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# Strategy for the Investigation of Hazardous Substances in Industrial Effluents: IDA (Industrial Discharge Assessment)

by

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16. Zusammenfassung

Die Erfassung der Gehalte und Wirkungen gefährlicher Stoffe in Abwassereinleitungen der Industrie erfordert eine Kombination chemischer und biologischer Untersuchungen, die über das bisher in der AbwV festgelegte Maß hinausgeht. In dieser Studie wurde deshalb eine Untersuchungsstrategie für gefährliche Stoffe in Abwassereinleitungen entwickelt, die die Parameter Persistenz, Bioakkumulierbarkeit und Toxizität auch experimentell verknüpft.

Die Ausarbeitung erfolgte nach Auswertung der international bestehenden Untersuchungsstrategien sowie der zur Verfügung stehenden Testverfahren. Der Aufbau der Strategie ist modular angelegt zur Sicherung einer flexiblen Anpassung an die Gegebenheiten eines Abwassers. Nach der Bestimmung von akuter und chronischer Toxizität sowie Gentoxizität folgt ein biologischer Abbautest zur Gewinnung der persistenten Abwasserfraktion. Aus dieser werden die bioakkumulierbaren Stoffe mittels Festphasen-Extraktion bestimmt. In der Strategie sind Unterschiede zwischen Direkt- und Indirekteinleitern berücksichtigt; auch die partikuläre Phase findet Beachtung. Durch die modulare Verknüpfung kann ermittelt werden, ob ein Abwasser toxische und persistente und bioakkumulierbare Stoffe enthält, die ein bedeutendes Gefährdungspotenzial für die aquatische Umwelt darstellen.

Eine erste Anwendung der Strategie auf drei Abwässer der chemischen und metallbearbeitenden Industrie zeigte, dass die Untersuchungsstrategie in der geplanten Art und Weise eingesetzt werden kann.

17. Schlagwörter

Untersuchungsstrategie; Industrieabwasser; Abwassereinleitung; gefährliche Stoffe; Biotests; akute Toxizität; chronische Toxizität; Gentoxizität; Persistenz; biologischer Abbautest; potenziell bioakkumulierende Stoffe

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18. Abstract

The assessment of amounts and effects of hazardous substances in industrial effluents necessitates a combination of chemical and biological investigations. Therefore in this study a strategy for the investigation of hazardous substances in wastewater discharges was developed which connects the parameters persistence, bioaccumulation, and aquatic toxicity.

The strategy IDA (Industrial Discharge Assessment) was elaborated based on the evaluation of existing investigation strategies and the available test systems. The strategy has a modular structure to assure a flexible investigation with regard to the particularities of a given wastewater. After the determination of acute and chronic toxicity and genotoxicity a microbial degradation test is carried out to obtain the persistent wastewater fraction. The bioaccumulating substances are determined from this fraction by solid-phase extraction. Within the strategy differences between direct and indirect discharges are taken into account and particulate matter is considered as well. By the modular linking it can be determined if a wastewater contains toxic and persistent and bioaccumulating substances, which present an important potential of hazard for the aquatic environment.

The first application of the strategy on three wastewater samples of the chemical and the metal processing industry showed that the strategy can be used as planned.



19. Keywords

investigation strategy; industrial wastewater; wastewater discharge; hazardous compounds; bioassays; acute toxicity; chronic toxicity; genotoxicity; persistence; biological degradation test; potentially bioaccumulating compounds


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## Abbreviations

| | |
|---|---|
| AbwV | Abwasserverordnung (German Wastewater Ordinance) |
| Abs | Absorbance |
| AOX | Adsorbable organic halides |
| BCF | Bioconcentration factor |
| BODn | Biological oxygen demand over n days |
| COD | Chemical oxygen demand |
| TLC | Thin-layer chromatography |
| DOC | Dissolved organic carbon |
| DTA | Direct toxicity assessment |
| ECx | Effect concentration causing an effect of x % to the test organisms |
| ELS | Early life stage |
| EINECS | European Inventory of Commercial Chemical Substances |
| EOCl | Extractable organic bound chlorine |
| FNU | Formazine Nephelometric Units |
| $G_X$ | equivalent to LID (Lowest ineffective dilution), dilution level expressed as the reciprocal of the volume fraction of wastewater in the test sample where the effect is less than it is specified in the test method, e.g. less than 10 % immobilisation of daphnia (x denominates the test organism) |
| GC/MS | Gas chromatography/mass spectrometry |
| HPLC | High-performance liquid chromatography |
| $K_{ow}$ | Octanol/water distribution coefficient |
| LOEC | Lowest observed effect concentration |
| $N_{tot}$ | Nitrogen, total (sum of NH4-N, NO3-N and NO2-N) |
| NOEC | No observed effect concentration |
| $P_{tot}$ | Phosphor, total |
| PAHs | Polycyclic aromatic hydrocarbons |
| PBS | Potentially bioaccumulating substances |
| PCBs | Polychlorinated biphenyls |
| PEC | Predicted environmental concentration |
| PNEC | Predicted no effect concentration |
| TSS | Total suspended solids |
| TIE | Toxicity identification evaluation |
| $TN_b$ | Total bound nitrogen |
| TOC | Total organic carbon |
| TOCl | Total organic bound chlorine |
| SPE | Solid-phase extraction |
| SPMD | Semipermeable extraction device |
| SPME | Solid-phase micro extraction |
| WEA | Whole effluent assessment |

# 1 Background and Goals of the Research Project

## 1.1 Political Background

An important pathway for the introduction of hazardous substances into the aquatic environment is the discharge of industrial wastewater into surface waters and oceans.

The currently applied methods for the detection of hazardous substances in industrial wastewater are generally based on the analysis of sum and group parameters as well as selected single substances. These methods are partially supplemented by the application of biological test methods. Industrial wastewaters tend to be very complex mixtures, consisting of up to several thousands of individual substances so that a complete qualitative and quantitative assessment of hazardous substances can often not be performed with the afore-mentioned investigation methods.

With regard to future demands on environmental policies, those strategies will gain importance, that combine different investigation methods with the aim of a comprehensive effect-related characterisation of a wastewater. The following political developments concerning water quality on international and national levels require the application of effect-oriented investigation strategies:

- ***INC, HELCOM, OSPAR*** – 'generation aim' to cease the discharge of hazardous substances into the marine environment

On the 4th International North Sea Protection Conference in Esbjerg (4th INC, 1995) the Ministers of Environment of the North Sea riparian states agreed upon a new long-term goal to protect the North Sea with regard to hazardous substances (article 17 of the 4th INC), the so-called 'generation aim':

'*... prevention of the pollution of the North Sea by continuously reducing discharges, emissions and losses of hazardous substances thereby moving towards the target of their cessation within one generation (25 years). The ultimate aim is to achieve concentrations in the environment near background concentration for naturally occurring substances and close to zero concentrations for man-made synthetic substances.*'

The implementation of this goal was extended to the Baltic Sea by recommendation 19/5 of the Helsinki-Commission in March 1998 (HELCOM, 1998) and adapted at the Ministerial meeting of the Oslo and Paris Commission in July 1998 for the North-East Atlantic (OSPAR, 1998).

Reducing the introduction of hazardous substances through industrial wastewater discharges is an important contribution to reach the generation goal. Up to now, the selection of hazardous substances that are to be treated with priority takes place according to a substance-related approach on the basis of hazardous substance properties and of exposure data of single substances. The extension of this approach as the sole measure in order to reach the generation aim appears to be very tedious due to the large number of compounds contained in wastewaters and will, finally, not be successful. On the other hand, a strategy, which allows an effect-oriented evaluation of the whole wastewater load, could provide a pragmatic contribution to this goal.

In this sense, the OSPAR Working Group for the discharge of hazardous substances from point sources (OSPAR POINT) is currently drawing up a background paper about the status of the effect-related wastewater investigation in the OSPAR treaty states. The document is supposed to show to what extent effect-related investigations can be applied for the establishment of the 'best available technique (BAT)' for industrial wastewater discharges.

- ***EU-Water Framework Directive*** – Strategies against water pollution (Article 16)

The EU-Water Framework Directive 2000/60/EC aims to protect all waters and defines specific environmental goals for surface waters, groundwater and so-called protected areas. As strategies against water pollution measures are provided based on the recording of individual pollutants or groups of pollutants that are in a list of priority substances. However, the commission may '*...work out strategies against water pollution by other pollutants or groups of pollutants.*' (Article 16.9).

Besides the described single substance approach, there are presently no further strategies at hand. An effect-oriented investigation strategy can contribute to the reduction of water pollution in this context as well.

- ***VCI*** – Self-commitment of the German Chemical Industry

The German chemical industry association (Verband der chemischen Industrie, VCI) proposed a self-commitment: 'Environmental goals water protection, chemical industry – discharge of wastewater' based on the goal '*that no harmful effects shall result from the discharge of wastewaters by the chemical industry*' [Anonymus, 1998].

In order to evaluate the hazardous effects of wastewater discharges by the chemical industry, it was suggested to use acute toxicity tests and a mutagenicity test such as they are applied for the permit of wastewater discharges from the chemical industry according to annex 22 of the German Wastewater Ordinance. Other harmful effects like for example chronic toxicity or endocrine effects are not mentioned, however. Here a strategy, which considers all relevant effect parameters for a comprehensive characterisation of industrial wastewaters, could be used as a starting point for the development of the self-commitment.

## 1.2 Substance and Effect-Related Wastewater Investigations

For an effective prevention of water pollution, it is important to be able to characterise the kind and extent of the pollution of industrial wastewaters with hazardous substances as fully as possible. For that purpose on the one hand substance-related procedures are applied that are capable of detecting the presence of single substances and substance groups relevant to the environment. On the other hand, effect-related procedures capable of providing information about undesired effects (toxicity, genotoxicity) or properties (persistence, bioaccumulation) of the total wastewater are used without knowing the wastewater constituents underlying the observed effect.

***Substance-related wastewater investigations*** are capable of providing detailed analytical results for a preselected and limited number of compounds so that appropriate measures may be taken to reduce the emission of these compounds, in case they pose a harm to the environment. These measures can range from the substitution of the relevant substances applied and the change of the production process to an improved wastewater treatment. However, with regard to a comprehensive characterisation of the wastewater constituents the substance-related approach bears several disadvantages. Limiting the investigation to priority pollutants does not enable a comprehensive evaluation of the wastewater pollution as only a limited number of substances is considered. Due to the multitude and diversity of wastewater constituents, the effort for an expansion of target analysis would not be practical. The alternatives are also connected with methodical shortcomings: (a) by screening analyses substances may be identified for which no information about their hazard potential are available, or substances are detected, which cannot be identified; (b) sum and group parameters for organic substances provide information about the extent of pollution of a specific wastewater, but they often do not specifically reflect hazardous properties or effects.

***Effect-related wastewater investigations*** have the essential advantage compared to substance-related investigations that it is possible to directly detect the deleterious effect of all constituents in a complex wastewater mixture summarily. It is, thus, possible to detect a potential hazard for the aquatic environment without having to identify the underlying substances. However, as information about the responsible compounds is not obtained, the measures to improve the discharge quality cannot be targeted as for known pollutants. In addition, each effect-related detection method can only allow an assessment of one hazardous effect. Therefore it is necessary to use a combination of several tests that are directed towards different effects.

Due to the described limitations of substance-related wastewater analysis, concepts for ***investigation strategies*** are presently being developed and tested in some countries, that aim at detecting the environmentally relevant wastewater pollution with effect-related investigation methods. These investigation strategies differ in the determination of other parameters besides the *acute toxicity* of wastewater. They partially consider *chronic toxicity* or other hazardous substance properties related to the aquatic ecosystem like *persistence* and *bioaccumulation.* The final goal of these strategies is to enable an assessment of the hazard of a respective wastewater discharge to the aquatic system. The results of these investigations can then be used as decision basis for the permission or control of wastewater discharges and serve as a guide for measures to reduce the wastewater pollution through hazardous substances.

The stage of development of the effect-related test procedures that can be utilised in investigation strategies varies greatly: it ranges from standardised and validated test procedures for the determination of *acute toxic effects* with organisms of all four trophic levels to procedures for the detection of *potentially bioaccumulating wastewater constituents* that are all still under development or testing and which have only been exemplarily applied to selected samples by individual laboratories.

Another approach for a wastewater investigation strategy would be the combination of physico-chemical fractionation methods with substance or effect-related testing of the obtained fractions. Via the combination '*Fractionation and sum parameter determination*' chemically defined group parameters can be determined, which represent substance properties relevant to the environment. The methods currently proved for the determination of 'potentially bioaccumulating compounds' are based on this approach, for example. The combination '*Fractionation and biological effect testing*' can ease the identification of those substances from a complex wastewater that are responsible for an observed hazardous effect. The concept of 'toxicity identification and evaluation' of the American Environmental Protection Agency uses this approach, for example [USEPA, 1991a, 1993a, 1993b].

Thus, different methods for the detection of a hazardous substance property in wastewater exists as well as different concepts for the combination of the respective methods into an investigation strategy for hazardous substances in industrial effluents. The investigation strategies themselves differ finally in the selection and number of the methods applied as well as in the sequence of the methods applied in gradated strategy proposals. An integration of fractionation methods into investigation strategies is yet rarely found.

## 1.3 Aim of the Research Project

The aim of this research project is to develop a concept for an assessment strategy, which allows a comprehensive and practicable characterisation of the hazardous wastewater constituents by combining different investigation methods.

The assessment strategy to be worked out should cover the three major hazardous properties of wastewater constituents, such as the aquatic toxicity, the potential to bioaccumulate and to persist in the aquatic environment. If necessary, additional parameters should also be considered, which are deemed necessary for a comprehensive characterisation of the type and extent of the environmental hazard potential.

Due to its effort this investigation strategy should not serve for the regular control of industrial wastewater discharges. Rather, it should enable to assess the potential hazard of a wastewater discharge to the aquatic ecosystem of the receiving water. The strategy should enable to detect effluents that require an improved wastewater treatment with regard to water quality control. It should also allow to detect the effects of changed production processes in a plant or of a modified wastewater treatment process on the quality of the wastewater to be discharged.

In this sense, the investigation strategy could become a helpful instrument also in the context of permitting processes.

## 1.4 Procedure

The process of developing the '*Industrial Discharge Assessment*' strategy (IDA) can basically be divided into four steps:

1. Initially, an in-depth evaluation of the existing strategies for the characterisation of hazardous substances in industrial wastewaters introduced on an international level (chapter 2) and of the current state of research regarding the investigation methods for the detection of single hazardous properties has to be performed (chapter 3).
2. Based on this evaluation, the concept of the assessment strategy has to be drawn up afterwards and a set of tests from the pool of established test methods has to be selected, which will serve for the detection of singular effects deemed important (chapter 4).
3. The key steps of this strategy have to be applied exemplarily on industrial wastewater discharges in order to test the feasibility of the methods and the reasonability of the selected linkages in the assessment strategy (chapter 5). By this way it shall be avoided that a strategy is drawn up and propagated, which finally turns out to be untenable.
4. Based on these laboratory tests, their results and the practical experiences, finally the critical points of the methodology and any unanswered questions in the investigation strategy will be worked out which need to be checked further (chapter 6). In addition questions will be treated that may result from the evaluation of the literature and which might require further investigation from the authors' points of view.

# 2 Investigation of Industrial Wastewater Discharges for Hazardous Substances

## 2.1 Characterisation of Hazardous Materials in Industrial Wastewater Discharges

### 2.1.1 General

In the following chapters a short description of the different approaches and methods for the characterisation of hazardous substances in industrial wastewaters is given in order to ease the understanding of the investigation strategies developed so far. The advantages and disadvantages of the substance-specific and the effect-oriented investigation approach for wastewater are summarised and the assessment of the potential adverse effects of wastewater discharges on the receiving water according to the emission and immission-related approach is introduced.

The parameters which have to be considered for a comprehensive characterisation of the hazardous properties of wastewater discharges result form the definition of the term '*hazardous substances*' in connection with the legal regulations (Table 1).

**Table 1:** Definitions of the term 'hazardous' in different regulations.

| Regulation | Context | Term | Definition |
|---|---|---|---|
| EC-Water Framework Directive [WRRL, 2000] | Strategies against water pollution (Maintenance and protection of the aquatic environment in the Community) | Hazardous substances | In Article 2, 29: Substances or groups of substances that are toxic, persistent and liable to bioaccumulate, and other substances or groups of substances which give rise to an equivalent level of concern |
| OSPAR - Strategy with regard to hazardous substances [OSPAR, 1998] | Protection of the marine environment against the introduction of hazardous compounds | Hazardous substances | In Annex I: Substances or groups of substances that are toxic, persistent and liable to bioaccumulate (toxic is defined via the following effects of substances on organisms:<br>- reduction of survival, growth and reproduction<br>- carcinogenicity, mutagenicity or teratogenicity<br>- adverse effects as result of endocrine disruption) |

As a rule, the following properties are inherent to hazardous substances: toxicity (acute and chronic), enrichment capability in the organism (bioaccumulation), longevity in the environment (persistence), genotoxicity, carcinogenicity or teratogenicity as well as the impact on the hormonal system (endocrine disruptors). In the maximum case, all these parameters would have to be considered in the investigation strategy and determined for the whole wastewater.

### 2.1.2 Single Substance and Whole Wastewater Investigations

Industrial wastewater discharges are generally very complex mixtures of numerous substances. The European Inventory of Existing Commercial Substances List (EINECS) contains over 100,000 chemicals available on the European market (http://ecb.ei.jrc.it/existing-chemicals) and more chemicals are added each year. Besides these intentionally produced substances that can potentially be found in wastewater discharges, unknown substances occur, which have been formed as by-products in the production process or which were generated during wastewater treatment. The exact composition of a wastewater is usually unknown.

The discharge of known hazardous substances can be monitored by target analysis. For a stringent discharge control it would be desirable to have detailed information on the type and quantity of all wastewater constituents as well as on their ecotoxicological properties. However, this is impossible to achieve. In addition, for many substances no suitable and standardised analytical methods are available. Organic wastewater constituents can be detected with the help of sum and group parameter methods like the DOC, COD, BOD or AOX. This allows a broad but coarse characterisation of the organic load of a wastewater and provides information about the efficacy of wastewater treatment measures.

Biological test procedures are increasingly applied for the ecotoxicological investigation of wastewaters in order to assess the potential effects of wastewater discharges on the aquatic environment. Their application is prescribed in some countries in the context of discharge permits for specific types of wastewater. But presently the chemical-specific approach for the detection of hazardous pollutants is still more widely used.

In the following table (Table 2) the advantages and disadvantages of single substance investigations by means of target analyses and of whole wastewater investigations with ecotoxicological methods are summarised.

Both investigation approaches have their limits. The most important drawback of target analysis is its high analytical effort whereas in whole wastewater investigations it is often difficult to allocate an observed effect to a specific substance.

In the last years it became more widely recognised that the traditional approach to assess the hazard potential of the discharge by detecting individual compounds and investigating the environmental hazard potential of these compounds cannot provide a comprehensive assessment of the ecotoxicological properties of a discharge. Therefore supplementary methods for the investigation of hazardous effects of wastewaters have to be applied.

An approach combining chemical analysis and the detection of hazardous effects significantly expands the available database for and increases the quality of the assessment of the environmental hazard potential of a wastewater discharge [Villars, 1995]. The assessment strategy for industrial wastewater discharges (IDA) took this into account.

**Table 2:** Advantages and disadvantages of single substance and total wastewater investigations in the context of the authorisation and control of industrial wastewater discharges.

| | Advantages | Disadvantages |
|---|---|---|
| **Single substance investigations** | ▪ Measures for the reduction of the discharge of hazardous substances can be more targeted (production process, wastewater treatment, legislation)<br>▪ The toxicity of problematic substances is often better documented than the toxicity of wastewater, existing data and quality criteria can be applied.<br>▪ The evaluation of the human exposure and the possible effects is possible.<br>▪ Environmental administrations are more used to regulate individual substances. | ▪ Even with the largest possible analytical effort only a small part of the wastewater constituents and possible degradation products can be detected.<br>▪ The correlation of the variability of the single substance concentration with the variability of wastewater toxicity cannot be presumed.<br>▪ Protective goals for single substances do not result in the same protection level for the wastewater toxicity.<br>▪ There are effect-based water quality criteria for less than 150 substances. For other substances, there is no or only very little data available. |
| **Whole wastewater investigations** | ▪ The total toxicity of a wastewater is measured without having to know the identity of the wastewater constituents.<br>▪ Synergistic and antagonistic effects are taken into account.<br>▪ An indication of the content of bio-available toxic matter is given. | ▪ An effect can hardly be allocated to a specific wastewater constituent without a large investigation effort. |

### 2.1.3 Hazard and Risk Assessment of Wastewater Discharges

The investigation strategies for industrial wastewater discharges described in more detail in chapter 2.2 provide the basis for evaluating these discharges, as they inform about the kind and extent of their hazard or risk potential.

The development of these assessment strategies for wastewater usually followed the principles of environmental hazard assessment of single substances. Therefore a brief description of those principles as well as of their transfer to the assessment of wastewater discharges follows.

The environmental hazard assessment of single substances can take place in different tiers. In general, hazard identification, hazard assessment and risk assessment have to be distinguished (Fig. 1). Depending on the purpose of the assessment (e.g. classification, ranking or individual evaluation of substances) and its final result (hazard, risk), the type and extent of the necessary information regarding the exposure and effect as well as the data evaluation method differs.

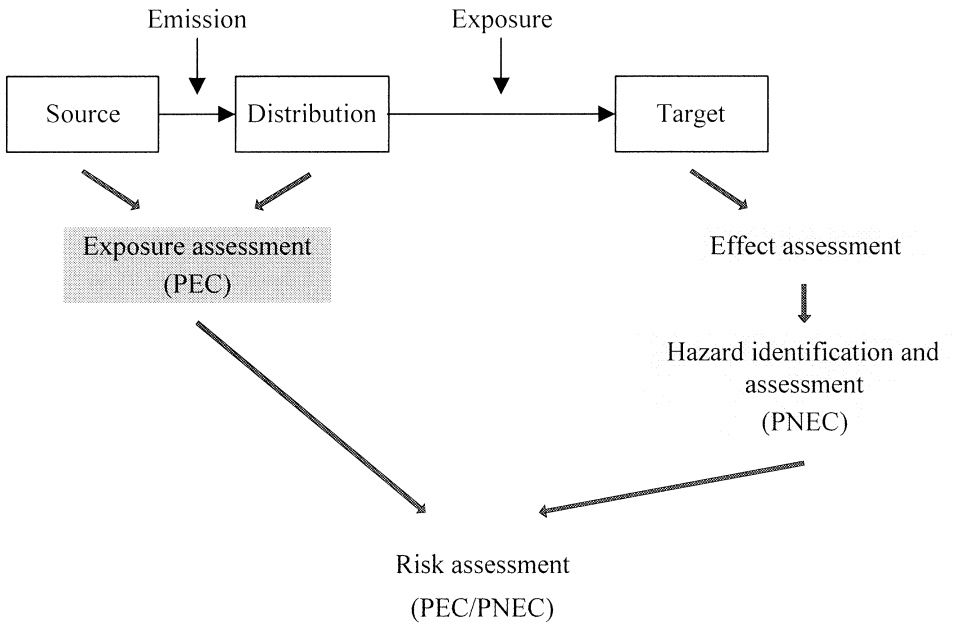



**Fig. 1:** Connection between hazard and risk.

The *hazard identification* includes the determination of hazardous substance properties (physico-chemical properties, ecotoxicity, persistence, bioaccumulation potential) and allows a first evaluation of the substance-related environmental hazard potential.

During the *hazard assessment* the hazard connected related with the use of a substance is determined in case that the substance is regularly emitted into the environment. Based on the determined ecotoxicity data a maximum concentration level is derived, which is supposed to have no effect on aquatic organisms (PNEC, predicted no effect concentration). As far as tests were carried out with organisms that are characteristic and significant for the respective aquatic system, the hazard assessment can be performed with some security.

Thus, the hazard assessment follows an emission-related point of view of water protection [Stortelder & van de Guchte, 1995]. Transferred to the investigation of wastewater, this would imply that the type and extent of hazardous properties of a wastewater discharge has to be detected. Only the quality of a wastewater discharge is finally decisive for answering the question whether there is further need to improve the discharge quality in order to protect the receiving water or not.

This contrasts the environmental *risk assessment*, which considers the hazard potential and the probability of its occurrence. For single substances an exposure analysis has to be additionally conducted. For that purpose, the actual environmental concentration must be determined via monitoring, or it must be estimated with a mathematical model for a specific scenario of use, emission and dilution. This calculation is based on the prospective extent and frequency of a substances entry into the environment, as well as on its basic environmental properties (PEC, predicted environmental concentration). Many assumptions have to be made for the risk assessment, as not all data are readily available. Therefore, the outcome of a risk assessment is generally less certain than that of a hazard assessment.

During the process of risk assessment for new and old substances in the EC [European Commission, 1996], the ratio of PEC to PNEC is calculated. The more extensive the database about the effects and the knowledge of the exposure of a substance is, the more exactly can the risk connected with its use be determined. A PEC/PNEC > 1 suggests that hazardous effects may be connected with the regular use and emission of a substance. After checking whether additional information about exposure or effects could reduce the PEC/PNEC-relation, either an improved assessment of the PEC and the PNEC with this additional information is carried out or it is decided on hazard-reducing measures. This process can be continued until there is sufficient information available for a final risk assessment.

Such a risk assessment can be a tedious process. Therefore simplified methods may be applied depending on the assessment purpose. For a comparative assessment of the risk of a selection of substance a ranking based on their relative environmental risk may be carried out. This was undertaken in the process of preparing the recommendation concerning the list of priority substances for the EU-water framework directive [UBA, 1999].

The risk assessment corresponds to an immission-related point of view. Contrary to the emission-related point of view and with regard to wastewater discharges this means that not only the effluents quality is considered but also the situation of the receiving water. During the risk assessment, the information about the runoff-quality is put into relation to the information about the receiving waters quality and quantity. Following this approach the discharge of a toxic wastewater would pose a risk to the environment only if the dilution in a defined catchment area of the receiving water is insufficient to decrease the compounds concentration to below the PNEC.

Advantages and disadvantages of the *emission and immission-oriented assessment* of wastewater discharges [Stortelder & van de Guchte, 1995]:

The emission-based wastewater assessment is generally regarded as the more strict approach that is committed to the precautionary principle. It is a technology-based approach and it is oriented on the technical possibilities of wastewater treatment and thus promotes its development and it ensures a steady demand for improved treatment techniques. Because only the quality of the discharge is decisive the quality standards derived are location-independent and are valid for all industrial enterprises of a certain sector (e.g. German Wastewater Ordinance), regardless of the state of the water body. Though the emission-based approach is generally considered more strict, it may be the less strict approach in those cases, where a receiving water is already highly polluted as it takes not into account of the impact of the sum of the discharges and also diffuse sources within a specific catchment area.

The immission-related approach first of all requires the definition of quality criteria to be applied to the surface waters. Based on these quality criteria the quality of the receiving water has to be evaluated and determines which additional immission can be tolerated to meet the defined quality criteria. Following this approach, the requirements on the discharger will generally vary largely from location to location. Additionally, this approach tends to consider dilution as the solution for wastewater pollution. Because of the local reference immanent to this approach, the

success of an immission-related water protection policy depends more on on-site decisions and may, thus, be subjected to local political and economic pressures.

Nevertheless, the emission- and the immission-based approach are not incompatible. As shown below, attempts are being made to connect both approaches. Moreover, the novel EC water framework directive also includes the so-called 'combined approach' (Article 10), which calls for the control of water pollution for the priority substances through the definition of emission limits as well as through environmental quality standards.

## 2.2 Wastewater Investigation Strategies in Different States

### 2.2.1 General

In this chapter the wastewater investigation strategies developed, currently tested or proposed in different countries are presented. These investigation strategies may serve different needs:

- Determination of the environmental hazard potential of an existing plant through its wastewater discharge in order to find out whether and how the quality of the wastewater need to be improved
- Grant of discharge permits
- Characterisation of the wastewaters from different industrial sectors
- Priority setting for measures to reduce the emission of hazardous substances
- Definition of control programmes for the regular control of wastewater discharges
- Planning of new plants, new processes or modifications of existing processes during which wastewater occurs.

### 2.2.2 Denmark

**Background**

In Denmark the discharge of industrial wastewater into watercourses requires authorisation and the responsibility lies with the county councils (Environmental Protection Law *625/1997*). In granting the permit limit values are defined for the wastewater discharge considering the 'best available technique' as well as the potential ecotoxicological risk of the discharge. According to the guidelines of the Danish Environmental Protection Agency on Receiving Water Quality Planning (Administrative guideline no. 2, 1983), there shall be no acute toxicity outside of the initial mixing zone and no chronic toxicity outside of a limited area around the discharge location [Pedersen et al., 1993]. Although these guidelines are more directed towards single substances, their validity is also generally recognised for whole wastewater investigations with biological test procedures.

With the implementation of the EU-directive 76/464/EEC (Discharge of hazardous substances) in 1996, water quality standards were established in Denmark for approximately 120 priority substances of the lists 1 and 2. The concentration of these substances in the receiving water after the initial dilution of the wastewater discharge has to be below the concentration levels defined in the water quality standards. The application of biological tests is not generally prescribed for the authorisation and control of industrial wastewaters; the decision is left to the respective licensing authority. Therefore, ecotoxicological investigations of industrial wastewaters were conducted in very different extents for most of the important industrial dischargers since the first large investigation in 1977-78 and were evaluated differently in the context of the permitting process.

In order to develop long-term uniform standards for the authorisation of industrial wastewater discharges, the Danish Environmental Protection Agency compiled a report in 1992, which summarises the experiences gained during ecotoxicological investigations and assessments of industrial wastewater in Denmark and other states (Ecotoxicological evaluation of industrial wastewaters, Pedersen et al. (1994)). Based on these experiences and the available methods, a strategy was proposed in the report for the ecotoxicological investigation, characterisation and evaluation of industrial wastewaters. On this basis a guidance document for the risk assessment of industrial wastewaters was introduced in 1995, which describes the selection and application of the different methods in an exemplary way [Pedersen et al., 1995].

**Danish Strategy Concept 'Ecotoxicological Evaluation of Industrial Wastewaters'**
[Pedersen et al., 1994]

The strategy concept represents a tiered investigation and evaluation strategy, which includes and combines whole wastewater and single substance investigations, depending on the status of the information available on hazardous substances. As depicted in Fig. 2 the strategy consists of a basic level and three successive levels. After each level, the results are evaluated as to whether they are sufficient for an administrative decision (discharge authorisation) about the wastewater discharge or whether additional information is required.

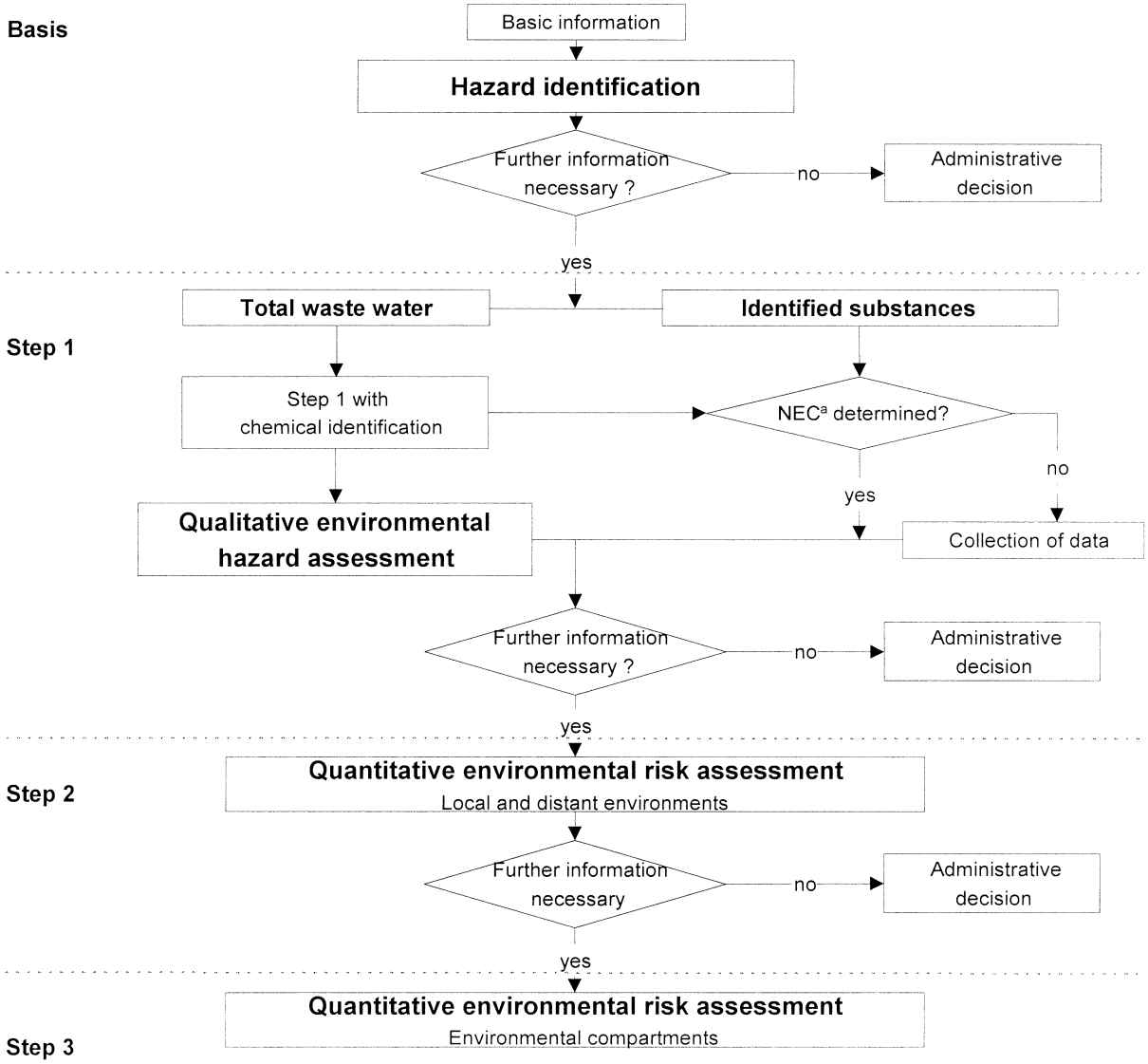



**Fig. 2:** Strategy proposal of the Danish EPA for the investigation and evaluation of industrial wastewaters ([a]NEC: no effect concentration).

The evaluation of a wastewater discharge is on the one hand based on the determination of the wastewater dilution or the concentration of specific single substances in the receiving water (C, exposure analysis) and on the other hand on the determination of the dilution or concentration at which an unacceptable ecotoxicological effect is no longer to be expected (NEC, no effect concentration, effect analysis). In line with the international practice for the evaluation of substances, it is assumed that there is no ecotoxicological impact on the environment if C is lower than NEC. Therefore, the comparison of C with NEC is used as a measure for the expected hazardous effect of the wastewater discharge on the receiving water. As neither C nor NEC are commonly known, it is necessary to estimate these concentrations via the determination of the PEC (predicted environmental concentration) and the PNEC (predicted no effect concentration) values. For this purpose the strategy provides more comprehensive and detailed wastewater investigations from step to step in order to (a) enable an increasingly precise prediction about the potential hazard to the environment and (b) to reduce the existing insecurities in the evaluation as the knowledge of the wastewater characteristics becomes more and more profound.

In order to obtain the information necessary for the evaluation, the strategy concept comprises methods for the determination of the effect parameters acute and chronic toxicity, bioaccumulation and persistence, the accompanying determination of physico-chemical parameters and sum parameters as well as the application of spread and distribution models of wastewater discharges in the receiving water. The following proceedings are intended for the individual steps:

*Basis step: Wastewater hazard identification*

In the basis step the available information for a first assessment is collected and it is evaluated whether the wastewater may cause ecotoxicological effects in the receiving water. Information is collected on:

- used or produced substances, drawn up in a mass balance to estimate which hazardous substances may be present in the wastewater
- the measured quantities of the substances in the wastewater
- physico-chemical properties of the substances
- biological properties of the substances like biodegradability, bioaccumulative potential, toxicity and genotoxicity
- physico-chemical properties, degradability and persistence of the whole wastewater
- currents and possible spreading patterns in the environment
- uncertainties and quality of the data used in the above mentioned points

In addition, sum parameters may be determined and a wastewater screening with biological methods could be considered (inhibition tests with activated sludge or toxicity tests as so-called limit tests: the original wastewater is tested only (without dilution steps)).

*Step 1: Wastewater hazard assessment*

In this step the data for a *qualitative* environmental hazard assessment have to be determined to enable a detailed assessment of the potential of a wastewater discharge to cause an ecotoxicological effect in the receiving, water.

Initially, an investigation of the *whole wastewater* is carried out to obtain a minimum data set for the hazard assessment. This data shall be sufficient in order to describe the *toxicity* (acute toxicity on fish, crustacean and algae, NOEC-values) as well as the *exposure-relevant parameters* (biodegradability, bioaccumulation, physico-chemical data, dilution pattern, PEC-values). If the wastewater is not treated prior to its discharge, a so-called aerobic stabilisation of the wastewater may be performed (biodegradability test) and its effects on the microorganisms as well as the toxicity after the stabilisation (persistent toxicity) may be determined.

Parallel to the whole wastewater investigations, information are compiled about the identified *single substances* (from measurements or data collections) in order to estimate their hazard potential (maybe they have already been classified as hazardous and there are *limits values* or *water quality criteria* (NEC, no effect concentration); if not available, it should be searched for relevant data in the literature).

The first two steps of this strategy concept are directed towards the discharging industrial plant (production, wastewater characteristics). If it is possible to derive the NEC (no effect concentration) and C (concentration in the environment) values from the collected data, then potential effects on the environment can be assessed. If these cannot be determined, also less precise parameters can be used provisionally. For the evaluation of the whole wastewater, the NOEC divided by an uncertainty factor can be used for an estimation of the NEC. For single substances, the calculated ECL (environmental concern level) can be used and instead of C the concentration after the first mixing of the wastewater discharge in the receiving water can be employed.

After these steps a decision regarding the necessary measures for quality improvements can be made; these measures may aim at a reduction of the discharge of specific substances, of sum parameters or a reduction of the amount of wastewater to be discharged by modifications of production processes, replacement of certain production chemicals or by improved wastewater treatment. If it proves necessary to obtain further insight into the toxic wastewater constituents, source-tracking methods (determination of the toxicity origin through separate waste stream investigations) or toxicity identification evaluation (TIE) may be applied.

*Step 2: Wastewater risk assessment - local and distant environment*

In the second step the data for a *quantitative* environmental risk assessment have to be determined by conducting an ecotoxicological test programme, which enables the calculation of NEC and C from statistically confirmed data. Then it is possible to establish the discharge criteria for the whole wastewater or for single substances.

In this step acute toxicity tests with a larger number of organisms or tests with a longer duration for sub-lethal effects can be conducted, namely if the wastewater contains bioaccumulating or persistent compounds. These tests can be conducted with the whole wastewater, relevant components or identified substances. Additional analyses and experiments may become necessary in order to determine the fate of the wastewater constituents after their discharge. Thereby sufficient data should be obtained to be able to quantify the wastewater concentrations in the receiving water.

*Step 3: Wastewater risk assessment - environmental compartments*

If necessary, special investigations of the wastewater/water situation are carried out in the third step, for example for specific effects of problematic substances of the wastewater mixture, the transfer and the effects in other compartments (sediment) or within the food chain, for interactions between the species, water studies or water controls.

