zahn-Wellens test" according to EN ISO 9888). In Germany this test is widely used in wastewater and product evaluation with respect to their treatability. The inoculum concentration has been fixed at 1000 mg/l suspended solids. The elimination in a parallel "abiotic control" describes removal of volatile substances by evaporation, and the ”three hour value”, i.e. the DOC/COD-elimination measured after three hours test duration in the test vessel, is used to estimate sorption processes. Nevertheless a disadvantage of the test design is that no distinction can be made between adsorption and biodegradation.

It is stressed that the conditions defined in the Technical Guidance Document on Risk Assessment (EC 2003) (“The TGD”) should always be fulfilled:

- The pass level should be reached within 7 d;
- The lag phase should be no longer than 3 d;
- Any removal in the test before biodegradation occurs should be below 15 %.

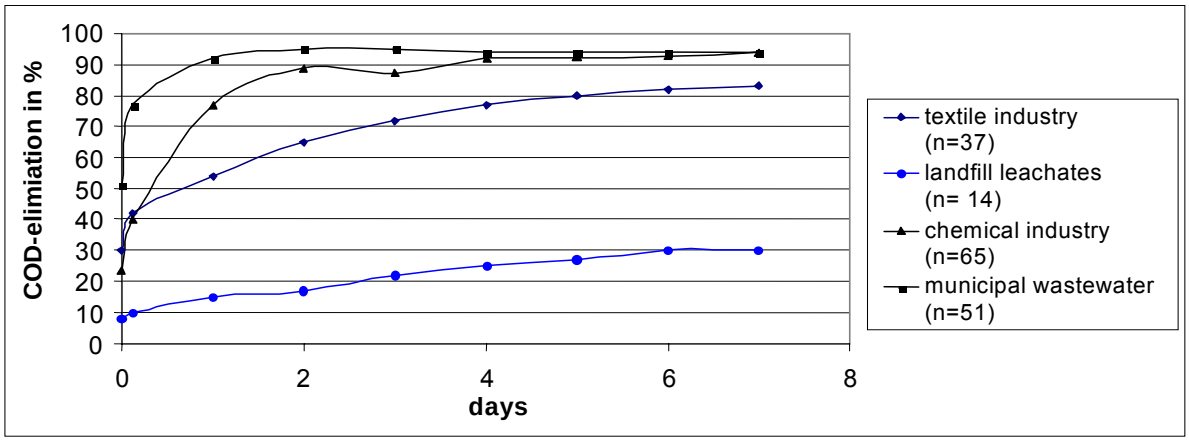


Figure 1. Bioelimination of wastewater in the Zahn-Wellens test

As a rule a COD/DOC-elimination of 80% in 7 days (corrected for the part eliminated in the abiotic control) is considered a sufficient criterion for biological treatability in municipal sewage treatment plants. Sometimes the test duration is prolonged up to 28 days in order to reach the plateau-phase of the degradation curve in order to predict the behaviour in industrial sewage treatment plants (Pagga 1995, Stucki 2000). Therefore the TGD defines the specific criteria to be fulfilled for inherent substances being degraded in sewage treatment plants, which were just mentioned. Some results with wastewater samples from different sectors are shown in figure 1 above (data taken from Gartiser and co-authors 1996 and supplemented with non-published data of Hydrotox GmbH).

The Zahn-Wellens test has been used to calculate the contribution of single process waters to determine the recalcitrant portion of COD or DOC (resistant to degradation, also described as "refractory" COD/DOC) in the

effluent of real treatment plants. Stuhlfauth (1995) found that the recalcitrant DOC of 63 process waters from a chemical industry company, as calculated from the Zahn-Wellens test results, was nearly identical with the corresponding value determined in the real biological treatment plant of the company. Similarly Killer *et al*. (1993) determined the bioelimination rates of textile and domestic wastewater, as the principal dischargers of a municipal treatment plant, in the Zahn-Wellens test and confirmed the additivity of recalcitrant DOC loads by comparison with the real municipal treatment plant. Thus 84% of the recalcitrant DOC load could be attributed to four textile mills. The DOC elimination of several single chemicals also showed comparable results in the Zahn-Wellens test and in real biological treatment plants (Pagga 1995).

The influence of the test duration, adaptation of the inoculum and biomass growth on test results and predictability of bioelimination is currently discussed. Adaptation of the inoculum has a decisive influence on the degradation kinetics and therefore on suitable test durations. Although the inoculum used usually derives from the activated sludge plant, where the wastewater is treated, the need for adaptation in the test is inevitable, as the partial volume of the wastewater tested usually amounts to only a few percent of the total flow through the respective biological treatment plant (exception: domestic wastewater). Nevertheless the recalcitrant DOC at the plateau phase seems quite independent of activated sludge source and test duration (Gartiser *et al.*1996).

The Zahn-Wellens test has been successfully applied to predict the recalcitrant or refractory COD/TOC-loads of different wastewater streams in biological treatment plants (industrial or municipal) especially in the chemical industry (EC 2002) and therefore is an effective management tool in stream inventories. The test has been extended to assess the elimination of other relevant wastewater parameters such as AOX, heavy metals and ecotoxicity (Gartiser *et al.*1997). Cross-media effects must be considered by suitable measurements (three hour value and abiotic control vessels) in order to assess other emission routes to air and fields. It can be concluded that the Zahn-Wellens test is a suitable test to assess the treatability of industrial effluents discharged to municipal wastewater treatment plants.

2.5 Comparison of the methods: accuracy and precision

The CO_2 evolution method gives the most direct evidence of oxidation of organic carbon during biodegradation; the removal of DOC can be due to processes other than biodegradation and the uptake of oxygen is only an indirect measurement for assessing biodegradability. As said in 2.1 above, it can be interpreted as biodegradation at least when a typical growth curve is followed. Of the three measurements, only by using DOC, either in the DOC die-away test or as additional determinations in the other two methods, an indication can be obtained of the formation of any recalcitrant intermediate metabolites (OECD 1995).

The inconsistency and unpredictability of the inocula, which have to be used, adversely affect the overall accuracy, precision and reproducibility of the various methods. For chemicals, which are very easily biodegraded, such as the reference chemicals, very high values of % DOC removal approaching the theoretical of 100 % are consistently obtained with high precision and reproducibility. However, the % ThOD and % $ThCO_2$ obtained are always lower than % DOC removal (for all chemicals, not just the very easily degradable chemicals) because some of the carbon is converted to biomass. The proportion of the carbon used for cell synthesis varies both between species of bacteria and between chemicals so that the % $ThCO_2$ and % ThOD will vary from test to test. The precision with which very easily degradable chemicals are assessed in these tests is high, but not so reproducible either between tests using inocula from different sources or as for % DOC removals. For wastewater samples containing substances, which are not so easily degraded and that may require longer lag periods, the precision in the various tests is lower than with single chemicals, especially when low cell densities are used.

2.6 Member state experiences

Denmark

In two studies (Kristensen *et al*. 1992, Pedersen *et al*. 1994) the OECD methodology is referenced. According to the background document (OSPAR Commission 2000) the publication from 1994 is used as an unofficial handbook.

Germany

In Germany biodegradability of wastewater is most commonly estimated by determining the biological oxygen demand over 5 days (BOD) but in recent years the application of this test has been declining. Due to the short term of 5 days only a part of COD (a BOD:COD about 50% for municipal waste water is considered favourable) is converted into BOD. Therefore the test result provides more of an estimation of the treatability of wastewater in municipal treatment plants than an estimation of persistence of the wastewater itself.

As a second tool, the Zahn-Wellens test method (EN ISO 9888) is applied to wastewater from several sectors including chemical and pharmaceutical industry and landfill leaches. The inoculum concentration is fixed at 1000 mg/l suspended solids. Often local authorities require this test for wastewater from sectors such as textile industry and printing industry. As mentioned above a COD/DOC-elimination of 80 % in 7 days is considered a sufficient criterion for biological treatability. The test period may be extended to 28 days for landfill leaches. In addition the use of chemicals for special applications such as chelating agents in cooling water is limited by the "Wastewater Ordinance" if a pass level of e.g. 80 % COD- or DOC-elimination in 28 d is not reached.

The coupling of degradation and ecotoxicity tests has been practised in routine measurements (Annex 51, German Wastewater Ordinance) and research projects (Gartiser *et al.*1997). Usually laboratory flow-through activated sludge plants and/or the Zahn-Wellens test are used together with acute toxicity tests (fish, *Daphnia magna, Vibrio fischeri).*

The Netherlands

The Netherlands has developed a method, which is a modification of OECD 301E. The method is ready, but more experience should be gained. In 1995 and 1996 a first extensive study was performed with 10 large volume effluents. Persistence of chronic toxicity, mutagenicity and bioaccumulation was one of the studied subjects. Additional degradation (i.e. in addition to what was achieved in a preceding STP) (or persistence) of organic substances was determined based on DOC and aniline concentration.

Chronic toxicity was tested after the degradation step. Mutagenicity and bioaccumulation were determined before and after the additional degradation step.

Based on DOC additional degradation occurred in 4 out of 10 effluents. Based on the concentration of PBS (potentially bioaccumulating substances) additional degradation occurred even more frequently. In 5 out of 5 effluents a decrease of 85% of the PBS concentration was detected.

The same approach is used for both fresh and marine water. In a study with marine water one effluent was used to test a marine version of the persistence/degradation step. In the development phase acute toxicity was tested before and after the additional degradation step. The results showed that a strong reduction of acute toxicity occurred after the degradation, while DOC did not show any decrease in concentration. Therefore, performing tests for this short-term effect before the degradation step is advised, while chronic toxicity, being a long-term effect, should be tested after the additional degradation step.