**Investigation Programme**

Based on the above-described investigation and evaluation strategy, an investigation programme was suggested for each step. It is not a fixed programme for each wastewater discharge, rather the suitable steps should be selected depending on the type of wastewater and the previous knowledge. Neither the whole wastewater investigation nor the single substance investigation

need to be performed in all cases. Considering the difficulties connected with the single substance approach it may often be more practical to focus on whole wastewater investigations.

In the following, a short survey of the suggested methods for the whole wastewater investigation given:

In the basis step no tests or analyses are intended besides the above-mentioned screening investigations, but the collection and evaluation of existing data. Physico-chemical parameters and sum parameters and their temporal variability are determined in the steps 1 and 2. Further tests for toxicity, bioaccumulation and biodegradability are conducted in these steps and the spreading and distribution of the wastewater in the receiving water is estimated. A survey of the methods applied in the steps 1 and 2 is depicted in Table 3.

More complex investigations for the determination of environmental effects of a wastewater discharge (e.g. field studies) are conducted in step 3. A genotoxicity test is not included in this strategic concept but it may be considered in future.

**Table 3:** Whole wastewater investigations of the steps 1 and 2 within the Danish strategy concept.

| | **Parameters** | **Step 1: Qualitative hazard assessment** | **Step 2: Quantitative risk assessment** |
|---|---|---|---|
| **Exposure parameters** | Wastewater composition and variability | Physico-chemical parameters<br>Sum parameters<br>Discharged quantity (24h/week test)<br>(T, pH, density, suspended matter, DOC, AOX etc.) | Evaluation of the variability (peaks) of specific test parameters with time or the operation of the enterprise |
| | Spreading and distribution | Mass balances, simple spread models | Models for local and distant environmental areas |
| | Biodegradation | BOD, COD<br>Simulation of wastewater treatment (aerobic) stabilisation (then, if necessary, investigations for persistent toxicity or bioaccumulation) | Simulation of water conditions (batch tests) |
| | Bioaccumulation | HPLC or TLC screening<br>(if necessary, chemical analysis for the identification of the bioaccumulating fraction, bioaccumulating substances that are toxic or persistent are especially relevant) | Bioaccumulation tests with organisms |
| **Effect parameters** | Toxicity*<br>(* the organisms selected for the tests are listed in annex 1) | Three acute toxicity tests:<br>Fish 96 h $LC_{50}$<br>Crustacea 48/96 h $LC_{50}$<br>Algae 72 h $EC_{50}$<br>(for the determination of the NOEC-values)<br>Selection of the organisms depending on the water into which the wastewater is discharged (freshwater, saltwater or brackish water) | Two additional acute tests with other species (e.g. microorganisms, protozoan, molluscs) or chronic tests with fish, crustacean or algae<br>(the chronic tests are especially relevant if persistent or bioaccumulating fractions are present in the wastewater) |

***In summary, the Danish strategy concept can be characterised as follows:***

- Immission-oriented, tiered and flexible, but extensive investigation programme.
- Purpose is the determination of the risk of water pollution through a wastewater discharge.
- Whole wastewater and single substance investigations depending on the individual case.
- Effect parameters: acute and chronic toxicity, persistence, bioaccumulation.
- Chronic tests are applied when there are indications of persistent or bioaccumulating fractions.
- Evaluation of the whole wastewater toxicity via the PEC/PNEC approach.
- Discharge permits can be granted on the basis of the guidance document for the risk assessment of industrial wastewater discharges, but this is not legally prescribed.

### 2.2.3 United Kingdom

**Background**

The United Kingdom follows an immission-oriented approach for the control of wastewater discharges. The legal requirements are presently limited to chemical parameters and priority hazardous substances. Discharge authorisation is based on environmental quality standards established for surface waters. Since ten years, there are attempts to apply also the toxicity detection as an integrated approach to improve the effluent quality as well as the surface water quality [Mackay et al., 1989].

**The British Strategy Concept**

The procedure developed is called Direct Toxicity Assessment (DTA) and is shown in Fig. 3. It is currently being tested for its applicability in several pilot studies by the responsible authorities, executive organisations and the dischargers (industrial enterprises) [Hayward, 1999]. The DTA is supposed to enable the permitting authorities to detect wastewater discharges that cannot be sufficiently controlled with the chemical methods applied so far and which require special attention in the future. Nationally and internationally standardised toxicity test methods were selected for the DTA.

In the *first step* (discharger prioritisation), chemical, biological and ecotoxicological data are used to decide whether a specific wastewater discharge needs an additional toxicity-based permit. Decision criteria are the complexity of the wastewater, its ecotoxicity as well as the effect of the discharge on the receiving water observed in biological studies. A distinction is made between discharges into surface waters (freshwater) and oceans (saltwater) and it is intended to use toxicity tests with luminescent bacteria (*V. fischeri*, luminescence inhibition, fresh- and saltwater), daphnia (*D. magna* immobilisation, 24 h, freshwater) or oysters (*C. gigas*, embryo-larvae-development, 24 h, saltwater).

A characterisation of the toxicity and the variability of the wastewater discharges takes place in the *second step* (discharger characterisation). For this purpose the wastewater discharges are examined repeatedly with a battery of tests using three trophic levels and considering the organisms in the respective receiving water (algae, invertebrate and fish). The following tests can be selected:

Freshwater: *S. capricornutum* (algae), 72 h, growth inhibition
*D. magna* (daphnia), 48 h, immobilisation
*O. mykiss (*fish), 96 h, lethality

Saltwater: *P. tricornutum* (algae), 72 h, growth inhibition
*C. gigas* (oyster), 24 h, embryo-larvae-development
*S. maximus (*fish), 96 h, lethality

The results of the most sensitive test organism are used to set a concentration (dilution) at which no toxic effect occurs (PNEC).

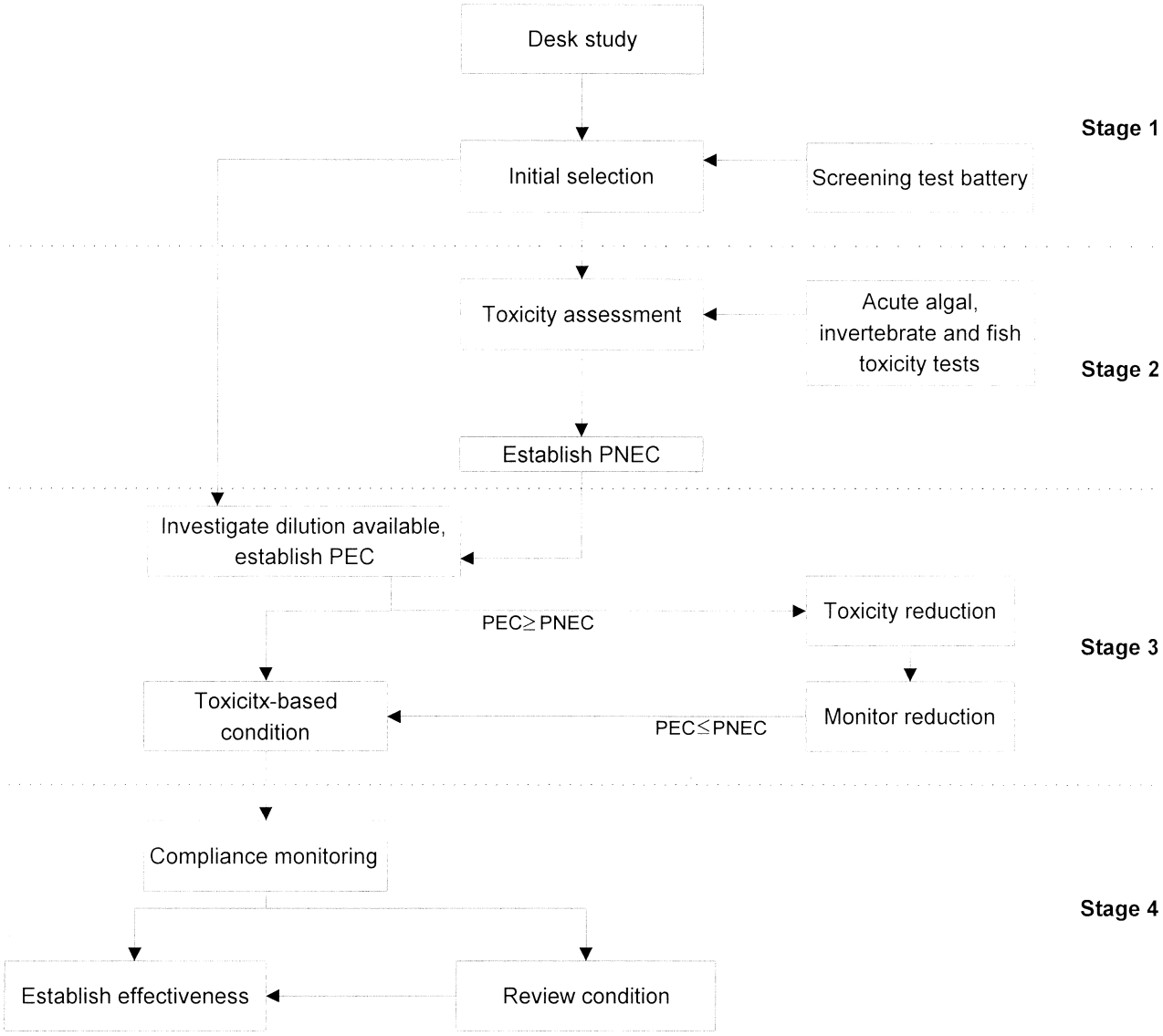



**Fig. 3:** Procedure of the Direct Toxicity Assessment (DTA) in Great Britain for the control of wastewater discharges [according to Johnson et al., 1996].

In the *third step* (reduction of toxicity and toxicity permit) the concentration of the wastewater after mixing in the surface water is estimated (PEC) based on the amount of wastewater being discharged and the dilution and dispersion in the receiving water. If the PEC-value exceeds the PNEC-value (PEC/PNEC > 1) outside of the mixing zone, measures in order to reduce toxicity are necessary before a discharge permit is granted. Toxic effects are, thus, tolerated at the place of discharge and inside the mixing zone.

The *fourth and last step* (permit control) is the monitoring of the wastewater with regard to the granted permit.

**Pilot Study**

In a pilot study of the British Environmental Agency (EA) on the feasibility of the DTA-protocol, 12 out of 46 wastewater discharges were selected as potentially relevant for a toxicity-based permit after the first investigation step and were treated according to steps 2-4 [Johnson et al., 1996].

Finally, only two of the discharges examined had PEC/PNEC-values less than 1, implying that they will not cause any toxicity in the receiving water after dilution and dispersion in the mixing zone. The other ten effluents did not fulfil these criteria and were likely to cause toxic effects in the receiving water body. If DTA was put into force, it would be necessary to reduce their toxicity before a permit is granted. With regard to the sensitivity of the toxicity tests, it was found in this study of the EA that the daphnia and the oyster tests were always as sensitive or more sensitive than the algae growth or fish lethality tests.

In the pilot projects for the DTA attention was driven towards the comparability of different tests for acute and chronic toxicity as well as towards standardisation, quality control and statistic evaluation [Wharfe, 1996].

As the introduction of toxicity tests in permit and control procedures is still in the trial stage in the United Kingdom further aspects of effluent quality such as the presence of persistent or bioaccumulating wastewater constituents do not yet play a role.

### *In summary the British strategy can be characterised as follows:*

- Strict immission-orientation in the definition of any kind of quality standards for wastewater discharges.
- Criteria are related to the concentration in the receiving water after dilution and dispersion.
- Toxicity tests (DTA) in the pilot stage as supplement to the chemical analyses; their incorporation into discharge permits is intended.
- Acute toxicity tests are in the foreground, in the long range chronic tests, sub-lethal endpoints and mutagenicity shall be integrated.
- Other parameters besides ecotoxicity are not considered.

### 2.2.4 The Netherlands

**Background**

In the Netherlands, wastewater discharges are evaluated according to an emission-oriented approach. Discharge permissions intend to reduce the emission of specific hazardous substances according to the precautionary principle. In addition, the discharger has to apply reduction measures for these substances according to the best available technique (BAT) and/or best environmental practice (BEP). A strategy for a whole effluent assessment (WEA) is currently being tested, and is supposed in long term to be integrated into the Dutch procedure for the granting of discharge permits in future [Tonkes et al., 1998].

The ***entire permit procedure*** will comprise four phases after the integration of the WEA. The phases 1, 2 and 4 are already in application [Tonkes et al., 1995]:

In *phase 1* (limiting pollution) the production processes are evaluated with regard to the possibility of reducing wastewater pollution.

In *phase 2* (chemical-specific evaluation) data on the chemical properties and potential effects on the aquatic system are collected and assessed for the substances found in phase 1 as far as they are wastewater-relevant. If sufficient information about the substance properties and their possible effects (toxicity, persistence, bioaccumulation) is available, it is decided, which improvement measures have to be performed. Hazardous substances have to be replaced with less hazardous ones. It can be assumed that normally the available information will not be sufficient for an effluent assessment. In this case, the wastewater itself has to be investigated (phase 3, WEA integration).

In this *phase 3* (WEA), the chemical and the effect parameters of the wastewater will be experimentally determined. The procedure for the WEA-assessment is described below in more detail.

After improving the discharge quality, its success will be monitored in *phase 4* (evaluating the discharge remnant). For this purpose the quality of the receiving water will be observed with a limited number of physico-chemical parameters. This phase introduces an immission orientation into the strategy. If the surface water quality turns out to be insufficient, it is proposed to return to phase 3 and to further improve the effluent quality by improving its treatment or applying other measures in the production process (reduction of emission).

**Dutch Strategy Concept for the Whole Wastewater Assessment (WEA)**

The parameters acute and chronic toxicity, genotoxicity, persistence and bioaccumulation are included in the WEA. All parameters are regarded equally relevant for the hazard potential of industrial wastewater discharges and, thus, a discharge is classified as being environmentally harmless only when all parameters are below limits that still have to be defined. [Tonkes & Baltus, 1997]. The procedure of the whole wastewater assessment is shown in Fig. 4.

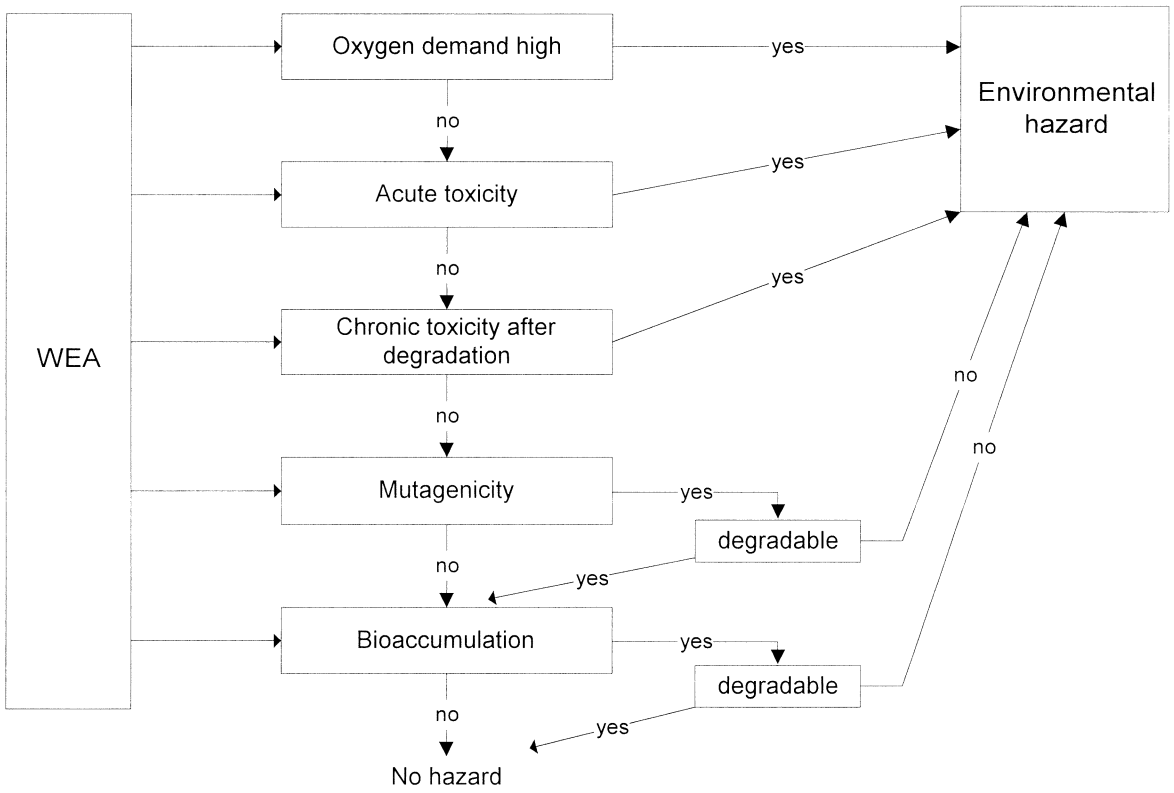



**Fig. 4:** Dutch strategy concept of the whole effluent assessment (WEA) [Tonkes & Baltus, 1997].

The development of the WEA-concept started in the 1990s with a first investigation of methods suitable for the assessment of the whole wastewater (toxicity, bioaccumulation) in addition to the existing limits of the substance-specific assessment approach for wastewater discharges [Pols, 1988]. The tests used for future application in the WEA were then either selected from the available nationally or internationally standardised or proven tests (toxicity and genotoxicity) or existing methods were modified (persistence and bioaccumulation).

In a first study in 1994, 17 wastewater discharges were investigated with acute toxicity tests of the four trophic levels in order to gain first practical experiences [Tonkes et al., 1999]. For 15 wastewaters, at least one test showed an acute toxic effect. In eight cases the toxic effect could not be explained by means of the physical and chemical data that were also determined. The algae test was found to be the most sensitive of the tests applied. The complete WEA-investigation programme was first tested in 1996 in a pilot study on grab samples of ten wastewater discharges [Tonkes & Baltus, 1997].

## Pilot Study

The following test methods were applied in the pilot study:

| | |
|---|---|
| Acute toxicity | Fish, daphnia and algae test with limnic organisms, luminescence inhibition test and toxicity test kits (rotifers) |
| Chronic toxicity | Fish test (early life stage), daphnia test (reproduction) |
| Genotoxicity | Muta-chromoplate kit (miniaturised Ames test) |
| Persistence | Simulation of the degradation in the water, 28 days, DOC-reduction |
| Bioaccumulation | HPLC-method, biomimetric extraction |

Additionally, the BOD test is included to detect a potential lack of oxygen in the receiving water.

Of the ten wastewater discharges examined, eight were acutely and six chronically toxic. Mutagenicity was detected in all samples and 5 wastewater samples contained a higher amount of bioaccumulating substances. An additional biological degradation was observed in three samples.

Based on these experiences and on further method developments, some tests were recently included or replaced [Tonkes, 2000]. Tests with saline organisms were chosen for the investigation of wastewater discharges into marine environment. The luminescence inhibition test over 22 hours was included as a chronic test. The umu test was chosen for the detection of genotoxic effects and a SPME-procedure for the determination of potentially bioaccumulating substances. The selected tests and test organisms are listed in the following table (Table 4).

**Table 4:** Biological tests applied in the Dutch investigation strategy.

| | **Trophic level** | **Freshwater** | **Saltwater** |
|---|---|---|---|
| Acute toxicity | Fish | *Brachydanio rerio,* 96 h | *Poecilia reticulata,* 96 h |
| | Crustacean | *Daphnia magna,* 48 h | *Acartia tonsa,* 48 h |
| | Algae | *Raphidocelis subcapitata,* 72 h | *Phaeodactylum tricornutum,* 72 h |
| | Bacteria | *Vibrio fischeri,* 30 min. | *Vibrio fischeri,* 30 min. |
| Chronic toxicity | Fish | *Brachydanio rerio,* 8 d | *Scophthalmus maximus,* 48 h |
| | Sea urchin | - | *Psammechinus miliaris,* fertility |
| | Crustaceans | *Daphnia magna,* 21 d | *Acartia tonsa,* 48 h |
| | Algae | *Raphidocelis subcapitata,* 96 h | *Phaeodactylum tricornutum,* 96 h |
| | Bacteria | *Vibrio fischeri,* 22 h | *Vibrio fischeri,* 22 h |

The implementation of the WEA into the permit procedure of wastewater discharges is currently planned for the year 2006 [Tonkes, 2000]. Until then the practical experiences with the investigation strategy have to be intensified and the selected tests has to be validated. Finally, decision values have to be developed, which allow for the differentiation between a positive and a negative result of a certain test (yes/no decisions).

### *In summary the Dutch strategy proposal can be characterised as follows:*

- Application of the WEA for the reduction of emissions and in granting of wastewater discharge permits.
- Emission-oriented approach with a conclusive evaluation of the water quality of the receiving water as a control of success.
- Chronic toxicity, persistence and bioaccumulation are integrated in the hazard assessment.
- All effect parameters are regarded as equally relevant.

### 2.2.5 Sweden

**Background**

Different projects for the biological and chemical characterisation of industrial wastewater discharges were conducted in Sweden in the 1980s in order to obtain the background information allowing for their later application in monitoring and in permit procedures [Bengtsson & Renberg, 1986]. The CID-methodology (characterisation of industrial discharges, CID) was developed and a guideline for its realisation was published in 1990 by the Swedish EPA [Swedish Environmental Protection Agency, 1990]. The guideline combines biological tests with chemical analyses and is regarded as an effective aid to detect the presence of hazardous substances in wastewaters and to evaluate their significance. The CID has been applied for the investigation of wastewaters from different industrial sectors, municipal wastewater as well as for leachates.

In the context of the STORK-project (STORK: persistent organic pollutants in chemical industry effluents) investigations for the biological and chemical characterisation of wastewater discharges from the chemical industry were conducted between 1989 and 1994 with this methodology [Swedish Environmental Protection Agency, 1997]. The purpose of this project was to determine the discharge of organic compounds that are persistent, bioaccumulatative and toxic under particular consideration of halogenated compounds from plants of the chemical industry and other significant dischargers.

**The Swedish Strategy Concept (CID)**

The *aim* of the CID-methodology is to characterise the hazardous properties of a wastewater as a basis for the assessment of the environmental risk of its discharge. The CID-methodology follows the immission-oriented approach of water protection.

The CID is intended to determine the presence of persistent, potentially bioaccumulating and toxic substances as well as relevant single substances or groups of substances by combining different biological tests and chemical analyses. The investigation programme consists of several steps; in the first step different sum parameters are determined and toxicity tests are performed. If additional information is required to make a decision about the necessity of environmental protection measures, additional investigations can be conducted on separate waste streams or with more specific analysis and test methods. As an alternative to the CID, it is also possible to conduct single substance investigations in cases where a wastewater should only contain very few substances.

CID-investigations include up to three steps which are all based on a basic step of data collection on the industrial process, the wastewater and the receiving water. The same effect parameters are considered in all steps, but the intensity of the investigations increases with each step. An examination takes place after each step in order to determine whether the data obtained for the evaluation of the environmental risk are sufficient.

In the *basic step* information is collected about the industrial process, the raw materials used, the products and by-products, the wastewater characteristics, the wastewater quantity, known

relevant single substances, the characteristics of the receiving water as well as the results of previous investigations. In the *first step* investigations are planned for a first chemical characterisation of the wastewater, of the biodegradability via BOD- and COD-determinations, bioaccumulation and of the acute toxicity of the wastewater. In the *second step* biodegradability tests, chronic toxicity tests, tests for mutagenicity, measurements of the bioaccumulation potential and the toxicity after the biodegradation as well as more specific chemical analyses are applied. The methods used in the steps 1 and 2 are presented in Table 5.

Steps 1 and 2 serve for an enhanced characterisation of the wastewater; step 3 is directed towards the determination of the effects to the receiving water caused by the wastewater discharge. For this purpose, different methods are provided depending on the results of step 2, which range from more specific tests in the laboratory to the investigation of organisms from receiving waters. Among these methods are investigations of the biodegradability with microorganisms from the receiving water (simulation tests), biological investigations of water and sediment organisms, the bioaccumulation potential in fish and mussels, as well as additional chemical analysis depending on the wastewater.

**Table 5:** Wastewater investigations in the steps 1 and 2 of the Swedish strategy concept.

| **Parameters** | **Step 1:** | **Step 2:** |
|---|---|---|
| Chemical characterisation | Physico-chemical parameters<br>Sum parameters<br>(COD, $BOD_7$, TOC, pH, suspended matter, conductivity, nitrogen phosphorous)<br>*Alternative*<br>DOC<br>Analysis of known or suspected hazardous single substances<br>Hydrocarbons, TOCl, EOCl, AOX | Further wastewater investigations with special techniques (GC/MS, HPLC with fluorescence or electrochemical detection)<br>Specific single substance investigations |
| Biodegradation | | Tests with tracking of TOC or DOC-degradation (Die Away) or respirometric tests with methods for the ready or inherent degradability of organic compounds;<br>aerobic stabilisation of the sample followed by the determination of toxicity and bioaccumulation as well as chemical analyses |
| Bioaccumulation | TLC-investigations for the presence of lipophilic compounds upon suspicion | TLC-investigations for lipophilic compounds in the stabilised sample |
| Biological characterisation | Acute toxicity tests:<br>Fish 96 h $LC_{50}$<br>Crustacean 48/96 h $LC_{50}$<br>Algae 3 d $EC_{50}$<br>Higher developed plants 5 d $EC_{50}$<br>Microorganisms: activated sludge (if biological treatment of the wastewater takes place first), luminescent bacteria test as pre-screening test | Depending on the results of the first step, selection of one or two of the following tests:<br>Chronic toxicity with fish, crustacean, mussels or algae, or genotoxicity with the Ames test |

The application of step 1 of the CID-methodology is regarded as a 'standard investigation program', which can also be applied for comparative investigations of different wastewater discharges. Steps 2 and 3 are to be designed in a flexible way depending on the individual case.

A part of the risk assessment according to the CID-methodology is the consideration of the local conditions at the place of discharge. This comprises the wastewater quantity and its temporal variation, the dilution and the water flow-rate of the receiving water, the chemical characteristics of the surface water (hardness, pH etc.), additional wastewater dischargers in the surrounding area, as well as special uses of the receiving water. An initial simple classification of the acute toxicity resulting from step 1 takes place as follows: a wastewater is defined as being acutely toxic if the dilution at the place of discharge leads to a wastewater concentration, which is higher than 1/10 of the $LC_{50}$- or $EC_{50}$ -values of the acute toxicity test. In this case immediate measures to improve the discharge should be taken. If the wastewater concentration ranges between 1/100 and 1/10 of the $LC_{50}$-or $EC_{50}$ -values, the step 2 investigations should be initiated.

Substances with a bioconcentration factor (BCF) > 100 or with an octanol/water partition coefficient ($\log K_{ow}$) > 3 are regarded as bioaccumulating; if they are present in a discharge, additional tests should be conducted. An important aspect for the evaluation is the determination of the portion of persistent substances in the wastewater. Up to now, standardised degradation tests are available only for single substances. Their evaluation criteria such as the 60-70 % DOC-reduction required for 'ready degradability' cannot be directly transferred to complex wastewaters. Additional information about the properties of the persistent fraction is needed to evaluate its environmental significance, such as its toxicity, the content of bioaccumulating substances or its AOX.

**The Stork-Project**

The purpose of the Stork-project (Swedish Environmental Protection Agency, 1997; conducted 1989-1994) was to characterise and compile persistent organic substances in wastewater discharges of the chemical industry with a minimal investigation programme. It was based on step 1 of the CID-methodology supplemented by a biodegradability test (Fig. 5). Direct as well as indirect dischargers were examined.

The results of the investigation of 53 industrial plants showed that individual parameters were of variable importance for the evaluation of the different wastewaters. Moreover, the quality of discharges varied strongly in one industrial branch due to different production processes, raw materials or wastewater treatment measures.

In many plants the results of the characterisation led to reduction of the wastewater pollution. If the cause of a high toxicity or high potentially bioaccumulating or persistent portions could not be explained with the data in hand, the separate waste streams of a plant should be examined to narrow down the emission source in a production process. Only if this does not serve to identify the source an attempt of a chemical-analytical identification should be made.

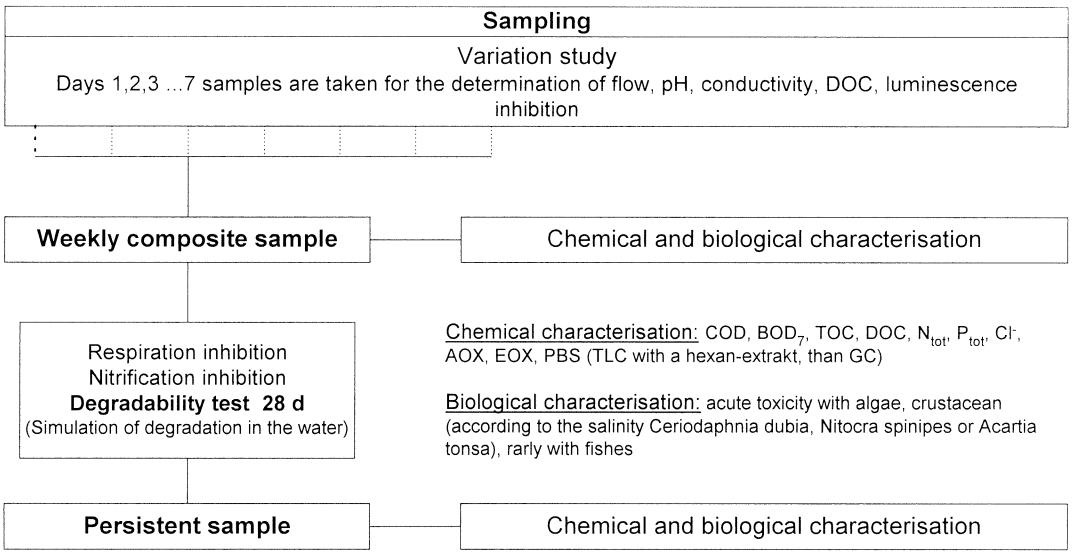



**Fig. 5:** Investigation program of the Swedish STORK-project, chemical and biological characterisation of industrial wastewater discharges.

## *In summary the Swedish strategy concept can be characterised as follows:*

- Tiered and flexible investigation program, immission-oriented.
- Minimum program can be applied for the initial characterisation of wastewater discharges.
- Whole wastewater investigations, also single substance investigations depending on the individual case.
- Applied effect parameters: acute and chronic toxicity, persistence, bioaccumulation, genotoxicity.
- Use of chronic tests if there are indications of persistent or bioaccumulating fractions.
- Can be applied for the discharge permit but this is not obligatory.

### 2.2.6 USA

Since the 1980s biological test procedures are a part of the legal requirements in the wastewater sector in the USA. The regulations of the water quality in the USA are based on the Clean Water Act (CWA), which demands the protection of the aquatic system and the population from toxic substances. In order to reach these aims the American Environmental Protection Agency (US-EPA) followed a strategy to implement water quality standards and to control toxic discharges (water quality-based toxics control); for this purpose chemical, ecotoxicological and biological control approaches were integrated [USEPA, 1991b]. In application of this strategy, the states have established quality standards for their waters. The control of point sources is assured by the grant of discharge permits in the context of the National Pollutant Discharge Elimination System (NPDES).

The grant of a discharge permit is preceded by the quality control through an integrative investigation strategy, in which selected chemical analysis procedures and toxicity tests are applied in parallel. The focus is on the chemical analysis for wastewaters that contain only a small number of (known) pollutants; if a wastewater is very complex toxicity test are of special relevance. These wastewater investigations are supplemented by investigations of the receiving water with regard to the organisms therein (biosurvey). The toxicity tests that are applied to the wastewater are known as WET tests (whole effluent toxicity). The US-EPA has issued very detailed guidelines regarding the implementation, validation and evaluation of the WET tests [USEPA, 1993c, 1994a, 1994b].

*Derivation of the quality criteria for wastewater discharges:* according to the immission orientation (water quality-based approach) of the approach it is necessary to derive the qualitative requirements for a discharge; these requirements are based on predefined quality criteria for the receiving water body and on the specific situation of a discharge location. The acceptable load for a receiving water is defined for various parameters including also chemical parameters taking into consideration the mixing ratios (waste load allocation, WLA). By this approach it is intended to ensure that wastewater discharges hardly cause any hazardous effect outside the mixing zone.

If a discharge does not fulfil the quality requirements with regard to toxicity, the supervising authorities can demand the performance of a TI/TR-investigation (toxicity identification/toxicity reduction). These investigations aim at identifying the chemical substances causing the toxicity of a wastewater (toxicity identification) and shall demonstrate treatment methods, which help to reduce the wastewater toxicity to an acceptable level (toxicity reduction).

The grant of a discharge permit is then related with the adherence of the wastewater quality and the regular execution of the above-described integrative quality controls (chemical analyses and toxicity tests).

*Toxicity tests:* the mixing ratio of the wastewater discharge and the receiving water determines the aquatic toxicity tests to be applied. A high dilution after discharge (> 1000 at the lowest water level) requires three acute toxicity tests (algae, daphnia and fish), dilution factors between 100 and 1000 necessitate the performance of either acute or chronic toxicity tests. Even more adverse conditions with only a slight dilution (< 100) oblige the performance of chronic toxicity tests. Thus, the American procedure extensively applies toxicity tests with marine and limnic organisms and considers acute as well as chronic effects, which normally comprise three trophic levels (algae, daphnia, fish).

In addition to the immission-based requirements, there are also emission standards oriented to the best available technology, which refer to chemical waste load parameters.

*Toxicity Identification Evaluation*: the TIE (toxicity identification evaluation) is probably the first legally binding procedure of toxicity-oriented wastewater investigations world-wide. The substance responsible for an acute or chronic effect in a wastewater is to be identified by a sequence of extraction and fractionation steps followed by an identification phase. After the identification the results need to be verified to prove that the identified substance in the detected concentration is the cause of the toxic effect [USEPA, 1991a, 1993a, 1993b]. This procedure is based on the assumption that it should be comparatively easy to stop the emission of the identified toxic substance by means of operational measures and, thus, to eliminate the toxicity of the wastewater. As long as the chemical identity of the compound causing a toxic effect remains unknown, this appears to be a much more difficult task.

But actually, the past years have shown that the identification of toxic substances, especially in the case of complex wastewaters, is extremely difficult as well as time and cost intensive. This is especially true when higher developed organisms like fish are used as test organisms. For this reason the toxicity identification procedure appears to be less applied in the process of granting discharge permits.

The (older) procedure of 'Toxicity Reduction Evaluation' [USEPA, 1989] is much easier to perform. In this procedure, the chemical nature of toxic wastewater constituents and at the same time the possibility of their elimination is narrowed down by simple sample manipulations in the laboratory (changes to the pH-value, purging, adding of complexation agents or solid-phase extraction) followed by repeated toxicity tests.

The so-called 'toxicity tracking' is an alternative method with the intention to avoid the entry of toxic substances into the industrial wastewater mixture. Here, a toxic effect detected in the effluent is traced back through the treatment plant up to the separate industrial waste streams with the corresponding biotest. This allows to localise the source of the toxic effect in a production process. Once this has succeeded, the chemical cause of the toxic effect can often be determined without requiring additional investigations according to the 'Toxicity Identification Evaluation'. It may then be possible to avoid the emission of the toxic substance into the wastewater and the receiving water by changing the production process (e.g. substitution of substances) without the costly need to modify the wastewater treatment.

Over one decade of discharge permits it was possible to strongly reduce the emission through wastewater discharges and to significantly enhance the quality of the surface waters [Ausley, 2000]. However, the success of this strategy also shows its weakness: with the reduction of point sources through the NPDES diffuse discharges are gaining importance but these are not considered in the calculations and quality assessments so far [Ho, 1997].

Recognising this shortcoming a more intensive ecological risk assessment is intended. This should also include the consideration of synergistic effects of different discharges with variable loads after their mixture in the receiving water body [Ausley, 2000].

***In summary the US-American strategy can be characterised as follows:***

- Stepwise procedure with integration of the toxicity, immission-oriented.
- Consideration of acute and chronic toxicity.
- Discharge permit requires the adherence to the standards derived from the situation of the receiving water.
- Detailed identification procedure (TIE) in the case the toxicity exceeds the WLA.
- So far no integration of other hazardous properties (persistence, bioaccumulation).

### 2.2.7 Germany

The German wastewater legislation is emission-oriented. According to the Federal Water Act (Wasserhaushaltsgesetz, WHG, §7a) a permit for the discharge of wastewater is only granted if the load of hazardous substance is kept as low as possible by applying the appropriate treatment procedures according to the status of technology [WHG, 1996]. The Wastewater Ordinance (Abwasserverordnung, AbwV) and its annexes are uniformly applied in all Federal States and define the minimum requirements on the quality of wastewater that correspond to the state of technology according to the legislation [AbwV, 1999]. The annexes of the Wastewater Ordinance are sector-specific so that not all industrial wastewater discharges have to fulfil the same quality requirements. In general the limits for chemical sum, group or single parameters are fixed in the annexes.

*Toxicity*

In the German wastewater legislation the toxicity of directly discharged wastewaters is considered in two ways:

The Wastewater Charges Act (Abwasserabgabengesetz, AbwAG, § 3, section 1) states that the wastewater charge is assessed according to hazard units, which also consider the acute toxicity of a wastewater towards fish.

For 22 of the 57 individual branches specified in its annexes the Wastewater Ordinance limits the acute fish toxicity. Other toxicity tests are considered in three of the annexes only:

- Annex 22 (chemical industry) sets toxicity limits for the fish, daphnia, algae and luminescence inhibition tests. Furthermore, the genotoxicity test (umu test) has to show a negative result.
- Annex 51 (waste storage) requires the performance of fish, algae and luminescence inhibition tests prior to mixing the landfill leachate with other wastewaters.
- Annex 57 (wool laundries) limits the fish and daphnia toxicity at the point of discharge into receiving water.

The toxicity limits listed in the annexes of the Wastewater Ordinance are given as G-values. The G-value describes the lowest dilution level of wastewater in a biotest where the effect is less than it is specified in the test method. The G-value is the reciprocal of the respective wastewater volume fraction in the test sample.

The following organisms are used according to the annexes of the Wastewater Ordinance in the toxicity tests:

- *Vibrio fischeri* in the luminescent bacteria test (luminescence inhibition, 30 min)
- *Daphnia magna* in the daphnia test (immobilisation, 24 h)
- *Scenedesmus subspicatus* in the algae test (growth inhibition, 72 h)
- *Leuciscus idus* in the fish test (lethality, 48 h)

Recently the Federal Environmental Agency collected and evaluated a large set of toxicity data of industrial discharges that originate from the control of wastewater discharges by the

responsible water authorities and included luminescence inhibition, daphnia and algae tests data besides the fish test results. Typical discharge toxicities for a wide variety of industrial branches could be deduced [Diehl et al., 1999] and it was, thus possible to show the benefits of detecting the acute toxicity with other tests and also in other sectors as those considered in the Wastewater Ordinance so far. The authors concluded that the traditional fish test should be complemented by other biotests to adequately detect toxic effects in some branches.

*Persistence*

The second criterion determining a potential hazard, the persistence, is only indirectly considered. In numerous of the branch-specific annexes of the Wastewater Ordinance the BOD and the COD of a discharge are limited. However, these regulations aim at limiting the risk of oxygen depletion in the receiving water, which may occur through the discharge of insufficiently treated wastewater. As the $BOD_5$-determination is also a degradability test, it may appear that the persistence is also taken into account in the existing statutory regulations. The determination of the $BOD_5$ is not suitable to take account of the aspect of persistence as the test period is too short (5 days instead of the usual 28 days or longer), as the sample matrix is altered by extensive dilutions and as finally the detection parameter ($O_2$-consumption) does not provide any information about the quantity of organic substances remaining at the end of the degradation test.