Norway

Biodegradation studies are performed in connection with ecotoxicological characterisation of complex wastewaters in order to assess the treatability of the wastewaters by biological processes and the discharge of non-readily degradable organic constituencies to the receiving waters.

Two test methods are employed for this purpose, the respirometric test (OECD 301F) and the DOC die-away test (OECD 301A). The latter is primarily used, when further characterisation of the wastewater after degradation, e.g. by toxicity tests, shall be done. In such cases, normally both methods are applied; the 301F to obtain a complete degradation curve over 28 days, and the 301A, using a larger volume, sufficient for the subsequent toxicity tests. Also, the 301A allows the use of higher wastewater concentration, which may be necessary to detect any changes in toxicity caused by degradation.

The results are interpreted in a qualitative way, recognising the inherent limitations of these tests for studying degradation of complex mixtures. For those wastewaters that show a slow and incomplete degradation within 28 days further characterisation is generally required to identify the non-degradable components.

Toxicity tests carried out before and after the degradation test have been used to investigate the removal of toxic components by degradation. With this technique it has been possible to show if toxicity is reduced or not, but seldom a quantification of the toxicity reduction has been possible. One problem with this approach is that the degradation test has to be performed at a concentration that is not toxic to the microorganisms in the inoculum. The possibility to detect changes in toxicity then depends on the difference in sensitivity between these degrading organisms and the organisms used in the toxicity test (usually algae).

Sweden

The test method to be used is an adaptation of the International Standard ISO 7827 (ISO 9/94). The method has been in regular use since the late 1980s for the assessment of whether a wastewater discharge is adequately treated, as an optional part of the permitting process, although often prescribed by the licensing body as part of the permit.

The purpose of the test is both to determine the degradation in terms of change in the dissolved organic carbon concentration and to prepare a solution of any remaining persistent chemicals and degradation products for further characterisation (the stabilised sample). The method was described in section 2.3.

The projects run in Sweden in the early 90's pointed out the following remaining problems, which need be considered in a continued development of the methodology:

- Biodegradation under standardised conditions. One factor that is difficult to standardise is the inoculum. If adapted sludge is available, the test will generate more relevant results, than a test with sludge from a municipal wastewater treatment plant that does not have this type of industrial influx. If the discharge goes to a surface water recipient, the preference was that the inoculum should be prepared from that water. Such individual solutions will be relevant for the individual industry, but for comparison of the performance of different industries, MSTP sludge has to be used.
- A corresponding argument applies to the temperature in the degradation process. The standard 20 ^{o}C invites comparison, but does not say what actually happens in the recipient water, where perhaps 10 ^{o}C is a reasonable mean value, at least in the northern part of the convention area. In the sewage treatment plant the normal temperatures are considerably higher instead. Again a choice has to be made between local relevance and comparability.
- The diluted sample problem has been mentioned. There have been successful trials with evaporation of samples after the degradation test, but this technique needs further development although promising for the characterisation of stabilised wastewater samples.
- The inoculum in the degradation test may influence the PBS estimation in the stabilised water. The solution was to use a very light inoculum, such as in the modified ISO 7827.

Thus there are some irreconcilable factors, but each one can of course be decided upon in an individual test case. The problem of interpretation of results, whether the pass level is put at 80 % or even higher is more fundamental. As said by Norway in above, the results can only be interpreted qualitatively, but it is our experience that in combination with chemical tests it is often possible assess whether the wastewater can be or has been satisfactorily treated in a biological wastewater treatment plant. A degradation test according to ISO 7827/OECD 301A (modified) or comparative schemes is also required for the important study of the treated – stabilised – wastewater.

United Kingdom

Based on a survey of several relevant British laboratories the following was found: The most common methods occurring were parts of OECD methods 301, particularly Part F, the Manometric Respirometry Test measuring oxygen uptake as a screen of chemicals for 'ready' biodegradability in an aerobic aqueous medium. A variant of part E, the Modified Screening Test, is also used for assessing the biodegradability of substances in seawater (now referred to as OECD method 306). Another method occurring was OECD method 303A, to simulate the biodegradation in sewage treatment plants.

2.7 Conclusions on the present situation

The report to the UK Dept of the Environment, Transport and Regions by Johnson & Watts (2001) concluded that at present no screening procedure is used routinely to measure the persistence of substances in effluents and as such there are no extensive datasets with which to compare different approaches. Different European countries apparently use different approaches (Table 1), some Contracting Parties have considerable experience and data associated with the method selected by them for this application. Johnson and Watts (2001) also found that shake flask (open bottle) tests are most appropriate for effluent testing. The IEG concludes that the Zahn-Wellens test is suitable for the assessment of the degradability of wastewater sent to a biological treatment plant. For the application of directly discharged wastewater one of the DOC removal methods is recommended, such as the OECD 301A or E.[2] Based on Norwegian and Swedish experiences special care is necessary if the stabilised sample (i.e. after degradation) is to be used for further determination of toxicity or bioaccumulation. This application requires bigger stirred vessels in order to generate sufficient volumes for further analysis.

[2] France has a reservation against the procedure agreed in the IEG, and does not support its conclusions

3 Persistence – methods under development

Germany

Experience with effect-based test systems will be collected and evaluated in a forthcoming research project, in order to make (un)published studies by industry, governmental agencies and experts from other institutions available. Those involved in current research projects and other experts will be contacted in order to show the current situation in the selection of test methods and an evaluation of results will be summarised. Finally a test strategy for the use of biological tests in wastewater management will be developed.

Several projects are in progress in which the Zahn-Wellens test with COD/DOC-analysis is combined with other determinations such as CO_2 evolution and oxygen respiration. The inoculum concentration is usually set to 200 mg/l suspended solids in order to lower the respective blank values of controls. Meinecke *et al*. (2001) used a closed system with air circulation, Gartiser and Urich (2001) an open system with carbon dioxide free air according to the "Sturm-test". Thus a better estimation of the elimination factors biodegradation and sorption is possible, whereas evaporation can be measured only in open systems. Neither research group has applied their test system to wastewater samples so far but is planning to do so.

Recently Reemtsma and Klinkow (2001) presented a testing strategy for dangerous substances in wastewater. Here a DOC die-away test according to OECD 301A with an inoculum concentration of 30 mg/l suspended solids discharges has been proposed. According to Reemtsma other measurements such as the determination of CO_2 evolution would be interesting, but do not seem practicable due to the greater effort needed. Since DOC elimination measurement will not distinguish between biodegradation and adsorption, part of the total carbon will often not be found in the filtrate.

Spain

The current objective is to develop a system with a capacity to quantify the environmental hazard of effluents by means of the integration of the three main parameters: toxicity, persistence and bioaccumulation potential. To this end two independent studies are in progress. One will be directed to establish a procedure to estimate the persistence of the toxic components of the effluent, and the overall toxicity of the effluent itself. For this study, an artificial degradation system will be implemented, simulating degradation under natural conditions. The result will be used in a system dynamics model to estimate the evolution of the effluent along the river course.

4 The assessment of potentially bioaccumulating substances (PBS)

Effluents will often contain a complex combination of substances, some of which will have the potential to accumulate (and possibly biomagnify) in biota and others, which will not bioaccumulate, irrespective of the exposure conditions. The extent to which substances accumulate in biota will depend on (Johnson & Watts 2001):

- the physico-chemical characteristics of substances (for example accumulation is likely to be significant where the octanol/water partition coefficient log K_{ow} > 4);
- the persistence of the substance in the environment since a chemical has to be present for a sufficient period to allow uptake to occur;
- the exposure scenario in an organism in terms of whether uptake occurs via the water column and/or through ingestion of contaminated particles (such as sediment) or food.

Note also that metabolic elimination cannot be accounted for by chemical screening either, this potential short-coming is shared by the two alternative routes.

Whereas pelagic organisms will primarily accumulate chemicals via the water column, sediment-dwelling organisms may accumulate hydrophobic chemicals via ingested contaminated particles. Uptake via food can become important for organisms higher in the food web, particularly for substances (such as mercury and organic substances with a log K_{ow} of 5-8), many of which have the potential to biomagnify.

The use of *in situ* methods (and laboratory-based whole organism tests) may provide the most realistic assessment of the nature of potentially bioaccumulating substances in effluents since the organisms used have the capacity to concentrate all such substances (metals and organic chemicals) in a non-selective manner. However, it should also be remembered that these methods are usually applied to determine the extent to which particular substances of interest accumulate in biota. Therefore, for reasons of cost and

practicality these approaches should not be used in a screening role but rather as a confirmatory tool for local environmental impact assessments or as part of a general quality assessment framework for water bodies. Consequently chemical methods including the possible use of so-called summary variables represent more cost-effective and practical screening options for assessing the presence of potentially bioaccumulating substances in effluents. This is true if they generate environmentally realistic data, which reflect the nature of uptake in whole organisms.

The inherent limitations of all chemical methods used to screen for biological qualities should be stressed. There is no molecular size cut-off as caused by biological membranes, and there is a lack of metabolic elimination routes, as compared to biological accumulation.

4.1 Selectivity of methods

Laboratory-based chemical methods for assessing potentially bioaccumulating (organic) substances (PBS) in effluents typically combine extraction with separation techniques and quantify PBS against a standard, usually within a log K_{ow}-based 'window of lipophilicity', which is considered to comprise organic substances likely to bioaccumulate in biota.

Chemical methods for assessing PBS must be able to consider as many target organic compounds as possible and, ideally, should consider both substances which are found in the aqueous phase and which are associated with particulate matter. To achieve this objective the key factors are:

- The selectivity of the method.
- The level of recovery of target compounds.
- The limits of detection of the quantification techniques.

If the selectivity of the method is low, interfering material (that is non-bioaccumulating substances) is extracted and will lead to an overestimation of PBS. In contrast if the method selectivity is high, not all potentially bioaccumulating substances will be extracted leading to an underestimation of the amount of PBS.

The achievement of accurate PBS quantification requires a high recovery of target compounds since certain procedural steps may lead to a loss of volatile PBS. If there is only a low recovery of these compounds there will be an underestimate of PBS. In complex mixtures such as effluents where the constituents are often unknown, quantitative recovery of a wide range of PBS is needed to avoid underestimation.

In certain industrial sectors naturally derived lipophilic compounds, which are part of a complex raw material may constitute a major part of the PBS. This does not per se invalidate the analysis, since abnormal concentrations of such material, possibly with microbiological transformations from the wastewater treatment, may be an aberration to consider for the recipient.

4.2 Screening tests for assessing potentially bioaccumulating substances

Screening tests assessing the PBS in effluents may comprise a number of procedural steps, namely pH adjustment or filtration, extraction and separation, and finally analysis of the extract. Note that some operations may be seen as unrealistic as compared to what occurs in the recipient water.

Table 3 (adapted from Johnson & Watts 2001) summarises the approaches adopted in each procedural step in a series of potential screening methods and the table in Annex 3 discusses their advantages and limitations in more detail.

The procedures for the extraction of the organic substances from effluents vary in duration from 1 day for the Solid Phase Micro-Extraction (SPME) method to 14 days or longer for the C_{18} Empore disc and the Semi-permeable Polymeric Membrane Device (SPMD). The staff time involved in conducting the procedures varies from approximately 1 hour for the SPME method to 1 day for the HPLC, SPE and Empore disc methods.

All the extraction methods have been shown to be effective at removing organic substances from effluents but no inter-calibration data is available which would allow their relative efficiencies to be compared. The biomimetic procedures (C_{18} Empore disc or column, SPMD and SPME) all extract highly bioaccumulating compounds to a greater extent than less bioaccumulating substances, which is a more realistic approach than the exhaustive liquid-liquid extraction procedure used in certain methods. Extraction of aqueous samples containing suspended solids may cause problems or anomalies, which is why pre-treatment by filtration may be a necessity. Since bioaccumulating material is often attached to the particles, one should be

aware of the fact that this might result in an underestimate of the discharge. The strength of the attachment of course determines the environmental impact.

Different analytical detection methods will be capable of identifying and quantifying different types of substances and the challenge is to provide procedures, which cover a wide range of target compounds. Aqueous separation systems such as HPLC may lack some of the 'universal detection' qualities of techniques, such as FID or mass spectroscopy generally used with gas chromatography (see Annex 3). It is also important to define the extent to which the screening test actually needs to identify the PBS or if it is sufficient to be able to define the total magnitude of PBS and determine whether this exceeds a pre-defined threshold (Johnson & Watts 2001).

4.2.1 Liquid extraction with thin layer or high performance liquid chromatography determination

An early reference to such a technique is the method presented by Renberg and co-authors (1985). To accomplish a more standardised and uniform determination of the samples the method was modified and described in detail by Adolfsson-Erici and Wahlberg (1992). A cyclohexane extract of the stabilised sample is applied to a hydrophobic thin layer plate (TLC) together with a mixture of reference substances. When the plate is eluted with an acetone-water mixture the substances are separated according to how fat-soluble they are. The most lipophilic fractions, which may be assumed to contain potentially bioaccumulating substances, are then isolated and quantitatively estimated using gas chromatography. In early investigations, the PBS that accompanied the inoculum material in the stabilisation test caused disturbances, and hence this PBS had to be determined separately to enable a correction. Hynning (1996) developed the method was further, reflecting development in chromatographic instrumentation and methodology. Here the extract is fractionated by semi-preparative HPLC and then identified by GC-MS and quantified by GC-FID.

Comparison of the results for reference compounds using the semi-preparative HPLC method and the Thin Layer Chromatography (TLC) method (Renberg *et al.* 1985) used in the Swedish Environmental Protection Agency STORK Project (SEPA 1997) showed satisfactory agreement in terms of the differentiation of compounds into groups of different log K_{ow} values. Some results are presented in 4.3, Sweden.

4.2.2 EGOM

As a faster, pre-screening method the full organic content of the extract has been determined as "extractable gas-chromatographable organic material (EGOM)", using squalane as an empirical standard. If the quantity of EGOM justifies it, a full PBS determination can be carried out. This sequential approach saves time and money, where the PBS content is found to be low or negligible. Some Swedish PBS/EGOM experiences may be found in the paper by Tarkpea *et al*. (1998) and in the STORK report (SEPA 1997).

4.2.3 Solid phase micro-extraction with gas chromatography

How to run solid phase extraction methods can be summarised as follows:

The solid phase extraction (SPE)/high pressure liquid chromatography (HPLC) fractionation method used in the study utilised the following stages:

- Filtration of sample through glass fibre filter
- SPE extraction for dissolved substances and soxhlet extraction for particulate substances
- Separation of substances on reverse phase HPLC and collection of compounds
- Drying of the PBS fraction.

Information on the solid phase micro-extraction (SPME) is summarised in figure 2. Organic bioaccumulating compounds are extracted with a fibre that is coated with a polymeric coating simulating the characteristics of animal lipids. The method is fast, cheap and representative for the uptake by animals, because the fibre coating only extracts freely dissolved bioavailable compounds. It is a biomimetic extraction technique in that compounds that accumulate highly in organisms also accumulate in the coating.

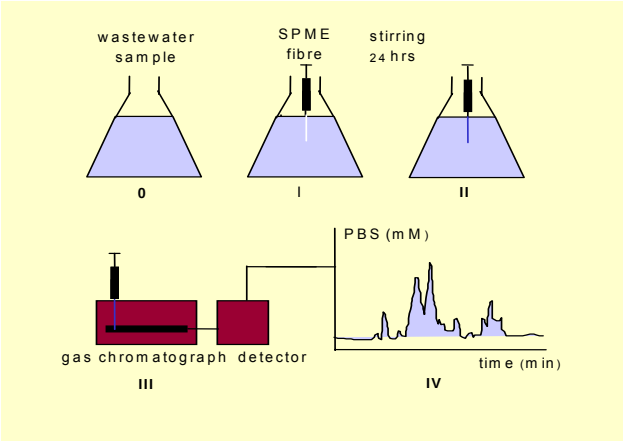


Figure 2. Solid phase micro-extraction (SPME) of effluents: a SPME fibre is inserted into an effluent sample (I); the sample is stirred vigorously over a 24 h period to obtain an equilibrium between the coating and the freely dissolved compounds in the sample after which analysis can be started (II); the fibre is directly inserted into the injection chamber of a gas chromatograph (III); a mass spectrometer detector is used to determine the total concentration of PBS, but can also be used to identify and quantify individual bioaccumulating compounds (IV)

The quantification of the PBS concentration in millimoles is based on the use of 2,3,5-trichlorotoluene as an external standard. The error that is made by assuming a constant response factor for all compounds contributing to the total PBS concentration is relatively small (de Maagd 2000). A short (10 m) GC-column is used in order to enable a more precise integration of the total area of peaks.

The two methods adopted fundamentally different approaches to assessing potentially bioaccumulating substances. The SPME method only measured the freely dissolved fraction, whereas the SPE/HPLC method provided a total extraction of substances and measured free and particle-bound compounds. The SPME procedure is less time consuming than the SPE/HPLC procedure, but the SPE/HPLC procedure may be able to provide more information. A comparison of the methods is presented in section 5, The Netherlands.

The main bias of a chemical method is that biotransformation as an elimination route is not included in the simulation. A second but in practice less relevant bias is that there is no cut-off in molecular size in chemical methods including the SPME method. Compounds with an effective diameter > 10 Å are normally considered unable to pass biological membranes, but can still be extracted by the fibre coating.

4.3 Bioaccumulation – member state experiences

Denmark

For first stage tests, HPLC screening with respect to K_{ow} is recommended (Pedersen *et al*. 1994).

The Netherlands

RIZA is confident that the SPME procedure, which is described above, can be used routinely in the future. The main concern at this moment is to reduce variation in test results. The opinion is that the large variation that was met with during interlaboratory testing (see section 5) is not an inherent shortcoming of the method, but rather a general challenge in the analytical standardisation of a test procedure. Another concern is that the extent to which some compounds partition to the fibre can be greatly affected by the presence of salts. This must be considered when examining marine samples.

Norway

In Norway, assessments of potentially bioaccumulating substances in industrial wastewaters have been assessed sporadically during the last ten years. The method that has been used is essentially as described by Renberg *et al*. (1985).

The wastewater samples are acidified to pH 2 and extracted twice in cyclohexane. Emulsions are broken by freezing or addition of salt. The extract is washed with water and dried over sodium sulphate. After evaporation to a small volume (1-5 ml) the extract is fractionated on a TLC plate. Using reference substances with known K_{ow}, fractions containing different K_{ow}-ranges are scraped off the TLC plate. An internal standard is added and the fractions extracted in hexane or cyclohexane/isopropanol. The extracts are analysed on GC/FID. The area under each peak on the chromatogram is related to the internal standard

($C_{18}H_{38}$ or $C_{14}H_{30}$) for quantification. Three different laboratories have been performing these analyses, and the fractions separated are not consistent, depending on the reference substances used. Usually three fractions are reported in the low (< ca. 3), medium (ca. 3 - ca. 6) and high >ca. 6) log K_{ow} ranges.