If one assumes that treated industrial wastewater does not contain any biodegradable substances, the COD or the DOC of the treated discharge would be a measure for the amount of persistent substances in a wastewater. However, this is only partially true. Thus, it must be concluded that the parameter 'persistence' is not yet considered in German Wastewater legislation.

In addition to that, there are currently no other investigation strategies for wastewater discharges in Germany that apply toxicity tests on a broader basis or that consider other hazardous properties such as bioaccumulation, persistence or sublethal effects.

However, methods of toxicity testing and a procedure for the determination of potentially bioaccumulating substances are currently being developed with the support of the Federal Environment Agency (see chapter 3).

### 2.2.8 Comparison of the Investigation Strategies

*Emission vs. immission*: One basic difference between the presented strategies for evaluating and regulating industrial discharges is their emission or immission-orientation. While the immission-oriented concepts consider the conditions at the place of discharge and the surrounding environment for evaluating the environmental risk potential (e.g. Denmark, Sweden, England and the USA), the emission-oriented concepts focus and regulate the quality of the wastewater to be discharged (e.g. Netherlands, Germany).

Generally, immission-oriented investigation strategies are more laborious and time-consuming than emission-oriented approaches, as they have to investigate the receiving water and need much more supplementary information to determine the PEC values.

*Investigation parameters*: The investigation strategies in Denmark, Sweden and The Netherlands comprise the parameters acute and chronic toxicity, genotoxicity, persistence and the potential of bioaccumulation. In other countries like England and the United States, only the acute and the chronic toxicity of the wastewater is considered so far.

In the Dutch concept for whole wastewater investigations, the determination of the chronic toxicity is carried out after a biological degradation test (simulation of biodegradation in the water). A similar coupling of degradation tests with toxicity tests can also be found in the Danish and the Swedish strategy. Chronic toxicity tests are especially important when persistent and bioaccumulating wastewater fractions occur. These tests can be carried out before or after a biodegradation test.

The parameters toxicity, persistence and bioaccumulation are regarded as being hazard parameters of equal relevance in the emission-oriented Dutch concept. The presence of persistent and toxic or persistent and bioaccumulating wastewater fractions requires more detailed investigations in immission-oriented concepts as these fractions may evoke long-lasting effects in the receiving water.

*Test procedures:* Nationally or internationally standardised test procedures are used to determine toxicity or genotoxicity in all concepts. Biodegradation experiments for determining the persistent fraction are partially conducted according to OECD or ISO guidelines. These guidelines were, however, standardised for single substance investigations rather than for wastewater investigations. Finally, the available methods to determine potentially bioaccumulating compounds vary strongly and are hardly comparable.

*Concluding remarks*

The existing investigation strategies coincide in the kind of hazardous effects considered, the application of largely standardised biotests and the chemical characterisation of the wastewater prior to the detection of effect parameters. The present methods to detect persistent and potentially bioaccumulating substances appear to be connected with a high degree of uncertainty. Besides that indirect dischargers are not considered except for the Danish concept.

None of the investigation strategies developed so far considers hazardous substances that are sorbed to particulate matter. Although it could be argued that the particulate phase is not a part of the (dissolved) water phase and does, therefore, not represent a water contamination, hazardous substances sorbed to particles may cause a hazard in the receiving water, if they are ingested by aquatic organisms or if substances desorb and pass into the water phase.

An important point that remained open in most assessment strategies is the quantitative aspect of the determination of persistent and bioaccumulating compounds. Only in the Swedish concept a first attempt to set a quantitative limit for the persistent compounds has been made. However, due to the immission-orientation of this concept, this attempt is only of limited help.

Therefore, it is still unclear which concentration of persistent or bioaccumulating compounds can be regarded as harmless and at which concentration level they have to be classified as hazardous, thus requiring measures to improve the quality of a discharge.

# 3 Methods for the Determination of the Effect Parameters Toxicity, Bioaccumulation and Persistence

## 3.1 Ecotoxicity

### 3.1.1 Basics

Biological tests with aquatic organisms are used to determine potential hazardous effects of substances that may be introduced into the aquatic environment. The main goal of a toxicity test in quantitative terms is to determine that concentration of the test compound that does not evoke a toxic effect in the test organism. It is believed, that the organisms in its natural environment will have similar sensitivity against the test compound and that concentrations below this critical value will ensure the survival and the normal development of the aquatic organisms and the functioning of the biocenosis in receiving water.

Laboratory test are generally conducted with individual species under well defined test conditions. This leads to a limited ecological relevance of the results, as environmental influences and the functioning of the whole ecosystems are not considered in the test system. Since each test species has its own sensitivity towards a substance, the transfer of the test results from one species to another may be problematic. However, the important advantages of laboratory tests is the possibility to standardise the test procedure and, thus, to obtain reproducible results [Rudolph, 1992]. An effect detected in a laboratory test may also occur in the environment, but it is very difficult to establish a causality between the concentration of a substance and the deleterious effect to an organism in the environment as numerous other and unknown influences may affect the species (e.g. the joint effect of different substances, abiotic factors).

Various biological endpoints are used for the detection of toxic effects on aquatic organisms, ranging from the observation of functions of the entire organism down to the biomolecular level. Among these endpoints are the mortality, reproduction, behaviour and growth of organisms but also the alteration of cell functions and enzyme activities.

The extent of a toxic effect depends on the exposure concentration of the hazardous substance and on the duration of the exposure. Acute toxicity tests detect effects after a short period of exposure related to the test organism's life span. A typical endpoint in acute organismic tests is the survival rate of the test organisms. The growth inhibition or special biological functions are often detected in tests with microorganisms and the test result may be given in form of the lethal concentration (LC) or the effect concentration (EC) at which 50% of the test organisms show an effect ($LC_{50}$ or $EC_{50}$). In a chronic toxicity test, however, the exposure period is longer as compared to the life span of the test organism; this allows to record also sublethal effects. Particular life stages like the early development and processes such as reproduction are regarded as being especially sensitive and may, thus, be used to test chronic effects. The result is normally given as the lowest test concentration at which a first effects become apparent (LOEC; lowest observed effect concentration) or as the highest test concentration which does not evoke a significant effect (NOEC; no observed effect concentration).

The extent of a toxic effect depends upon the hazardous substance and on the test organism or the test system itself. The physico-chemical properties of the test compound determine its distribution in the organism and the site and the kind of effect by which it interferes with the functions of the organism. Depending on the metabolism and elimination mechanisms of the test organism, an effect may occur at different concentrations, after different exposure times and at different levels of the organism. This is also the reason for different sensitivities of different species of different trophic levels as well as within one trophic level.

Biological test procedures may be used for different investigation purposes, such as substance testing, the control of wastewater discharges or the monitoring of the water quality. The choice of an appropriate test depends upon the purpose of the investigation and on the material that has to be tested [Nusch, 1991]. Therefore a large number of organismic and suborganismic tests has been developed; the latter measure an effect on the cellular or the biomolecular level *in vivo* or *in vitro*. Among the suborganismic tests are also tests for mutagenic effects (genotoxicity).

Wastewater discharges are usually very complex mixtures of numerous unknown compounds. By using biological test procedures for wastewater control, the joint toxic effect of all wastewater constituents can be measured and the overall toxicity can be regulated. This contrasts the single substance approach, by which only a limited number of (toxic) substances can be regulated.

In order to be applicable to the control of wastewater discharges, bioassays have to fulfil specific requirements [Steinhäuser, 1996a]:

- Standardised test procedure.
- Good reproducibility and practicability (performance, exposure time, costs, test organisms available throughout the year).
- Quantitative interrelationship between the test concentration and the biological response.
- Unequivocal test results (appeal-proven).

In the following those standardised test systems are presented, that have been applied for wastewater investigations. A tabular survey including the references can be found in annex 2.

### 3.1.2 Acute Toxicity Tests

*Fish and Crustaceans*

The most widely applied tests for acute toxicity testing in wastewater investigations are tests with *fish* and *daphnia* under freshwater conditions. They are nationally as well as internationally standardised (e.g. ISO, OECD, US-EPA). The test procedures are very similar and differ only in the selected fish or daphnia species. Fish tests are performed with 1 to 14 days old or older fish, depending on the species, over a period of 48-96 h, with mortality as endpoint. In daphnia tests, daphnia < 24 h old are exposed over a period of 24-48 h and the number of immobile daphnia is evaluated. Both tests are carried out at different dilution steps and the result is given in $LC_{50}$, $EC_{50}$ or G-values (lowest non-effective dilution level).

Tests under brackish or saltwater conditions are applied less frequently, but for many marine fish standardised procedures were issued by the US-EPA. The ISO is currently preparing a guideline for a marine fish test. For investigations under saltwater conditions, *copepods* or *mysids* are used instead of daphnia. An ISO procedure was recently released for copepods (ISO 14669, 1999). After 24 and 48 h exposure in various dilutions the mortality is detected and the $LC_{50}$ determined. Mysids are used in an US-EPA procedure where mortality is determined after 48 and 96 h as the $LC_{50}$-value [USEPA, 1993c].

*Rotifers and protozoans*

Tests with rotifers and protozoans were rarely used for wastewater investigations, but more often for single substance testing. The different tests have been reviewed by Snell & Janssen (1998)for rotifer tests and by Gilron & Lynn (1998) for protozoan tests. So far, there are no internationally standardised procedures, but an ASTM-method is available for rotifers (fresh- and saltwater, 24 h lethality). Rotifers are increasingly applied in test kits because they produce cysts that can be reactivated over a long period of time (see page 41). A DIN-draft for a protozoan test is being developed in German.

*Mussels*

There are US-EPA procedures (ASTM) for tests with mussels under saltwater conditions. A test with oysters (*Crassostrea gigas*) is currently being tested in England in the context of the Direct Toxicity Assessment (chapter 2.2.3). The embryo-larvae-development is tracked here by exposing embryos shortly after their fertilisation over a period of 48 h to determine atypical larval development and mortality.

### 3.1.3 Chronic Toxicity Tests

Chronic toxicity tests are generally divided into short and long-term tests. Chronic short-term tests do not last longer than seven days. The test endpoints here are survival, growth and reproduction. Most test procedures suitable for wastewater investigations were developed by the US-EPA since the end of the 1980s.

#### *Chronic Short-Term Tests*

*Fish*

The USEPA has developed two short-term fish tests [USEPA, 1994a, 1994b]. The mortality and growth of fish larvae after an exposure over 7 days is determined in the first test. In the second test the mortality of fish embryos ranging from shortly after fertilisation to hatching (approximately 4 days) is tracked. Then the larvae are exposed for another 4 days to detect teratogenic substances by morphological mutations.

*Amphibians*

The ASTM has developed a test with South African clawed frogs (*Xenopus laevis*) for the detection of teratogenic substances, the so-called FETAX test (Frog Embryo Teratogenesis Assay-Xenopus, ASTM 1439-98). Frog embryos in a specific initial development stage are exposed over a period of 96 h. Then the number of malformed embryos is evaluated under the microscope. Environmental samples (surface waters, sediment extracts) were examined for example by Fort et al. (1999)with the FETAX test.

*Crustaceans*

Chronic short-term tests are increasingly carried out with *Ceriodaphnia dubia*, as they have a shorter reproduction span (3 to 5 days) than *Daphnia magna* (9 to 11 days). In the test procedures of the USEPA for *C. dubia*, newly hatched daphnids are exposed up to eight days in the wastewater, then the rate of survival and reproduction is determined [USEPA, 1994b].

*Rotifers*

Rotifers reproduce parthenogenetically like daphnids and have a very short reproduction span compared to other organisms, which makes them interesting for chronic short-term tests. Currently a rotifer reproduction test with *Brachionus calyciflorus* over 48 h is being tested for wastewater investigations in an inter-laboratory study in France. At the beginning of the test one rotifer (< 2 h old) is placed in each of the wells of a microtiter plate. After the exposure, the number of rotifers per well is determined.

*Algae*

An often applied and internationally standardised method is the determination of the growth inhibition of algae. Cultures of single-cell green algae (mostly *Scenedesmus subspicatus*) are exposed over a period of 72 or 96 h and the cell concentration is measured at least daily. Growth inhibition and growth rate are evaluated [OECD, ISO, USEPA 1994a,b].

*Higher-developed plants*

Various national procedures are available for chronic tests over a period of 7 days with different *lemna* species (ASTM, EPA, OECD-draft). Growth inhibition is determined via the total number of fronds, the growth rate and/or the alteration of the fronds. Presently, a DIN-draft is under development in Germany.

***Chronic Long-Term Tests***

Chronic long-term tests are applied less frequently for the investigation of wastewater discharges. The Danish investigation strategy proposes the application of chronic fish and daphnia tests in the second investigation step. In the Dutch strategy, these tests are applied after the biodegradation test. The tests are performed according to the OECD-guidelines.

Long-term fish tests can be carried out as prolonged toxicity tests with adult fish up to 14 days (mortality, changes in behaviour) or as tests with early developmental stages. In the latter,

fertilised fish-eggs are used and the test is continued until the yolk sac of the control fish has been resorbed. Lethal and sublethal effects are observed [OECD].

The chronic reproduction test with *Daphnia magna* lasts for a period of 21 days and measures the number of living offspring per daphnia [OECD 202]. The ASTM has released a procedure in which the chronic short-term tests with *C. dubia* is prolonged for up to 15 days to detect the effects on the daphnia hatched during the test.

### 3.1.4 Microorganisms

***Luminescence Bacteria***

The acute luminescence bacteria test is among the most widely applied bioassays for wastewater and it is internationally standardised [ISO]. The method is based on the detection of the luminescence inhibition of *Vibrio fischeri*, which is generally determined after a test duration of 30 minutes at 15° C. In the standard procedures, this test is conducted as a cuvette-test.

A chronic test with luminescent bacteria, where the chronic effect is measured after 22 hours incubation at 27° C, is currently in the trial phase. After the incubation the decrease of the luminescence intensity is measured which is induced by the exhaustion of the cell metabolism. This test is included in the Dutch investigation strategy as a test for chronic toxicity [Tonkes, 2000].

In an investigation of 25 hazardous substances the chronic luminescent bacteria test, the 48-hour reproduction test with the rotifer *B. calyciflorus* and the *D. magna* reproduction test over 21 days were compared [Radix et al., 1999]. Although the rotifer test was found to be somewhat less sensitive than the daphnia test, it still showed a rather good correlation. However, the chronic luminescent bacteria test showed a much worse correlation while having the same sensitivity as the daphnia. Both tests, the chronic luminescent bacteria test and the rotifer test, were regarded in this study as being suitable for the screening of chronic effects. In comparative wastewater investigations of the chronic effect in the 22-h luminescence inhibition test with the chronic toxicity of *C. dubia* and *P. promelas*, the NOEC-values showed a good correlation of the chronic luminescent bacteria test with the daphnia test, whereby the NOEC-values of the daphnia test were somewhat higher, however [Sweet et al., 1997].

In Germany a DIN-method was developed, which serves to determine the growth inhibition of *V. fischeri* over 7 h (DIN 38412-37). In a comparison of the two endpoints growth (7 h) and luminescence inhibition (24 h) with single substances the growth inhibition test was clearly less sensitive to heavy metals [Gellert, 2000] and also less sensitive to organic toxic compounds. This may be due to the high concentration of nutrients in the growth inhibition test as compared to the 24h-luminescence inhibition test: the high salt content may induce the loss of test compounds by adsorption and it also leads to a much higher reproduction rate, rendering the test less sensitive [Froehner et al., 2000].

***Pseudomonas Putida***

Internationally standardised test procedures are available for the determination of acute or chronic effects with *Pseudomonas putida* (representative for heterotrophic microorganisms in surface waters). In acute tests the respiration inhibition is detected after 30 min (DIN) and in chronic tests the growth inhibition after 16 h (ISO).

***Other Test Systems***

Other tests with microorganisms are often applied for the investigation of indirect wastewater discharges with the aim to protect the sewage treatment plant from disturbances due to inhibitory substances in these wastewaters. These tests may use activated sludge in order to determine an inhibition of respiration (ISO, OECD) or nitrification (DIN). An ISO-draft describes an anaerobic screening-test for the detection of toxic effects from wastewater constituents on the anaerobic gas production during the digestion (ISO).

### 3.1.5 Non-Standardised Organismic Tests

***Fish-egg Test***

A fish-egg test was developed in Germany for the determination of acute toxic effects of wastewater discharges on the embryonal development. In this test fertilised fish-eggs are exposed over a period of 48 h. The test endpoints are on the one hand parameters that are suggested to correspond to the lethality of adult fishes, and on the other hand parameters, which indicate specific effects. A comparison of this test with the acute fish test showed a higher sensitivity of the fish-egg test and an increased indicative value, as information about specific effect mechanisms can be obtained [Friccius et al., 1995; Lange et al., 1995]. At present, this test is in the final stage of the standardisation procedure. The future DIN-method, however, considers only the evaluation of impairments, which result in death as a definite disruption of the embryonic development (DIN 38415-6 draft 2000). This test was developed and will be used as a substitute for the acute fish test.

***Toxicity Test Kits***

So-called toxicity test kits are increasingly applied. These are commercial tests, which are prepared by the manufacturer in such a way that the tests can be carried out in every laboratory without requiring an extensive preparation or equipment. The test kit contains test organisms as cysts and the media necessary for their revitalisation. The tests are easily performed and no organisms have to be cultured.

Up to now, the following tests kits are available:

| | |
|---|---|
| Rotoxkit F: | freshwater rotifer *Brachionus calyciflorus* |
| Rotoxkit M: | saltwater rotifer *Brachionus plicatilis* |
| Thamnotoxkit F: | freshwater crustacean *Thamnocephalus platyurus* |
| Streptoxkit F: | freshwater crustacean *Streptocephalus proboscideus* |
| Artoxkit M: | saltwater crustacean *Artemia salina.* |

The toxicity test kits Rotoxkit F, Streptoxkit F and Thamnotoxkit F were compared by Latif et al. (1995) with the acute *D. magna* test (24 h) in an investigation of 42 municipal and industrial wastewater discharges. The test kits turned out to be just as sensitive as the daphnia-test; *T. platyurus* was more sensitive to 75 % of the toxic samples than *D. magna*. The test kits Rotoxkit F and M, Thamnotoxkit F as well as Artoxkit M were included in the first investigations for the development of the Dutch investigation strategy [Tonkes & Baltus, 1997]. However, these were found to be less sensitive compared with the acute luminescent bacteria and daphnia tests, which were also performed.

***Miniaturised Test Systems***

Miniaturised test systems are mainly developed with regard to the automation of existing test methods or with the intention to minimise the sample volume required for a test.

Some examples are the miniaturised luminescent bacteria test in microtiter plates (100 µl sample; Fiehn et al., 1997) as well as the use of daphnia (*D. magna* < 24 h old) or freshly hatched fish larvae (*P. promelas*) in 48-well microtiter plates (2 ml sample; Powell et al., 1996). The algae growth inhibition test can also be conducted in a miniaturised form [e.g. Höhne, 1991; Blaise et al., 1998].

As the miniaturised tests work with the same organisms, endpoints and mostly also with the same media as their not-miniaturised predecessors, their sensitivity is expected to be comparable. However, a validation against the initial test is necessary.

### 3.1.6 Suborganismic Tests

Suborganismic test systems detect toxic effects on a cellular, enzymatic or biomolecular level. Their underlying principle is the simulation of biomolecular target sites in organisms leading to a manifestation of toxic effects due to their interaction with hazardous substances.

However, the uptake, the metabolism and elimination mechanisms available to an organisms can not be represented by these tests. Therefore, suborganismic test can only provide a first indication of a potential hazard to the whole organism and have to be compared with organismic tests if they are to be used as a replacement for these tests.

Suborganismic tests together with miniaturised versions of organismic tests are commonly referred to as microbiotests [Blaise, 1998].

***Fish Cell Tests***

Different test systems with fish cells were developed in order to be able to reduce the number of fish tests or to abandon them completely. However, none of the tests appears to be generally established so far [Denizeau, 1998]. As fish cell lines often RTG-2 fibroblasts (rainbow trout gonads) or BF-2 fibroblasts (bluegill sunfish) are used. In addition, primary cell cultures, e.g. rainbow trout hepatocytes, are also utilised. These tests are carried out in microtiter plates with an incubation time of 24 h. Mostly the cell vitality is determined with the neutral-red-test (impairment of the cell and lyosome membrane) or the MTT-test (inhibition of enzymes in the

mitochondria) by colour reactions. Other detected endpoints besides the cytotoxicity are the cell morphology and the cell adhesion.

The RTG-2 fish cell test was less sensitive compared to the acute fish and the fish-egg test in an investigation of single substances [Lange et al., 1995]. Gagné & Blaise (1998a) conducted comparative wastewater investigations with two cytotoxicity tests (rainbow trout hepatocytes as well as fibroblasts) and the acute fish-test (rainbow trout). The test with hepatocytes showed a better correlation with the fish test for the examined wastewaters. In a subsequent inter-laboratory test this test was found to be reproducible and transferable to other laboratories. [Gagné et al., 1999].

***P450-Induction-Test***

The enzyme cytochrome P450, which is present in the liver cells of fish and other vertebrates, is used as a biomarker for the exposure to hazardous organic substances. In the P450 induction test, the induction of detoxification enzymes is measured via the cytochrome P-dependant enzymatic activity of 7-ethoxyresorufin-O-deethylase (EROD) in the liver of rainbow trout. The fish are exposed over a period of 96 h; afterwards liver homogenates are prepared for the enzymatic assay. For example this test was applied by Burnison et al. (1996) and Garric et al. (1996) for the investigation of wastewater samples.

***Enzymatic Tests***

Enzymatic test systems detect the inhibition of enzyme activities in vitro. There are test systems for the detection of the cholinesterase, urease or aldehyde dehydrogenase inhibition [Obst et al., 1998]. In Germany a standardised procedure for the cholinesterase inhibition test is available [DIN 38415-1]. This test serves for the targeted detection of organophosphate and carbamate pesticides as it measures the cholinesterase inhibition and thus the effect on the nervous system. This test was hardly ever applied for wastewater investigations; its main application so far was the monitoring of surface waters.

***Immunoassays***

Immunoassays are based on the measurement of the occupation of the binding sites of antibodies through the target analytes. As the binding reaction itself does not provides an easily detectable signal, various markers are used for the detection of the immune response. Depending on the type of detection, there are for example radio immunoassays (RIA), enzyme-linked immunosorbent assays (ELISA) or fluorescence immunoassays (FIA). With antibodies test systems for specific analytes can be developed. These exist for a large number of pesticides as well as PAHs and PCBs and are applied for the investigation of surface waters [Dankwardt et al., 1998]. In Germany, a general framework for the performance of immunoassays for the determination of pesticides is given in a DIN-method [DIN 38415-2].

Immunoassays seem rather unsuitable for the investigation of industrial wastewater due to the complex matrix and the risk of cross-reactions. In addition, they do not detect a toxic effect but are directed towards the chemical structure of the target analytes. Insofar they cannot be regarded as toxicity tests but as a supplement or replacement of chemical analyses.

### 3.1.7 Selection of Possible Toxicity Tests for the IDA Strategy

Standardised short-term tests with organisms of all four trophic levels for the determination of the aquatic toxicity are available in Germany. With the fish, daphnia and luminescent bacteria tests the acute toxicity is measured and with the algae test also chronic toxic effects are determined. A large experience has been gained with the application of these tests for the investigation of wastewater discharges [Diehl & Hagendorf, 1998].

It is generally recognised that these test can only reflect a part of the possible detrimental effects on aquatic communities. At the same time, the search for the most sensitive test will not be successful due to the large number of factors influencing the sensitivity of organisms (see chapter 3.1.1). These four standardised tests are therefore generally regarded as being necessary and sufficient for a safe water quality control [Steinhäuser, 1996b]. Therefore there is no need to include additional tests with other organisms into the IDA strategy for the detection of acute effects.

Instead of the fish test, the suitability of the fish-egg tests or of fish cell cultures as screening tests has been examined as a replacement for animal protection reasons. The fish-egg test is currently in the standardisation phase in Germany [DIN 38415-6 draft]. However in anticipation of this procedure, the fish-egg test is already incorporated into the IDA strategy as a substitute of the fish test. This is also advantageous for practical reasons, as this test requires a much smaller sample volume.

Significantly fewer experiences exists in Germany with regard to the application of other chronic toxicity tests than the algae test for wastewater discharges. Even the chronic algae test is not as frequently applied for wastewater discharge investigations as the three acute tests [Diehl et al., 2000]. But the application of chronic tests becomes increasingly important, as only these tests are suitable to detect also a long-term ecotoxicological hazard potential of wastewater discharges.

It is therefore reasonable to consider also the chronic hazard potential in the IDA strategy. Short-term tests to detect chronic effects are of special interest, as it is difficult to keep wastewater samples stable over a longer period of time. A standardised test is already available with the algae test. Other tests that could be considered are the chronic daphnia test (with *C. dubia*), the chronic rotifer and the chronic luminescence test. These tests would require a more detailed testing, examination and validation prior to being considered for inclusion in the IDA strategy. In addition, a prolonged fish-egg test (> 48 h), corresponding to the chronic short-tem tests of the USEPA, could also be of interest.

### 3.1.8 Genotoxicity

***Basics***

The term genotoxicity generally comprises all effects, which cause DNA-damages. Initial DNA-damages can however be repaired enzymatically, so that they do not inevitably lead to mutations, meaning changes to the DNA-sequence, or are transmitted to daughter-cells.

Today's state of knowledge does not allow to establish a connection between a positive result in a genotoxicity test and effects on the ecosystem level. However the genotoxic effect detected in a bioassay is an accepted effect in toxicity testing and a genotoxicity test has already been included in that annex of the German Wastewater Ordinance that regulates the quality of effluents of the chemical industry (AbwV, annex 22).

The general importance of the detection of genotoxic effects is substantiated by the following [Helma & Knasmüller, 1997]:

- genotoxic substances in waters may have direct impacts on human health if persons get into contact with these waters (especially via drinking water)
- genotoxicity can reduce the reproduction of organisms
- mutations caused by DNA-damages can increase the instability of ecosystems by altering the population structure

As already mentioned, there are enzymatic mechanisms for the repair of DNA-damages in cells. Therefore, DNA-damages may be temporary in nature and do not necessarily have any additional consequences. Based on that, different endpoints can be detected in genotoxicity tests even on a cellular level. These are: (a) gene mutations, (b) primary DNA-damages and other chromosome damages like (c) clastogenesis (chromosomal breaks and gain, loss, or rearrangement of pieces of chromosomes) and (d) aneuploidy (abnormal chromosome numbers in the nucleus). A correlation with effects on a higher biological or even ecosystem level, which would be of great relevance for the hazard assessment, is still missing today.

***Genotoxicity Tests for Wastewaters***

There are numerous different tests for the detection of genotoxic effects. The number of tests applied to water and wastewater is, however, substantially lower [Helma et al., 1994].

*Bacterial Tests*

The *Ames test* detects gene mutations and is based on the exposure of a histidine-requiring strain of *Salmonella typhimurium* generated by mutation. The measure for the mutagenic activity is the number of histidine-independent colonies formed by reversion. The Ames test is internationally (OECD) and nationally standardised and was applied very frequently also for wastewater investigations since its introduction by Ames in 1973.

The most widely applied tests besides the Ames test are tests for the detection of primary DNA-damages with bacteria, like the SOS chromotest (*Eschericia coli*), the umu test (*Salmonella typhimurium*) and the Mutatox test (*Vibrio fischeri*).

In the *SOS chromotest* and the *umu test* the activity of the SOS-gene, which is required for the repair of DNA-damages, is detected indirectly. The extent of the induction of this gene serves as a measure for the primary DNA-damage that has occurred. An ISO-procedure was developed for the umu test and there is also a DIN-procedure available (DIN 38415-3).

In the *Mutatox test* a dark (non-luminescent) strain of the luminescence bacteria is used. DNA-damages are detected by the reappearing light production in this strain due the activity of the SOS-gene in the presence of genotoxic agents.

These tests are commercially available as test kits and are comparatively easy to perform without requiring extensive biochemical equipment [Legault & Blaise, 1994].

*Tests with Eukaryotic Cells*

For the detection of chromosomal damages cells of higher organisms are used. The major tests used in wastewater investigations are the sister chromatid-exchange test and the comet assay.

The *comet assay* detects breaks in DNA-strands: in the gel electrophoretic separation the DNA-fragments form a comet-like tail. The length of this tail serves as a measure for the extent of a damage. Advantageously this test can be conducted with different cells, e.g. of aquatic organisms like fish [Mitchelmore & Chipman, 1998].

The *sister chromatid-exchange test* tracks the symmetric exchange of DNA-fragments between the chromatids of a chromosome. This exchange is detected under the microscope by observing the transfer of a chromatid marked with a coloured DNA-base in the chromosome.

These tests for the detection of primary DNA-damages necessitate larger efforts and higher costs than bacterial tests and so far they have not shown any advantage over the former tests. Thus, bacterial tests are preferred for the IDA strategy.

One of the first genotoxicity tests developed, the bacterial mutagenicity test by Ames (*Ames test)* has lost its attractiveness for wastewater investigations in the past years. Several times false positive results were found and it was shown that typical wastewater constituents like amino acids and nutrients could have caused these results. Therefore an over-estimation of the genotoxic potential is expected when the Ames test is used.

Although comparisons were made between different tests [de Maagd & Tonkes, 2000a], it is yet not possible to make a general statement about the sensitivity of the afore mentioned tests. In addition, not all tests can be applied quantitatively in terms of a clear dose-effect relation.

Due to the different detectable end points, the application of a test battery for the comprehensive detection of genotoxic effects has been discussed. But since genotoxicity is just one of the toxic effects to be detected in the IDA strategy, only one genotoxicity test can be incorporated.

***Discussion***

In view of their wide application and the reduced test effort, it appears to be advantageous to apply a bacterial test for the detection of primary DNA-damages in the IDA strategy. As the umu

test is standardised in Germany (test guideline No. 410 in the Appendix of the Wastewater Ordinance) and as it is already included in the wastewater legislation, its incorporation into the IDA strategy seems reasonable. Furthermore, a substantial database of genotoxicity tests results from wastewater with the umu test is available, which will ease the classification of future experimental data.

### 3.1.9 Endocrine Disruptors

***Basics***

Endocrine disruptors are generally defined as substances that alter the normal functions of the endocrine (hormonal) system and thus impair the different physiological processes controlled by hormones. Endocrine disruptors can affect the hormonal system via different mechanisms. A direct effect can be initiated through antagonistic binding to hormone receptors. Indirect effects may be caused by influencing the biosynthesis or the metabolism of hormones. The effects on the reproductive system are regarded as being especially significant. In most investigations, the focus was on oestrogen-like (feminising) effects, but androgen effects were also proven in selected cases.

Besides natural and synthetic hormones, various chemicals are also classified as having endocrine-disrupting effects (xenoestrogens). These include industrial chemicals like alkyl phenols, bisphenol A or phthalates, but also certain insecticides, herbicides and fungicides. In addition, plant hormones have met growing attention. Different methods were developed in the past years to determine the endocrine effect of single substances or substance mixtures in environmental samples like surface waters or wastewaters. Different in vitro assays were suggested as screening tests especially for estrogenic effects, which are all based on one of the following effect mechanisms of steroid hormones:

- measuring the activity of enzymes involved in steroid synthesis
- competitive ligand binding assays with receptors
- cell proliferation assays
- gene expression assays in mammal cells and yeast

A discussion of the advantages and limits of these test systems with regard to the evaluation of the estrogenic effect of chemicals can be found in Zacharewski (1997).

***Tests for Wastewater***

The determination of the *induction of the vitellogenin-synthesis*, which can be conducted in vivo and in vitro, has proven to be a suitable measure for estrogenic effects. Male fish are used for in vivo tests, as their vitellogenin expression in the liver is not active. An increased vitellogenin level caused by an induction can be detected by blood tests. In in vitro vitellogenin-tests, the hepatocytes (liver cells that synthesise vitellogenin) mainly of rainbow trout but also of other fish are exposed. Recently, first investigations of amphibian hepatocytes (*Xenopus laevis*) were

also performed [Kloas et al., 1999]. A vitellogenin formation can be detected in the cell assays by the determination of the extra-cellular vitellogenin concentration or by the quantification of the vitellogenin-mRNA. Different detection methods were developed for that purpose.

Purdom et al. (1994) conducted first investigations of the vitellogenin induction in fish in 1987/89 after their exposure in the effluents of sewage treatment plants (measured with a radio-immunoassay, RIA). Hansen et al. (1998) performed a similar investigation and compared the vitellogenin-induction in fish determined with an ELISA (enzyme linked immunosorbent assay) with the induction of detected estrogenic substances in an in vitro hepatocytes test. Besides the in vitro induced vitellogenin-content in hepatocytes through municipal and industrial wastewater discharges, Gagné & Blaise (1998b) also determined the vitellogenin-mRNA-expression with a chemoluminescence in situ hybridisation-assay (CISH). Islinger et al. (1999) on the other hand developed a non-radioactive dot/blot/ RNAse protection-assay for the analysis of the vitellogenin-mRNA-expression and applied it also for wastewater investigations.

Other in vitro tests simulate the hormone-caused activation of the *estrogen receptor* . Suitable test systems are for example hormone-dependent human breast cancer cells (MCF-7). The test with MCF-7-cells (also called E-screen-test) is based on the cell growth in the presence of estrogenic substances. So far, no false positive or negative results were obtained for substances with known estrogenic effect.

So-called *recombined yeast cell-tests* were developed that use genetically modified yeast cells into which the human estrogen receptor was incorporated and which is used for competitive ligand binding tests. The binding of a substance to the receptor was linked with the expression of the ß-galactosidase enzyme, so that an effect can be measured photometrically via the ß-galactosidase activity. The yeast assay was shown to detect natural estrogens and known xenoestrogens.

The MCF-7-test was applied by Körner et al. (1999, 2000) for the investigation of wastewater extracts obtained by SPE. The cell proliferation was quantified through the detection of the total protein content with a photometric method. The recombined yeast assay was used in different investigations of complex mixtures relevant to the environment. Wastewater discharges were examined by Desbrow et al. (1998) and Wegener et al. (1999) as well as activated sludge extracts by Rehmann et al. (1999). Desbrow et al. (1998) applied the yeast cell assay in a 'toxicity identification evaluation' (TIE) procedure for the identification of estrogenic substances in treated municipal wastewater. Balaguer et al. (1999) developed a recombinant assay with MCF-7 cell lines with an estrogen-regulated luciferase gene, which measures the binding to the estrogen receptor via the luciferase-activity in a luminometer. Finally, there are attempts to apply an estrogen-receptor for the selective extraction of estrogen-active substances from wastewater [Seifert et al., 1999].

***Discussion***

For practical reasons no in vivo tests can be considered for the application in the IDA strategy. All three in vitro test systems (MCF-7, yeast assay and hepatocyte assay) detect estrogenic effects and are performed in microtiter plates. The duration of the tests varies from seven days (E-screen), 48 hours (hepatocyte assay) to 4 hours (yeast assay). Therefore these tests appear suitable for the IDA strategy due to the required sample volume, the degree of their automation and their duration. But none of the in vitro tests has been standardised so far.

Contrary to these technical aspects, other important aspects have not been clarified yet. To ensure the environmental relevance of an effect detected in vitro, it must be possible to confirm this effect with in vivo tests. Thus, in order to be able to apply cell assays as screening tests, it is important that these in vitro tests are calibrated against in vivo tests. Comparative investigations regarding the sensitivity of the different in vitro test systems for wastewater investigations have not been performed. Therefore, it is not possible to select the most sensitive test system. In a comparison of eight in vitro tests with 20 endocrine disrupting substances, high effects were detected in all test systems for natural and synthetic estrogens. The results for the tested xenoestrogens varied, however [Andersen et al., 1999]. In addition it became evident that additional standardisation and validation work is required in order to increase the reliability of the in vitro tests.

In conclusion, it appears that the ecological relevance of estrogenic effects of wastewaters or 'environmental chemicals' detected with in vitro biotests is still unclear, similar to those of genotoxicity tests (chapter 3.1.8). However, as some laboratory investigations clearly indicated the potential of estrogenic compounds to affect the reproductive system of higher organisms [Danzo, 1998] it may be necessary to integrate an in vitro test for the detection of endocrine effects into the IDA strategy in due course. Further test comparison, standardisation and validation is required before.

At present, the EU is conducting a community research programme on environmental hormones and endocrine disruptors (COMPREHEND, 1999-2001). It focuses on the detection of the extent of the pollution of municipal and industrial wastewater discharges by endocrine disrupting substances and their effect on the organisms in the receiving waters in various Northern European countries [Pickering & et al., 2000]. In this context it is also intended to perform a comparison of the above mentioned in vitro test systems, so that more detailed information about the suitability of the various tests for wastewater investigations may become available.

## 3.2 Bioaccumulation

### 3.2.1 Basics

***Bioaccumulation, Bioconcentration and Bioavailability of Substances***

The term bioaccumulation describes the enrichment of a substance in a living organism from its environment. Regarding aquatic organisms this process takes place via direct uptake from the water or via ingestion with the food. Kinetically, an enrichment occurs only when the uptake rate into the organism is higher than the elimination rate from it. The bioaccumulation of a substance can vary largely between different species, as various biological and physiological properties influence the extent of the uptake of a substance and the ability to eliminate it.

The direct, passive enrichment of a substance from the aqueous phase is called bioconcentration; the tendency of a compound to become enriched by bioconcentration is mathematically described by the bioconcentration factor (BCF). The BCF is defined as the ratio of the concentration of a chemical in the organism to the concentration in the surrounding water and refers to the state of equilibrium when the rate of uptake equals the rate of elimination. The BCF can refer to the wet weight of organisms or to their fat content. It is experimentally determined via the exposure of organisms (mainly fish) in a static or continuous test system. Compartment models are used for the mathematical description of the uptake and the elimination of chemicals. In the simplest model, the one-compartment-model, the organism is regarded as a homogenous lipid body. The enrichment is then described as distribution process between a water and a lipid phase, which takes place via passive diffusion.

Of all the physico-chemical properties of a substance that influence its bioconcentration, the hydrophobicity is the most important. It largely determines the tendency of a substance to partition into the double-lipid-layer of the cell membrane and to be transported further into the organism. The distribution coefficient between 1-octanol and water ($K_{ow}$) is a comparatively easily measurable parameter and a direct proportionality between it and the bioconcentration factor was found. Thus, the tendency of a compound to bioaccumulate can be assessed from its $K_{ow}$-value and it increases with a compounds hydrophobicity and $K_{ow}$-value.

Bioconcentration factors can be lower than expected from the $K_{ow}$, if a substance is metabolised in and excreted from an organism. Deviations in the correlation between BCF and $K_{ow}$ are also observed for substances with very high $K_{ow}$-values (> 6), when the BCF no longer increases with the $K_{ow}$ and the bioconcentration potential derived from the $K_{ow}$ is overestimated. This is due to the limited permeability of the membrane double layer for these very hydrophobic compounds. Finally, the uptake of a substance with a molecular weight of more than 500 u or a molecule diameter of approximately 1 nm is diminished due to sterical hindrance, which reduces its bioconcentration. An additional factor, which influences the uptake of a substance is its degree of ionisation. Generally, compounds bearing an electric load cannot diffuse through a cell membrane but have to be actively transported. Therefore, the pH of the medium and the p$K_a$-value of the ionizable substance determine whether and to what extent it is taken up [Fent, 1998].