In some of the studies, wastewater samples are analysed before and after biological stabilisation in a 28 days biodegradation test.

Table 3 Summary of the procedural steps adopted with different types of laboratory-based chemical methods for assessing potentially bioaccumulating substances

Method and reference	Procedural stage				
	Pre-treatment	Extraction	Pre-treatment before analysis	Separation	Detection
Thin-layer chromatography (TLC) Renberg *et al* (1985)	Adjustment of sample to pH < 2	Extraction with hexane	Evaporation under nitrogen	3 zones collected by TLC separation, extracted with hexane, evaporated under nitrogen and separated by gas chromatography	Flame ionisation
High performance liquid chromatography (HPLC) Burkhard and Sheedy (1995)	-	Extraction with hexane	Sample cleaned up using silica gel, sodium sulphate, sulphuric acid on celite and sodium sulphate Evaporation using Kuderna Danish method Evaporation under nitrogen	3 fractions collected from solid phase extraction C_{18} column, dried under nitrogen, evaporated under nitrogen and separated by gas chromatography	Mass spectrometry
High performance liquid chromatography (HPLC) Klamer and Beekman (1995)	Adjustment of sample to pH < 2	Extraction with hexane	Sample cleaned up using aluminium oxide, silica gel chromatography and preparative gel permeation chromatography Evaporation using Kuderna Danish method Evaporation under nitrogen	Collection of fractions from C_{18} HPLC separation	Fluorescence and UV/VIS detection[1]
High performance liquid chromatography (HPLC) Stenz *et al* (1999)	Filtration	Solid phase extraction	Sample cleaned up with ultra- filtration and preparative gel permeation chromatography	Collection of fractions from C_{18} HPLC separation, lyophilisation of fractions	Gravimetric and TOC analysis
Empore disc method Van Loon *et al.,* (1996)	Adjustment of sample to pH < 2 and addition of bactericide	Exposure of disc to sample for 14 days[2] Extract disc with cyclohexane	Evaporation under nitrogen	Minimal separation by gas chromatography	Mass spectro-metry or vapour pressure osmometry

Method and reference	Procedural stage				
	Pre-treatment	Extraction	Pre-treatment before analysis	Separation	Detection
Semipermeable Membrane device (SPMD) Sodergren (1987)	-	Exposure of sample to hexane filled SPMD for ≥ 14 days	-	Separation by gas chromatography	Electron capture detector
Solid Phase Micro-Extraction (SPME) Verbruggen (1999)	Adjustment of sample to pH 7,5	Exposure of poly-acrylate SPME to sample for 1 day	-	Minimal separation by gas chromatography	Mass spectro-metry

[1] Not suitable for unknowns where we do not know whether a chromophore is in place

[2] The empore disc is exposed to the sample for 7 days before renewal of the sample and exposure for a further 7 days. The long duration has been questioned.

Table 4. Norwegian experiences

Waste-water	Category	total extracted [mg/l]	High log K_{ow}(> ca. 6) [mg/l]	Medium log K_{ow} (ca. 3 - ca. 6) [mg/l]
6	Chemical (paint)	150	-	5,8*
12	Chemical polystyrene)	2,89	0,22	0,79
13	Chemical (tall oil)	135	2,5	2,4
13	" after biodeg.	1,1	0,1	0,1
1	Chemical (VCM)	0,13	n.d.	n.d.
2	Pharmaceutical	1,3	0,84	0,1
3	Pharmaceutical	1,5	1,3	0,3
4	Pharmaceutical	5,2	5,2	n.d.
5	Pharmaceutical	1,3	1,1	n.d.
11	Pharmaceutical	1,2	0,009	0,048
7	Pulp & paper (TMP)	-		1,34*
7	" after biodeg.	-	-	0,5*
14	Pulp & paper (TMP)	n.d.	n.d.	n.d.
8	Pulp & paper	-	-	1,48*
8	" after biodeg.	-	-	0,13*
9	Pulp & paper	10,1	0,8	1,76
9	" after biodeg.	0,42	n.d.	0,004
10	Pulp & paper	2,3	0,28	0,15
10	" after biodeg.	0,074	n.d.	0,004

*Only fraction log K_{ow} >3 reported.

The wastewaters that have been characterised for bioaccumulating substances belong to different categories as shown in Table 4. The experience from the use of this technique is that interpretation is not straightforward. As a guideline, the criteria proposed in a Swedish programme for wastewater characterisation (SEPA 1997) have been used to identify those wastewaters where further actions should be considered.

Pulp & paper industry has been shown to discharge low concentrations of lipophilic components. Because of the large volumes of wastewater, the total discharge of these components may be significant. However, it is assumed that these components originate from the timber, and that they may not constitute an environmental risk. This means that detection of potentially bioaccumulating components in wastewaters will normally call for an identification of the components. When the nature of the wastewater is known, specific methods may be applied to further characterise the TLC fractions. In one case, it was shown that the lipophilic fraction contained bromine, an indication that brominated flame retardant was discharged.

Spain

The whole effluent, the organic fraction, and/or fractions obtained by HPLC using an analytical column are assayed for toxicity on fish, daphnia and algae. The most substantial improvement has been the modification of existing OECD methods or the development of alternative methods reducing drastically the amount of sample required for each test. The capability for testing very small amounts (a few microlitres) reducing dilution as much as possible has made feasible the direct fractionation of the organic extract by analytical grade reverse-phase HPLC in a single step. The achieved resolution and the use of an analytical grade column allow the characterisation of the toxic fraction by its retention time in HPLC. The toxic substances

can then be analysed determining their chemical structure by GC/MS (Vega *et al*. 1994, 1996) and their toxicity by *in vitro* test on fish cell lines (Tarazona *et al*. 1993), avoiding the use of vertebrates.

The general protocol contains among other items the following:

- Whole effluent toxicity tests with *Daphnia magna*, C*hlorella vulgaris* and fish *in vitro* approach test.
- Organic extraction by SPE or liquid-liquid procedures.
- Toxicity monitoring of both organic and aqueous fractions, using *Daphnia magna, Chlorella vulgaris* and fish *in vitro* or the most sensitive species.
- HPLC fractionation of the whole organic extract (90 min water/acetonitrile gradient)
- *in vitro* cytotoxicity monitoring of HPLC fraction, using RTG-2 fish cell line and a battery of end points.
- GC-MS of toxic HPLC fractions (compounds identification). When the structural identification of the toxic substance was not possible, risk assessment was performed directly comparing the toxicity of the whole effluent or its toxic fractions with the dilution ratio, considering the toxicity related to organic fraction as bioaccumulable.

Using this general protocol, more than 50 effluents have been assayed (Tarazona *et al*. 1990, Muñoz *et al*. 1994, Vega *et al*. 1994, 1996, Pablos 1999). It is the opinion of these authors that the combination of whole effluent toxicity testing (WETT) and the toxicity monitoring of the HPLC fractions is an efficient solution, giving both T and B information.

Sweden

The content of potentially bioaccumulating substances in the stabilised sample (after degradation) is determined according to the method described by Renberg and co-authors (1985), see section 4.2.

The highest concentrations and largest discharge quantities of potentially bioaccumulating substances in stabilised wastewater were measured at a textile mill (4,3 mg/l), pharmaceutical plants (maximum value 8,6 mg/l), organic chemical plants (max. 210 mg/l) and paint and varnish plants (max 18 mg/l). Dominating quantities were found in a paper mill (26 kg/d), a kraft pulp mill (18 kg/d) and a textile mill (8 kg/d) (SEPA 1997, where more results may be found). The project group considered persistent and bioaccumulating material in quantities below 0,01 kg/d or concentrations below 0,1 mg PBS/l to be safe. A more recent example is found in Annex 1 to this report.

United Kingdom

The only whole animal bioaccumulation test that arose in an enquiry was OECD method 305 parts A-F; Bioconcentration: Flow-through fish test, and one mention of the MITI equivalent. This is used for the assessment of bioaccumulation of single substances. There does not seem to be much use of surrogates - only one instance of SPMD assessments as a surrogate for fish in a single substance mesocosm test. It is more common to use a chemical estimation such as derivation of a partition co-efficient by shake flask method (e.g. OECD method 107) or by HPLC estimation (e.g. OECD method 117).

4.4 Conclusions on the present situation

Some Contracting Parties have used methods that determine the octanol-water partition coefficient through liquid extraction, chromatographic separation and detection. Although results may be difficult to interpret they have nevertheless proven useful. Newer solid-phase extraction techniques use fewer steps, and may possibly give more relevant information. An alternative approach to faster analysis is the partial PBS estimation through "EGOM", a total extract analysis.

5 Bioaccumulation – development

Germany

Several research projects have been run with the aim to assess potential methods for PBS measurement in wastewater. The main objective has been to include not only the water-soluble fraction, but also PBS adsorbed to suspended solids. Additionally variables such as weight or DOC were studied.

Within the framework of a recent research project a “summary parameter” has been developed to determine potentially bioaccumulative substances (PBS) in wastewater. The crucial step of the method is the separation of the PBS by semi-preparative reverse-phase HPLC. All organic compounds within a defined “lipophilicity window”, ($3<\log K_{ow}<8$) are collected, lyophilised and finally quantified gravimetrically with a

semi-microbalance. The method is applicable both to PBS in the liquid phase and PBS adsorbed to suspended solids. The PBS of several effluents of municipal and industrial wastewaters has been determined (Stenz & Metzger 2001). In a joint study by Germany and The Netherlands this SPE/HPLC method has been compared with the SPME method (see The Netherlands below).