Additionally, environmental factors can have a large influence on the bioavailability of a substance. A substance is defined as being bioavailable when it is freely dissolved in the aqueous

phase and can be directly taken up by organisms, i.e. when it is membrane-permeable. The most important process that influences the bioavailability of hydrophobic substances is the sorption to suspended particles, sediment, humic substances, and other macromolecules [Geyer et al., 2000]. Here, the pH of the water (degree of ionisation of the substance) and the temperature (respiration rate of cold-blooded organisms) also play a role. Thus, the bioavailability may determine the actual extent of the bioconcentration in the aquatic environment.

In addition to the process of bioconcentration, bioaccumulation also includes the active uptake of substances via food and other ingested material such as sediment and suspended matter. The contribution of the active uptake to the bioaccumulation is still a matter of discussion. For most substances, the bioconcentration has been shown to predominate. For very hydrophobic substances ($\log K_{ow} > 6$), however, with a strong tendency to sorb onto particulate matter the uptake with the food can become most important [Qiao et al., 2000].

***Determination of the Bioaccumulation Potential of Substances***

The $K_{ow}$-value is generally used for a first assessment of the bioaccumulation potential of substances. The $K_{ow}$ of single substances can be experimentally determined via partitioning between an octanol and a water phase or by reversed-phase liquid chromatography with standardised procedures (OECD 107 and 117). Another possibility is to calculate the $K_{ow}$ via quantitative-structure-activity-relationships (QSAR). Due to the different determination methods the $K_{ow}$-values compiled for a substance may vary [Ritter et al., 1995].

In Germany, the $K_{ow}$-value is used for estimating the bioaccumulation potential of chemicals in the approval procedure. Substances with a $\log K_{ow} < 3$ are regarded as not being bioaccumulative, substances with a $\log K_{ow}$ between 3 and 6 are classified as bioaccumulative and substances with a $\log K_{ow} > 6$ are bioaccumulative if their molecular weight is below 500 u. If a more detailed assessment is necessary, the BCF has to be experimentally determined in a fish test. Generally, substances with $\log K_{ow}$-values of > 3 are internationally regarded as potentially bioaccumulating [Beek et al., 2000].

### 3.2.2 Determination Procedures for Wastewater

In order to obtain a comprehensive overview on the load of a wastewater with bioaccumulating substances, various detection procedures were developed. They determine the total amount of so-called 'potentially bioaccumulating substances' by using the $K_{ow}$ as the guiding parameter parallel to single-substance evaluations. As the procedures based on the $K_{ow}$-value do only consider the passive enrichment, but neither the active uptake nor a metabolic transformation and release, they can only determine a bioaccumulation 'potential'. The existing procedures for determining the potentially bioaccumulating fraction in wastewater were recently reviewed [de Maagd, 2000].

Most of the procedures consist of a sample pre-treatment, an extraction step in which the fraction of interest is separated from the wastewater, followed by a purification step to remove matrix

**Table 6:** Survey of the experiences available so far for the determination of potentially bioaccumulating compounds.

| Method | Pre-treatment | Extraction and reprocessing | Separation/detection |
|---|---|---|---|
| TLC [Renberg et al., 1985] | pH = 2 | Liquid-liquid-extraction (hexane), concentration | RP-TLC/GC-FID |
| HPLC [Klamer & Beekman, 1995] | pH = 2 | Liquid-liquid-extraction (hexane), Extract purification via aluminium oxide and silica gel column, removal of high molecular weight substances by GPC, enrichment | RP-HPLC/UV |
| Preparative HPLC [Metzger et al., 2000] | pH of the wastewater, filtration | Solid-phase-extraction C18 (elution with MeOH and THF) and soxhlet-extraction of the filter with toluene | RP-HPLC, collection of fractions, concentration, weighing resp. $C_{org}$-detection |
| Empore Disk [Verbruggen et al., 1999a; Verhaar et al., 1995] | pH = 7.5 | C18-coated teflon filter disk | Vapour-pressure osmometry GC/MS |
| SPME [de Maagd & Tonkes, 2000b] | pH = 7.5 | Polymer-coated fibre, C8 | GC/MS, HPLC/MS |
| SPMD [Verbruggen et al., 2000] | pH of the wastewater, filtration | Polyethylene-tubing filled with hexane or lipids | GC |

compounds and a final separation connected with the detection of the potentially bioaccumulating compounds (Table 6).

Each of these procedural steps has a significant influence on the spectrum of compounds, which is finally detected as potentially bioaccumulating and, thus, on the selectivity of the method as a whole. Besides the analytical procedure it also depends upon the sample matrix, which portion of the organic load is determined as being potentially bioaccumulating. For example, the hydrophobicity of dissolved anionic organic substances may change depending on the pH-value and the cationic composition of a sample. Organic compounds may occur in a freely dissolved state or associated with organic macromolecules (humic substances) and this association may affect the $K_{ow}$-value of the compound and thus the bioaccumulation potential, but also its bioavailability.

Finally, one of the fundamental difficulties when developing or comparing procedures to determine potentially bioaccumulating compounds is that their true amount in a sample is generally unknown. It is possible to obtain different results with different procedures, but it is impossible to decide, which one is closest to the true value.

In the following the different methods and the individual steps are described in more detail.

***Liquid Extraction and Thin Layer Chromatography (TLC)***

The first method for the detection of potentially bioaccumulating compounds in wastewater was introduced in 1985 by Renberg et al. (1985) .The less polar dissolved substances were extracted from a wastewater sample by liquid-liquid-extraction with hexane. The extract was separated according to its polarity via reversed-phase thin layer chromatography. Then the band on the thin layer plate that contains the hydrophobic substances was scraped off and the potentially bioaccumulating compounds were desorbed with a suitable solvent. Its amount was finally estimated via GC-FID analysis. This method is applied in the Swedish investigation strategy [Swedish Environmental Protection Agency, 1997].

***High-Pressure Liquid Chromatography (HPLC)***

In 1995, Klamer & Beekman (1995) published the first method based on a HPLC-separation for the assessment of potentially bioaccumulating compounds in wastewater samples. After a hexane extraction (see above), the crude extract is cleaned and higher molecular substances are separated by GPC (gel permeation chromatography). The separation and detection of the potentially bioaccumulating compounds was performed by reversed-phase-HPLC with UV-detection. With the gradient elution applied. the chromatographic retention time and the $\log K_{ow}$-values of 55 reference substances over a $\log K_{ow}$-range of 0.9 - 8.3 correlated.

***Solid-Phase Extraction - Preparative HPLC***

On the basis of a preparative reversed-phase HPLC separation, a method is currently being developed in Germany for the determination of potentially bioaccumulating compounds [Metzger et al., 2000]. The filtered wastewater samples are extracted by solid-phase extraction (SPE), the extract is eluted from the solid phase with a solvent mixture and separated according to the $K_{ow}$-values via a preparative RP-HPLC. The fraction eluting from the column in the corresponding time range of the desired $\log K_{ow}$-range 3-8 is collected, the solvent is removed and then the weight of this potentially bioaccumulating fraction is determined. Additionally, by measuring the organic carbon content ($C_{org}$) of this material the portion of the wastewater DOC assigned to the bioaccumulating fraction can be determined.

When the particles removed by filtration are extracted with toluene, this extract can be processed accordingly. In this way the particle-bound fraction of potentially bioaccumulating compounds can be determined.

***Empore Disc - Vapour Pressure respectively Gas Chromatography***

Verhaar et al. (1995) introduced a method for the so-called biomimetric extraction to assess the baseline toxicity of substances (narcosis) via a simulation of the bioconcentration process. The uptake of substances is simulated by the exposure of a hydrophobic extraction disk (C18-empore disk) in large volumes of water over a period of up to two weeks. Analogous to the natural

process of bioconcentration an equilibrium is established between water and a hydrophobic phase during this 'biomimetric' extraction process.

After this time, the substances adsorbed on the disk are extracted and the potentially bioaccumulating compounds are detected by vapour-pressure osmometry. Alternatively, Verbruggen et al. (1999a) also applied gas chromatography-mass spectroscopy for the detection of the adsorbed substances.

***Solid-Phase Microextraction - Gas Chromatography***

The solid-phase microextraction (SPME) is based on the adsorption of dissolved substances on a polymer-coated fibre, which is immersed into the sample. This kind of extraction is also based on the establishment of an equilibrium between the two phases and it is also non exhaustive as the empore disk extraction; but the SPME requires less time and a reduced sample volume. After the extraction (24 hour stirring), the fibre can be used directly as an injection needle for the GC so that a solvent extraction is not necessary. This procedure is being tested for its application in the Dutch investigation strategy [de Maagd & Tonkes, 2000b]. The quantification is made by evaluating the peak areas after mass spectrometric detection.

***SPMD-Gas Chromatography***

A semi-permeable polyethylene-tubing filled with hexane [Verbruggen et al., 2000] or another hydrophobic material like fish lipids is used for SPMD (semi-permeable membrane device) enrichment [Södergren, 1987]. The device is immersed into a body of water or a wastewater discharge over a longer period of time (several weeks) for the detection of hydrophobic substances [Kot et al., 2000]. Similar to the bioconcentration process the uptake into the tubing occurs by passive diffusion and depends upon the $K_{ow}$ of the substances [Petty et al., 2000]. Verbruggen et al. (2000) was the first to apply the SPMD for the passive extraction of potentially bioaccumulating compounds in wastewater discharges. After the exposure of the SPMD over a period of two weeks, substances were re-extracted and determined by GC.

### 3.2.3 Method Comparison

As this short summary shows, very different methods are currently proposed for determining potentially bioaccumulating compounds from wastewater. These are now discussed successively:

*Sample Pre-treatment*: In some cases the samples are acidified prior to the extraction [Renberg et al., 1985; Halder & Ahne, 1990]. This causes a protonation of acid groups and thus a reduction of the polarity for many anionic organic substances. On one hand, this appears to be a systematic error, as this protonation will hardly occur at the pH-value of the surface water and a larger mount of potentially bioaccumulating compounds will be detected after acidification than without. On the other hand, the polarity of anionic organic substances in water can be significantly reduced by the formation of ion pairs with multivalent cations. An acidification pushes this effect back and thus makes the investigation result less dependent on a variable wastewater matrix. A certain independence from the wastewater matrix appears to be important, as this matrix changes completely upon mixing of a discharge with the receiving water.

In many cases the particulate matter is initially removed by filtration; this process entails the risk of loosing potentially bioaccumulating compounds by adsorption onto the filter and the filter cake. Centrifugation should be applied instead, as this process avoids losses of hydrophobic compounds.

*Extraction*: Extractions with organic solvents (liquid-liquid extractions) are time consuming and are, nowadays, largely replaced by solid-phase extractions. New procedures for the detection of potentially bioaccumulating compounds should also reflect this trend. The use of the SPME (resp. the extraction disks) seems attractive as it simulates the 'natural' process of bioconcentration experimentally. In contrast, the SPE is an exhaustive extraction method. Although it is also based on hydrophobic interactions with the wastewater components like the bioconcentration, an distribution equilibrium is not established as in the case of the bioconcentration and the SPME. However, the extent of the exhaustive extraction will be less influenced by the sample matrix and the speciation of the organic substances within a sample as in procedures based on equilibration.

*Clean-up*: The primary aim of the clean-up steps is to remove higher molecular weight substances, which may have fallen into the targeted $K_{ow}$-range, but which are not permeable and not bioaccumulative due to their size (see above). A purification is not necessary if the subsequent steps cannot detect these components (e.g. GC).

*Separation*: All exhaustive extraction methods require a chromatographic separation of the extracted components prior to their detection to focus further on the $K_{ow}$-range of interest [Renberg et al., 1985; Halder & Ahne, 1990; Metzger et al., 2000]. Liquid chromatography (LC) is preferred over gas chromatography (GC), as it also detects nonvolatile, thermally less stable and more highly functionalised compounds that may be lost in a GC separation. However, GC eliminates interferences from high molecular weight components because these are just not sufficiently volatile.

*Detection*: GC-separation is usually coupled to mass spectrometric or flame-ionisation detection. These detectors are considered as being mass sensitive. The response of these detectors, i.e. the relation of the signal intensity to the substance quantity, is however substance-related, so that a quantification of unknown substances is not possible. At least this procedure enables a quantitative assessment as the response factors normally do not vary by more than a factor of 2.

With regard to HPLC, UV-detection was used until now. However, this detection method is undesirably selective as only UV-active substances are detected. At very low wavelengths (200-220 nm) non-aromatic compounds may also absorb light, but the response is much more variable than in a MS- or FI-detection. For the quantification of unknown substances or even substance mixtures, HPLC with UV-detection cannot be applied. A relative comparison of samples, obtained with the same procedure and analysed with the same method, may be possible.

Insofar the approach chosen by Metzger, to quantify the chromatographically purified fraction of bioaccumulating substances by weighing or $C_{org}$-determination, is reasonable. Especially the organic carbon determination is attractive, as it can be put into relation with typical wastewater parameters like the TOC or DOC. In comparison, the informative value of the osmometry, which

provides a molar concentration, is much lower. Besides that, this detection method seems to be too insensitive [de Maagd, 2000].

Up to now, no comparative investigations with two or more of the methods described above have been carried out.

The informative value of the sum parameter 'potentially bioaccumulating substances' needs further debate. As it can only be defined operationally, it will be necessary to decide on one determination procedure in order to come up with comparable data. However, this will not yet improve the informative value.

Another subject to decide upon is to which extent the speciation of dissolved organic substances has to be considered when determining potentially bioaccumulating compounds. A more emission-related approach would justify such a consideration. However, it must be taken into account that the water matrix containing the potentially bioaccumulating compounds will drastically change after the discharge. Thus, in many cases the mixing with surface water will be accompanied by a reduction of the ionic strength and this matrix change will likely have a strong influence on the speciation of organic compounds. Anionic organic substances bound onto particles via cationic bridges could be desorbed and transferred into the dissolved phase, colloids and other agglomerates may disaggregate and the compounds may pass into the dissolved state when cations that reduce electrostatic repulsion are lost. Against this background, it would also be appropriate to push back the influence of the wastewater matrix and to neglect questions of speciation during the determination of the potentially bioaccumulating substances.

Finally, the existing and future procedures for the determination of potentially bioaccumulating compounds also have to be evaluated with regard to their compatibility to a multistage investigation strategy. This aspect was not considered in previous method developments.

## 3.3 Persistence

### 3.3.1 Basics

***Persistence***

After the entry of an organic substance into the aquatic environment its persistence is one of the decisive factors influencing the duration of its stay in the environment and the resulting concentration in a compartment. Substances are generally regarded as being persistent if they can only be decomposed slowly or not at all by natural elimination processes. Their final elimination can only take place through mineralisation, i.e. if they are degraded into carbon dioxide, water and inorganic components.

Persistent xenobiotic substances are undesirable in the environment, as they may be widely distributed and may enrich in different environmental compartments. Especially nonpolar persistent substances can be enriched in aquatic organisms due to their bioaccumulation potential and can migrate along the food chain. In the long term, this can lead to damages of the respective organisms. Therefore investigations of the persistence of organic substances are included in the process of evaluating the risk potential of the introduction of organic substances into the environment.

***Elimination and Microbial Degradation of Organic Substances***

The elimination of organic substances in the environment can take place via abiotic (physico-chemical) and biotic (biological) processes. Physico-chemical processes like oxidation, hydrolytic and photolytic reactions may lead to the transformation of a substances but not to its mineralisation. For the considered substances abiotic transformation processes are mostly of inferior importance due to kinetic inhibition.

The predominant process for the elimination of organic substances in aquatic systems is the biological degradation through microorganisms. The microorganisms use the organic carbon and the energy ultimately released by the transformation process for their growth and the maintenance of their metabolic processes. The biodegradation may take place under aerobic (surface water) or anaerobic conditions (sediment).

Whether, how fast and to what extent an organic compound is degraded depends upon its chemical structure and concentration. Additionally, the environmental conditions in the respective compartment influence the biodegradation, such as the number and the composition of microorganisms, the concentration of nutrients, the type and concentration of other organic substances, the oxygen content, the pH-value, and the temperature as well as the presence of particles. The biodegradation process can vary depending on whether the substance is present alone or as a mixture together with other substances. In receiving waters, pollutants use to be present in small concentrations compared to natural substances. Their biodegradation often occurs co-metabolically, so that the compound is transformed or mineralised without being used as a source for carbon or energy in the presence of other substrates, which can maintain the growth [Fritsche, 1999].

***Standardised Biodegradation Tests***

Different laboratory tests were developed for the assessment of the biodegradability of organic substances in aquatic systems and sewage treatment plants. The biodegradation in laboratory systems is influenced by the same factors as the biodegradation in aquatic systems (see above), which results in numerous possible test factors.

The properties of the test system, e.g. temperature, nutrients concentration, substrate content, co-substrate, and test duration can be defined in laboratory tests. However a standardisation of the inoculum, i.e. the source of the microorganisms, their composition and state of adaptation, is difficult to perform. Therefore, mixed cultures are widely used and the inoculum is defined by its origin. Generally, activated sludge from sewage treatment plants, the effluent of municipal wastewater treatments or surface water is used as inoculum, partially also sediment is added or the biomass is mixed from different sources. Due to the numerous factors can influence the results of a biodegradation test, the reproducibility of the test results achieved by different test systems can be limited. It is not possible to establish a 'true' or reference method for the assessment of the biodegradability [Pagga, 1997].

Different analytical methods can be used to follow the degradation process: sum parameters such as the $CO_2$-production, the $O_2$-consumption or the DOC-reduction may be used and single substance analysis can be applied. $CO_2$- or $O_2$-measurements provide information about the degree of mineralisation, but do not consider the transformation into biomass. The final degree of degradation is also determined with DOC-measurements. With this parameter, however, it is not possible to distinguish between biodegradation and abiotic removal through sorption onto the biomass, as both processes diminish the DOC-content. The primary degradation (loss of the initial chemical identity) can be determined via single substance analysis. This approach is required to follow degradation at low substrate concentrations.

Generally, degradation tests are applied to assess the extent of degradation in a natural or technical environment (aquatic system or sewage treatment plant). The test method used should simulate this environment to a certain degree. The more precise a degradation behaviour has to be predicted the closer must the test conditions reflect the respective environment and the higher becomes the effort to perform a test. In contrast a simple test system is sufficient to evaluate whether a substance can principally be degraded or not. Therefore different standardised tests were developed for different investigation purposes.

The OECD developed a test hierarchy for the investigation of the biodegradability of organic substances. To enable a cost-efficient assessment of their degradation behaviour different test steps from simple to complex tests can be applied depending. This test strategy is generally accepted and the OECD tests correspond to the tests of the EU respectively to the ISO and DIN-procedures except minor deviations [Merrettig-Bruns, 2000]. It consists of the following three steps:

1. Step: Tests for ready biodegradability (OECD 301 A - F)
2. Step: Tests for inherent biodegradability (OECD 302 A - C)
3. Step: Simulation tests (OECD 303 A and B)

The applied tests are all directed towards the aerobic biodegradability in freshwater systems. The first step can be used to screen for biodegradability. The test conditions do not favour the biodegradation and are characterised by a low inoculum density (high ratio of test substance to microorganisms) and non-adapted microorganisms. The tests D and E have a lower biodegradation potential with approximately $10^2$ cells/mL (final effluent as inoculum) than the other four tests with $10^4$-$10^5$ cells/mL (activated sludge as inoculum) and with substrate contents of 10-40 mg/L. Besides that the tests differ in the applied detection method. A substance is regarded as being readily biodegradable if 70% of the DOC is removed or 60% of the theoretical $CO_2$-production or $O_2$-consumption occurs within 10 days after the end of the lag-phase (starting phase up to a degradation of 10 %); the entire test duration must not exceed 28 days. A positive result implies that this substance degrades rapidly in the aquatic environment and during biological wastewater treatment. A negative result does not exclude a degradation in the environment; therefore in the next step the inherent biodegradability is tested.

The tests of the second step are more favourable for a biodegradation, as a higher cell number in relation to the substrate is used. In the Zahn-Wellens-Test (OECD 302 B) a cell number of $10^6$ - $10^7$ cells/mL is used for a substrate content of 50 - 400 mg/L. In addition, a pre-adaptation of the microorganisms is possible and the test can be extended beyond 28 days. With the obtained biodegradation results, substances can be classified into non-biodegradable (biodegradation > 20 %) or potentially biodegradable (biodegradation > 20 %). If more than 70 % removal occurs the test substance can be expected to be completely removed in an adapted sewage treatment plant.

Simulation tests of the third step are provided, if the rate of the microbial degradation is to be assessed for a special environment. A typical example is the need to assess the residual concentration in the environment for a risk assessment. Simulation tests are standardised by the OECD especially for the degradation during the biological wastewater treatment (OECD 303). The ISO is currently developing a simple test for the assessment of biodegradation kinetics in surface waters with low test concentrations [Ingerslev & Nyholm, 2000].

A tabulation of the applied methods in the test hierarchy can be found in annex 3. There are also other standardised tests listed concerning biodegradation in marine systems or under anaerobic conditions. A review of test systems for the assessment of the biodegradability of organic substances and a detailed discussion of the different factors that influence the test results is given in a report of the OECD (1995).

### 3.3.2 Biodegradability Tests for Wastewater

In this chapter an overview of biodegradability tests that have been used to investigate wastewaters is provided. It focuses on tests that determine the residual biodegradability of biologically treated wastewater in natural waters, as this kind of tests used to be included into investigation strategy for hazardous substances in wastewater discharges. For the same reason, biodegradability tests for untreated wastewater are described only shortly.

***Biodegradability Tests for Untreated Wastewater (Indirect Discharges)***

The application of a biodegradability test for untreated wastewater may be necessary if an indirect discharge has to be examined. These tests simulate the biodegradation in a sewage treatment plant and are intended to provide a wastewater quality that is comparable to the effluent of a municipal sewage treatment plant.

For this purpose, a test with a high inoculation density is applied. A suitable test is the static Zahn-Wellens-Test (inherent test, OECD 203), that covers all elimination mechanisms of a sewage treatment, being biodegradation, sorption and stripping [Schönberger, 1991]. In Germany it can be applied in accordance with the Wastewater Ordinance for the detection of the aerobic biodegradability of wastewater in biological treatment plants. (Wastewater Ordinance, Appendix 4, guideline No. 407/408, equivalent to DIN EN 29888). When using an activated sludge inoculum of 1g/L dry matter, a DOC- or COD-reduction of 80 % indicates the treatability of a wastewater in municipal sewage treatment plants. Due to the high biomass concentration, a certain portion of the wastewater constituents can also be eliminated through sorption. This portion can be estimated through the decrease in concentration within the first 3 hours of the test ('3-hour-sample'); it is assumed that sorption processes occur much faster than biodegradation processes [Gartiser et al., 1996]. In order to follow the mineralisation of compounds that are sorbed to the activated sludge or that are poorly soluble, a test system detecting the $CO_2$-production and the $O_2$-consumption is currently being examined for its standardisation as an ISO-directive.

Up to now, the biodegradation potential of raw wastewaters is most frequently characterised by its $BOD_5$/COD or $BOD_5$/TOC ratio. The $BOD_5$-value of raw wastewaters is an important parameter to be considered in the dimensioning of sewage treatment plants. In effluent discharges the $BOD_5$-value indicates the burden for the oxygen budget of the receiving water. This test is, however, not suitable as a biodegradation test in an investigation strategy for wastewater discharges, since the effluent matrix is drastically altered by the addition of the dilution water and since the test duration is not sufficiently long. If the test duration is extended, the $O_2$-consumption based on nitrification processes interferes with the mineralisation of the organic matter. Other tests are, thus, required.

***Biodegradation Tests for Treated Wastewater (Direct Discharges)***

The incorporation of a biodegradation tests into investigation strategies for the characterisation of hazardous wastewater constituents follows two intentions: (a) to assess that fraction of effluent constituents which must be expected to persist in the receiving water and (b) to determine the qualitative changes of the effluent due to microbial degradation processes in the receiving water. It is especially important to examine, whether the hazardous properties detected in a wastewater discharge (toxicity, bioaccumulation) are likely to persist in the receiving water. A suitable test system should therefore be able to roughly simulate the biodegradation conditions in the receiving water and to provide a wastewater fraction that contains substances that are either hardly biodegradable or not biodegradable at all.

It was outlined above, that the results of a biodegradation test are influenced by the test conditions. However, there are no standards available for assessing the biodegradability of wastewater discharges with a test simulating the conditions of receiving waters, except the aforementioned $BOD_5$-test. For this reason, standard tests for the determination of the ready biodegradability of single substances have been used to investigate the discharges in the investigation strategies described in chapter 2 (Sweden, The Netherlands, Denmark).

In the following some test systems that have previously been applied to wastewater discharges and that have been coupled with a subsequent characterisation of its persistent fraction (ecotoxicity, potentially bioaccumulating compounds) are described and discussed in more detail. The respective test conditions are summarised in Table 7. Based on the results reported for the respective tests the test conditions are then compared with regard to their degradation potential. Based on this comparison, a test system suitable for incorporation into the IDA strategy will be selected.

In the *Swedish investigation strategy (CID)* for the comparison of the wastewater discharges of different industrial sectors, a modified ISO-test (ISO 7827 corresponds to OECD 301 A, DOC die-away-test) was applied as degradation test [Swedish Environmental Protection Agency, 1997]. The aim of this test was to obtain a fraction that contains all persistent compounds and degradation products for its further characterisation. If inhibitory effects were detected prior to the degradation test, the wastewater samples were diluted as much as necessary to avoid this inhibition (see Table 7).

Municipal effluent was used as inoculum in concentrations that reflect the conditions of the receiving water. The content of potentially bioaccumulating compounds of the inoculum was determined in order to consider a possible sample contamination by the inoculum. DOC-reductions of 10-90 % were observed in the numerous samples examined. In case of direct discharges, an insufficient wastewater treatment was indicated by a high degradability (DOC-removal) in this test.

Treated municipal effluent was chosen as inoculum because a better comparability of the results should be ensured due to its uniformity, even if different laboratories conduct the test. However, it remains unclear to what extent the degradation process then corresponds to the conditions in the receiving water. Concerning indirect discharges, the authors concluded that the test system provided a weaker biodegradation potential than it would have been achievable in adapted sewage treatment plants. They propose to use the uniform sewage treatment plant effluent for the comparison of different discharges. When the investigation focuses on the quality of one discharge, the sediment of the receiving water or the sludge of the respective treatment plant may be used.

In the *Dutch investigation strategy (WEA)* a modified OECD-test (OECD 301 E, modified OECD-screening-test) was used to assess the degradability of wastewater discharges in the surface waters [Tonkes & Baltus, 1997]. Ten biologically treated wastewater samples from different industrial origins were examined. After 28 days, three of the samples showed an additional degradability of up to 45 % DOC, which even increased when the test was prolonged. The authors called for standardised test procedures. Moreover, it may be necessary to first

investigate the surface water used as inoculum with the methods of the investigation strategy to establish a correction factor if necessary.

In the *Danish Guideline for the Risk Assessment of Industrial Wastewater Discharges* a so-called aerobic stabilisation is carried out based on the OECD-test 301 E in the first investigation step (screening) [Pedersen et al., 1995]. This test is especially designed for samples that were not sufficiently pre-treated prior to their discharge. In the second investigation step tests are

**Table 7:** Biodegradation tests applied in the whole wastewater investigations.

| Strategy resp. author | Samples | Tests | Test conditions | Test duration | Sum parameters |
|---|---|---|---|---|---|
| Sweden<br>CID [Swedish Environmental Protection Agency, 1997] | Industrial wastewater discharges, direct and indirect dischargers | EN ISO 7827 (OECD 301 A) modified | Test volume 15 L, Inoculum: 1 mL/L settled municipal effluent, addition of mineral nutrient solutions, T = 20°C | 28 days or until the DOC-decrease is less than 10 % in 4 days (partly up to 80 days) | Tracking of the decrease via DOC, $BOD_7$ in regular intervals, COD, TOC at the beginning and the end |
| | Wastewater samples were diluted prior to the test if a sludge respiration inhibitory test (ISO 8912) indicated an inhibitory effect of the wastewater. The dilution was carried out such that an inhibitory effect was no longer discernible. | | | | |
| The Netherlands<br>WEA [Tonkes & Baltus, 1997] | Industrial wastewater discharges, direct dischargers | OECD 301 E modified | Test volume 10 L for 28 d, sample 1:1 diluted with surface water as inoculum | 28 days (at times up to 84 days)<br>15°C, aeration in the dark | DOC |
| Denmark<br>Guideline [Pedersen et al., 1995] | Industrial wastewater discharges, direct and indirect dischargers | Step 1: aerobic stabilisation according to OECD 301 E<br>Level 2: OECD 306 | Inoculum: final effluent or surface water<br>Wastewater diluted with sea-water | 2-4 weeks<br>Several weeks | DOC<br>Tracking of the degradation of the relevant effect parameter |
| Nyholm<br>[Nyholm, 1996] | Industrial wastewater discharges, direct and indirect dischargers | 1. aerobic stabilisation according to OECD-tests for ready degradability<br>2. easy degradability | Inoculum: final effluent or surface water, sample 1:1 diluted, addition of mineral nutritive medium<br>Same conditions as 1., but higher dilution | 1-3 months until DOC or toxicity remain constant | DOC<br>DOC, $O_2$, $CO_2$ |
| Germany<br>Wastewater Ordinance [AbwV, 1999] | Industrial wastewater discharges, indirect dischargers, separate streams | No. 407/408 of Appendix 4 of the Wastewater Ordinance (Zahn-Wellens-Test) | Inoculum: activated sludge | 7 days | COD or DOC |

proposed that are more closely related to environmental conditions, e.g. the OECD 306 test (degradation test for sea-water).

*Nyholm* (1996) suggested a combination of biodegradation tests with toxicity tests. The toxicity serves as a guiding parameter in order to be able to distinguish between biodegradable and persistent toxic wastewater constituents. This scheme is used for direct as well as indirect discharges. First an aerobic stabilisation is performed, for which the sample is diluted at least 1:1 with the test medium. The persistent fraction thus obtained is expected to be sufficiently concentrated for a further characterisation. Activated sludge (general characterisation) or receiving water (higher simulation character) can be used as inoculum and the biomass density should not exceed the level of tests for easy degradability. For the second test the stabilised sample is diluted, as further substances may be degraded at a lower concentration. In this way, the ready biodegradability of the wastewater constituents is determined and the degradation is tracked with sum parameters. The method was exemplarily applied to a highly polluted pulp mill effluent.

Comparative investigations regarding the influence of the test conditions (type and quantity of inoculum) on the degradation kinetics of biologically treated *wastewater discharges* have rarely been carried out. Some examples are given by Khan et al. (1999) and Percherancier et al. (1996), who investigated the effect of experimental conditions on the determination of the so-called BDOC (biodegradable DOC) of municipal effluents. Khan et al. (1999) varied the type and quantity of the inoculum (effluent or activated sludge from municipal sewage treatment plants) in order to optimise the speed of the degradation over a degradation period of 28 days. Increasing the inoculum density by adding activated sludge led to a pronounced acceleration of degradation and also to a higher degrees of degradation in some cases. Percherancier et al. (1996) used different natural inoculi at the same density ($10^3$ cells/mL) for the determination of the BDOC over a period of 8 days. The inoculum from oligothrophic river water provided a faster degradation than the inoculum from sediments of eutrophic rivers, but the final degree of degradation was identical.

When the biodegradation of *single substances* was compared with different tests for ready biodegradability [Gotvajn & Zagorc-Koncan, 1996; Koziollek et al., 1996], it was found that some substances can only be classified as being readily biodegradable in a test with a higher inoculum density according to the OECD-criteria. In tests with a lower inoculation density, they may either degrade slower, so that the 10-day criterion is not fulfilled, or to a lesser extent. Therefore the biodegradability may be underestimated, when a low inoculum density is used.

### 3.3.3 Discussion

The test-systems that have been used to determine the persistent fraction of effluents are based on OECD-tests for ready biodegradability. These tests provide a low degradation potential which was expected to be comparable to the degradation potential of the receiving water. These tests should remove only easily degradable compounds from the wastewater sample. In all cases, the biodegradation was followed by a DOC-determination. In order to achieve a higher conformance

with the natural conditions, final effluent was used at a low inoculum density. In the Dutch strategy the sample was diluted 1:1 with surface water without adding any additional biomass.

To date, the influence of these factors on the final degree of biodegradation, the degradation rate and the parameters determined afterwards (ecotoxicity, bioaccumulating compounds) has not been examined. The different OECD-tests for ready degradability allow a certain variability in the inoculum density, depending on whether final effluent or activated sludge is applied. The tests presented here partly have a very long duration of up to 2 or 3 months; it appears attractive to accelerate the degradation by using activated sludge (30 mg/L TSS) as inoculum while obtaining the same degree of final degradation. This would reduce the extent of the investigation and the results would be available much faster.

# 4 Concept of the IDA Strategy

## 4.1 Requirements for a new strategy

Based on the existing investigation strategies, and the analysis of their advantages and disadvantages (chapter 2), the following requirements should be fulfilled by a new assessment strategy for industrial discharges:

- The three criteria toxicity, persistence and bioaccumulation should be considered for a comprehensive investigation of the environmental hazard potential of industrial wastewater discharges.
- These effect parameters should be regarded as being equally relevant.
- Besides the acute toxicity, the chronic toxicity and genotoxicity should also be considered. It should be possible to integrate new test procedures or additional biological effect parameters, for example for sub-lethal effects, if required.
- The strategy should be emission oriented according to the precautionary principle and corresponding to the German water legislation.
- The strategy should also consider particle-bound hazardous substances even though their relevance can presently not be evaluated.
- It should consider the principal differences between direct and indirect discharges.
- As in the other concepts, the strategy should start with a chemical characterisation of the wastewater in order to obtain fundamental data about the wastewater properties.
- The strategy should be flexible to allow its adaptation to different objectives and wastewaters and, thus, to avoid unnecessary expenses.
- The strategy should have a modular design, so that the investigation can be suspended if sufficient information was gained.
- Finally standardised or at least well established test methods should be incorporated as far as possible as their sensitivity, feasibility and reproducibility is well documented.

Considering these requirements the following modular strategy was developed for the investigation of hazardous substances in industrial wastewater discharges.

## 4.2 The IDA-Concept

The novel modular strategy for industrial discharges assessment (IDA) is divided into several modules. This modular design provides the required flexibility of the strategy. Each of these modules consists of and links several investigation processes, such as e.g. the determination of two effect parameters and of chemical parameters.

Depending on the *type of wastewater* to be examined, some modules can be incorporated in or excluded from the assessment. In addition, the investigation can be suspended after certain modules depending on the *purpose* of the examination. Finally, the modular structure allows to decide in individual cases whether the investigation should be continued after the execution of a module depending on its *results* or whether the detected effects demand measures to improve the wastewater quality. The succession of the modules is illustrated in Fig. 6.

The **module 'Sampling and Characterisation'** combines the initial tasks of the investigation starting with the sampling. Generally a thorough characterisation of the wastewater is carried out at first; it may be based on the compilation of available data and supplemented with additional analyses if necessary.

In the following **module 'Toxicity'** acute and chronic toxic effects and genotoxic effects are determined. If pronounced effects are seen, the examination may be suspended and measures for the improvement of the wastewater quality may be initiated.

In the **module 'Persistence'** that fraction of the wastewater is experimentally determined, which is not amenable to further mineralisation in the receiving water. This fraction is regarded as the persistent fraction.

The third fundamental hazard parameter is then recorded in the **module 'Bioaccumulation'** by fractionating the dissolved organic wastewater constituents according to their polarity. Those compounds exhibiting a $\log K_{ow}$-value > 3 are regarded as being potentially bioaccumulating.

With these modules the three fundamental hazardous effects persistence, bioaccumulation, and toxicity (PBT) are determined. An important feature of this strategy is that these three hazard parameters are not determined independently from each other by parallel investigations, but that they are experimentally linked according to their logic relationship. This enables the detection of the toxic and persistent wastewater fraction (in the module 'Persistence') as well as that of the toxic, persistent and bioaccumulative fraction (in the module 'Bioaccumulation').

Two additional modules are provided to meet the afore mentioned requirements (chapter 4.1):

The **module 'Particle Load'** can be inserted after the module 'Sampling and Characterisation', if the discharge contains a significant amount of particulate matter. This module allows for a first assessment of whether this particulate phase evokes a toxic effect. The particulate matter is then examined separately in this module.

Besides that, the **module 'Indirect Discharges'** has to be applied when indirect discharges are investigated. This module simulates the biological treatment of an indirect discharge in a municipal sewage treatment plant. Afterwards the effluent sample is forwarded to the module 'Sampling and Characterisation' and then processed as a direct discharge sample.

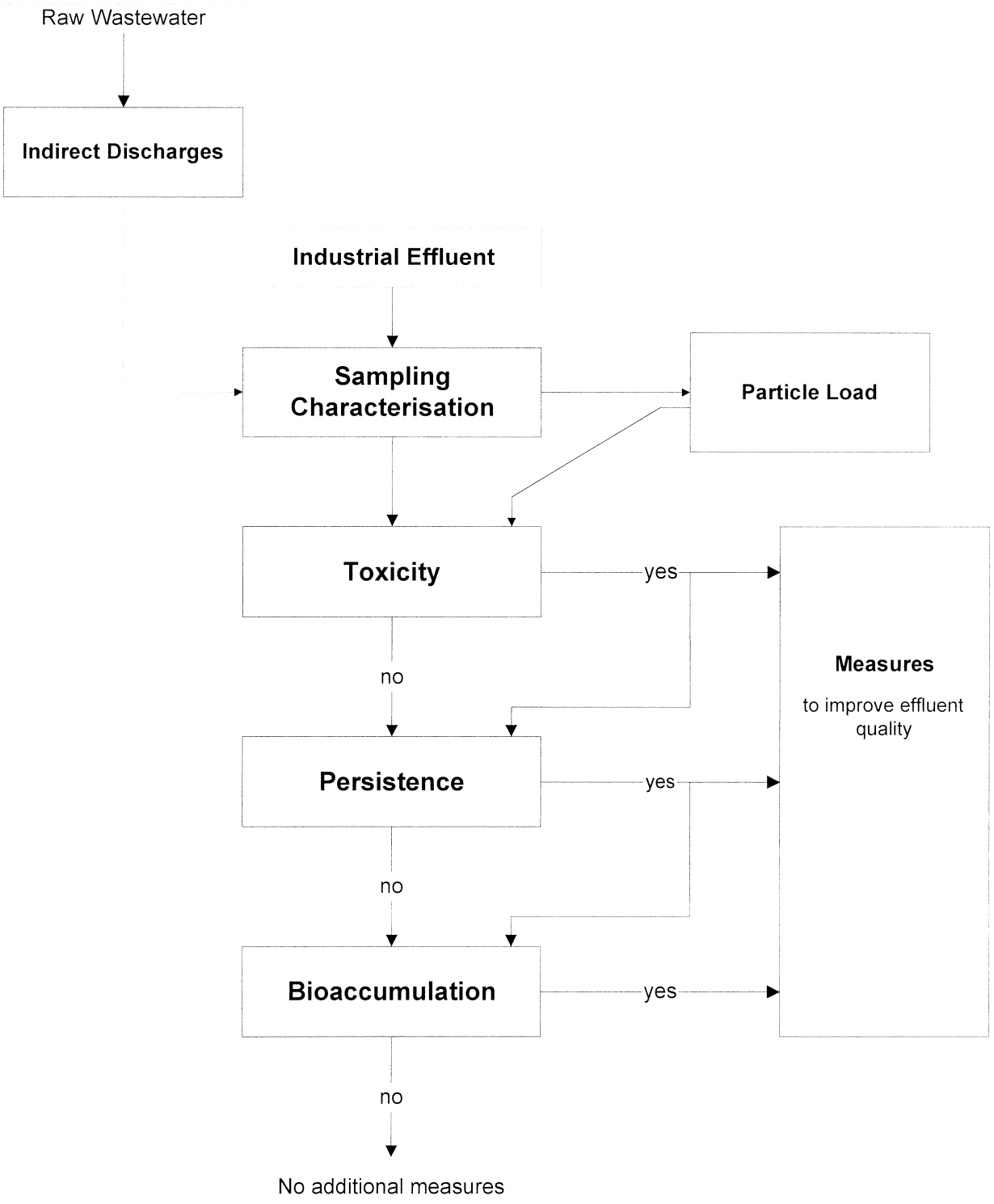



**Fig. 6:** Succession of the modules in the IDA strategy.

## 4.3 Description of the Modules

The individual modules, their internal structure and logic connections as well as the detection methods to be applied in the modules are now described in more detail. The experimental details can be found in annex 9.

### 4.3.1 Module 'Sampling and Characterisation'

The first step of the IDA strategy is the collection of samples at the discharging plant. Samples of indirect discharges are then transferred to the module 'Indirect Discharges' (see below), while the wastewater of direct dischargers is directly subjected to the chemical characterisation.

This characterisation uses existing data obtained in other investigations, e.g. during the operation of a treatment plant or from the regular discharge supervision. The characterisation is not only important for an exact description of the discharge to be assessed; it also indicates necessary investigations in the context of the strategy (e.g. the determination of the turbidity for the module 'Particle Load'). Finally the data obtained here serve as the initial set of analytical data, which is needed for comparison throughout the assessment strategy. Several analytical parameters determined in this module are repeatedly used in other modules, namely the DOC-content and the UV-absorbance.

Methods:

*Sampling*

According to the annexes of the German Wastewater Ordinance so-called 'qualified grab samples' or two-hour composite samples are used for quality control purposes. The same type of sampling could, thus, be used in this assessment strategy in order to ensure compatibility.

On the other hand, many examinations show that the quality of industrial wastewater discharges, whether direct or indirect, can vary greatly in time, as the majority of production processes are discontinuous. These quality fluctuations are probably the higher the smaller the plant being investigated is. With regard to the considerable expense and the potential consequences of the assessment strategy it appears important to obtain representative samples that integrate the temporal variation of the respective discharge; the sampling strategy may then deviate from that used in permit procedures. Whether it requires one-day or several day composite samples to adequately reflect the quality of the discharge depends upon the examined discharge (direct/indirect) and the company behind it (e.g. size, number of separate streams and production processes). The Swedish strategy generally calls for 1-week composite samples (chapter 2.2.5). Obviously a generally applicable collection time for preparing a representative composite sample can hardly be deduced.

*Characterisation*

The characterisation process aims at detecting the inorganic and organic load of a wastewater sample based on the established parameters of the German Wastewater Ordinance. Basic parameters are the pH-value, the conductivity, the suspended solids and the turbidity followed by

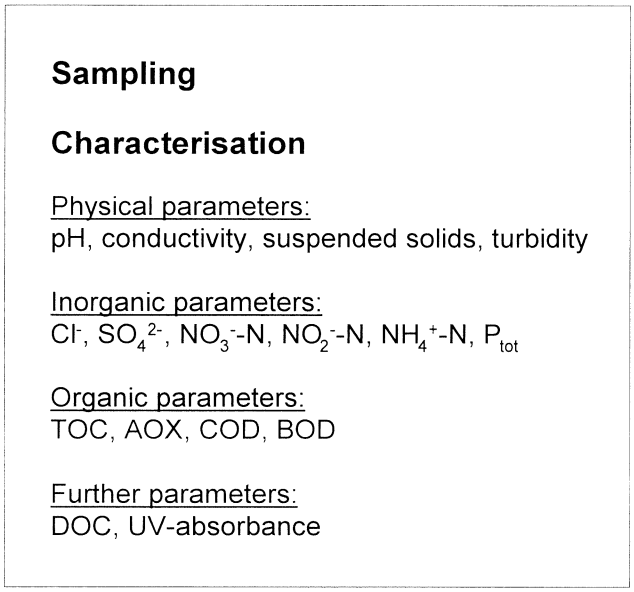



**Fig. 7:** Module 'Sampling and Characterisation'.

parameters that describe the inorganic load such as the chloride, nitrate, sulfate and the ammonia content as well as organic parameters like AOX, COD, TOC, and $TN_b$. The final selection of parameters depends upon the properties of the samples to be examined. Two additional parameters that are necessary for the following investigations are the DOC-content and the UV-absorbance (see Fig. 7). Depending on the branch of the discharge there may be other control parameters of the respective annex of the Wastewater Ordinance that have to be determined.

### 4.3.2 Module 'Toxicity'

This module starts with the determination of the acute toxicity and the genotoxicity (step 1, Fig. 8). If the wastewater exhibits a considerable acutely toxic and/or genotoxic effect, it may be advisable to suspend the investigation and to directly initiate measures for the reduction of this effect and for an improvement of the wastewater quality.

Chronic toxicity need not be determined if a sample is acutely toxic. However, in the absence of acute toxicity or genotoxicity, a sample should be investigated for chronic toxicity (step 2, Fig. 8). In case of chronic effects it may, again, be appropriate to suspend further investigations and to focus on a quality improvement of the effluent.

Methods:

Standardised toxicity tests for wastewater samples with organisms of all four trophic levels are available in Germany (Appendix II, section 4, Wastewater Ordinance): Luminescent bacteria (luminescence inhibition, test guideline no. 404), algae (chlorophyll-fluorescence-test, no. 403), daphnia (immobility, no. 402) and fish (mortality, no. 401). When performing toxicity tests guideline no. 400 of the appendix should also be considered ('Guideline for Sampling and Performing of Biological Test Procedures').

It is generally accepted that a battery of toxicity tests covering all four trophic levels should be used to detect toxic effects (chapter 3.1.1). The assessment strategy follows this trend.

Of the tests mentioned, all but the algae test are directed towards an acute toxic effect. As a substitute for the fish test, a fish-egg test will be available in due time which is currently in the last standardisation phase in Germany. This test is selected in the IDA strategy to detect acute toxic effects together with the luminescence inhibition and the daphnia test.

The algae test can be defined as a short term chronic toxicity test. Short-term tests are more suitable for wastewater investigations since they are more easily performed and since it is often difficult to keep a wastewater samples stable throughout the test duration of a long-term toxicity test. Finally, it may be useful to incorporate a second chronic short-term test with organisms of another trophic level into the IDA strategy in a later stage of development.

As all these tests except the luminescence inhibition test use freshwater organisms, they serve for the detection of toxic effects of a wastewater being discharged into the limnic system (freshwater). Though it uses a marine bacteria (*V. fischeri*), the luminescence inhibition test is generally accepted for investigating wastewater discharges into limnic and marine systems (brackish or saltwater). In the investigation strategies mentioned in chapter 2, marine organisms are used for investigating either wastewater discharges with a high salt content (e.g. The Netherlands) or discharges into the marine environment (e.g. Great Britain). There are only a few standardised or established test procedures available for discharges into the marine environment. As only very few direct discharges into the marine environment can be found in Germany, toxicity tests with marine organisms have not been included in the permit procedure so far. In the case of elevated contents of chloride or sulfate in a discharge the Wastewater Ordinance allows for an increased dilution factor in the toxicity test (guideline No. 505 of the Appendix to the Wastewater Ordinance). Therefore, no tests with marine organisms were incorporated into the IDA strategy.

For the detection of *genotoxicity* two standardised tests, the um test and the Ames test are available. The umu test detects primary DNA-damages and it is already applied to test wastewater discharges of the chemical industry according to annex 22 of the Wastewater Ordinance (test guideline No. 410 of the appendix), this test was also selected for the IDA strategy.

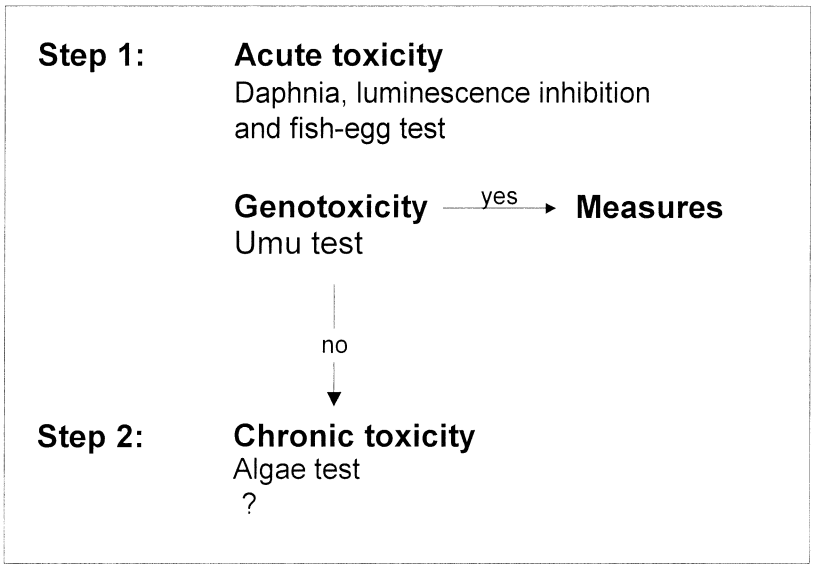



**Fig. 8:** Module 'Toxicity'.

### 4.3.3 Module 'Persistence'

In order to detect the persistent fraction of the wastewater constituents, a degradation test is conducted, which is supposed to simulate the biodegradation potential of a receiving water (Fig. 9). The persistent wastewater fraction is detected after the degradation test in the biologically treated sample by determining the DOC-content and UV-absorbance. By comparing these two values with the initial data recorded in the module 'Sampling and Characterisation', the portion of the persistent fraction can be detected.

A certain decrease of the organic wastewater load in this biodegradation test can even be expected for discharges, that have been intensively treated before. Many compounds are not or not entirely eliminated in sewage treatment plants due to the limited hydraulic residence time (2-10 h) or the presence of other, more easily degradable substances [Pagga, 1987].

If pronounced effects were detected in the module 'Toxicity' (and the investigation was not stopped), the corresponding toxicity tests (acute and chronic toxicity, genotoxicity) should be conducted again after the biodegradation test. This allows to determine whether the toxic constituents are persistent or whether they can be expected to decrease through biodegradation after discharge into the receiving water.

The interpretation of the sum parameters determined after the biodegradation experiment of a complex wastewater is less straightforward than in a test with a single substance. In the latter case, a compound is considered as being readily biodegradable if more than 70% of its DOC are removed in a period of 10 days. In the case of a wastewater sample, 70% DOC removal means that 30% of the organic wastewater load can be regarded as being persistent. The subsequent toxicity tests help to characterise the quality of this residual organic matter.

Analogous to the module 'Toxicity', the discharge assessment may be suspended in favour of quality improvement measures in case that a certain amount of persistent organic matter was determined in the module 'Persistence'.

With these two modules 'Toxicity' and 'Persistence', it can not only be determined whether a wastewater discharge contains toxic as well as persistent substances, but also whether there is a toxic and persistent fraction. This is of special importance for the final hazard assessment.

Methods:

When classifying the degradation potential of single substances, a distinction is made between ready (low cell density, degradation under comparatively disadvantageous conditions) and inherent (high cell density, advantageous degradation conditions) degradability. There are different OECD and ISO-directives (chapter 3.3.1) for the corresponding tests. Some of them have already been applied, partially in modified form, to detect persistent substances from wastewaters (chapter 3.3.2).

In the context of the IDA strategy a test on ready biodegradability is more useful. Thus, the OECD 301A-test was selected, in which activated sludge serves as inoculum and the degradation is monitored via the decrease in DOC (die-away-test).

On one hand, the lower biomass density applied in this die-away-test comes closer to the conditions in receiving waters than the high biomass density of tests for inherent degradability. On the other hand the biomass density of the OECD 301A test is still higher than in tests for ready biodegradability that use surface water or biologically treated wastewater as inoculum (chapter 3.3.1). One could, thus, assume that this test may overestimate the biodegradation potential of the surface water. However, comparative investigations with very low and moderate biomass densities have shown, that the speed of mineralisation rather than its extent differs between the two. It is, thus, likely that using the die-away test results in a similar persistent fraction than with a test employing a lower biomass density, but that the test result can be obtained in a shorter period of time.

It would also be interesting to follow the microbiological mineralisation by monitoring the $CO_2$-production. The corresponding tests, however, are comparatively difficult to perform and therefore appear almost inapplicable in the context of this strategy.

Often a ratio of 1/10 is used as a rough estimate of the portion of wastewater found in many surface waters. Correspondingly, the use of a 1/10 dilution of the wastewater with surface water in the biodegradation test would result in conditions that are close to many 'natural' situations. It remains open, whether or not this would stimulate the microbial degradation of the organic wastewater constituents. For three reasons a pre-dilution was not proposed in the IDA strategy: (a) it contradicts the emission-orientation that is inherent for the IDA-strategy, (b) it would significantly complicate the following analytical work as the wastewater constituents are strongly diluted and (c) toxic effects could only be detected if they exceed the dilution step of $G > 8$.

For the interpretation of the biodegradation test, it is of some importance whether a compound was truly mineralised or whether its concentrations decreased due to sorption onto the biomass. Sorbed substances would be attributable to the persistent fraction but they are no longer analytically amenable by the DOC-determination. Furthermore, sorbed compounds would be missing in the subsequent determination of the 'potentially bioaccumulating fraction' in the module 'Bioaccumulation' (see below). In order to differentiate between mineralisation and removal by sorption, a sample is taken about 3-4 hours after the start of the biodegradation experiment. As in the Zahn-Wellens-Test (guideline No. 404 of the Appendix of the Wastewater Ordinance; see chapter 3.3.2) it is assumed that sorption of hydrophobic substances onto the biomass is comparatively fast, while biodegradation is much slower. A concentration decrease within the first three hours of the experiment is, thus, attributed to sorption, while a later decrease reflects microbial degradation.

**Biological degradation test**
**DOC-Die away test**

**Determination:** Sum parameters (DOC, UV)

further characterisation: toxicity

**Fig. 9:** Module 'Persistence'.

### 4.3.4 Module 'Bioaccumulation'

In this module, the third hazard parameter besides toxicity and persistence is determined. For this purpose the biologically treated sample from the module 'Persistence' is used. The positioning of the module 'Bioaccumulation' in series after the degradation test is unusual in comparison with other strategies (chapter 2.2).

There are two reasons for this positioning:

- In order to become enriched in aquatic organisms, substances do not only have to have elevated $\log K_{ow}$-values but they also have to persist in the environment. Otherwise they would be degraded prior to their enrichment and would, thus, not give rise to concerns. Therefore, the determination of potentially bioaccumulating compounds in the biologically treated sample, which contains the persistent compounds only, is reasonable.
- In addition, the integrity of the sample is destroyed through the analytic detection of potentially bioaccumulating substances. Therefore, the sample volume, which is used in the module 'Bioaccumulation', can not be used in any other module. The selected sequence of modules, therefore, saves sample volume and keeps the analytical effort to a minimum.

As already mentioned above this sequence of modules may give rise to the risk of losing potentially bioaccumulating substances, which by definition have higher $\log K_{ow}$-values (> 3), through sorption processes in early examination stages. This problem was taken into account in the module 'Persistence' through the examination of the 3-hour-sample. Another measure to minimise substance losses by sorption during the course of the investigation is to avoid filtration throughout the procedures. Separations of the dissolved and particular phase have to be performed by centrifugation instead.

In critical cases it is also possible to determine potentially bioaccumulating substances prior to conducting the module 'Persistence'. The amount of potentially bioaccumulating compounds lost in the biodegradation test can then be detected by comparing the results before and after the degradation test. With regard to the much increased investigation effort, this option should only be considered in very critical cases.

The fraction of potentially bioaccumulating substances is defined through its $\log K_{ow}$-values > 3 (chapter 3.2.1). As described later on in more detail, this fraction is selectively removed from the discharge sample by the detection procedure and the routine parameters DOC-content and UV-absorbance are subsequently determined. The potentially bioaccumulating fraction is calculated as the difference to the initial values.

As far as toxic or genotoxic effects have been determined in the biologically treated sample of the module 'Persistence', the corresponding toxicity tests should also be conducted with the remaining sample after removal of the potentially bioaccumulating compounds in the module 'Bioaccumulation'. Then, this linkage of the modules allows to detect the fraction in a wastewater that is persistent, bioaccumulative and toxic at the same time.

Methods:

Different separation and detection methods are applied in the existing strategy concepts to detect potentially bioaccumulating compounds (see chapter 3.2.2). Among them is the separation of compounds obtained by hexane extraction of the wastewater by means of thin-layer chromatography (Sweden) or HPLC (Denmark) as well as the solid phase micro-extraction or the biomimetric extraction (The Netherlands). In Germany, a method is currently being developed, which applies a preparative HPLC for the separation and quantification of the wastewater constituents with $\log K_{ow}$-values > 3 after concentration via solid-phase extraction [Metzger et al., 2000].

Similar to this method solid-phase extraction is proposed to be used in the IDA strategy. The major advantage of the SPE procedure is, that it does not only allow to detect the potentially bioaccumulating fraction, but to get it in hand. Thus, additional properties of this fraction can be determined such as its toxicity.

It was initially intended to selectively extract substances with $\log K_{ow}$-values > 3 by SPE. This approach could have been used to remove the potentially bioaccumulating compounds from the sample and the DOC of this fraction would have been obtained easily through the determination of the DOC difference before and after the SPE. For a further characterisation of the potentially bioaccumulating fraction it could be eluted from the solid phase by a suitable solvent.

However, no hydrophobic solid-phase material could be found, that exclusively extracted organic material with a $\log K_{ow}$-values > 3. Therefore, the investigation strategy had to be designed as illustrated in Fig. 10. Initially, the sample is sucked through a suitable SPE-cartridge. The majority of the polar substances remain in the sample and can be found in the so-called 'SPE-filtrate'. A certain amount of substances with $\log K_{ow}$-values < 3 is 'erroneously' fixed on the solid-phase together with those compounds exhibiting $\log K_{ow}$-values > 3, which are intentionally sorbed to the solid-phase material. By elution of the solid phase with a suitable solvent mixture, the substances with $\log K_{ow}$-values < 3, which are not attributable to the potentially bioaccumulating fraction, are eluted from the solid phase and collected, while the potentially bioaccumulating compounds remain sorbed. This eluate can then either be investigated separately or it can be combined with the SPE-filtrate, as these two together comprise the non-bioaccumulating fraction. By comparing its DOC with that of the sample prior to the SPE, the DOC-contribution of the potentially bioaccumulating fraction can be detected. In critical cases the potentially bioaccumulating fraction can be eluted from the solid-phase material and its amount and properties (e.g. toxicity) can be determined directly.

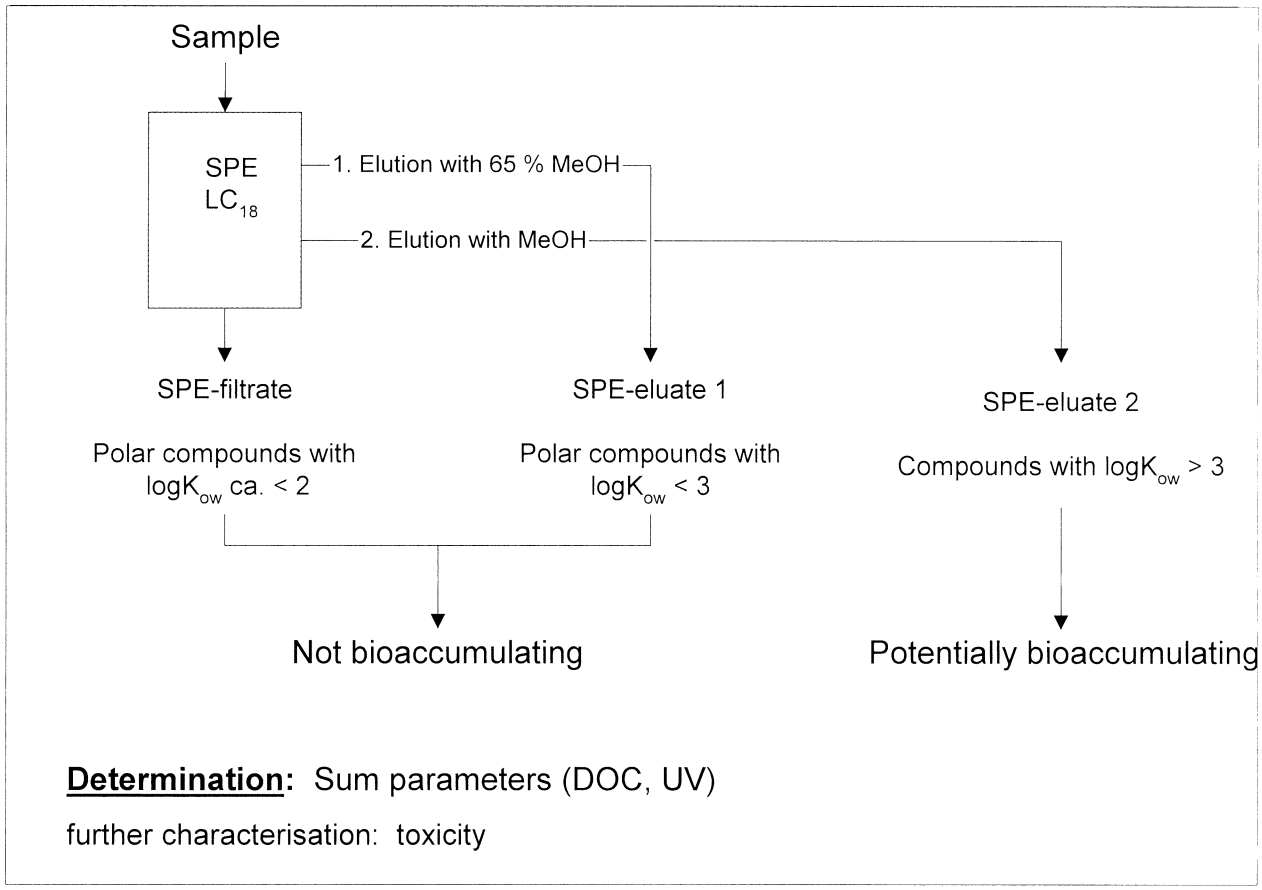



**Fig. 10:** Module 'Bioaccumulation'.

### 4.3.5 Module 'Particle Load'

This module was designed to assess the importance of a particulate wastewater load with regard to hazardous substances and to examine this particulate fraction in more detail. This is an optional module and it should only be conducted if there is a significant particle load or if other indications for the importance of the particulate fraction for the hazardous potential of the discharge have been obtained.

This module is necessary, since the hazardous properties of particle bound substances cannot be detected in other modules of the IDA strategy. For example, it is not possible to detect the persistence of particulate organic compounds with the degradation test included in the module 'Persistence', as the mineralisation there is determined by the organic carbon content of the dissolved phase (DOC). Therefore, the module 'Persistence' uses the particle-free centrifuged wastewater sample and not the entire sample.

Similar problems arise in the module 'Bioaccumulation', in which particles would be removed from the sample during solid-phase extraction but could not be detected separately. These two examples illustrate that the particulate phase must be examined separately.

According to Fig. 11 the importance of the particulate phase is initially assessed by determining the turbidity or suspended solids content of the discharge. Its significance with regard to the hazardous potential is assessed by comparing the toxicity of the homogenised, particle-

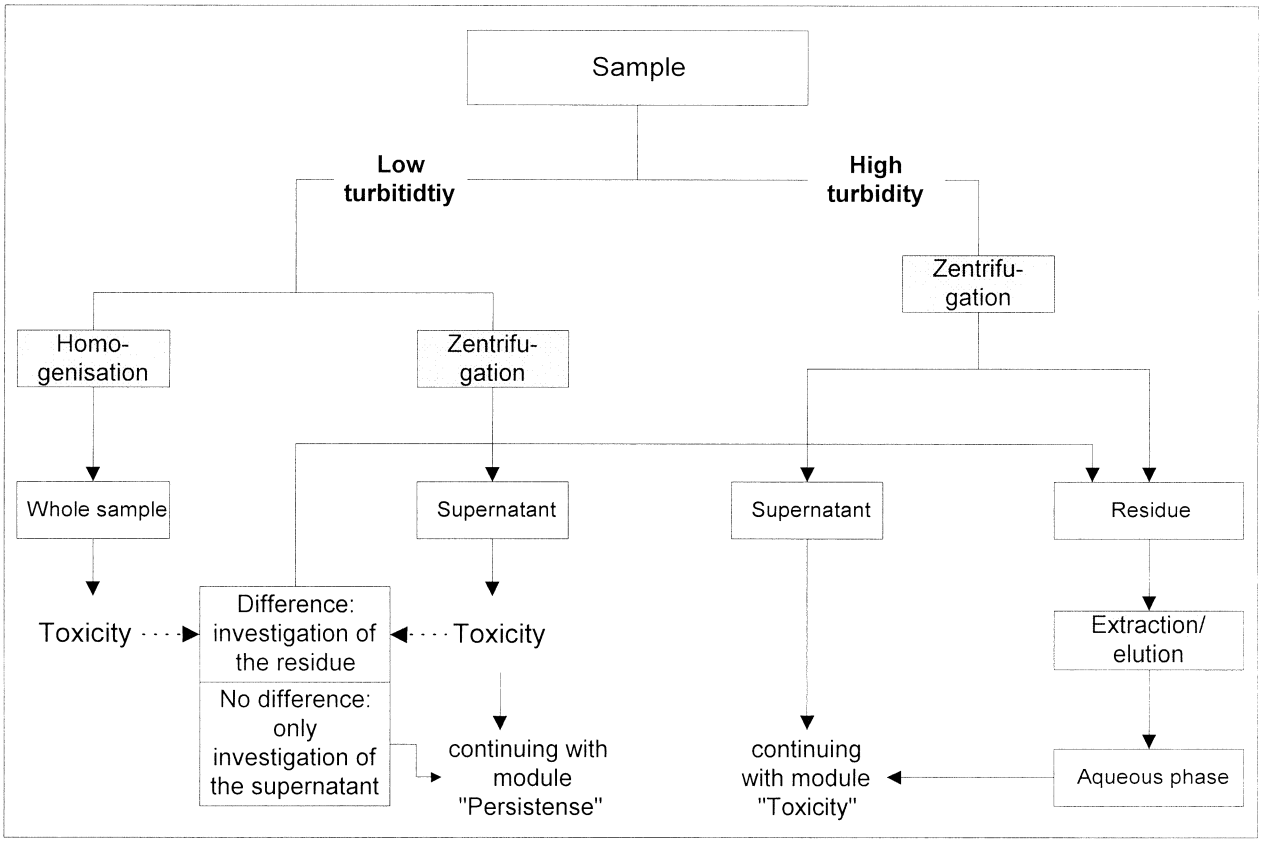



**Fig. 11:** Module 'Particle Load'.

containing sample with the toxicity of the centrifuged, particle-free sample. If differences in the biological effects of these two samples cannot be detected, it is not necessary to examine the particulate phase further in the IDA strategy and only the dissolved wastewater fraction is examined (after centrifugation). A general separate investigation of the particulate phase for hazardous properties is not justified with regard to the doubled expense for the investigation. Furthermore, an investigation of the particulate phase becomes difficult for samples with a low content of suspended solids, as large sample volumes would have to be centrifuged in order to provide sufficient material.

The particle content of a wastewater may be so high, that it disturbs the toxicity testing of the homogenised sample; then, the particulate phase has to be removed by centrifugation. In those cases the investigation may either be suspended and measures may be induced to improve the particle removal in the wastewater treatment, or eluates or extracts of the particulate phase are prepared and these are investigated parallel to the dissolved phase in the IDA strategy to assess their hazard potential.

The selection of the toxicity parameter for the general assessment of the relevance of the particulate phase has several reasons:

- Only in the toxicity testing can an effect of particle bonded substances be detected.
- The other two hazard parameters, persistence and potential bioaccumulation are of dubious relevance with regard to particulate matter. This makes toxicity the hazard parameter of highest priority for the particulate phase.

It is presently unknown, up to which particle content (detected as turbidity or suspended solids content) the toxicity tests work without disturbances. It appears likely, that this strongly depends upon the respective test and its detection principle.

### 4.3.6 Module 'Indirect Discharges'

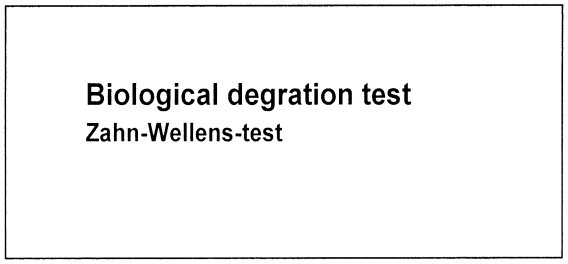
In the IDA strategy, a distinction is made between directly and indirectly discharged wastewaters, as this determines the treatment a wastewater has undergone prior to or will undergo after its discharge. Indirect discharges become biologically treated in municipal treatment plants after samples have been taken for investigation with the IDA strategy, while directly discharged wastewater has already been treated prior to the sampling. Therefore, the module 'Indirect Discharges' provides a microbial degradation test that is supposed to simulate the municipal wastewater treatment of the indirectly discharged wastewater. It is only then examined further in the module 'Sampling and Characterisation'.

One has to keep in mind that wastewater from combined sewer systems can enter surface water without prior treatment through combined sewer overflows, mainly after heavy rainfalls [Reemtsma et al., 2000] and that the wastewater of indirect industrial discharges will reach the receiving water without a biological treatment in this case. This path for the entry of hazardous wastewater constituents is not considered in the IDA strategy, as such overflows are irregular and likely to become significantly reduced in the future due to an improved retention in the sewer system [e.g. Engel, 1998].

Methods:

An aerobic degradation test has to be applied for the pre-treatment of indirect discharges. This test has to simulate sewage treatment plant conditions, implying that it has to work with a high biomass density (guideline No. 407 of the appendix to the Wastewater Ordinance, or OECD 302b, see Fig. 12). Despite the differences of this degradation test compared to a sewage treatment plant, previous studies have shown that these tests agree sufficiently with a municipal wastewater treatment with regard to the elimination of single substances and organic sum parameters [Steinhäuser, 1996a].

It must, however, be clarified, whether the same agreement in the elimination efficacy between the laboratory test and a municipal wastewater treatment can also be obtained, when the industrial wastewater is applied undiluted as intended. In the municipal sewage system a strong dilution by sanitary wastewater will take place, that could possibly have an influence on the degradation of the wastewater constituents in the municipal wastewater treatment.




In case that the undiluted wastewater exceeds the limits for the DOC (400 mg/L) or the COD (1 g/L) in the degradation test, a dilution has to be performed.

**Fig. 12:** Module 'Indirect discharges'.

# 5 Experimental Work

## 5.1 Objectives

In order to test the applicability of the IDA strategy it was exemplarily applied to three different industrial wastewater discharges.

With these laboratory experiments it was intended to verify whether the linkage of the modules is useful and practicable with regard to the intentions of the IDA strategy. Moreover, the test procedure for the determination of potentially bioaccumulating compounds in the module 'Bioaccumulation' had to be developed. The approach suggested for this purpose is new and experimental details had to be established. In addition the module 'Bioaccumulation' required a fundamental evaluation regarding its feasibility in the context of the IDA strategy. The laboratory experiments served for the testing of some critical steps in the experimental sequence of the modules. Special attention was paid to the linkage between the modules 'Persistence' and 'Bioaccumulation'. More detailed questions to be answered by these experiments are outlined below in the chapters describing the respective module experiments. Although they consist of established procedures and therefore did not require experimental testing, the modules 'Sampling and Characterisation' and 'Toxicity' were processed as well, as they are inevitable parts of the IDA strategy.

The laboratory experiments are also supposed to hint on critical aspects and any other remaining methodical questions that need to be developed further. They are discussed in chapter 6.

The further development and if necessary modification of the IDA strategy is important in order to derive a robust and well applicable method. Besides laboratory experiments intensive discussion will contribute to its development.

## 5.2 Wastewater Origin

The three wastewater samples used are grab samples from direct industrial dischargers which were obtained from a state authority. As listed in Table 8, the samples stem from two sectors, the chemical and the metal processing industry. All samples are effluents of end-of-pipe biological wastewater treatment plants. Each of the treatment plants received wastewater from different production lines, which were partially pre-treated by physico-chemical means.

**Table 8:** Origin and treatment of the examined wastewater discharges.

| Wastewater | A | B | C |
|---|---|---|---|
| Origin | Production of herbicides, medical products and paints | Production of herbicides, intermediate pharmaceutical products and paints | Surface treatment and metal finishing |
| Type of treatment | Biological treatment | Biological treatment supplemented by activated carbon | Biological treatment |

## 5.3 Module 'Sampling and Characterisation'

Initially, the three samples had to be characterised in detail, according to the parameters suggested in the IDA strategy. A comparison of different sampling strategies (grab samples, mixed samples) was not part of these investigations.

The first analyses of the samples showed that they are of very different quality with regard to their organic load, salinity and contamination with particulate matter (Table 9).

Samples A and B both stem from the chemical industry, from which sample B is much less loaded with organic substances. This could result from the intensive biological treatment that was improved by addition of activated carbon (Table 8). Both samples have a very low turbidity and a high salinity.

On the contrary, the effluent sample from the metal finishing industry (sample C) is very turbid and contains 142 mg/L of suspended solids. This sample illustrates that there may be cases in which the particle load can be of great importance concerning the industrial discharges quality.

**Table 9:** Physico-chemical characterisation of the wastewater samples.

| **Sample** | **A** | **B** | **C** |
|---|---|---|---|
| pH | 6.8 | 7.5 | 7.9 |
| Conductivity [mS/m] | 6.2 | 5.5 | 1.7 |
| **Turbidity[FNU[a]]** | **6.4** | **2.2** | **142** |
| Suspended solids [mg/L] | 8.6 | 5.8 | 257 |
| Colour | Salmon | None | Slightly yellow and dark brown particles |
| $Abs_{254}$[b] [$m^{-1}$] | 107 | 5.2 | 31 |
| $Abs_{436}$ [$m^{-1}$] | 6.2 | 0.3 | 1.1 |
| **DOC centrifuged [mg/L]** | **29.6** | **2.6** | **18.7** |
| COD centrifuged [mg/L] | 88 | 13 | 55 |
| COD homogenises [mg/L] | 115 | 16 | 233 |
| $Abs_{254}$/DOC | 3.6 | 2.0 | 1.7 |
| COD/DOC centrifuged | 3.0 | 4.7 | 2.9 |
| $NH_4$-N [mg/L] | 1.5 | 2.8 | 0.04 |
| $NO_3$-N [mg/L] | 6.5 | 6.3 | 9.3 |
| **Chloride [mg/L]** | **803** | **1066** | **215** |
| **Sulphate [mg/L]** | **1606** | **1054** | **181** |
| Phosphate [mg/L] | < 1 | < 1 | < 1 |

[a] FNU: Formazine Nephelometric Units
[b] Abs: UV-absorbance

In this sample the majority of the organic load is found in particulate form: from the total COD of 233 mg/L, the COD of the centrifuged sample (55 mg/L) is less than 1/4, while more than 3/4 of the COD originate from the particulate matter.

The three grab samples are, thus, of very different quality and comprise a good basis for the following method evaluation. The data obtained in the module 'Sampling and Characterisation' provide a first insight into the characteristics of each of the effluents.

## 5.4 Module 'Toxicity'

Procedure:

Here the toxic effects of the centrifuged samples were determined in the luminescence inhibition test, in the daphnia, fish-egg, and algae test as well as the genotoxicity in the umu test. The luminescence inhibition test was conducted over a period of 30 minutes, the daphnia and the fish-egg test over a period of 48 hours and the algae test as a growth inhibition test over a period of 96 hours. The latter one detects also chronic effects. The detailed procedure of each of the tests and the establishment of the G-values are described in annex 4.2.

Results:

None of the three samples showed an effect in the daphnia test ($G_D = 1$), while sample A only showed a slight effect (18 % at a dilution of 1:1, $G_A = 2$) in the algae test. Sample A was also the only one with an effect in the luminescence inhibition test ($EC_{50} = 0.56$, $G_L = 6$) and in the fish-egg test ($G_{Ei} = 3$). No genotoxic effect was detected in any of the samples according to the umu test ($G_{EU} = 1.5$). The results of the umu test are shown in annex 5.2.

Only sample A is classified as being slightly toxic with regard to the dissolved substances in the luminescence inhibition test and the fish-egg test [Dannenberg, 1994]. According to annex 22 of the Wastewater Ordinance, which regulates the discharges of the chemical industry ($G_F$ 2, $G_D$ 8, $G_A$ 16, $G_L$ 32 and $G_{EU}$1.5), this sample does not comply with the requirements of the fish test ($G_F$) or the future fish-egg test, respectively.

## 5.5 Module 'Particle Load'

This module was applied in order to test its applicability and to determine difficult aspects.

### 5.5.1 Procedure

In addition to the tests used in the module 'Toxicity', for which the dissolved fraction obtained by centrifugation was used, all samples were additionally tested in this module as homogenised, particle-containing samples.

### 5.5.2 Results

A difference between the centrifuged and the homogenised sample was not detected in any of the tests for any of the samples; Fig. 13 reflects this for the luminescence inhibition test: the inhibition curves of the particle-free and the particle-containing samples run completely parallel in all three cases. The analogous inhibitory curves of the algae test are depicted in annex 5.2.

Thus, a toxic effect originating from the particular phase was not discernible for any of the three samples in these biotests. For samples A and B this was already expected in view of their low suspended solids contents. The good agreement of the results obtained for the homogenised and the centrifuged variant of sample C shows, additionally, that particle contents of up to 250 mg/L need not disturb the toxicity tests. However, the extent of disturbances may generally be influenced by the type of particles (settling, suspended) and the measuring principle of the test. If the major fraction of the particulate substances settles to the bottom of the test container during the test, this does apparently not disturb the detection in the luminescent inhibition, the algae and the daphnia test.

Because no toxic effects of particle bonded substance were detected, the particulate phase of the three samples was not examined further.

## 5.6 Module 'Persistence'

The most important question to be investigated here was, whether the biodegradation test for treated effluents could be coupled with the determination of sum parameters and, moreover, with toxicity testing. A special aspect was the compatibility of the biodegradation experiment with the subsequent toxicity test. In addition it was examined, whether the 3-hour sample is suitable to detect the sorption of more hydrophobic compounds onto the biomass and, thus, to distinguish between removal of dissolved compounds by sorption and by biodegradation.