Reemtsma *et al*. (2001) suggested a quantitative estimation of potentially bioaccumulating compounds in wastewater by DOC difference before and after solid-phase extraction and a two-step elution. By the first elution of the solid phase with a methanol-water mixture (65:35) substances with log K_{ow}<3, which are not attributable to the PBS fraction, are removed while the PBS fraction remains sorbed. By the second elution with 100% methanol the PBS fraction is collected.

The Netherlands

Scientists from the Ministry of Transport, Public Works and Water Management (RIZA) in the Netherlands and the German Ministry of Environment, Nature Protection and Nuclear Safety have carried out an assessment of the suitability of two types of tests for screening potentially bioaccumulating substances in complex effluents, the SPE/HPLC and SPME methods (de Maagd *et al* 2000, Stenz *et al* 2000). In all, tests were conducted on seven industrial discharge types in the Netherlands, as listed in Table 5. The methods were briefly described in section 4.2 above.

Table 5. Summary of the data for the different PBS screening tests for complex effluents used in the collaborative Netherlands/German study

Effluent type	DOC (mg/l) (% of TOC)	TOC (mg/l)	SPME data (mM)	SPE/HPLC data (mg/l)	
				Liquid	Particulates
Waste incineration	75 (89%)	84	91	2,2	24
Paper production	47 (87%)	54	120	0,87	43
Food oil production	10 (77%)	13	217	0,36	74
Smelting plant	60 (100%)	58	37,5	0,26	53
Pesticide production	33 (97%)	34	9,4	0,14	19
Chemical production	6,0 (76%)	7,9	12,5	0,25	12
Metal plating	43 (88%)	49	350	14,0	62

Table 5 above shows the results of tests on seven complex effluents from different industrial plants. On the basis of freely dissolved substances both methods discriminated between the different effluents and identified the metal plating plant and pesticide production plant as having the highest and lowest levels of potentially bioaccumulating substances respectively. However, the tests showed a different ranking order (highest to lowest) for bioavailable bioaccumulating substances.

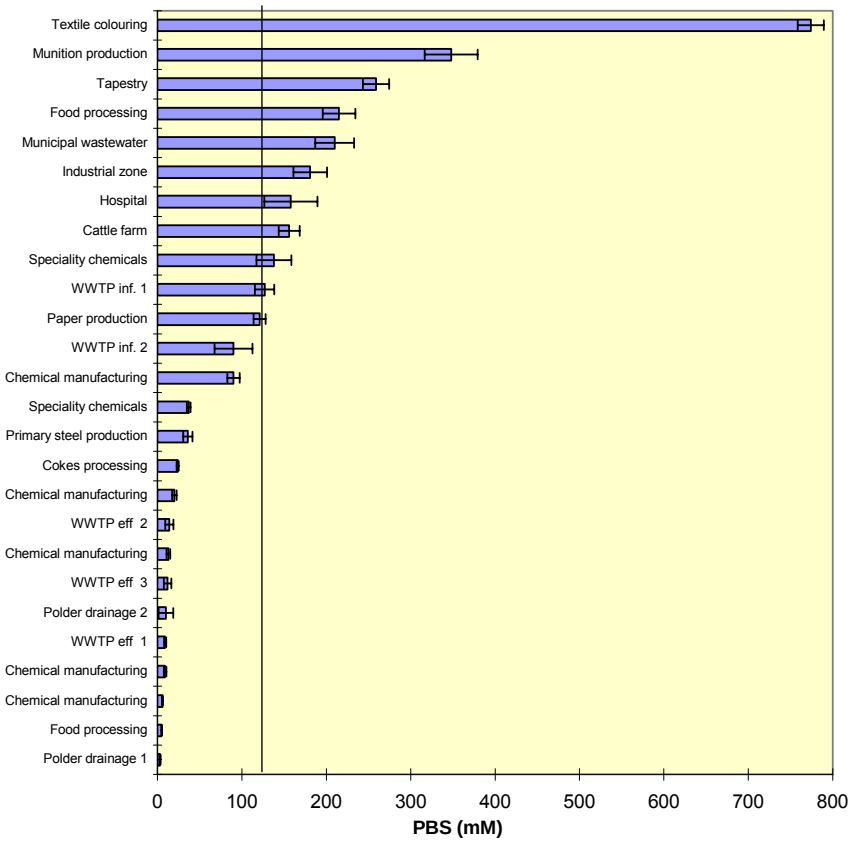
The SPE/HPLC method indicated that the amount of PBS associated with the particulate phase from each effluent was considerably higher than that measured for the liquid phase, though this finding needs to be evaluated with regard to bioavailability to aquatic biota.

The response from the SPME fibres can vary considerably, usual compensation is by calibration. The fibres need several conditioning exposures to samples before ‘settling’. This may account for some of the variation observed in Table 5. Also, there may be a salt effect, i.e. partitioning between fibre and liquid phase (examples are found in the marine environment).

Development history. Verbruggen and co-workers developed SPME for the measurement of PBS at the Research Institute for Toxicology, Utrecht University. Early studies showed that

- The method is able to distinguish effluents, surface water and a blank from each other on their PBS contents in a reproducible way.
- The C8-fibres that were used were not of a constant quality. Within samples a co-variation was found between the used fibre and the amount of PBS that was measured.

Figure 3. PBS concentration data for different effluent types in the Netherlands



In a larger study polyacrylate fibres were selected because the partition coefficients of accumulating compounds for polyacrylate correlated well with their bioconcentration factors. Furthermore, the supplier of the fibres could guarantee a more constant quality for the polyacrylate fibres than for the C8 fibres. The variation coefficients in PBS content within triplicate measurement overall were < 15% (figure 3).

In a third step an interlaboratory ring test was performed in 2000, which involved a series of six laboratories measuring four replicates of seven samples, namely:

- a freshwater control
- a seawater control
- four industrial effluents
- a synthetic effluent comprising ten standard compounds of varying $K_{ow.}$ This was used to determine the accuracy by which the laboratories could identify unknown compounds based on their mass spectrum
- an external standard containing 2,3,5-trichlorotoluene.

The results of the ring test (figure 4) show that, although the relative amount in PBS concentrations are to a certain extent in agreement between laboratories, i.e. certain samples contain the highest PBS concentrations according to all participants, variation is still very high. In subsequent discussions it was concluded that the main source of variation is probably caused by inconsistencies in the experimental protocol. For example, the procedure suggested by the supplier to clean up the fibres before use was shown to be insufficient. One of the laboratories adjusted that procedure in order to solve this problem. Another problem is to describe the integration procedure in the experimental protocol so as to enable every laboratory to perform the integration in an identical way. Subsequent development intends to remedy these shortcomings.

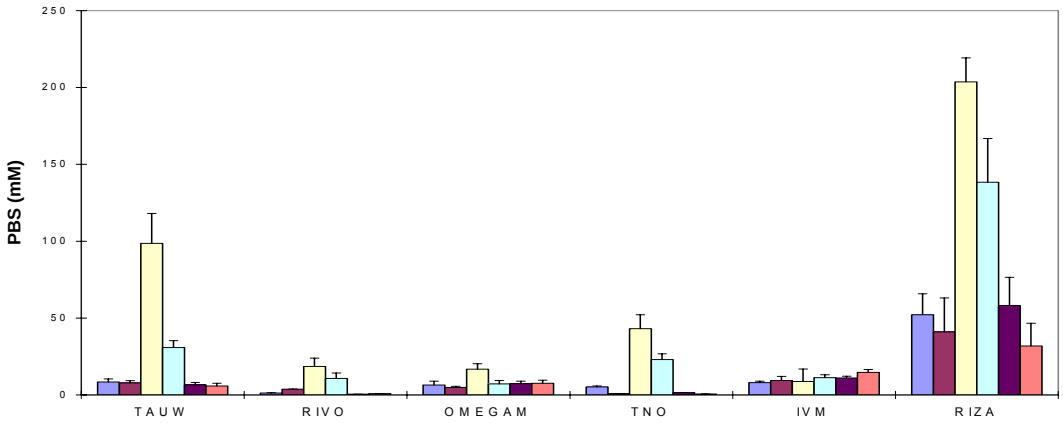


Figure 4. Summary of the results from the inter-laboratory ring test of the SPME method (Samples 1-4 are effluents and samples 5-6 are reference samples)

Validation. At RIZA an additional validation was performed on the ability of the SPME procedure to predict the concentration of bioaccumulated organic compounds in Daphnids after exposure to effluents. In order to do so a dilution series of an effluent was made. In each dilution the PBS concentration was measured with SPME, which was matched to the bioaccumulated organic compounds accumulated in Daphnids exposed to the same dilution. Daphnids were extracted with hexane, and subsequently the extract was analysed. As expected the organic compounds of the Daphnids themselves were dominant in the extract. However, by looking at specific masses that were also found in the SPME extract a comparison could be made between SPME and Daphnids. A good correlation between concentrations of effluent related masses in Daphnids and SPME was found. This suggests that SPME validation can also be performed on the basis of Daphnid body burdens.

Potential for routine application of SPME. Four regional directorates of the Ministry of Transport, Public Works and Water Management, Rijkswaterstaat, participated in a study in which each directorate had five effluents from its region measured on PBS with the SPME method. For each directorate the effluent with the highest PBS content was studied in more detail within the RIZA laboratory in order to identify compounds that had a relevant contribution to the total PBS content in the sample. This detailed study included analysis of SPME extracts on a much longer GC-column. On this column the analysis of unknown compounds in surface waters is performed. This enabled us to measure a standard containing 122 compounds in parallel, so that identification could be made both on mass spectra and Kovatt's retention indices. Doing so, between 23 and 52% of the total concentration of PBS could be identified.