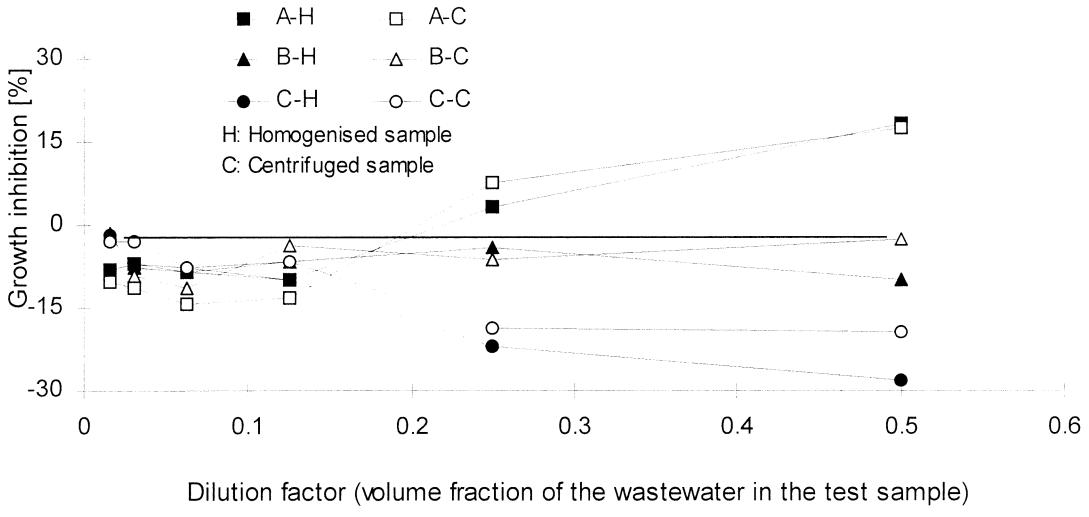



**Fig. 13:** Inhibition curves obtained in luminescent inhibition tests of the wastewater samples A, B and C homogenised and centrifuged.

### 5.6.1 Procedure

A test for ready degradability with municipal activated sludge (30 mg/L TSS) was used. Besides the effluent samples, aniline was applied as a control substrate in parallel. At the end of the biodegradation test the DOC-content and the UV-absorbance of the biologically treated samples were determined and the samples were examined in the luminescence inhibition test, the algae growth test and the umu test. The detailed procedure is described in annex 4.3.

### 5.6.2 Results

Persistence

The results of the degradation test for the three samples are summarised in Table 10. The decrease of the dissolved organic carbon content up to the end of the experiment after 28 days is listed together with the removal after three hours of testing (3h-sample).

**Table 10:** Reduction of the organic load of the three wastewater samples and the aniline control in the test for ready biodegradation.

| Sample | Initial DOC [mg/L] | Initial $Abs_{254}$ [$m^{-1}$] | Reduction after 3 hours | | Reduction after 28 days | |
|---|---|---|---|---|---|---|
| | | | DOC [%] | $Abs_{254}$ [%] | DOC [%] | $Abs_{254}$ [%] |
| A | 29.7 | 107 | 6.0 | -1 | 29 | 12 |
| B | 2.6 | 6.1 | 15.8 | 39 | 15 | 48 |
| C | 18.3 | 31 | 2.4 | 13 | 15 | 26 |
| Aniline | 34.3 | 31 | 1.7 | -0.6 | 97 | 93 |

*Final values after 28 days:*

The strongest decrease of the organic load took place in sample A with 29 % of 29.7 mg/L DOC. In the less organically loaded samples B and C, only 15% of the DOC was removed (of 2.6 and 18.3 mg/L, respectively), while 26 - 48 % of the UV-absorbance was removed. However, the initial DOC content of sample B was too low as to detect any significant effects after biodegradation. However, it is important to note that its DOC did not increase during the degradation test and that no artificial DOC was introduced by the biomass.

These values show that the organic load of discharges can be diminished even after a very intensive wastewater treatment. With regard to the hazardous properties of the samples these results show, that between 21 mg/L DOC (sample A) and 2.2 mg/L DOC (sample B) of persistent organic substances are found in the industrial wastewater discharges.

The degradation curves for the samples A and C as well as for aniline are presented in Fig. 14. The curve for sample B is not shown due to its low absolute values. The decrease of DOC and the UV-absorbance ($Abs_{254}$) can be found in annex 6 for all tests.

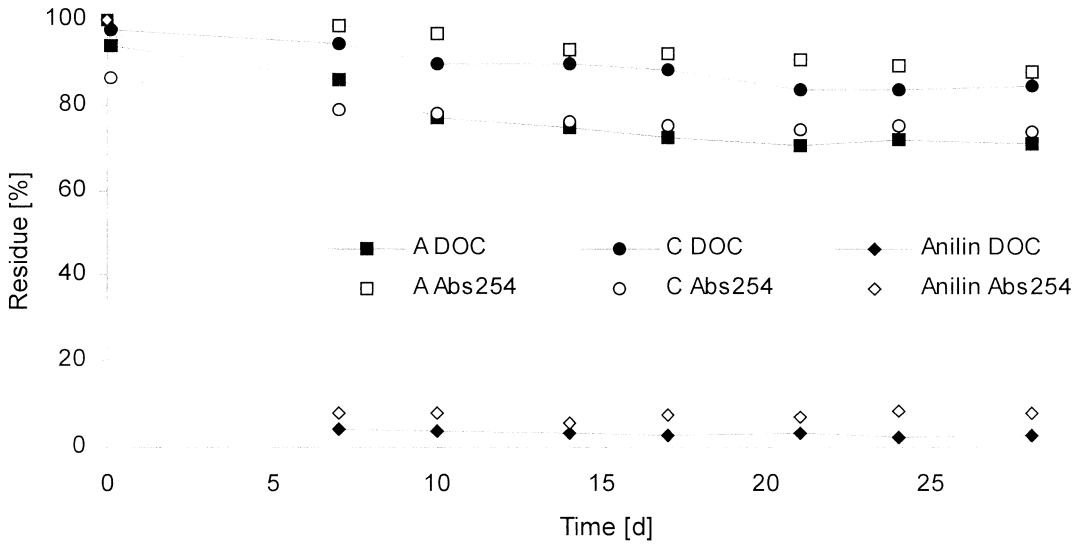



**Fig. 14:** Relative DOC- and $Abs_{254}$-values of the samples A and C as well as the aniline control in the degradation test over 28 days.

For the samples A and C a slight reduction of the organic load in the 28 days of the test can be discerned, with only small differences between the DOC-content and the UV-absorbance. The reference substance aniline, however, is rapidly and completely mineralised, reflecting the activity of the biomass used for the test.

*3h-sample:*

According to the data obtained for the 3h-sample the sorptive removal of organic wastewater constituents is insignificant for sample A. On the contrary, the reduction of the DOC-content and the UV-absorbance observed in sample B seems to be entirely due to sorption. The 3h-value of the DOC-content does not differ from the 28-day values. In the case of sample C, there is only a slight, sorption-related DOC-removal after 3 hours, while about 50% of the reduction of the UV-absorbance appears to be due to sorption.

These heterogeneous results do not allow to draw a final conclusion with regard to the suitability of the 3-hour sample for the detection of sorptive effects. But, nevertheless, the results have shown that there can be a very fast (< 3 h) removal of dissolved organic substances, followed by a very slow potentially microbial decrease during the following 28 days (Fig. 14). Insofar it is likely, that the fast removal is due to sorption and the temporal development of the degradation curves (e.g. 7-day value) lets the 3h-sample appear appropriate for detecting sorptive removal. The finding that the UV-absorbance of some samples is diminished to a larger extent than the DOC supports this assumption: as a rule of thumb, the UV-absorbing aromatic fraction of dissolved organic compounds is the less hydrophilic one and is, therefore, preferentially removed by sorption as compared to the not UV-absorbing aliphatic fraction.

From the data presented one could suggest to extend the equilibration time allowed for the sorption to about 12 hours prior to the first sampling, since the biomass concentration is significantly lower than in the Zahn-Wellens test and the samples are usually more stable than untreated effluent samples. However, the effect of an extended equilibration time would have to be a subject of additional investigations.

Toxicity Tests:

The biologically treated samples A to C (i.e. A-b, B-b and C-b) were then subjected to toxicity testing. Only sample A-b showed an effect in the luminescence inhibition test which was of the same extent as before the degradation test (Fig. 15). The weakly toxic constituents of sample A were, thus, not removed in the test for ready biodegradability and can be regarded as being persistent according to the understanding of the IDA strategy.

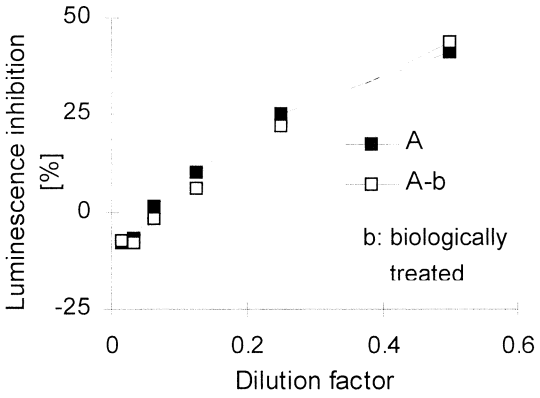



**Fig. 15:** Luminescence inhibition of sample A before and after the degradation test.

The degraded sample A was also examined in the umu test but no effect was detected (see annex 5.2).

With reference to the investigation methodology, this result together with the blank values also illustrate that the biodegradation test does not introduce artificial toxicity into the luminescence inhibition test and the umu test. The procedure of the degradation test is thus compatible with the subsequent toxicity tests. This result is of decisive importance for the overall strategy.

## 5.7 Module 'Bioaccumulation'

This module required the largest extent of methodical development. The initial question was, whether a sufficiently clear separation of the wastewater constituents into potentially bioaccumulating ($\log K_{ow} > 3$) and hydrophilic, non-accumulating ($\log K_{ow} < 3$) compounds could be obtained with the suggested detection method (chapter 4.3.4, Figure 10). Afterwards, the transfer of the procedure to real wastewater samples with variable matrix constituents had to be studied.

Thus, these studies are divided into methodological developments with standard substances (chapter 5.7.1 and annex 7) and into the application on the three industrial discharges (chapter 5.7.2).

### 5.7.1 Method Development

In the following, the detection procedure as well as the results of the method development are summarised. A detailed description and evaluation of the experimental results can be found in annex 7.

By means of a standard mixture consisting of ten aromatic substances covering a $\log K_{ow}$ range from 1.9 to 5.8 first the suitable conditions were determined for a separation around a $\log K_{ow} \approx 3$ (polarity-cutoff) by solid-phase extraction and two-step elution. This fractionation was followed by HPLC separation with diode-array-detection.

A methanol-water mixture with 65 % methanol (MeOH) turned out to be suitable for the first elution step to elute more polar compounds ($\log K_{ow} < 3$) that were erroneously sorbed to the SPE-material and 100 % MeOH was suitable to elute the potentially bioaccumulating compounds ($\log K_{ow} > 3$). Some uncertainty in the fractionation around a $\log K_{ow}$ of $3 \pm 0.2$ cannot be avoided. This is caused by the chromatographic effects in the solid-phase cartridge and by an uncertainty in the experimental determination or calculation of the $\log K_{ow}$-values in the literature. The nominal fractionation limit for this procedure is therefore set to $\log K_{ow} = 3 \pm 0.2$. During the application on wastewater samples, we worked with a MeOH-content of 68 % for the first elution. This can be regarded as being the utmost limit.

It is suggested to determine the amount of potentially bioaccumulating compounds in a sample by determining a DOC difference as outlined in chapter 4.3.4: the DOC-contents of the SPE-filtrate and the first SPE-eluate are subtracted from the DOC-content of the original sample. It is, therefore, essential to completely remove the solvent from the first eluate fraction, as otherwise, its DOC-contribution would be extremely overestimated. For this preliminary examination, we used biologically treated tannery wastewater as well as molasses wastewater.

The removal of MeOH from the first eluate fraction (68 % MeOH) prior to the determination by a simple evacuation step in a vacuum centrifuge turned out to be insufficient. A reduction of the residual methanol content to acceptable blank values (0.2 mg/L) required a threefold evacuation (with intermediate addition of water). This procedure was shown to be suitable for the combination with the DOC-determination of the eluate fraction.

### 5.7.2 Application on the Wastewater Samples A, B and C Before and After the Biodegradability Test

The method for determining the potentially bioaccumulating fraction via SPE, that was established with reference compounds dissolved in pure water was then applied to the samples A and C as well as on the samples derived after the biodegradability test in the module 'Persistence' (A-b and C-b). Each sample was processed twice during two different days in order to get information on the reproducibility of the procedure.

***Contents of potentially bioaccumulating compounds in samples A and C***

The results of the SPE of all four samples measured by the DOC-contents and $Abs_{254}$-values of the fractions are summarised in Fig. 16 as the mean of the two determinations. However, in two cases excessive values during one of the two extractions occurred and had to be excluded. The standard deviations of the two determinations of potentially bioaccumulating compounds and the distributions of organic carbon and UV-absorbance onto the different fractions are listed in annex 8.1. A first error estimation is also performed in annex 8.1.

Fig. 16 shows for both samples, that the portion of non-polar extractable compounds (eluate 1 and 2) decreases and the polar non-extracted fraction (SPE filtrate) relatively increases in the biodegradation test. This is especially evident in sample A where the DOC was diminished by almost a third in the biodegradation test. The content of potentially bioaccumulating substances

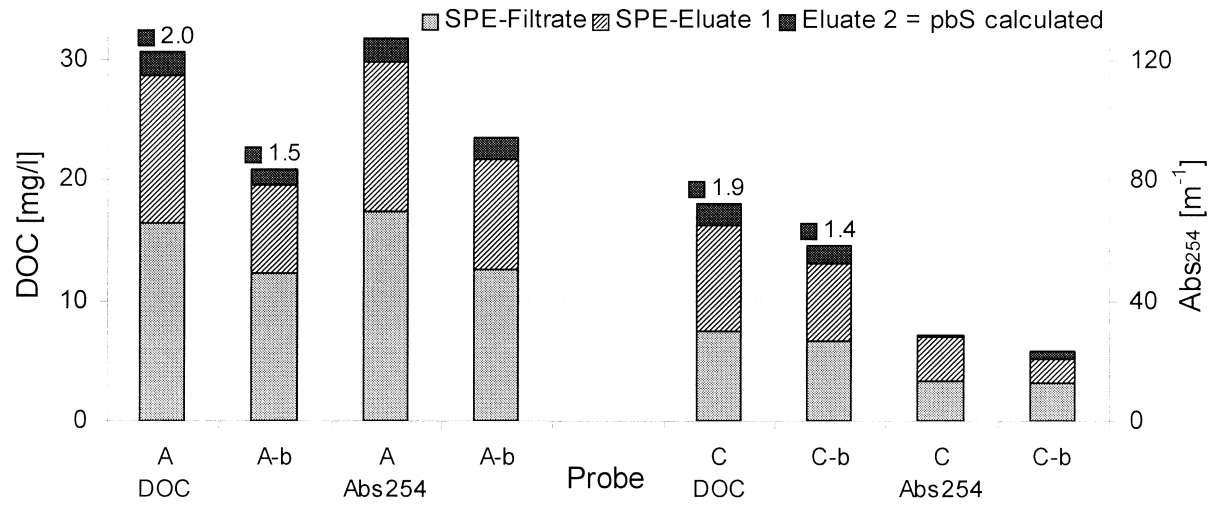



**Fig. 16:** SPE of the wastewater samples A and C before and after the biodegradation test (A-b and C-b) – DOC-contents and $Abs_{254}$-values of the fractions (potentially bioaccumulating fraction = sample - (filtrate + eluate 1)).

in all four samples ranges from 1.4 to 2.0 mg/L DOC. The contents prior to the biodegradation test (2.0 mg/L DOC, sample A and 1.9 mg/L DOC, sample C) are slightly higher than those after the test (1.5 and. 1.4 mg/L DOC, respectively).

The potentially bioaccumulating compounds comprise for 3-11 % of the DOC of sample A, 1-13% for sample A-b, and for 4-18% of the DOC in sample C and 1-18 % for sample C-b. However, the error estimation showed that the determination of the potentially bioaccumulative fraction by this approach results in an error of up to 1.3 mg/L DOC. This value adds up from the standard deviations of the DOC-contents of the original sample, the SPE-filtrates and the first eluates after blank value corrections. This error is of the same range as the absolute content of potentially bioaccumulating compounds determined in these effluent samples.

It must be considered, that this error was calculated from a comparatively small data set, in which the blank values of the SPE-filtrates varied in a relatively wide margin of 1.2 ± 0.5 mg/L DOC. For narrowing down the error of determination, one would have to extract a larger sample volume and to diminish the variability of the blank values. Finally, it will be necessary to experimentally determine a limit of detection for the potentially bioaccumulating compounds.

***Fractionation behaviour of the wastewater samples***

Based on the method development with standard substances of known $\log K_{ow}$-values (chap. 5.7.1 and annex 7.1) a relationship was established between the retention time of these compounds in HPLC-analysis and their $\log K_{ow}$-values. By means of this chromatographic 'grid' of standard compounds (Fig. 17b), the $\log K_{ow}$-values of unknown compounds in the wastewater samples can be estimated (Fig. 17 a and c). The HPLC analysis of the SPE-fractions, thus, allows to assess the suitability of the SPE-method for the fractionation of wastewater constituents according to their $\log K_{ow}$-value of 3.

The HPLC-chromatograms of the eluates of the wastewater samples as well as of the standard mixture obtained after the SPE with two-step elution are shown in Fig. 17. The second eluate

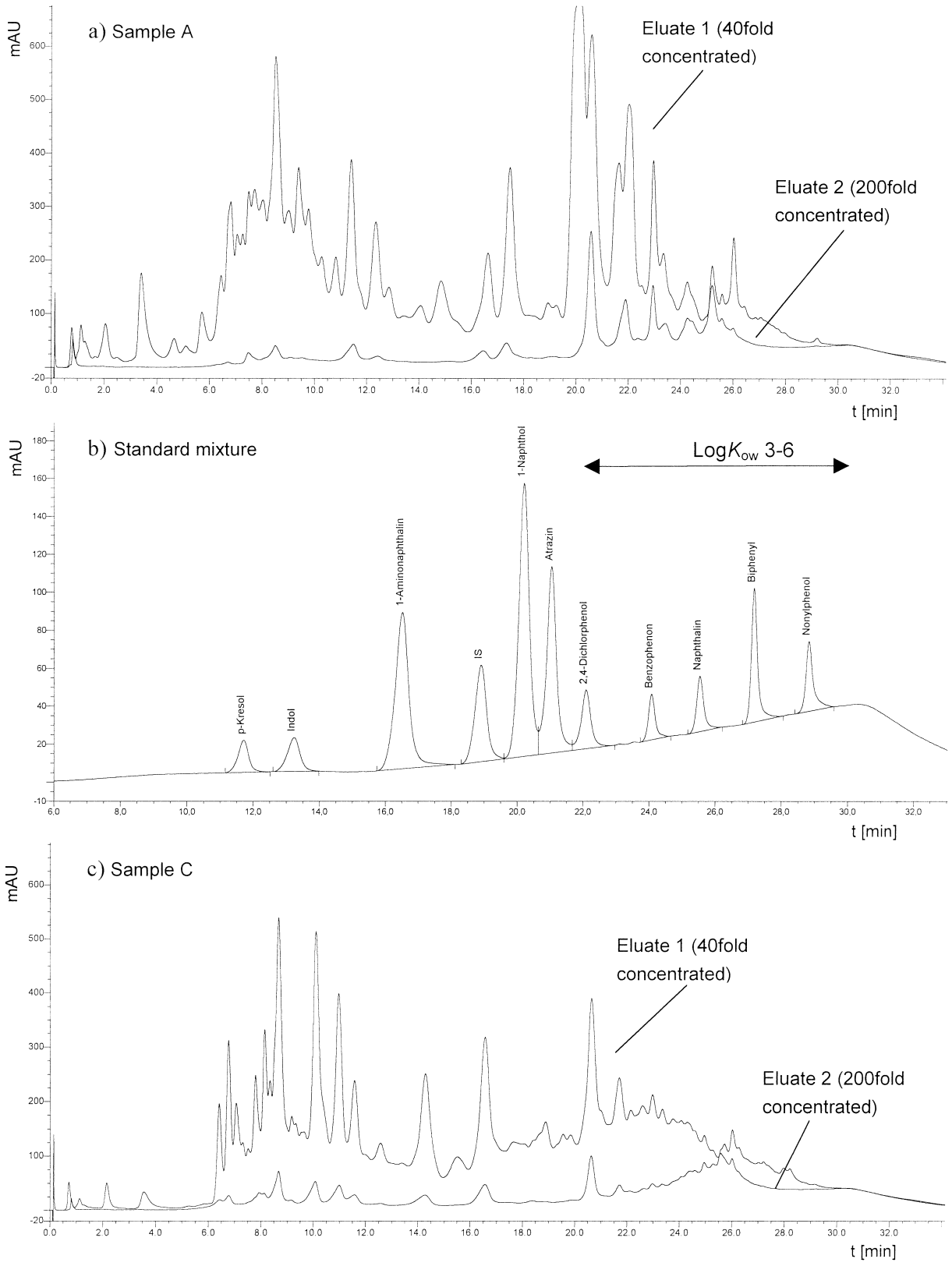



**Fig. 17:** HPLC-chromatograms of the sample A (a, top), of the standard mixture (b, middle) and of the sample C (c, bottom) after the SPE with two-step elution.
Eluate à 5 mL, eluate 1: 68 % methanol, eluate 2: 100 % methanol, $\lambda$ = 230 nm

(methanol fraction with a desired log$K_{ow}$ > 3) was analysed at a 5 times more concentrated level than the first eluate.

The samples A and C (Fig. 17 a and c) contain organic substances over the entire polarity range (retention time) with a significant contribution of polar substances that elute prior to p-cresol (log$K_{ow}$ ≈ 2). The second eluate, that is intended to represent the potentially bioaccumulating wastewater constituents, is less concentrated than the first eluate. The intended log$K_{ow}$-cut off of 3 is marked by the standard substances atrazin, 2,4-dichlorophenol and benzophenone and corresponds to a retention time of 21 to 24 min (Fig. 17 b). In this time range signals are found in both eluate fractions of the wastewater samples A and C (Fig. 17 a and c). With increasing retention times, the signal intensity of the first eluate fractions decreases and no compounds are detected in these fractions at a retention time of 27 minutes. The same fractionation was obtained for the samples taken after the degradation tests (see annex 8.2).

The separation of the wastewater extracts by HPLC shows that the polarity-cutoff obtained for the fractionation of the wastewater samples was shifted towards higher log$K_{ow}$-values as compared to the standard mixture. This would lead to the underestimation of the content of potentially bioaccumulating substances. In future work, the portion of methanol in the eluent used for the first elution of the SPE cartridges will be reduced from 68% to about 65% (see annex 7, Figure 4).

The HPLC analysis of sample C shows, that some components of the high polarity range (log$K_{ow}$ < 3; retention time < 21 min) also occur in the second eluate (Fig. 17 c). This points at an incomplete elution in the first step and can be easily avoided by increasing the elution volume.

***Combination with toxicity tests***

The IDA strategy intends to combine the determination of potentially bioaccumulating substances with the toxicity detection by biotests; this provides insight into the polarity range of the toxic substances of a wastewater sample. This combination, thus, allows to assess whether the potentially bioaccumulating compounds are also toxic. This would markedly increase their hazard potential.

The laboratory experiments were supposed to show whether the sample handling necessary in the module ‘Bioaccumulation’ (extraction and further processing) gives rise to artificial toxic effects in the following biotests. In addition, the practicability of the suggested procedure in the module ‘Bioaccumulation’ (chapter 4.3.4) was investigated.

With the aid of blank extractions, it was first determined whether the determination of potentially bioaccumulating substances is compatible with the subsequent biotests. For this purpose, eluates of the first elution step of the SPE cartridges were forwarded to the biotests after repeated evacuation and dissolution. The eluates of the second elution step were merely concentrated and adjusted to a methanol content of 2% prior to the toxicity tests; this methanol content does normally not cause a toxic effect.

The SPE-filtrates and eluates of the blank extractions neither showed a toxic effect in the daphnia nor in the algae test. Only the SPE-filtrate was tested in the umu test, but an effect did

not occur here either. The extraction conditions used for the determination of potentially bioaccumulating substances thus proved to be suitable for a combination with the biotests.

As only sample A showed a toxic effect in the luminescence inhibition test (chapter 5.4), this and the biologically treated sample A-b were the only samples incorporated into these investigations. Neither of the two samples showed a luminescence inhibition in the SPE-filtrate. The first SPE-eluate of both samples, however, showed a pronounced toxic effect with a 60 to 70% inhibition. The second eluate also showed toxic effects, but only after a concentration by a factor 2 - 4 (Fig. 18). It can be concluded, that the majority of substances responsible for the toxic effect of sample A are of moderate polarity (first eluate; $\log K_{ow} < 3$) and are not considered as being potentially bioaccumulating. However, some uncertainty remains for two reasons:

- The slight inhibitory effect in the second eluate may indicate the presence of substances with a $\log K_{ow}$ around 3, some of which may then exhibit a bioaccumulation potential.
- In addition, the HPLC-measurements (see above) have already indicated that under the selected conditions (68% of methanol in the first eluent), some of the potentially bioaccumulating substances were erroneously eluted in the first eluate. This may have also affected the elution of the toxic components.

However, it can be ruled out that inorganic sample constituents are responsible for the toxic effects encountered in sample A, as inorganic components pass through the SPE cartridge and inorganic toxic compounds would have provoked toxicity in the so-called 'SPE-filtrate'.

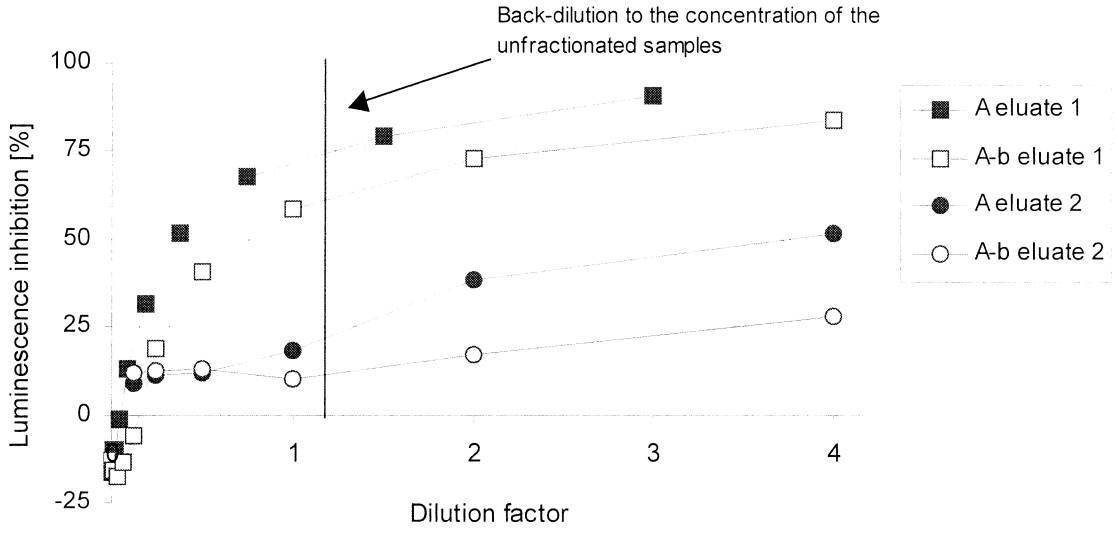



**Fig. 18:** Inhibitory curves of the eluates of the samples A and A-b in the luminescence inhibition test Eluate 1: 68 % methanol; eluate 2: 100 % methanol.

From these first applications of the module ‘bioaccumulation’ of the IDA-strategy it can be concluded, that the determination of potentially bioaccumulating substances from industrial discharges by means of SPE can safely be combined with the toxicity detection by the luminescence inhibition, the daphnia and the algae test.

This option of evaluating the potentially bioaccumulating compounds with respect to their aquatic toxicity is an essential advantage of the IDA strategy as compared to other assessment approaches.

# 6 Evaluation and Outlook

## 6.1 General Aspects

The investigations and their results as described in chapter 5 have exemplarily shown the practical applicability and the benefits of the IDA strategy for assessing the environmental hazard of industrial discharges.

It was possible to successfully conduct key steps of the methodology. This is especially important for those modules linking the detection of two different effect parameters, such as the coupling of the determination of persistent or potentially bioaccumulating substances with the determination of the residual DOC and with the toxicity tests (in the modules 'Persistence' and 'Bioaccumulation').

These couplings are an essential feature of the IDA strategy as they provide a linkage between the three hazard parameters bioaccumulation, persistence and toxicity. In this way the IDA strategy does not only provide information about the toxic effect of a discharge and about the quantity of persistent or potentially bioaccumulating substances. Rather, it allows to determine whether the persistent fraction is also bioaccumulating and/or toxic. With this unique characteristic the IDA strategy improves the assessment of industrial discharges.

Similar to other strategies (e.g. Denmark, Sweden, chapter 2.2) the IDA strategy calls for decisions about whether a specific test result is considered as being negative or positive. In case of a positive result, it is proposed to suspend further examinations and to initiate measures to improve the discharge quality. Decisions, however, require decision limits for the respective parameters; except for some toxicity tests applied in Germany in the context of the Wastewater Ordinance, decision limits are neither available nor generally accepted. Moreover, it is presently not possible to assess the consequences of a content of 20 mg/L of persistent substances in an industrial discharge for the ecosystem of the receiving water.

The IDA strategy, by linking the hazard parameters in and via the modules, significantly improves the basis for finding adequate decision limits: the final outcome of the assessment is not just the information that a discharge contains 20 mg/L of persistent substances, but also, whether potentially bioaccumulating and/or toxic compounds are part of the persistent fraction. However, finding appropriate decision-limits for the content of potentially bioaccumulating and of persistent substances requires a large data set for industrial discharges, which can only be obtained by a broad application of the strategy.

Generally, one has to keep in mind that only the key steps of the IDA strategy have yet been tested. Further tests for its robustness and repeatability as well as for the determination of detection limits have to be performed using mixtures of standard substance and industrial discharges. The further development of the IDA-strategy a robust and applicable method also necessitates further discussion of the parameters to be used, their linking and the selected test methods.

## 6.2 Individual Modules

### 6.2.1 Module 'Sampling and Characterisation'

Representative composite samples are required for the investigation with the IDA strategy. Due to the lack of good reference data, however, the sampling duration has not been established yet.

The analytical parameters adopted from the Wastewater Ordinance for characterising the total discharge do not raise any methodical questions. Based on data collected during the operation of industrial effluent treatment plants and their supervision by the authorities, the effort for characterising the discharges (organic load, salts, heavy metals) can probably be reduced even further.

### 6.2.2 Module 'Indirect Discharges'

Contrary to most of the examination strategies developed so far, the IDA strategy is also applicable to indirect discharges as its procedure differentiates indirect and direct discharges. In the module 'Indirect Discharges' of the IDA strategy the indirect discharges are to be treated with a high biomass concentration according to an established degradation test prior to all further examinations. This treatment is supposed to simulate the effects of a municipal sewage treatment plant.

A dilution of the discharge with sanitary wastewater, as in the municipal sewer systems, is not applicable. As this module was not tested yet, it remains open whether the biodegradation test without dilution adequately simulates the alteration of the discharge occurring in the municipal sewage treatment plant.

### 6.2.3 Module 'Particle Load'

The inclusion of the particulate material in the IDA strategy turned out to be a complex task. On the one hand, different modules like 'Persistence' and 'Bioaccumulation' are not accessible for particulate matter due to the detection methods used. On the other hand, the validity of the parameters persistence and bioaccumulation for particles is to be questioned.

Thus, the comparison of the toxic effects of the particle-containing and the particle-free sample appears to be the only parameter that can be used to assess the importance of the particulate fraction for the hazard assessment. It must be considered, however, that these biotests are directed towards dissolved substances; particle bound compounds will hardly evoke an effect in these tests. Only that portion of sorbed substances, which desorbs under the test conditions and during the test duration likely develops an effect onto the test organisms. This will probably be only a small portion of the total sorbed substances. However, in order to justify this approach, we can state that (a) the substances sorbed on particles do not truly represent a contamination of the dissolved water phase and that (b) an insufficient removal of particles from an effluent should give rise to an improvement of the particle removal rather than evoking a complex examination of the water quality and its potential environmental hazard.

Presently, it is not fully clear as to which extend the particle content (measured as turbidity or suspended solids content) will disturb the toxicity tests. If the particle content is too high to test homogenised samples, the particulate phase has to be examined separately. For that purpose one has to clarify whether an aqueous eluate or the aqueous dissolution of an organic solvent extract of the particulate phase is appropriate for toxicity testing. The separate examination of the particulate phase according to the IDA strategy has not yet been performed.

### 6.2.4 Module 'Toxicity'

The IDA strategy (chapter 4) makes use of established toxicity test such as those defined in the Appendixes of the Wastewater Ordinance; this will also be the case for the fish-egg test in due course. The overview on the present status of toxicity and biotests given in chapter 3.1 has shown that miniaturised and, thus, faster and cheaper test procedures are being developed to detect established parameters, and that new sub-lethal effects and tests are becoming increasingly important. A typical example is the testing for endocrine effects of environmental chemicals and wastewater discharges, which is promoted on an international level (e.g. OSPAR-convention regarding hazardous substances). Generally, chronic and sub-lethal effects are expected to become increasingly important in the context of persistence and bioaccumulation. Thus, the selection of biotests in the module 'Toxicity' may be subjected to change in due course.

The following criteria should be fulfilled by any test incorporated into the IDA strategy in the future: (a) the tests should have reached a well established level, (b) a sufficiently large number of positive results from industrial discharges should have been obtained and (c) widely accepted threshold values for the corresponding test should be established. As the IDA strategy provides three or even four stages of toxicity testing (in the modules 'Toxicity' and 'Particle Load' as well as in the modules 'Persistence' and 'Bioaccumulation'), one will always have to limit the number of different tests. However, the number of biotests that has to be conducted can be rapidly reduced due to the sequential structure of the strategy, as only those tests have to be performed in later modules, that exhibited positive results in the preceding modules. A selection of suitable biotests out of a larger collection of accepted tests according to the industrial branch being investigated may offer a suitable alternative.

### 6.2.5 Module 'Persistence'

The concept to couple a degradation test with the subsequent determination of the residual DOC and toxicity in order to determine the persistent fraction and its biological effects, appears suitable and valid. The selection of the degradation test from the large number of established procedures was also appropriate.

Nevertheless, following the microbial degradation by monitoring the $CO_2$-evolution would be an interesting supplement. This approach would allow to distinguish between sorption and mineralisation as DOC-reduction processes and would also allow to determine the degradation of particulate matter. However, it is instrumentally more difficult to detect the $CO_2$-evolution than to determine the DOC. Considering that the $CO_2$-production is subjected to a higher variability

than the DOC decrease and that only a few milligrams per litre of DOC were mineralised in the degradation test yet performed, it becomes evident that the detection limits presently available for the $CO_2$-formation do not meet these requirements.

Using the 3h-sample for the detection of the removal by sorption appears to be an appropriate approach. It may be possible to extend the equilibration time up to 12 hours without overestimating the sorption. With this prolonged sorption phase, it should also be possible to consider sorption processes with a slower kinetic.

Finally it should be noted that the term 'Persistence', as used in this study and elsewhere, is limited to the aerobic microbial degradability with mixed cultures. But the stability of a compound in the aquatic system can also be influenced by abiotic transformations like hydrolysis or (indirect) photolysis. Abiotic transformations do not lead to mineralisation, but they change the structure along with the polarity of the compounds, which may then have an effect on the microbial degradability. In addition, there are demands for considering the anaerobic degradability when assessing environmental properties of chemicals, namely of mass chemicals, as a substantial amount of material may be buried in anaerobic environments through sorption and deposition in the sediment.

Nevertheless, neither abiotic transformation nor anaerobic degradation is presently considered in the IDA strategy, since the general environmental significance of these processes could not be evaluated safely.

### 6.2.6 Module 'Bioaccumulation'

The procedure introduced here for the detection of the persistent and potentially bioaccumulating substances is currently the only one that can be combined with conventional and universal analytical parameters such as the DOC-determination and it can also be combined with toxicity testing. This is an important progress, as it allows to determine the third and final hazard parameter of a fraction that already fulfils the two other properties. As this approach was newly developed some methodical aspects of the module 'Bioaccumulation' deserve further attention.

For example it has to be ensured by additional investigations, that the positioning of the module 'Bioaccumulation' in series after the module 'Persistence' does not entail unacceptable losses of potentially bioaccumulating substances.

As the assessment of the $\log K_{ow}$-values of the extracted and re-eluted substances from the wastewater samples via HPLC suggests, the polarity cut-off of the fractionation procedure must be adopted to the wastewater matrix. The aspects of extraction capacity of the SPE cartridges and the influence of a variable amount and quality of the dissolved organic material on the fractionation need to be studied in future.

The undisputable advantage of detecting the potentially bioaccumulating compounds by its DOC content is presently connected with the disadvantage of an unsatisfactory detection limit. A further reduction of the blank DOC would lower the overall error and also the detection limit. Finally, the repeatability of the procedure when treating effluent discharges has to be proven.

# 7 Summary

The extensive detection of hazardous substances in wastewater discharges of the industry requires a concerted combination of chemical and biological analyses beyond those established in the German Wastewater Ordinance or any other European water regulation/legislation. For this reason, an emission-oriented assessment strategy for hazardous substances in industrial wastewater discharges (Industrial Discharge Assessment, IDA) was developed, which covers the hazard parameters persistence, bioaccumulation and toxicity and links them experimentally.

The IDA strategy was elaborated based upon an evaluation of the existing investigation strategies and the available procedures for determining toxicity, persistence and bioaccumulation in wastewaters. The strategy introduced here has a modular structure, which provides the flexibility to adopt the investigation to the specific quality of a given discharge or to the requirements of an industrial branch.

After an initial characterisation of the discharge, the acute and chronic toxicity as well as the genotoxicity are determined. This is followed by a degradation test to obtain the persistent wastewater fraction. From this persistent fraction potentially bioaccumulating substances are determined by means of a solid-phase extraction procedure. The IDA strategy considers a potential environmental hazard of particle-bound substances and it can be applied to indirect discharges. Due to its succession of modules, the IDA strategy allows to determine, whether a discharge contains compounds that are toxic and persistent and potentially bioaccumulating and which, thus, pose a significant threat to the aquatic environment.

The first application of the key modules of the IDA strategy on three wastewater discharges of the chemical and the metal processing industry has shown that it is possible to implement the strategy as suggested and that it provides far reaching information about the discharges that is essential to evaluate their hazard potential. Some method development and evaluation concerning selected test procedures of the strategy are required to accomplish the IDA strategy.

The IDA strategy may be used in future to assess industrial discharges in the course of permit procedures or when changes in the industrial production or treatment processes have to be evaluated with respect to their effect on the discharge quality.

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# Annex 1 Toxicity Tests used in Investigation Strategies of Different States

## 1.1 Toxicity Tests used in the Danish Strategy Concept 'Ecotoxicological Evaluation of Industrial Wastewaters'

The following methods are assigned for the investigation and evaluation of the hazard resp. risk for the aquatic environment (fresh-, brackish and saltwater) in the 'guideline for hazard assessment of industrial effluents' from the Danish environmental agency in 1993 (cited according to OECD (1998) and in the 'Guidance document for risk assessment of industrial waste water' from 1995 [Pedersen et al., 1995].

**Annex Tab. 1:** Toxicity tests used within the Danish investigation strategy.

| When to test? | Method | Recommended Species | Endpoint | Standardised Test Guideline |
|---|---|---|---|---|
| **Stage 1:**<br>Selection of each a fish, a crustacean and an algae species which are of relevance for the receiving water.<br>Estimation of $PNEC_{Acute}$ and $PNEC_{Chronis}$ applying assessment factors. | Acute fish toxicity | ***Freshwater***<br>Zebra fish (*Brachydanio rerio*), Rainbow trout (*Onchorhynchus mykiss*)<br>***Brackish water***<br>Flounder (*Platichthys flesus*), Herring (*Clupea harengus*)<br>***Saltwater***<br>*P. flesus, C. harengus*, turbot (*Scophtalmus maximus*) | 96 h $LC_{50}$ | Int. (OECD TG 203) |
| | Acute crustacean toxicity | ***Freshwater***<br>*Daphnia magna*<br>*Gammarus pulex*<br>***Brackish water***<br>*Nitocra spinipes*<br>***Saltwater***<br>*Acartia tonsa* | 48 h $EC_{50}$<br>96 h $EC_{50}$<br>96 h $EC_{50}$<br>48 h $EC_{50}$ | Int. (OECD TG 202)<br>Nat. (DS 2209)<br>Int. (ISO Draft) |
| | Algae toxicity | ***Freshwater***<br>*Nitzschia palea, Selenastrum capricornutum*<br>***Brackish- and saltwater***<br>*Skeletona costatum, Phaeodactylum tricornutum* | | Int. (OECD TG 201) |
| **Stage 2a**<br>$PNEC_{Acute}/PEC_{max} < 1$<br>Additional acute toxicity tests may be performed for the refinement of $PNEC_{Acute}$. | Acute toxicity to micro-organisms | ***Freshwater***<br>*Pseudomonas putida*<br>***Brackish- and saltwater***<br>*Photobacterium phosphoreum* (Microtox) | 72 h $EC_{50}$ growth inhibition<br>0.5 h $EC_{50}$ luminescence inhibition | Int. (ISO N111 Draft)<br>Int. (ISO N127 Draft) |
| | Acute protozoan toxicity | ***Freshwater***<br>*Tetrahymena* sp.<br>***Brackish- and saltwater***<br>*Uronema marinum* | 24 h $EC_{50}$ growth inhibition | |
| | Acute toxicity to higher plants | ***Freshwater***<br>*Lemna minor*<br>***Brackish- and saltwater***<br>*Zostera marina* | 7 d $EC_{50}$ growth inhibition<br>28 d $EC_{50}$ growth inhibition | |
| | Acute toxicity to insects | *Chironomus* sp., *Baetis rhodani, Cloën bipunctata* | 96 h $LC_{50}$ | |
| | Acute mollusc toxicity | ***Brackish- and saltwater***<br>larvae from blue mussel, oyster | 96 h $LC_{50}$ | |

| | | | | |
|---|---|---|---|---|
| **Stage 2b:**<br>$PNEC_{Chronic}/PEC_{Average} < 1$<br>One or more tests may be applied for the refinement of $PNEC_{Chronic}$. | Chronic fish toxicity | As for acute toxicity | Embryo-sacfry-test 7-11 d $EC_{50}$, NOE C, LOEC, survival, hatching, growth<br>FELS 28-60 d $EC_{50}$, NOEC, LOEC | Int. (OECD Draft)<br>Int. (OECD TG 210) |
| | Chronic crustacean toxicity | As for acute toxicity | Daphnia 21 d $EC_{50}$, NOEC, LOEC, survival, reproduction<br>*Nitocra*<br>*Acartia* | Int. (OECD TG 202)<br>Nat. (DS 2209)<br>Int. (ISO Draft) |
| | Chronic algae toxicity | As for acute toxicity | 72 h $EC_{50}$, NOEC, LOEC, growth inhibition | Int. (OECD TG 202, ISO 8692) |
| **Stage 3**:<br>One or more of these tests may be applied for the refinement of the PNEC for sediments. | Toxicity to: | | | |
| | Crustacean | ***Freshwater***<br>*Gammarus pulex*<br>***Brackish- and saltwater***<br>*Corophium voulutator*<br>*C. insidiosum* | <br><br><br>Coro. 10 d $LC_{50}$ | |
| | Molluscs | ***Freshwater***<br>*Unio* sp.<br>***Brackish- and saltwater***<br>*Abra alba, Macoma baltica* | <br><br><br>Abra 5 d $LC_{50}$ | |
| | Annelids | ***Freshwater***<br>*Tubifex tubifex*<br>***Brackish- and saltwater***<br>*Arenicola marina*<br>*Nereis virens* | <br><br><br>Areni. 10 d $LC_{50}$<br>Nereis 10 d $LC_{50}$ | |
| | Insects | *Chironomus* sp. | | Nat. (ASTM E 1383-90) |
| | Echinoderms | *Echinocardium cordatum* | 21 d $LC_{50}$, | |

## 1.2 Toxicity Tests used in the Swedish Strategy Concept 'CID Biological-chemical Characterisation of Industrial Waste Water'

The following methods are used for toxicity testing according to the guideline of the Swedish Environmental Protection Agency 'Biological-chemical characterisation of industrial waste water' [Swedish Environmental Protection Agency, 1990].

**Annex Tab. 2:** Toxicity tests used within the Swedish investigation strategy.

| When to test? | Method | Recommended species | Endpoint | Standardised Test Guideline |
|---|---|---|---|---|
| **Stage 1:** If available data or experience is insufficient for assessing the potential hazard/risk to the environment. | Acute fish toxicity | ***Freshwater*** Zebra fish (*Brachydanio rerio*) Rainbow trout (*Salmo gairdneri*,), Bleak (*Alburnus alburnus*), Fathead minnow (*Pimephales promelas*) ***Brackish Water*** Stickleback (*Gasterosteus aculeatus*), Dab (*Platichthys flesus*) ***Saltwater*** Cod (*Gadus morhua*), G. aculeatus, P. flesus | 96 h $LC_{50}$ | Nat. (SS 028162 for freshwater fish) Nat.(SS 02189 for saltwater fish) |
| | Acute crustacean toxicity | ***Freshwater*** *Daphnia sp, Ceriodaphnia dubia* ***Brackish water*** *Nitocra spinipes, Crangon crangon* ***Saltwater*** *Acartia tonsa* | 48 h $EC_{50}$/ 96 h $EC_{50}$ | Nat. (SS 028180 for daphnia) |
| | Algae toxicity | ***Freshwater*** *Selenastrum capricornutum, Monoraphidium griphitti, Chlorella vulgaris, Scenedesmus subspicatus* ***Brackish- and saltwater*** *Skeletona costatum* | 5 d $EC_{50}$ | Int. (OECD TG 201) |
| | Toxicity to higher plants | Duckweed (*Lemna minor*), Onion (*Allium cepa*), Lentil (*Lens culinaris*) | 5 d $EC_{50}$ | |
| | Microorganism toxicity | Inhibition of respiration and nitrification of activated sludge *Vibrio fischeri* (Microtox) as pre-screening test | 3 h $EC_{50}$ | Int. (ISO 8192 draft, ISO/DIS 9509) |
| **Stage 2:** In dependence of the results in the 1. stage, especially when $LC_{50}$/PEC <1, further testing with the most sensitive species of stages. | Chronic fish toxicity | Zebra fish<br>As acute toxicity<br>Fathead minnow<br>Salmon, rainbow trout, brown trout, perch | Embryo-larvae survival 11 d $EC_{50}$<br>Survival 14 d prolonged exposure $LC_{50}$<br>Fry growth test 7 d $EC_{50}$<br>Physiological effects | Nat. (SS 028193)<br>Int. (OECD TG 204) |
| | Chronic crustacean toxicity | *Daphnia*<br>*Ceriodaphnia*<br>*Nitocra* | *Daphnia* 21 d<br>*Ceriodaphnia* 7 d<br>*Nitocra* 14 d $EC_{50}$, | Int. (OECD TG 202) |
| | Mussels | *Mytilus edulis* | larvae survival | |
| | Chronic algae-toxicity | Algae test battery | | |
| | Genotoxicity | Ames test | | |
| **Stage 3**: Confirmatory tests | Extended chronic toxicity studies with fishes in laboratory and cages experiments, studies of changes of caught wild fish, sediment toxicity tests. | | | |

# Annex 2 Standardised Toxicity Tests for Wastewater Investigations

## 2.1 Acute Toxicity Tests with Freshwater Organisms

**Annex Tab. 3**: Acute toxicity tests with freshwater organisms.

| Organism | Species | End point | Test duration | Result | References | | |
|---|---|---|---|---|---|---|---|
| | | | | | ISO | OECD | National standard |
| **Fish** | *Brachydanio rerio* and other fish species | Mortality | 24-96 h | LC | 7346-1, -2, -3 (1996) | 203 (1992) | EPA/OPPTS 850.1075 (1996)<br>DIN EN ISO 7346-1, -2, -3 (1998) |
| | *Pimephales promelas*<br>*Oncorhynxhus mykiss* | Mortality | 24-96 h | LC | | | EPA/600/4-90/027F (1993) |
| | *Leuciscus idus* | Mortality | 48-96 h | LC or $G_F$ | | | DIN 38412-31 (1989) |
| | *Leuciscus idus* | Embryo mortality and deformations | 48 h | LC or $G_{Ei}$ | | | DIN 38415-6 (Draft 2000) |
| **Crustacean** | *Daphnia magna* | Immobilisation | 48 h | EC | 6341 (1996) | 202 (1984) | DIN EN ISO 6341 (1996) |
| | *Daphnia magna* | Immobilisation | 24 h | EC or $G_D$ | | | DIN 38412-30 (1989) |
| | *Daphnia magna*<br>*Daphnia pulex*<br>*Ceriodaphnia dubia* | Mortality | 24, 48 or 96 h | EC | | | EPA/600/4-90/027F (1993) |
| **Rotifers** | *Brachionus calyciflorus* | Mortality | 24 h | EC | | | ASTM E 1440-91 (1998) |
| **Higher plants** | *Lemna gibba* | Growth inhibition | 7 d | EC or NOEC | | 221 (Draft 2000) | ASTM E 1415-91 (1998) |
| | *Lemna gibba* | Growth inhibition | 7 d | EC or NOEC | | | |
| | *Lemna minor* | Growth inhibition | 7 d | EC or NOEC | | | EPA/OPPTS 850.4400 (1996) |

LC/EC: Lethal concentration/effective concentration

G: Reciprocal of the volume fraction in the test where no effect in terms of the test system is observed (LID = lowest ineffective dilution)

NOEC: No observed effect concentration (highest concentration without a significant effect)

## 2.2 Acute Toxicity Tests with Salt and Brackish Water Organisms

**Annex Tab. 4**: Acute toxicity tests with salt and brackish water organisms.

| Organism | Species | End point | Test duration | Result | References | |
|---|---|---|---|---|---|---|
| | | | | | ISO | National standard |
| **Fish** | *Scopthalmus maximus* | Mortality | 72 h | LC or NOEC | ISO/WD 15990 | |
| | *Cyprinodon variegatus* and other species | Mortality | 96 h | LC or NOEC | | EPA/600/4-90/027F (1993) |
| **Crustacean** | *Acartia tonsa* and other species | Mortality | 48 h | LC | 14669 (1999) | |
| | *Mysidopsis bahia* | Mortality | 48-96 h | LC | | EPA/600/4-90/027F (1993) |
| **Mussels (embryo-larvae)** | *Crassostrea virginica*<br>*Grassostrea gigas*<br>*Mytilus edilus* | Mortality and deformations | 48 h | EC or NOEC | | EPA/OPPTS 850.1055 (1996 Draft) |
| **Rotifers** | *Brachionus plicatilis* | Mortality | 24 h | EC | | ASTM E 1440-91 (1998) |

## 2.3 Toxicity Tests with Microorganisms

**Annex Tab. 5**: Toxicity tests with microorganisms.

| Species | Method | End point | Test duration | Result | References | | |
|---|---|---|---|---|---|---|---|
| | | | | | ISO | OECD | National standard |
| *Vibrio fischeri* | Acute | Luminescence inhibition | 0.5 h | EC or $G_L$ | 11348-1,-2,-3 (1998) | | DIN EN ISO 11348-1,-2,-3 (1999) |
| | Chronic | Growth inhibition | 7 h | EC | | | DIN 38412-37 (1999) |
| *Pseudomonas putida* | Acute | Respiration inhibition | 0.5 h | EC | | | DIN 38412-27 (1992) |
| | Chronic | Growth inhibition | 16 h | EC | 10712 (1995) | | DIN EN ISO 10712 (1995) |
| Activated sludge | Acute | Respiration inhibition | 0.5 h | EC | 8192 (1986) | 209 (1984) | DIN EN ISO 8192 (1995) |
| | Acute | Nitrification inhibition | 4 h | EC | 9509 (1989) | | DIN EN ISO 9509 (1995) |
| | Acute | Growth inhibition | 6 h | EC | 15522 (1999) | | |
| Anaerobic digested sludge | Chronic | Inhibition of the gas production | 3-7 d | EC | ISO/DIS 13641-1,2 | | |

## 2.4 Chronic Short and Long Term Tests

**Annex Tab. 6**: Chronic short and long term tests.

| Organisms | Species | End point | Test duration | Result | References | | |
|---|---|---|---|---|---|---|---|
| | | | | | ISO | OECD | National standard |
| **Fish** Freshwater | *Pimephales promelas* | Larval growth and survival | 7 d | NOEC | | | EPA/600/4-91/002 (1994) Method 1000.0 |
| | *Pimephales promelas* | Embryo-larval survival and teratogenicity | 7 d | NOEC | | | EPA/600/4-91/002 (1994) Method 1001.0 |
| | *Leusiscus idus* | Embryo-larval development and survival | 8-10 d | LC/EC or NOEC | | 212 (1998) | |
| | *Brachydanio rerio* and other | Mortality | 14 d | NOEC | | 204 (1984) | |
| | *Oncorhynchus mykiss* | Growth | 14 d | EC/NOEC | 10229 (1994) | | |
| | *Oncorhynchus mykiss* | Growth | 28 d | EC/NOEC | | 215 (2000) | |
| | Various species | Embryo-larval survival | 30-60 d | NOEC | 12890 (1999) | 210 (1992) | |
| Saltwater | *Cyprinodon variegatus* | Larval growth and survival | 7 d | LC/EC or NOEC | | | EPA/600/4-91/003 (1994) Method 1004.0 |
| **Crustacean** Freshwater | *Ceriodaphnia dubia* | Immobilisation, reproduction | 8 d | NOEC | | | EPA/600/4-91/002 (1994) Method 1002.0 |
| | *Ceriodaphnia dubia* | Reproduction | 7 d | NOEC | | | NF T90-376 (2000) |
| | *Daphnia magna* | Reproduction | 21 d | NOEC | 10706 (2000) | 211 (1998) | |
| Saltwater | *Mysidopsis bahia* | Survival, growth, reproduction | 7 d | | | | EPA/600/4-91/003 (1994) Method 1007.0 |
| **Algae** Freshwater | *Scenedesmus subspicatus Selenastrum capricornutum* | Growth inhibition | 72 h | EC or NOEC | 8692 (1989) | 201 (1098) | DIN EN 28692 (1993) |
| | *Scenedesmus subspicatus* | Growth inhibition | 72 h | $G_A$ | | | DIN 38412-33 (1991) |
| | *Selenastrum capricornutum* | Growth inhibition | 96 h | EC/NOEC | | | EPA/600/4-91/002 (1994) Method 1003.0 |
| Saltwater | *Skeletonema costatum Phaeodactylum tricornutum* | Growth inhibition | 72 h | EC | 10253 (1995) | | DIN EN ISO 10253 (1998) |
| | *Champia parvula* | Reproduction | 7-9 d | EC/NOEC | | | EPA/600/4-91/003 (1994), Method 1009.0 |
| **Rotifers** Freshwater | *Brachionus calyciflorus* | Reproduction | 2 d | EC | | | NF T90-377 (2000) |

# Annex 3 Biological Degradation Tests

**Annex Tab. 7:** International standardised biodegradation tests.

| | Test | Test concentration | Inoculum | Measured parameter | Test duration | References |
|---|---|---|---|---|---|---|
| **OECD-Tests on ready degradability** | DOC Die-away test | 10-40 mg/L DOC | Moderate bacteria concentration, activated sludge 30 mg/L TSS | DOC | 28 d | OECD 301 A (1992)<br>ISO 7827 (1994)<br>DIN EN ISO 7827 (1996) |
| | $CO_2$ Evolution test | 10-20 mg/L DOC | Moderate bacteria concentration, activated sludge 30 mg/L TSS | $CO_2$, DOC | 28 d | OECD 301 B (1992)<br>ISO 9439 (1999)<br>DIN EN ISO 9439 (2000) |
| | Modified MITI test (I) | 100 mg/L substance | Moderate bacteria concentration, activated sludge 30 mg/L TSS | $O_2$, $CO_2$ | 28 d | OECD 301 C (1992) |
| | Closed bottle test | 2-10 mg/L substance | Low bacteria concentration, up to 5 mL/L ? municipal effluent | $O_2$ | 28 d | OECD 301 D (1992)<br>ISO 10707 (1994)<br>DIN EN ISO 10707 (1998) |
| | Modified OECD screening test | 10-40 mg/L DOC | Low bacteria concentration, 0.5 mL/L ? municipal effluent, surface water | DOC | 28 d | OECD 301 E (1992)<br>ISO 7827 (1994) |
| | Manometric respirometry test | 100 mg/L substance | Moderate bacteria concentration, activated sludge 30 mg/L TSS | $O_2$ | 28 d | OECD 301 F (1992)<br>ISO 9408 (1999)<br>DIN EN ISO 9408 (1999) |
| | Biodegradability in sea water | According to the Die-away or closed bottle test method | Sea water without further inoculum | | 28 d | OECD 306 (1992) |
| **OECD-Tests on inherent degradability** | Modified SCAS test | 20-50 mg/L DOC | High bacteria concentration, activated sludge 1-4 g/L TSS | DOC | up to 26 weeks | OECD 302 A (1981)<br>ISO 9887 (1992)<br>DIN EN ISO 9887 (1994) |
| | Zahn-Wellens/EMPA test | 50-400 mg/L DOC | High bacteria concentration, activated sludge 0.2-1 g/L TSS | DOC<br>COD | up to 28 d | OECD 302 B (1992)<br>ISO 9888 (1999)<br>DIN EN ISO 9888 (1999) |
| | Modified MITI test (II) | 30 mg/L substance | Activated sludge, 100 mg/L TSS | $O_2$ | 28 d | OECD 302 C (1981) |

| | Test | Test concentration | Inoculum | Measured parameter | Test duration | References |
|---|---|---|---|---|---|---|
| **OECD-Simulation tests** | Simulation test – Aerobic wastewater treatment: coupled units test | 10-50 mg/L DOC | 2.5-3 g/L TSS | DOC | Up to 12 weeks | OECD 303 A (1981)<br>ISO 11733 (1995) |
| | Simulation test – Aerobic wastewater treatment: biofilms | | Biofilm | | | OECD 303 B (2000 draft) |
| **Further ISO tests** | Ready biodegradability $CO_2$ headspace test | 2-40 mg/L DOC | Activated sludge<br>4 mg/L TSS | $CO_2$ | 28 d | ISO 14593 (1999) |
| | Ready biodegradability two-phase closed bottle test | 100 mg/L COD | Activated sludge<br>30 mg/L TSS | $O_2$ | 28 d | ISO 10708 (1997) |
| | Low concentrations in water, simulation of a static (shake flask method) or dynamic (river simulation method) aerobic surface water system | <100 µg/L | Surface water with or without sediment | $^{14}C$-labelled substances | | ISO/DIS 14592-1,-2 |
| | Anaerobic degradation test of substances in digested sludge | 20-100 mg/L TOC | Anaerobic microorganisms<br>10 Vol-% digested sludge | Biogas production ($CO_2$ and $CH_4$) | < 60 d | ISO 11734 (1995) |

# Annex 4 Materials and Methods

## 4.1 Physico-chemical Wastewater Characterisation

The following physico-chemical parameters were determined:

Conductivity, pH, suspended solids (TSS), turbidity, DOC, COD, UV-absorbance at 254 and 436 nm, $Cl^-$, $SO_4^{2-}$, $NO_3^-$, $PO_4^{3-}$, $NH_4^+$.

*Total suspended solids* were determined according to DIN EN 872. Appropriate sample volumes were filtered over rinsed and dried glass fibre filters (13400-50Q; Sartorius, Göttingen, Germany). The filters were dried for 2 h at 105°C and than weighed.

The *turbidity* was measured with the Hach 2100N Turbidimeter (Dr. Bruno Lange, Düsseldorf, Germany) calibrated with formazin-standards.

The *DOC* was measured with the LiquiTOC 2100 MB (Foss-Heraeus, Hanau, Germany) by UV/persulfate oxidation.

The *COD-determination* was performed with the Hach dichromate test system (DR/2000 Photometer, COD reactor and vials for a COD range of 0-150 mg/L, Dr. Bruno Lange, Düsseldorf, Germany).

The *UV-absorbance* was determined with a Lambda-2 UV/Vis-Spectrophotometer (Perkin-Elmer, Überlingen, Germany) at the wavelengths 254 nm and 436 nm.

The anions $Cl^-$, $SO_4^{2-}$, $NO_3^-$, and $PO_4^{3-}$ were measured by ion chromatography with a Dionex-system and conductivity detection (autosampler AS50, chromatography module AS50, gradient pump GP50, conductivity detector CD20, Dionex, Idstein, Germany). The separation was performed isocratically with a Dionex IonPac AS11-column (4×250 mm) with 13 mmol NaOH as eluent and a flow of 1 mL/min within 25 min.

$NH_4^+$ was determined photometrically after transformation with salicylate and hypochlorite ions in the presence of $Na_2Fe(CN)_5NO{\cdot}2H_2O$ according to DIN 38406-5.

## 4.2 Biological Tests (Ecotoxicity)

All tests were performed according to the following standardised test methods:

| Biotest | Test organism | $G_X$: lowest value of G in the test where | Test method |
|---|---|---|---|
| Luminescent bacteria | *Vibrio fischeri* | the luminescence is inhibited to < 20 % ($G_L$) | according to DIN EN ISO 11348-3 |
| Daphnia test | *Daphnia magna* | all daphnia keep their ability to swim ($G_D$) | DIN 38412-30 |
| Fish-egg test | *Brachydanio rerio* | at least 90 % of the embryos show no effects* in the sense of the method ($G_{Ei}$) | DIN draft 38415-6 |
| Algae test | *Scenedesmus subspicatus* | the biomass production is inhibited to < 20 % ($G_A$) | according to DIN 38412-33 |
| Umu test | *Salmonella typhimurium* | no more genotoxic effects are measured ($G_{EU}$) | DIN 38415-3 |

*these effects are: coagulated eggs, no somites, no tail separation, no heartbeat

The luminescence inhibition test and the algae test were performed in miniaturised form in microtiter-plates. At first 100 µL of the sample solutions were given into the wells of the microtiter-plate and then 100 µL of the luminescent bacteria resp. of the algae suspension were added automatically. The luminescence inhibition of *V. fischeri* was measured with a microtiter-plate luminometer (Lucy-1, Anthos, Salzburg, Austria) after an exposure of 30 min at 19°C. The results of this test procedure showed a good correlation with the test performed according to the DIN procedure in cuvette and a comparable sensitivity [Fiehn et al., 1997].

The algae chlorophyll fluorescence was determined according to Höhne (1991) with a microtiter-plate fluorimeter (Fluoroscan II, Labsystems, Frankfurt/M, Germany). The algae were incubated at 20° in an incubator with permanent light. The growth inhibition was determined every 24 h after the start for 96 h (according to the DIN procedure the data evaluation is performed after 72 h).

The number of immobilised daphnia was determined after 24 h according to the DIN method as well as after an exposure of 48 h.

## 4.3 Aerobic Biodegradation Test (Persistence)

The biodegradation test was performed according to EN ISO 7827 (corresponds to the die-away-test OECD 301 A). Substrate concentrations of 10-40 mg/L DOC as well as municipal activated sludge with 30 mg/L TSS as inoculum is applied. The biodegradation is monitored by DOC measurements.

In parallel to the wastewater samples a blank sample (inoculum blank) and a control sample (inoculum activity) were prepared. The test volumes were 1.5 L. At first 1 L of centrifuged wastewater was given into the test vessel. Then the mineral solutions were filled up to 0.5 L with centrifuged wastewater and transferred into the test vessel. Thereafter the inoculum was added and the test solution was well mixed immediately.

Mineral solutions:

A 8.5 g $KH_2PO_4$, 21.75 g $K_2HPO_4$, 33.4 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.5 g $NH_4Cl$

B 22.5 g $MgSO_4 \cdot 7\ H_2O$

C 36.4 g $CaCl_2 \cdot 2\ H_2O$

D 0.25g $FeCl_3 \cdot 6\ H_2O$

each dissolved in 1 L Millipore water

Test medium: 10 mL A and 1 mL of B, C as well as D per litre test volume

Inoculum: 4 mL activated sludge per litre test volume with 7.55 g/L suspended solids from the municipal wastewater treatment plant Ruhleben (Berlin, Germany), rinsed twice with drinking water

The blank sample contained only the test medium (mineral solutions in millipore water) as well as the inoculum, and for the control samples aniline (34.3 mg/L DOC) was added as substrate. Immediately after the addition of the inoculum the first sample was taken (start sample), and after 3 h the second sample (adsorption control). Further samples were taken after 7, 10, 14, 17, 21, 24 and 28 days.

The test solutions were kept for 28 days in the dark at 20 ± 2 °C under aeration and stirring. Samples of 80 mL were taken regularly. Losses of water by evaporation was compensated prior to each sampling by adding the appropriate volume of millipore water.

The samples were filtered directly after the sampling (0,45 µm membrane filter, cellulose nitrate, Machery-Nagel, Düren, Germany) and the UV-absorbance as well as the DOC was detected from the filtered sample at the sampling day.

After the end of the degradation test the biomass of 400 mL of the test solutions (except of the control) was extracted three times with 6 mL MeOH 20 min in an ultrasonic bath. The combined extracts were then concentrated to 1 mL in a vacuum centrifuge for HPLC measurements.

## 4.4 Solid-phase Extraction and HPLC (Potential Bioaccumulation)

For the preparation of the stock solutions and the standard mixture, the conditioning and the elution of the SPE cartridges and as eluent for the HPLC ultra-pure water (ELGA Maxima-System, USF ELGA, Ransbach-Baumbach, Germany) and HPLC "Gradient-grade" MeOH (Merck, Darmstadt, Germany) were used.

*Standard mixture*

Stock solution of about 400 mg/L were prepared with p-cresol, indole, 1-aminonaphthalene, 1-naphthol and 2,4-dichlorophenol in water and with atrazin, benzophenone, naphthalene, biphenyl and 4-nonylphenol in MeOH (all substances were of p.a. quality). A mixture with 10 mg/L resp. 30 mg/L of naphthalene, biphenyl and nonylphenol was made as pre-dilution for the SPE and for the determination of the recovery by HPLC. This mixture was further diluted 200 fold for the SPE to as concentration of about 50 µg/L for most substances and to about 150 µg/L for naphthalene, biphenyl and nonylphenol.

*SPE*

The solid phase material Isolute C18(EC) from IST (Separtis, Grenzach-Wyhlen) with 1 g sorbent was used. The conditioning was performed with 10 mL MeOH followed by 15 mL water. Sample volumes of 200 mL (standard mixture or centrifuged wastewater) were then extracted at a flow of about 3-4 mL/min. The first 1.5 mL of the filtrate were discarded. After the extraction the cartridge was rinsed with 1.5 mL of water, which was added to the filtrate. The cartridge was sucked dry and eluted with different water/MeOH mixtures and/or MeOH. A vacuum centrifuge (Speedvac A160, Savant, Farmingdale, USA) was used for the sample concentration.

*HPLC*

The HPLC measurements were performed with a Merck system (gradient pump L-6200A, column oven T-6300, autosampler AS 2000 A; Merck, Darmstadt, Germany) with DAD-detection (Gynkotek, Munich, Germany). A RP-18 column (RP18endc. Supersphere-100 4 µm, 3×150 mm, Knauer, Berlin) was used at 40°C and the injection volume was 10 µL. The following binary gradient with 50 mmol $NaHPO_3$ in water (eluent A) and MeOH (eluent B) was used: start with 5 % B, 3 min 30 % B, 10 min isocratic, 16 min 47 % B, 26 min 95 % B, 12 min equilibration time, constant flow of 0.75 mL/min.

The SPE-eluates of the standard mixtures were concentrated to about 1 mL and measured after the addition of 10 µg methyl benzoate as internal standard (IS). For the determination of the recovery 1 mL volume of the standard solution containing 10 or 30 mg/L per substance was spiked with 10 µg IS and analysed parallel to the SPE-eluates.

*Wastewater extraction*

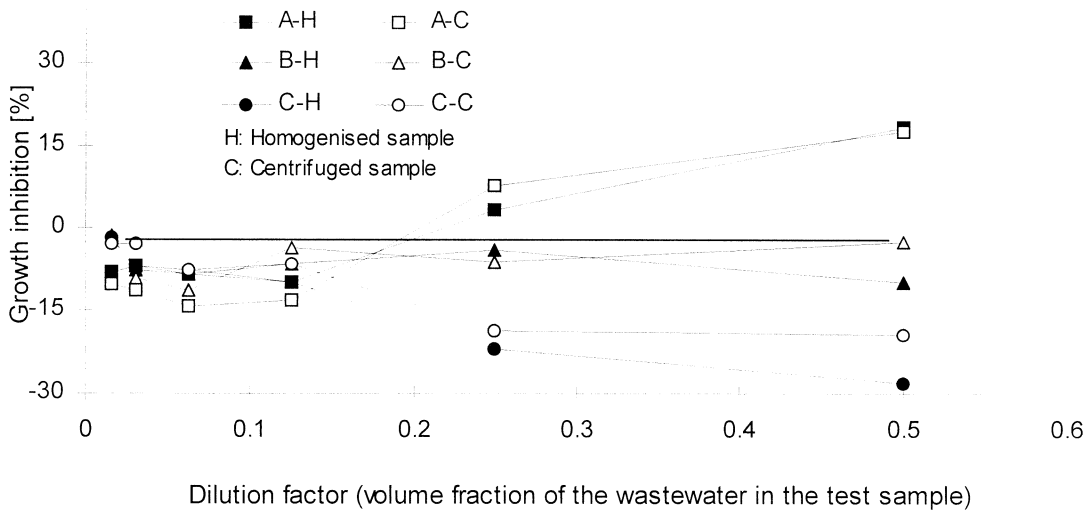
After some preliminary tests the wastewater samples were eluted in two steps: first with 5 mL MeOH/$H_20$ 68/32 (v/v) and then with 5 mL of pure MeOH.

In order to eliminate the MeOH prior to the DOC determination the first eluate was processed as follows: 2 mL of water were added to 2 mL of the eluate,the mixture was concentrated to 0.25 mL in the vacuum centrifuge and another 1.25 mL water was than added to a total volume of 1.5 mL. The concentration as well as the following addition of water was repeated twice and finally the eluate was concentrated to 1 mL. For the determination of the DOC and the UV-absorbance a volume of 0.3 mL or 0.4 mL of the concentrated eluate were taken (depending on the initial sample DOC) and diluted with water to 50 mL in a volumetric flask. In parallel to the wastewater samples ultra-pure water was extracted and processed as the wastewater samples to obtain the blank values.

# Annex 5 Results of the Biological Tests

## 5.1 Algae Test of the Wastewater Samples A, B and C

The samples were measured homogenised and centrifuged.




**Annex Fig. 1:** Growth inhibition of the wastewater samples A, B and C (homogenised and centrifuged) in the algae test.

## 5.2 Umu Test of the Wastewater Samples A, B and C

The samples A, B and C were measured in the umu test after centrifugation. Furthermore, the persistent fraction of sample A (A-b), the SPE-filtrate of the sample A-b as well as the SPE-filtrate of an ultra-pure water extraction as blank were measured.

**Annex Tab. 8:** Results of the umu tests.

| **Without S9** | Positive control 4-NQO IR = 3.54 | | | | | | Negative control FNU = 226.84 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **A centrifuged** | | **A-b (after the degradation )** | | **A-b SPE-filtrate** | | **B centrifuged** | | **C centrifuged** | | **Blank SPE - filtrate** | |
| Dilut. | G | IR | G | IR | G | IR | G | IR | G | IR | G | IR |
| 1:12 | 0.97 | 0.97 | 0.95 | 0.98 | 1.02 | 0.95 | 0.92 | 1.06 | 0.98 | 0.93 | 1.05 | 0.94 |
| 1:6 | 0.93 | 1.09 | 0.91 | 1.12 | 0.92 | 1.07 | 0.87 | 1.02 | 0.90 | 1.06 | 1.05 | 0.95 |
| 1:3 | 0.88 | 1.20 | 0.87 | 1.23 | 0.87 | 1.24 | 0.85 | 1.06 | 0.91 | 1.11 | 1.05 | 1.05 |
| 1:1.5 | 1.02 | 1.08 | 0.91 | 1.30 | 0.77 | 1.33 | 0.81 | 1.45 | 0.87 | 1.18 | 1.15 | 0.94 |

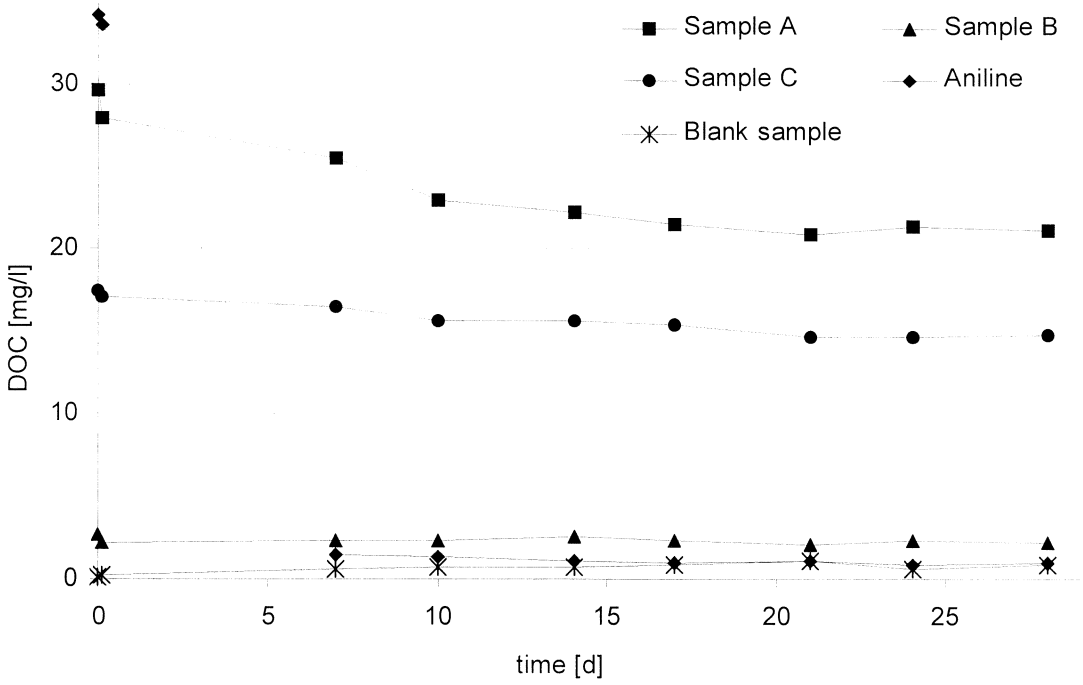
| **With S9** | Positive control 2-AA IR = 6.277 | | | | | | Negative control FNU = 279.69 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **A centrifuged** | | **A-b (after the degradation)** | | **A-b SPE-filtrate** | | **B centrifuged** | | **C centrifuged** | | **Blank SPE-filtrate** | |
| Dilut. | G | IR | G | IR | G | IR | G | IR | G | IR | G | IR |
| 1:12 | 1.04 | 0.92 | 0.99 | 0.92 | 1.01 | 1.00 | 1.14 | 1.13 | 0.99 | 0.92 | 1.01 | 1.06 |
| 1:6 | 1.04 | 0.98 | 1.02 | 0.99 | 1.17 | 1.08 | 1.00 | 1.02 | 1.07 | 1.20 | 0.99 | 1.42 |
| 1:3 | 1.05 | 0.80 | 1.07 | 0.95 | 1.16 | 0.85 | 0.95 | 0.97 | 1.08 | 1.18 | 0.94 | 1.00 |
| 1:1.5 | 1.30 | 0.93 | 1.11 | 0.78 | 1.03 | 0.76 | 0.99 | 1.38 | 1.32 | 0.91 | 1.07 | 0.79 |

**Validity criteria for the umu test:**

- The positive controls must reach at least an induction factor (IR) of 2.
- The negative controls must reach at least a turbidity of 140 FNU (formazin nephelometric units).
- The growth factor (G) must be greater than 0.5.
- A sample dilution is assessed as positive if the IR is larger than 1.5.

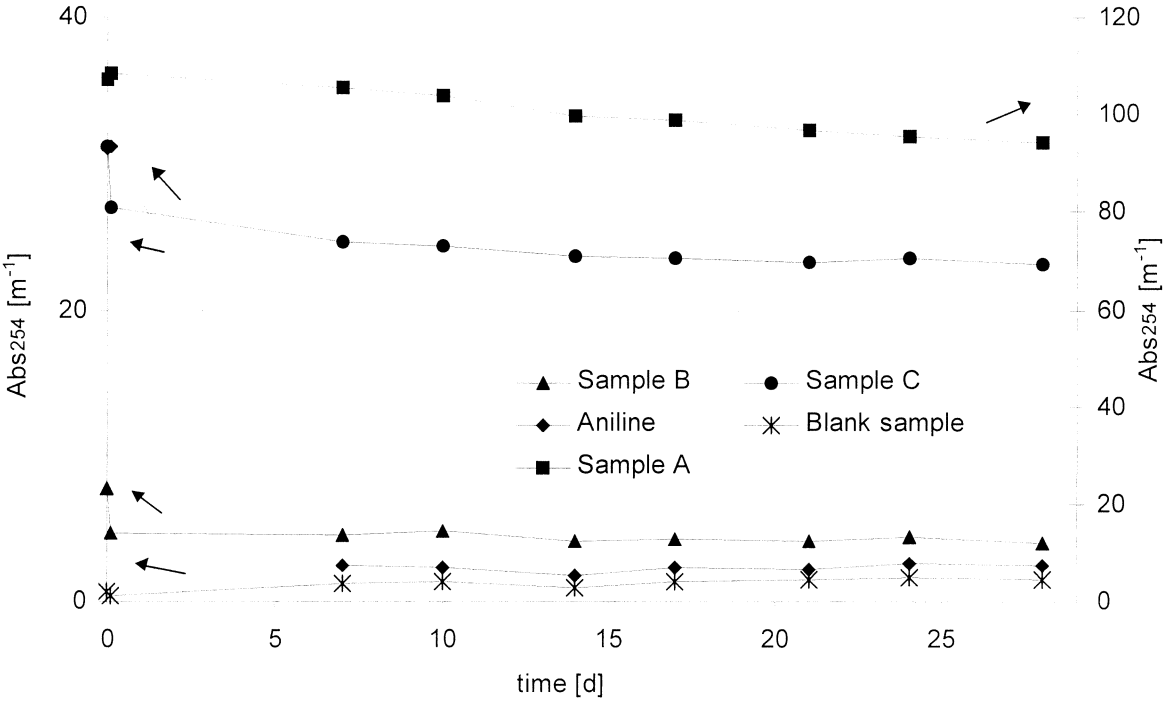
# Annex 6 Results of the Biological Degradation Tests

## 6.1 DOC Reduction




**Annex Fig. 2:** Reduction of the DOC.

## 6.2 Reduction of the UV-Absorbance at 254 nm




**Annex Fig. 3:** Reduction of the absorbance at 254 nm.

# Annex 7 Method Development for the Module 'Bioaccumulation'

## 7.1 Extraction of the Standard Mixture

Proceeding:

First suitable conditions for a fractionation of substances at a $\log K_{ow} \approx 3$ by SPE were established by means of a standard mixture of ten aromatic compounds ($\log K_{ow}$ 1.9-5.8, Annex Tab. 9). The sorbent used here for the SPE ($C_{18}$-Phase, end-capped) was the same as it was used by Metzger et al. (2000) for the determination of PBS by SPE and preparative HPLC. This sorbent was chosen by Metzger after comparative investigations of different sorbents of various suppliers and was reported to show the best recovery rates for the compounds in the $\log K_{ow}$-range 2.2 - 8.3. The analysis of the standard mixture and the eluates was performed by HPLC with diode-array-detection.