Spain

A common principle of our activity has been to develop an alternative to whole effluent toxicity testing (WETT) accounting for the bioaccumulation potential. The extraction of the organic (lipophilic) fraction and the assessment of its toxicity potential in effluent organic fraction toxicity testing (EOFTT) has been a constant objective. This research is ongoing, with the aim of obtaining quantitative information. The parallel assessment of persistence has been included as a main goal only recently.

Apart from the persistence study mentioned above, a second study is directed to assess the bioaccumulation potential. Again, parallel assessment for each toxic component and the overall toxicity of the effluent must be considered. The methodology accounts for the relationship between the lipophilic nature and capacity factor in HPLC for assessing the potential of individual fractions/components, and the ratio between the toxicity observed in WETT and EOFTT for the overall toxicity. Note, that this procedure only considers the toxic portion of a sample, not bioaccumulating compounds with other characteristics.

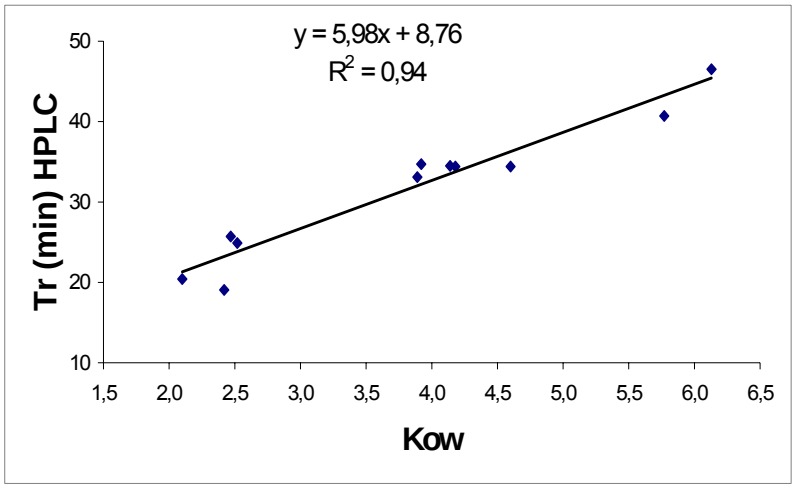


Figure 5. Correlation between HPLC retention time and K_{ow}

Finally, these two studies will be integrated in a system (toxicity, bioaccumulation, and persistence) that will provide a quantitative and realistic point of view on the environmental hazard of the effluents. These studies have already begun with the correlation of HPLC retention times and octanol/water partition coefficient. A good correlation between K_{ow} and HPLC retention times was found, as shown in figure 5.

The United Kingdom

There is a fair amount of work being performed in the UK to assess bioaccumulation from the sediment pathway, but as yet all methods used are under development.

6 References

- Adolfsson-Erici, M & Wahlberg, C (1992). Extraherbart gaskromatograferbart organiskt material (EGOM); Extraherbart organiskt bunden halogen (EOX); Potentiellt bioackumulerbara substanser (PBS). In Swedish. Report annexed to SEPA (1992)
- Ahtiainen, J, Pessala, P & Aalto, M (2003). Biodegradation of chemicals in a standardised test and in environmental conditions. Chemosphere, in press
- Battersby, NS (1997). The ISO headspace method biodegradation test. Chemosphere 34(8), 1813-1822
- Beek, B, Böhling, S, Franke, C, Jöhncke, U, Studinger, G, Thumm, E (2001). The assessment of biodegradation and persistence. In Biodegradation and persistence (B. Beek editor), Springer Verlag, Berlin
- Blok, J & Balk, F (1994). Guidance document for the interpretation of biodegradability test data. EC Commission contract no. B4-3040/93/00114
- Burkhard, L & Sheedy, BR (1995) Evaluation of screening procedures for bioconcentratable organic chemicals in effluents and sediments. *Environmental Toxicology and Chemistry*, 14, 697-711
- de Maagd, P G J (2000). Bioaccumulation tests applied in Whole Effluent Assessment: A review. *Environ. Toxicol. Chem.* 19(1), 25-35
- de Maagd, P G J, Stenz,G, Verbruggen, E M J, Tonkes, M, Hermens, J L M & Metzger, J W (2000). Comparison of a solid-phase micro-extraction/GC and a solid-phase extraction/HPLC-based method to assess the bioaccumulative potential of effluents. Pres. at 3rd Society of Environmental Toxicology and Chemistry (SETAC) World Congress, Brighton
- EC (1967). Council directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances
- EC (1988). Commission directive 88/302/EEC adapting to technical progress for the ninth time Council directive 67/548/EEC

- EC (1992). Commission directive 92/69/EEC adapting to technical progress for the seventeenth time Council directive 67/548/EEC
- EC (2002). Reference Document on Best Available Techniques in Common Waste Water and Waste Gas Treatment/Management Systems in the Chemical Sector. European IPPC Bureau Seville, Spain
- EC (2003). Technical guidance document in support of commission directive 93/67/EEC on risk assessment for new notified substances, commission regulation (EC) No 1488/94 on risk assessment for existing substances and directive 98/8/EC of the European Parliament and the Council concerning the placing of biocidal products on the market. European Communities
- Gartiser, S, Meyer, M & Jäger, I (1996). Zur Interpretation des Zahn-Wellens-Tests bei der Untersuchung von Abwasserproben. gwf-Wasser 137 (7), pp. 345 – 352.
- Gartiser, S, Meyer, M & Jäger, I (1997). Abbau ökotoxischer und mutagener Abwasserinhaltsstoffe im Zahn-Wellens-Test und Labor- bzw. technischen Kläranlagen. gwf-Wasser, 138, pp. 28–34.
- Gartiser, S & Urich, E (2001). Bestimmung der aeroben und anaeroben Abbaubarkeit sowie Hemmung von Antibiotika in genormten Testsystemen. Zwischenbericht zu Teil 4 des F&E-Vorhabens 29863722, Juni
- Hynning, P-Å (1996). Separation, identification and quantification of components of industrial effluents with bioconcentration potential. *Wat. Res.* 30(5) 1103-1108
- Ingerslev, F & Nyholm, N (2000). Shake-flask test for determination of biodegradation rates of ^{14}C-labeled chemicals at low concentrations in surface water systems. Ecotox. Environ. Safety 45, 274 - 283
- ISO (9/94). Evaluation in an aqueous medium of the „ultimate“ aerobic biodegradability of organic compounds – Method by analysis of dissolved organic carbon (DOC), International Standards Method 7827, (corresponds to DIN EN ISO 7827:96-04)
- ISO (10/94). Evaluation in an aqueous medium of the „ultimate“ aerobic biodegradability of organic compounds – Determination of biochemical oxygen demand (closed bottle test) International Standards Method 10707, (corresponds to DIN EN ISO 10707:98-03)
- ISO (1997). Water quality – Selection of tests for biodegradability, International Standards Method ISO/TR 15462, International Standards Organisation, Paris
- ISO (2001). ISO 16221: Guidance for determination of biodegradability in the marine environment. First edition 2001-02-01
- Johnson, I & Watts, C (2001). Review of potential screenings methods for the assessment of the persistence and bioaccumulation potential of effluents. Draft report to the Dept of Environment, transport and the regions, UK
- Killer, A & Schönberger, H (1993). Refraktäre Stoffe im Textilabwasser. Textilveredlung 28, Nr. 3., pp. 44-53
- Klamer, H J C & Beekman, M (1995). Estimating the 1-octanol/water partition coefficients (K_{ow}) and fish bioconcentration factors (BCF) of unknown compounds using a gradient HPLC method. *Toxicological Modelling*, 1, 169-179
- Kristensen, P, Pedersen, F, Damborg, A & Christensen, H W (1992). Økotoksikologisk vurdering af industrispildevand. Miljøprojekt nr. 188, Miljøstyrelsen
- Meinecke, S, Norr, C & Brackemann, H (2000). Prüfung der biologischen Abbaubarkeit von schwerlöslichen und oberflächenaktiven Stoffen in einem neuen Testsystem auf Basis des Zahn-Wellens-Tests. Vom Wasser 95, 177-190
- Muñoz, M J, Castaño, A, Blazquez, T, Vega, M, Carbonell, G, Ortiz, J A, Carballo, M & Tarazona, J V (1994). Toxicity identification evaluations for the investigation of fish kills: a case study. *Chemosphere* 29, 55-61
- Murty, A S (1986). Editor of *Toxicity of pesticides to fish*, Vol. 1, 69-116, CRC Publishing Co
- OECD (1992a). Guidelines for testing of chemicals. Section 3. Degradation and accumulation, 301 Ready biodegradability.

- OECD (1992b). Guidelines for testing of chemicals. Section 3. Degradation and accumulation, 302 Inherent biodegradability: Zahn-Wellens/EMPA test
- OECD (1995). Detailed review paper on biodegradability testing. Environment Monograph No. 98. Organisation for Economic co-operation and development, Paris
- OECD (2001). OECD series on testing and assessment. Harmonised integrated classification system for human health and environment hazards of chemical substances and mixtures. No. 33, 247 pp
- OSPAR Commission (2000). OSPAR Background Document concerning the elaboration of programmes and measures relation to Whole Effluent Assessment. OSPAR Commission, London
- Pablos, M V (1999). Incorporación de Bioensayos y sistemas de valoración toxicológica en el análisis de riesgo de la contaminación urbana y ganadera. Ph. D. Thesis, Veterinary Faculty. Complutense University, Madrid, Spain
- Pagga, U (1995). Prüfung und Bewertung von Abwässern auf biologische Abbaubarkeit. Korrespondenz Abwasser 42), pp. 263-270
- Pedersen, F, Damborg, A & Kristensen, P (1994). Industrispildevands miljøfarlighed, Miljøprojekt nr. 260, Miljøstyrelsen
- Reemtsma T & Klinkow, N (2001). Strategy for the Investigation of Hazardous Substances in Industrial Effluents: IDA (Industrial Discharge Assessment) Research Report 299 22 297 on behalf of the German Federal Environmental Agency, UBA-Texte 52/01
- Renberg, L, Sundstrom, S G & Rosén-Olofsson, A C (1985), The determination of Partition Coefficients of Organic Compounds in Technical Products and Waste Waters for the Estimation of Their Bioaccumulation Potential Using Reversed Phase Thin Layer Chromatography. *Toxicology and Environmental Chemistry*, 10, 333-349
- SEPA (1992). Utsläpp av stabila organiska ämnen från kemiindustrin. Undersökningar 1989 – 1991. In Swedish. Report 4103. Swedish Environmental Protection Agency, Stockholm
- SEPA (1997). Characterisation of discharges from the chemical industry. The STORK Project. Report 4766, Swedish Environmental Protection Agency, Stockholm
- Stenz, G & Metzger, J W (2001). Entwicklung, Optimierung und Validierung eines Summenparameters für potenziell bioakkumulierbare Stoffe (PBS) im Abwasser. Research Report Nr. 297 22 530 on behalf of the German environmental agency
- Stenz, G, Petrick, S & Metzger, J W (1999). A summative parameter to determine potentially bioaccumulative substances(PBS) in complex effluents – a HPLC-based method. Pres. at the Soc. Env. Tox. and Chem – Europe Meeting on effluent toxicology – a European perspective, Edinburgh, UK
- Stenz, G, Petrick, S, Kraub, X & Metzger, J W (2000). A summary parameter to quantify potentially bioaccumulative substances (PBS) in effluents of wastewater treatment plants. Presentation at the 3rd Society of Environmental Toxicology ands Chemistry (SETAC) World Congress, Brighton
- Stucki, G (2000) Zur Aussagekraft des biologischen Abbautests nach Zahn-Wellens zum Schicksal von chemischen Abwässern in Industriekläranlagen. gwf-Wasser Abwasser, 141, pp. 662-669
- Stuhlfauth, T (1995). Ecotoxicological Monitoring of industrial effluents. In: Richardson, M.: Environmental Toxicology Assessment. Taylor & Francis, Bristol
- Tarkpea, M, Andrén, C, Eklund, B, Gravenfors, E & Kukulska, Z (1998). A biological and chemical characterisation strategy for small and medium-sized industries connected to municipal sewage treatment plants. *Env. Tox. Chem.* 17(29), 234-250
- Tarazona, J V, Castaño, A & Gallego, A (1990). Detection of organic toxic pollutants in water and wastewater by liquid chromatography and *in vitro* cytotoxicity tests. *Analytica Chimica Acta* 234, 193-197
- Tarazona, J V, Cebrian, M & Castaño, A (1993). Development of *in vitro* cytotoxicity tests using fish cell lines. *In* A. Soares and P. Calow, eds. *Progress in Standardisation of Aquatic Toxicity Tests.* Lewis, Boca Raton, FL, 119-128
- UK Department for the Environment, Food & Rural Affairs: Economic Instruments for Water Pollution (1998), www.defra.gov.uk/environment/wqd/eiwp/index.htm

- van Loon, W M G M, Wijnker, F G, Verwoerd, M E & Hermens, J L M (1996). Quantitative determination of total molar concentrations of bioaccumulable organic micro-pollutants in water using C_{18} Empore disc and molar detection techniques. *Anal. Chem.* 68, 2916-2926
- Vega, M M, Castaño, A, Blazquez, T & Tarazona, J V (1994). Assessing organic toxic pollutants in fish processing wastes using fish cell lines. *Ecotoxicology*, 3, 79-88
- Vega, M M, Fernandez, C, Blázquez, T, Tarazona, J V & Castaño, A (1996). Biological and chemical tools in the toxicological risk assessment of Jarama river, Madrid, Spain. *Environ. Pollution* 93, 135-139
- Verbruggen, E M J (1999). Predicting hydrophobicity, bioconcentration and baseline toxicity of complex organic mixtures. PhD thesis, Utrecht University, Utrecht, The Netherlands
- Zahn, R & Wellens, H (1974). A simple method for testing the biodegradability of products and effluents. Chem.-Ztg 98, 228-232

Annex 1. Examples of PBT testing in Sweden

The dominant application of wastewater characterisation by chemical and biological means, including tests for persistence, potential, bioaccumulation and toxicity, is in the permitting process. This means that tests are performed either by the applicant as a demonstration of the status of the discharge from the site, or that the Environmental court demands that the operator demonstrate after introducing new processes or measures against the discharges that the intended improvements have indeed been achieved. A sometime third application may be the reporting requirement in one of the North Sea or Baltic Conventions.

Case 1. Resin acid production

A manufacturer of modified resin acids, which are used as adhesives in paint and glue manufacture, was requested by the licensing board in their recent permit to perform characterisation of their wastewater before and after the treatment plant. The primary reason was to verify that the treatment plant could take care of a new process line.

Among other findings the following was obtained:

Biodegradation. In the composite samples COD was reduced from 5671 to 319 mg/l, which means that there was a 94 % reduction in the treatment plant. In the 28 days stabilisation test (ISO 7827, adapted) the treated wastewater the remaining BOD_7, which was 7 mg/l, disappeared within 3 days. The DOC reduction over the sample period was only 8 %. Hence the biological treatment is taking care of what is biologically treatable, the persistent discharge amounted to 43 kg DOC/d.

Bioaccumulation. EGOM was determined to be 4,2 mg/l, that is more than 0,1 mg/l, which was seen as an acceptable content in a stabilised wastewater in the STORK project. Out of this the PBS portion was separated with HPLC, and constituted 2,6 mg/l ($\sum K_{ow}>3$). This corresponds to a discharge of 1,9 kg PBS/d to the Baltic Sea.

Toxicity. TU_{50} (toxic units at the EC50 level) estimated with Microtox (15 min) is 1,7 in the stabilised sample and 4 in the discharged wastewater. Based on this single estimate the wastewater was judged to be intermediate to low toxic.

On the basis of these findings the regional permitting authority concluded that the wastewater treatment plant performed as well as could reasonably be required, that the company should continue to study possible internal improvements in the processes, and that the implications of the B and T factors should be treated in the application scheduled for 2004, which will address the requirements under the IPPC directive.

Case 2. Urea/formaldehyde resin production

A production line for a urea/formaldehyde resin has proven to give a wastewater with inordinately recalcitrant content. A limited characterisation has been performed on the physically and chemically treated water in order to check on in-process developments.

Biodegradation. The wastewater was very toxic to the active sludge, and had to be diluted 20 times. The stabilisation process required 2 weeks, after which the DOC reduction was 85 %. This level was then constant until 28 d. The COD reduction was greater than 60 %.

Bioaccumulation. EGOM was determined to be 0,28 mg/l, which is lower than the 0,5 mg/l seen as an acceptable content in a wastewater as discharged.

Toxicity. Since there is a high content of NH_4-N it was judged impossible to use algae or other organisms. EC_{50} (15 min) with Microtox was 7,8, i.e. TU = 13. The sample was judged to be very toxic. After the stabilisation test the toxicity was reduced by more than 50 %. Because of the dilution required for the degradation test it is not possible to judge this with high accuracy. The consultant laboratory considered the toxicity to be caused by hydrogen peroxide and formaldehyde, which explains the treatability after proper dilution.

The wastewater is discharged after blending with other wastewater, before release to the Baltic Sea. There appears to be a potential for further treatment of such a blend, something that has not been possible to demonstrate in simulation runs. The permitting authority has not yet treated the quite recent results.

Case 3. Vinyl and polyvinyl chloride production

The Swedish manufacturer of vinyl and polyvinyl chloride performed a characterisation of their wastewater, after taking their thoroughly remodelled biological treatment plant on-stream:

Biodegradation. The treated water contained merely 6 mg BOD_7/l. It was judged uncalled for to perform a stabilisation test. A previous investigation had showed reduction in the stabilisation test to approximately the same TOC level as was now obtained in the treated wastewater.

Bioaccumulation. EGOM was determined to correspond to 0,33 mg OC/l or 0,39 mg/l calculated as $C_{20}H_{42}$, a reduction by 40 % from the situation after the old treatment plant. Since this is below 0,5 mg/l no PBS determination was performed.

Toxicity. TU_{50} and TU_{20} with Microtox (15 min) were below 1, that is no dilution should be necessary to alleviate acute toxicity at the outlet. A chronic test with *Nitocra spinipes* showed that reproduction was inhibited at lower dilutions than 3. Growth inhibition of the marine alga *Nephroselmis pyriformis* was strong in previous tests. EC_{50} was now 23 %, or TU_{50}=4,3, a considerable improvement. NOEC was determined to 10 %. Thus there is some inhibition of invertebrates and algae. Since there is a primary dilution at the outlet of more than 200-fold, no effect is expected in the recipient, a fjord of the Kattegat.

The environmental court accepted the presented arguments, but still decided on a trial period, during which the company should study possibilities to further reduce the discharge of suspended solids, in particular PVC.

Case 4. Oil and varnish production

This is also a producer of oils and varnishes, mainly for paint manufacture. A report has been generated for use in national reports to HELCOM. The plant has its own biological wastewater treatment after which it is further treated in the municipal treatment plant. Hence some of the ecotoxicological measurements are less important in this case.

Biodegradation. The further biodegradation over 28 d was as low as 3,5 %, indicating that the biotreatment is efficient. At the same time the reduction of TOC was as high as 52 %, depending on a continuous solubilisation of solid or emulsified material. There is reason to believe that further reduction is achieved in the municipal plant.

Bioaccumulation. EGOM was determined to be 4,9 mg/l. Out of this 1,2 mg/l were accounted for as PBS.

Toxicity. No inhibition of nitrification could be determined. EC_{50} with Microtox (15 min) was 57 % i.e. indicating a moderate acute toxicity. A test with *Ceriodaphnia dubia* indicated a similar toxicity range, but the only test of significance in this case is the nitrification inhibition.

The information is included as required in the Swedish report for Recommendation 20/6 for 2001.

Annex 2. Summary of the characteristics of ready, inherent and other biodegradability tests for single substances

Method	Test duration	Inoculum	Test conditions	Measurements	Endpoint
Ready and Inherent Biodegradability tests					
OECD 301A (ISO 7827)	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/ml) in surface waters, unchlorinated sewage treatment works effluents or activated sludge	Agitation in the dark or diffuse light under aerobic conditions at 20-24 ^oC	Dissolved organic carbon[1] (DOC)	DOC removal (%)
OECD 301B (ISO 9439)	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/ml) in surface waters, unchlorinated sewage treatment works effluents or activated sludge	Agitation in the dark or diffuse light under aerobic conditions at 20-24 ^oC	CO_2 production	% degradation
OECD 301C	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/ml) in a mixture from 10 different sites, incl. industrial sewage effluent. 1 – 3 month acclimation in lab!	Agitation in the dark under aerobic conditions at 24-26 ^oC	O_2 uptake	% degradation
OECD 301D (ISO 10707)	Up to 28 days	Micro-organisms (~10^5 cells/ml) in surface waters or unchlorinated sewage treatment works effluents	Agitation in the dark under aerobic conditions at 20-24 ^oC	O_2 uptake	% degradation
OECD 301E (ISO 7827)	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/ml) in unchlorinated sewage treatment works effluents	Agitation in the dark or diffuse light under aerobic conditions at 20-24 ^oC	Dissolved organic carbon[1] (DOC)	DOC removal (%)
OECD301F (ISO 9408)	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/ml) in surface waters, unchlorinated sewage treatment works effluents or activated sludge	Agitation in the dark or diffuse light under aerobic conditions at 20-24 ^oC	O_2 uptake	% degradation
OECD 302B	Up to 28 days	Washed activated sludge. Ratio between DOC of inoculum and test material 2,5:1 to 4:1; adaptation permitted	Aerated in the dark or with diffuse light. If necessary with agitation	Dissolved organic carbon[1] (DOC) or COD	DOC removal (%)
Seawater biodegradability test					
OECD 306 (ISO 7827 and 10707)	Up to 60 days	Micro-organisms[2] in test seawater	Agitation in the dark or diffuse light under aerobic conditions at 15-20 ^oC	Dissolved organic carbon[1] (DOC)	DOC removal (%)
OECD 309 (ISO 14592)	No fixed duration	Micro-organisms in surface water	Agitation in the dark or diffuse light under aerobic conditions at field temperature or 20 – 25 ^oC	Specific chemical or radiochemical analysis	Estimation of first order rate constant
OECD 310 draft (ISO 14593)	Up to 28 days	Activated sludge, suspended solids 4 mg/l, unchlorinated sewage effluent, surface water	Agitation in the dark or diffuse light under aerobic conditions	CO_2 production	% degradation

[1] Following membrane filtration or centrifugation and analysis by wet oxidation by persulphate/UV irradiation, wet oxidation by persulphate/elevated temperature (116-130 ^oC) or combustion

[2] Colony forming heterotrophic bacteria

Annex 3. Summary of the different approaches used for the procedural steps and comments on their suitability for the assessment of potentially bioaccumulating substances (PBS) in effluents

Procedure	Approaches	Comments
Pre-treatment	pH adjustment to 2 to bring phenolic and carboxylic compounds in an effluent to a neutral state allowing extraction with an organic solvent.	May decrease the selectivity of the method if non-bioaccumulable compounds are extracted and result in an over-estimate of PBS. Increases the costs associated with the analytical procedure.
	Filtration to remove particle bound compounds in an effluent.	May increase the selectivity of the method if bioavailable compounds are not extracted and under-estimate PBS. Increases the costs associated with the analytical procedure.
Extraction methods	*Liquid-liquid* in which effluent is extracted with an immiscible organic solvent to exhaustively remove both dissolved and particulate bound organic micro-pollutants.	May overestimate PBS since the procedure is exhaustive. The compounds extracted are dependent on the solvent used, the volume:volume ratio and the extraction time.
	Solid-phase using an empore disc or column, which partitions dissolved PBS onto the C_{18} disc coating. This biomimetic procedure extracts highly bioaccumulating compounds to a greater extent than less bioaccumulating compounds.	May underestimate PBS since particulate-bound substances are not considered. The long equilibration time of 2 weeks for the Empore disc reduces the practicality of the approach and increases the costs associated with the analytical procedure.
	Semi-permeable membrane device (SPMD) using a polyethylene tube filled with an organic solvent (or artificial lipid) to mimic the concentration of freely dissolved organic chemicals.	May underestimate PBS since particulate-bound substances are not considered. The long equilibration time of 2 weeks reduces the practicality of the approach and increases the costs associated with the analytical procedure.
	Solid-phase microextraction (SPME) which uses a polymer-coated fibre to extract freely dissolved organic chemicals. This biomimetic procedure extracts highly bioaccumulating compounds to a greater extent than less bioaccumulating compounds.	May underestimate PBS since particulate-bound substances are not considered. The method is practical and cost-effective due to the shorter equilibration times (compared to the Empore disc and SPMD methods) and the capability to avoid the use of solvents and minimise compound loss during pre-concentration by injecting the fibre into the gas chromatograph directly. However, limits of detection may be higher than for conventional solid-phase extraction.
Pre-treatment of extracts	*Cleanup* of wastewaters is a prerequisite for samples extracted by liquid-liquid extraction especially when gas chromatography is to be applied. Clean up can be performed with a range of techniques including the use of adsorbents such as fluorisil, (activated) silica and aluminium oxide.	May cause loss of PBS thus lowering target compound recovery.
	Pre-concentration is required to optimise the recovery of PBS from the large volumes of organic solvents during exhaustive extractions and generate sufficiently high concentrations for detection. Concentration is normally achieved by evaporating the solvent under nitrogen.	May lead to a loss of PBS (particularly semi-volatile bioaccumulating compounds) and low recoveries of target compounds.

Procedure	Approaches	Comments
	Derivatization is applied to increase the detection response of compounds that cannot be detected in their original form (for example carboxylic acids).	May lead to a loss of PBS (through volatilisation or decomposition) and low recoveries of target compounds.
Separation	*Thin layer chromatography (TLC)* is a form of liquid chromatography that uses a stationary phase coated onto a solid support, for example a glass plate, to separate compounds.	The method is fast and cost-effective and equipment costs are low (relative to HPLC). However, the method may be selective since it cannot measure relevant ionic compounds such as carboxylic acids and amines. In addition the system is not open to automation in the same way as GC and HPLC.
	High performance liquid chromatography (HPLC) separates hydrophobic compounds in the liquid phase. A high selectivity against interfering compounds (such as lipids and humic acids) is needed during extraction and cleanup.	The equipment costs of HPLC are high but automation means the approach is practical and unit costs are acceptable.
	Gas chromatography (GC) normally separates semi-volatile compounds with a boiling point of $\leq$ 400 ^{o}C	The equipment costs of GC are high but automation means the approach is practical and unit costs are acceptable. In general compounds with a low vapour pressure and/or thermal instability cannot be successfully eluted from a GC column.
Detection	*Ultraviolet and visible light detection* is based on the absorption of light in the ultraviolet or visible wavelength range. The technique can be applied directly to an extract but is normally combined with an HPLC system.	The method has broad selectivity and a wide variety of compounds can be successfully detected. However, response factors vary by orders of magnitude between compounds thereby limiting the possibility of reliable quantitative PBS assessment.
	Flame ionisation detection is used with GC.	The method can be regarded as a universal detection method for organic compounds. However, response factors vary by orders of magnitude between compounds thereby limiting the possibility of reliable quantitative PBS assessment.
	Mass spectrometry can be used to identify and quantify unknown PBS, which is valuable for complex mixtures such as effluents.	There are many ionisation methods available, which vary in sensitivity and specificity.
	Vapour pressure osmometry measures the total molar concentration of PBS and has been successfully applied without a separation step.	The method has almost universal selectivity and the analytical sensitivity allows the quantification of effluents (though probably not receiving waters).
	Gravimetric detection is applied following a preparative separation phase and measures the weight of the PBS and not the molar concentrations.	The method is likely to lose semi-volatile PBS and can have low selectivity if interfering high molecular weight non-bioaccumulable substances are not removed at the clean-up step prior to separation and detection. It requires large sample volumes due to the low analytical sensitivity.
	Fluorescence detection is used in connection with HPLC.	The method can only detect fluorescent compounds but is extremely sensitive to these compounds. The method can be used as part of a wider detection approach in which other techniques are also applied.
	Electron capture detection is used in connection with GC.	The method can only detect halogenated organic compounds but is extremely sensitive to these compounds.