As expected all compounds of the standard mixture were extracted with the used sorbent including the polar substances with $\log K_{ow}$-values below 3, and all compounds were eluted with methanol. The recovery rates ranged from 60 to 90 %. It was intended to find that mixture ratio of methanol and water that enables the elution of all standard substances with $\log K_{ow}$-values < 3 from the sorbent whereas all substances with $\log K_{ow}$-values > 3 should remain sorbed. The latter compounds should then be eluted in a second elution step with pure methanol. This would allow a fractionation of dissolved organic compounds at a $\log K_{ow}$-value of 3.

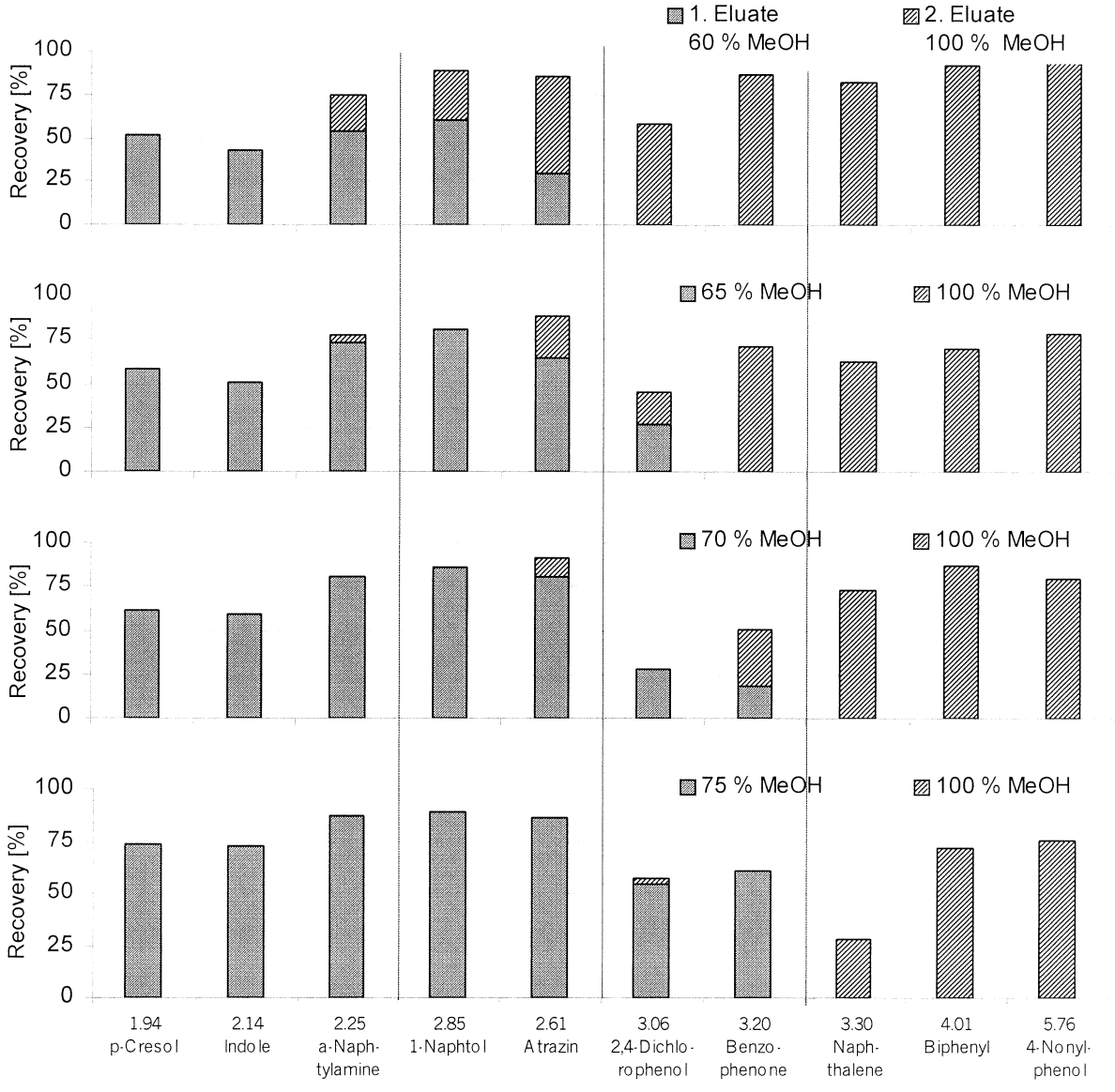
**Annex Tab. 9:** Physico-chemical data as well as HPLC retention times of the standard substances.

| Substance | Log $K_{ow}$ exp[a] | Log $K_{ow}$ calc[b] | p$K_a$[a] | water solubility [mg/l] [a] | Retention time [min][c] |
|---|---|---|---|---|---|
| p-Cresol | 1.94 | 2.06 | 10.3 | 2500 | 11.81 |
| Methyl benzoate (IS) | 2.12 | 1.83 | | 157 | 19.00 |
| Indole | 2.14 | 2.05 | -2.4 | 3560 | 13.35 |
| α-Naphtylamine | 2.25 | 2.25 | 3.9 | 1700 | 16.66 |
| Atrazin | 2.61 | 2.82 | 1.7 | 32 | 21.11 |
| 1-Naphtol | 2.85 | 2.69 | 9.3 | 866 | 20.29 |
| 2,4-Dichlorophenol | 3.06 | 2.80 | 7.9 | 1000 | 22.15 |
| Benzophenone | 3.20 | 3.15 | | <100 | 24.11 |
| Naphthalene | 3.30 | 3.17 | | 30 | 25.57 |
| Biphenyl | 4.01 | 3.76 | | 8 | 27.20 |
| 4-Nonylphenol | 5.76 | 5.99 | | 7 | 28.85 |

[a] The data were taken from the Hazardous Substances Data Bank (HSDB). [http://toxnet.nlm.nih.gov/]
[b] Calculated with KowWin (logKow) log P calculation [http://esc.syrres.com/interkow/logkow.htm]
[c] HPLC-conditions see annex 4.4.

Results:

With 20 % MeOH for the first elution no substances were eluted, and with 50 % MeOH about 20 % of the substances with the lowest log$K_{ow}$-values (1.9 resp. 2.1) were desorbed. At higher methanol amounts of 60-75 % MeOH for the first elution substances with higher log$K_{ow}$-values were increasingly recovered in the first eluate (Annex Fig. 4). With the stepwise increase of the methanol amounts the concentration of the five substances with a log$K_{ow}$-value ranging from 2.2 to 3.2 increased in the first eluate and decreased in the second eluate. These substances were completely eluted with 75 % MeOH. The test substances with a log$K_{ow}$ < 2.2 (p-cresol and indole) were always found in the first eluate and the three substances with a log$K_{ow}$ > 3.2




The substances are given in the sequence of their retention in the HPLC, there atrazin elutes despite a slightly lower log$K_{ow}$-value before naphthol.

1 g LC18(EC), Eluate á 5 mL, 200 mL extraction volume

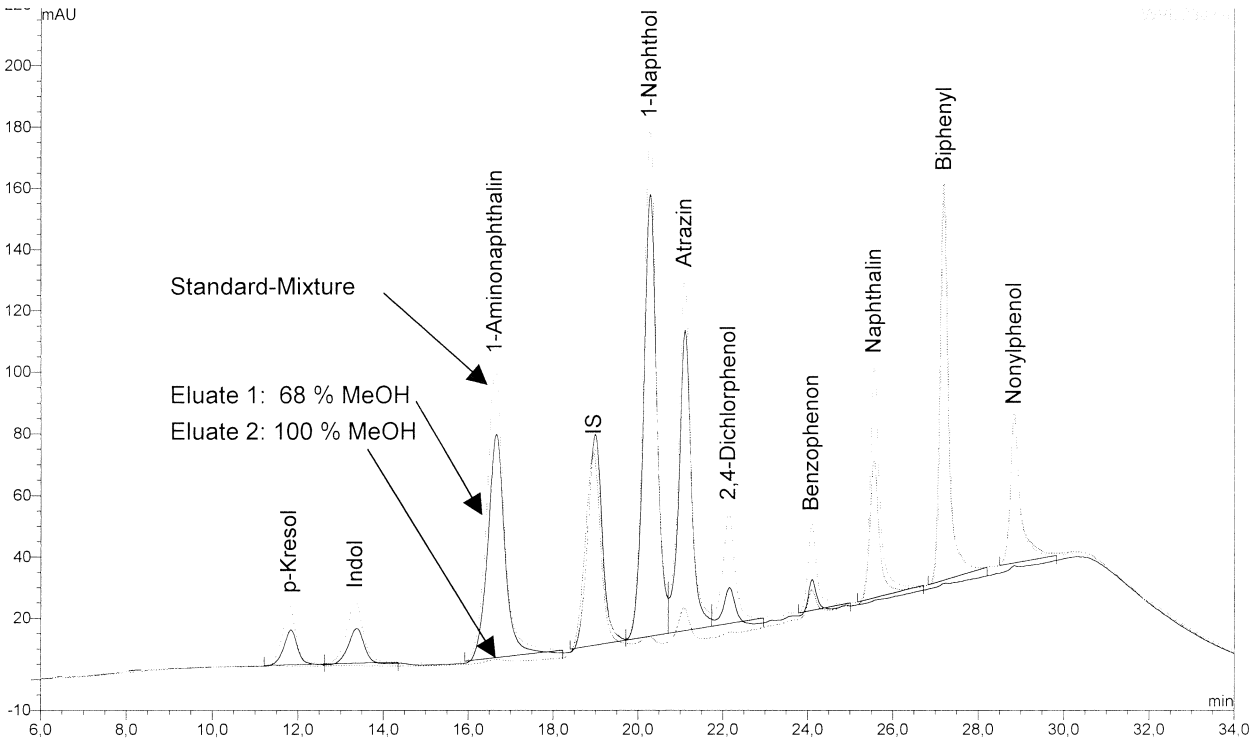
**Annex Fig. 4:** Two-step elution after the SPE of the standard mixture with MeOH amounts varying from 60 to 75 % in the first eluate and 100 % MeOH in the second eluate – Distribution of the standard substance to the eluate fractions.

(naphthalene, biphenyl and nonylphenol) always occurred in the second eluate. The best fractionation at a log$K_{ow}$ 3 was obtained with 65 % methanol in the first elution step. Only two substances (atrazin log$K_{ow}$ 2.61 and 2,4-dichlorophenol log$K_{ow}$ 3.06) in the log$K_{ow}$-range of 3 are present in both fractions to more than 5 %. Contrary to the data compiled in Annex Tab. 9 but according to the reversed-phase HPLC-chromatography (Annex Fig. 5) the log$K_{ow}$-value of naphthol is assumed to be lower than that of atrazin.

Annex Fig. 4 also shows that the fractionation around a log$K_{ow}$-value of 3 will never be totally focussed. This is partly due to a chromatographic effect in the extraction cartridge that cannot be suppressed. However it can be expected that the separation according to the log$K_{ow}$-value will be sharper for aliphatic compounds, as the aromatic compounds with their potential of forming strong л-interactions interactions with the hydrophobic SPE material are expected to be retained more strongly, than their log$K_{ow}$-value would expect.

Further uncertainty arises from the compiled log$K_{ow}$-values, which vary by an average of ± 0.2 [Verbruggen et al., 1999b], depending on the method that was used for the determination. For the standard compounds used here experimentally determined log$K_{ow}$-values were used. When calculated data would have been used (KowWin; Annex Tab. 9) the order of elution in the reversed-phase chromatography would have corresponded with the compiled data. The nominal separation limit is thus set to a log$K_{ow}$ of 3 ± 0.2.

Based on these results obtained with a mixture of standard compounds in pure water, a methanol amount of 68 % in the first eluate was selected for the following application of this method to wastewater samples. This methanol amount has to be regarded as the upper limit as 2,4-dichlorophenol (log$K_{ow}$ 2.8 to 3.06) is found in the first eluate under these conditions (see Annex Fig. 4).




**Annex Fig. 5:** HPLC-chromatograms of the eluates of the standard mixture after the SPE with two-step elution, eluate 1: 68 % MeOH, eluate 2: 100 % MeOH, detection at 230 nm.

## 7.2 Method Development with Real Samples, Solvent Blank Value

As outlined in chapter 4.3.4 the amount of potentially bioaccumulating wastewater constituents is to be determined via DOC-difference measurements. The amount of PBS corresponds to the difference in the DOC-content of the whole sample and the sum of the SPE-filtrate and its first eluate. The DOC of the second eluate was not determined. For the DOC-determination water was added to the eluates prior to the evaporation. Upon evaporating the second eluate to 1 mL large substance losses occurred. For the substances eluting in the first eluate, however, the recovery was not affected by the evaporation process.

Biologically treated tannery wastewater as well as molasses wastewater was used for these investigations as they have higher and more easily measurable DOC values as compared to the samples A, B, and C used later on.

The removal of methanol from the first eluate prior to the DOC determination turned out to be difficult. It was initially supposed that after the evaporation of 5 mL of the first eluate (68 % methanol amount) to 1 mL the methanol would be eliminated to a large extent and that after a subsequent back-dilution with water to the initial volume (50 mL) only a low DOC blank value would be present[1]. But this was not the case. A residual methanol content of about 4 % of the sample volume was estimated from the blank values extracted in parallel to the wastewater samples.

The DOC-amounts of the first eluates were twice as high as it was calculated by the DOC difference of the whole sample and the filtrate. Therefore, only the DOC-data of the filtrates are given in Annex Tab. 10. For these samples 31 % (sample 1) and 23 % (sample 2) of the DOC were extracted by the SPE and the amount of the extracted UV-active compounds was of the same range.

It was then investigated, whether repeated evaporation and re-addition of water can reduce the blank DOC of the first eluate. The best reduction of the blank values was obtained by

**Annex Tab. 10:** SPE of two biologically treated wastewater samples (determination of the DOC of the first eluates after single evaporation).

| | **Sample 1 – biologically treated tannery wastewater** | | **Sample 2 – biologically treated molasses wastewater** | |
|---|---|---|---|---|
| | DOC 60.1 mg/L | $Abs_{254}$ 118 $m^{-1}$ | DOC 60.5 mg/L | $Abs_{254}$ 180 $m^{-1}$ |
| **Fraction** | **% DOC** | **% $Abs_{254}$** | **% DOC** | **% $Abs_{254}$** |
| a) SPE-Filtrate | 69 | 70 | 77 | 75 |
| b) SPE-Eluate 1 | -[a] | 16 | -[a] | - |
| Difference => % PBS = 100 - (a +b) | - | 14 | - | - |

[a] not detectable as the methanol blank values were too high

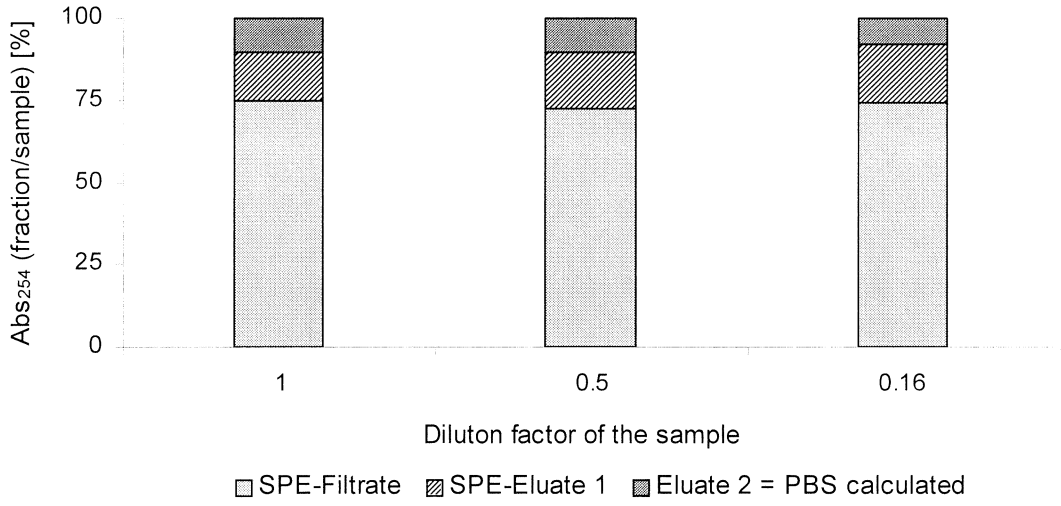
[1] 1 µL methanol in 1 mL water (0.297 mg C) results in a DOC of 1.5 mg/L at a back-dilution of the factor 200 which corresponds to the enrichment factor of the extraction of 200 ml sample.

evaporation to 0.25 mL, refilling to 2 mL with water and further evaporation to 1 mL. After the final back-dilution the DOC blank values were 0.84 ± 0.25 mg/L. This procedure was, however, not sufficient for the reduction of the residual methanol in the first eluates of the wastewater samples. Obviously organic constituents of the wastewater hampered the methanol evaporation.

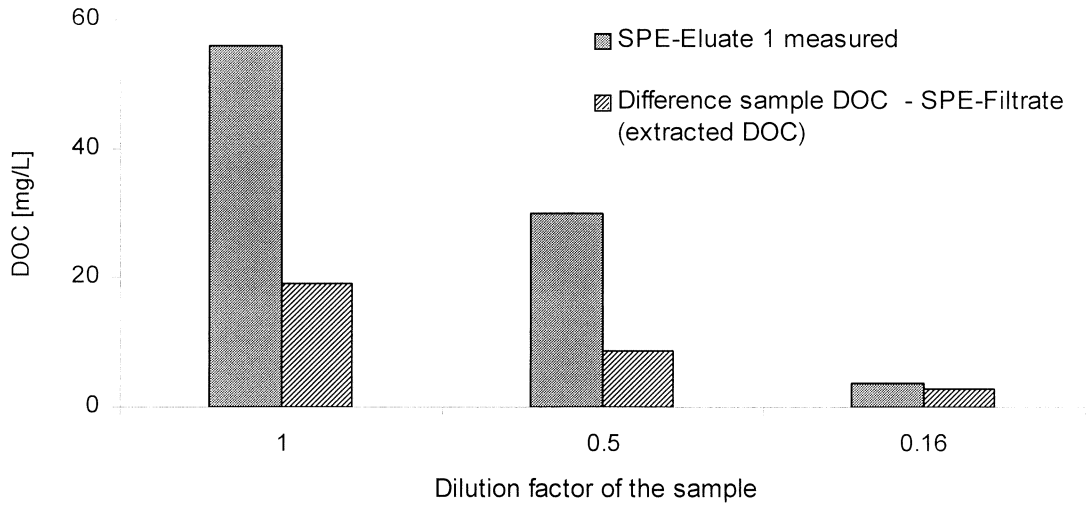
Additionally, a sample was processed after predilution in order to ascertain that the SPE cartridge that was used for the fractionation was not overloaded. The experimental data provided no evidence for this effect. (Annex Fig. 6 and 7). The distribution of the UV-absorbance ($Abs_{254}$) to the SPE fractions showed less differences between the different dilution steps and also the DOC-portion of the SPE-filtrates was very constant with 69, 71 and 68 % at increasing dilution. However it was found that the interfering residual methanol amount decreased with increasing dilution (Annex Fig. 7). This indicates in fact that with increasing DOC-content of the eluates increasingly more methanol is retained during the evaporation process.

Obviously, a repeated rinsing (evaporation and refilling with water) is necessary to ensure the elimination of methanol.

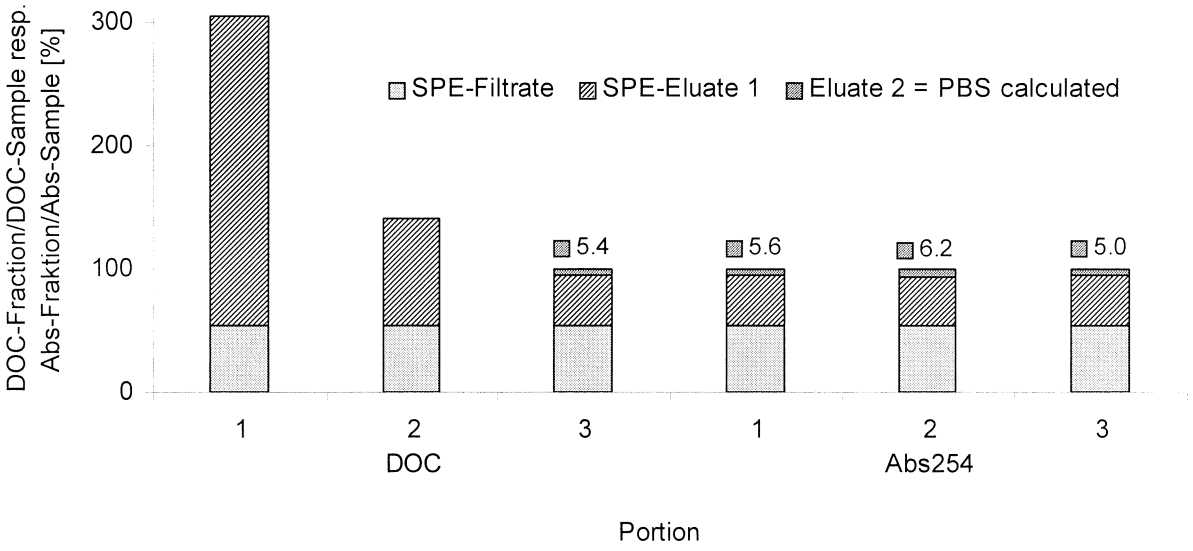
This approach was tested using a SPE eluate of the sample A which was split into three aliquots These three aliquots were then processed in different ways. Annex Fig. 8 shows that the 3rd procedure, a threefold evaporation to 0.25 mL, is well suited to eliminate the residual methanol from the eluate. The DOC blank values of this treatment was as low as 0.2 mg/L.




**Annex Fig. 6:** SPE of biologically treated tannery wastewater at different dilution steps – Distribution of the UV-active compounds onto the SPE-fractions (SPE conditions see annex 4.4).




**Annex Fig. 7:** SPE of biologically treated tannery wastewater (DOC = 60.1 mg/L) at different dilution steps – Comparison of the DOC of the 1. eluates after back-dilution with the extracted DOC (eluate 1, 5 ml 68 % MeOH, evaporation of the eluate to 0.25 mL, refilling to 2 ml and evaporation again to 1 ml, further extraction conditions see annex 4.4).

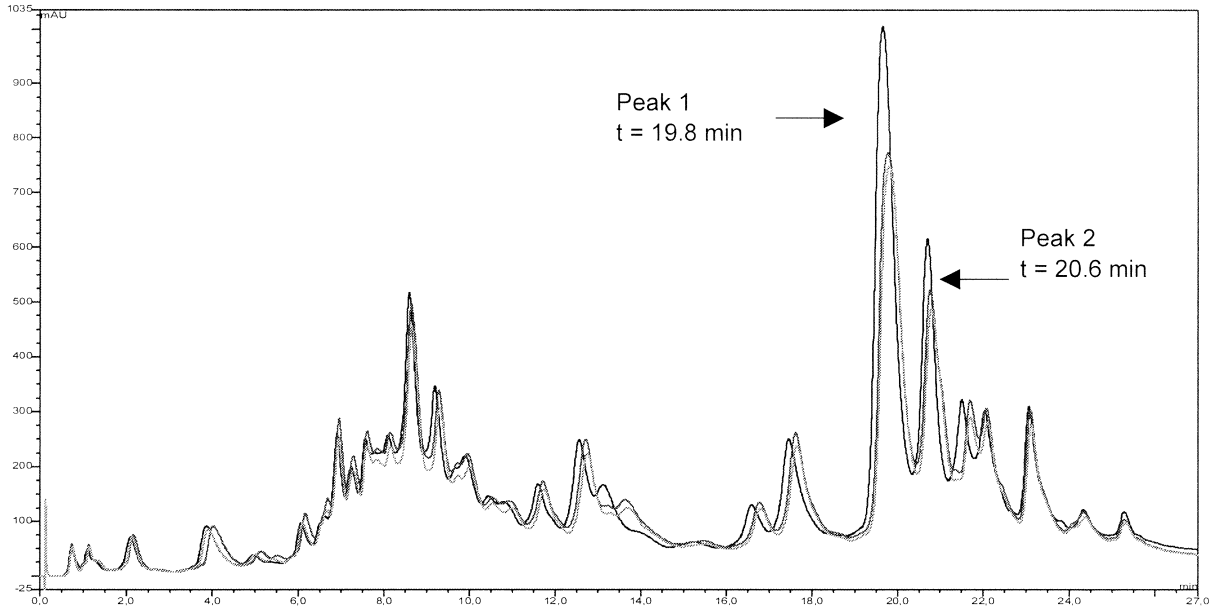



**Annex Fig. 8:** SPE of the sample A (DOC 30 mg/L) – Different processing of the first eluate (68 % methanol) after splitting in portions à 1.5 mL and refilling to 5 mL prior to the evaporation

1: Evaporation to 0.5 mL, addition of 1 mL water, evaporation to 1 mL
2: Evaporation to 0.5 mL, 3 fold addition of 1 mL water and evaporation again to 0.5 mL, finally to 1 mL
3: Evaporation to 0.25 mL, 3 fold addition of 1.25 mL water and evaporation again to 0.25 mL, finally to 1 mL

With this procedure a PBS-fraction of 5.4 % of the sample DOC was determined for sample A with the DOC-difference method. The amount of UV-active compounds in the first eluate was hardly influenced by the different evaporation procedures of the three aliquots (Annex Fig. 8, on the right). Moreover, the HPLC-chromatograms of the three aliquots showed that only low substance losses occurred despite the repeated strong evaporations (see Annex Fig. 9, two peaks). A PBS-portion of 5.6 ± 0.6 % can be calculated for the UV-absorbing substances. The total portion of extracted compounds was 45 % of the DOC of sample A.

These results show that the determination of potentially bioaccumulating compounds by SPE with fractionated elution can be coupled with the DOC determination.




**Annex Fig. 9:** SPE of the sample A– Different processing the first eluate (68 % MeOH) after splitting into three portions, HPLC-chromatograms of the three portions at 230 nm.

1: Evaporation to 0.5 mL, addition of 1 mL water, evaporation to 1 mL
2: Evaporation to 0.5 mL, 3 fold addition of 1 mL water and evaporation again to 0.5 mL, finally to 1 mL
3: Evaporation to 0.25 mL, 3 fold addition of 1.25 mL water and evaporation again to 0.25 mL, finally to 1 mL

# Annex 8 SPE of the Wastewater Samples A, B and C

## 8.1 SPE of the Wastewater Samples A, B and C before and after the Biodegradation Test – Results and First Error Estimation of the Method

The results of the SPE of the samples A and C before as well as after the biodegradation test (A-b and C-b) are presented in Annex Tab. 11. The eluates were treated as described in annex 4.4. The DOC contents and the absorbance at 254 nm are given for these samples as mean values of the results of the extraction at two different days.

**Annex Tab. 11:** SPE of the wastewater samples A and C for the determination of the PBS amount before and after the biodegradation test – Results of two determinations at different days. (Extraction and further treatment of the samples see annex 4.4)

| Parameter | Fraction | A | A-b[a] | C | C-b[a] |
|---|---|---|---|---|---|
| DOC [mg/L] | Sample | 30.8 ± 1.0 | 21.0 ± 0.3 | 18.1 ± 0.05 | 14.5 ± 0.4 |
| | SPE-Filtrate | 16.5 ± 1.0 | 12.3[b] | 7.4 ± 0.2 | 6.7 ± 0.02 |
| | SPE-Eluate 1 | 12.2 ± 0.3 | 7.2[b] | 8.8[b] | 6.4 ± 0.5 |
| | PBS calculated[c] | 2.0 ± 0.4 | 1.5 | 1.9 | 1.4 ± 0.9 |
| DOC [%] | SPE-Filtrate | 53.6 | 58.7 | 41.1 | 46.3 |
| | SPE-Eluate 1 | 39.7 | 34.3 | 48.4 | 44.4 |
| | PBS [%] | 6.6 | 7.0 | 10.5 | 9.4 |
| $Abs_{254}$ [$m^{-1}$] | Probe | 127 ± 0.6 | 93.9 ± 0.6 | 28.5 ± 0.7 | 23.5 ± 0.6 |
| | SPE-Filtrat | 69.9 | 50.6[b] | 13.4 ± 1.2 | 12.6 ± 0.3 |
| | SPE-Eluat 1 | 49.7 ± 3.6 | 36.2 ± 1.5 | 14.1[b] | 7.7 ± 2.0 |
| | PBS calculated[c] | 14.9 | 7.1 | 0.9 | 3.1 ± 1.7 |
| $Abs_{254}$ [%] | SPE-Filtrate | 55.0 | 53.9 | 47.2 | 53.7 |
| | SPE-Eluate 1 | 39.1 | 38.6 | 49.6 | 32.9 |
| | PBS [%] | 6.0 | 7.5 | 3.2 | 13.4 |

[a] after the biodegradation test
[b] only one value was used as the second showed too high blank values
[c] Difference: PBS = Sample - (SPE-Filtrate + SPE-Eluate 1)

In some cases, unrealistically high values were found in the first eluates or the SPE-filtrates although the corresponding blank values did not indicate a contamination. In these cases no standard deviation could be calculated. The blank values of the SPE-filtrates and the SPE-eluates were 1.2 ± 0.5 and 0.3 ± 0.2 mg/L DOC, respectively (mean values of five extractions). These blank values were subtracted from the values of the samples. For the samples B and B-b with their extremely low DOC contents no useful results were obtained.

In the following a first error estimation of the SPE method is undertaken. This estimation has a preliminary character, only, as the number of repetitions was limited.

- The standard deviation of the twofold determination of potentially bioaccumulating compounds can be used for this purpose. This is, however, only possible for two of the four samples and results in a standard deviations of ± 0.4 and ± 0.9 mg/L DOC for the samples A and C-b, respectively.

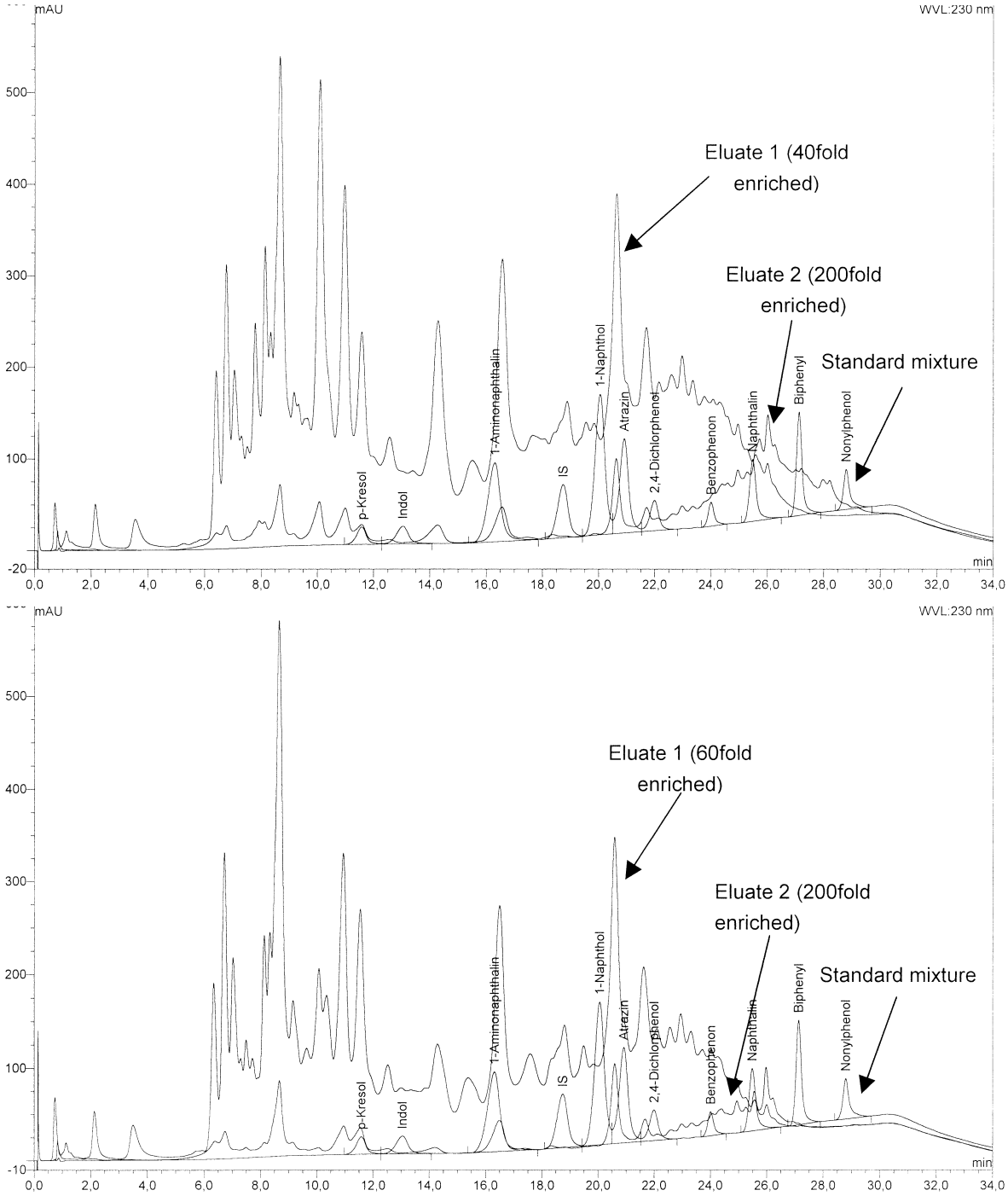
- A more general error estimation of the SPE-method can be performed by considering that the PBS content is calculated as the DOC-difference between the whole sample and the sum of the SPE-filtrate and its first eluate. The error of the PBS-determination result therefore from the sum of the standard deviations of the determination of these DOC of the sample, the filtrate and the eluate. By this approach an error of ± 1.3 mg/L DOC was determined for the determination of the PBS content.

This value is in the magnitude of the PBS contents determined in the effluent samples, which where in a range of 1.4-2.0 mg/L DOC. The PBS determination appears to be sensitive against errors, namely for samples with low PBS-amounts.

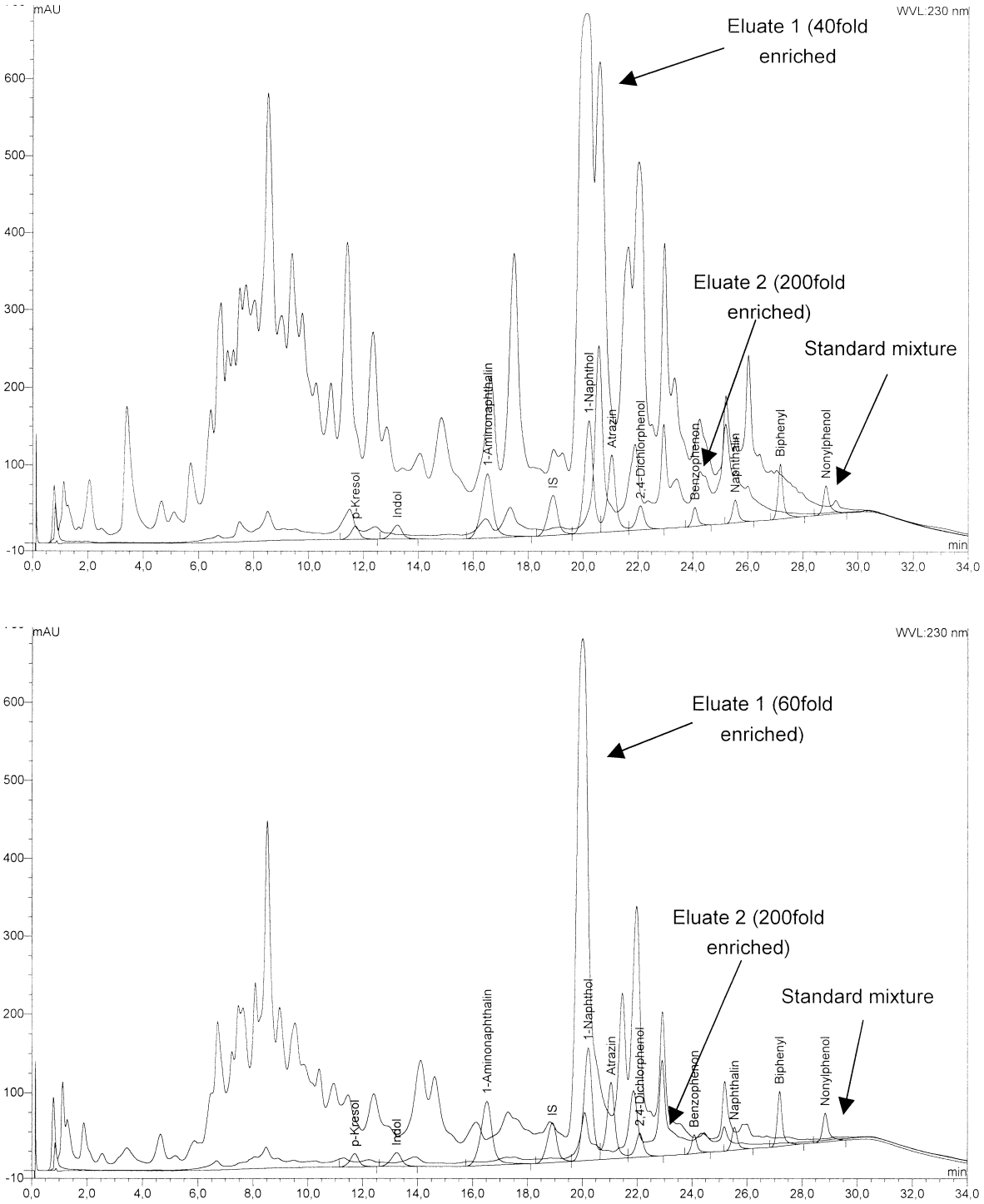
The error of the PBS-determination must therefore be determined more exactly prior to a further application of the SPE-method. It must be determined for which DOC range the method can be applied. In order to decrease the experimental error it is necessary to decrease the variability of the blank DOC. This may be achieved by a twofold conditioning of the solid-phase cartridges prior to their use.

## 8.2 SPE of the Wastewater Samples A and C before and after the Biodegradation Test – HPLC-Chromatograms of the Eluates 1 and 2

The following figures show the HPLC-chromatograms of the two eluates obtained after the SPE of the samples A and C before the biodegradation test as well as after the test. (Annex Fig. 10: and Annex Fig. 11). Additionally the separation of the standard mixture is presented. The log$K_{ow}$-range around 3 in which the substances atrazin, 2,4-dichlorophenol and benzophenone elute covers the retention times from 20.7 to 24.5 min.

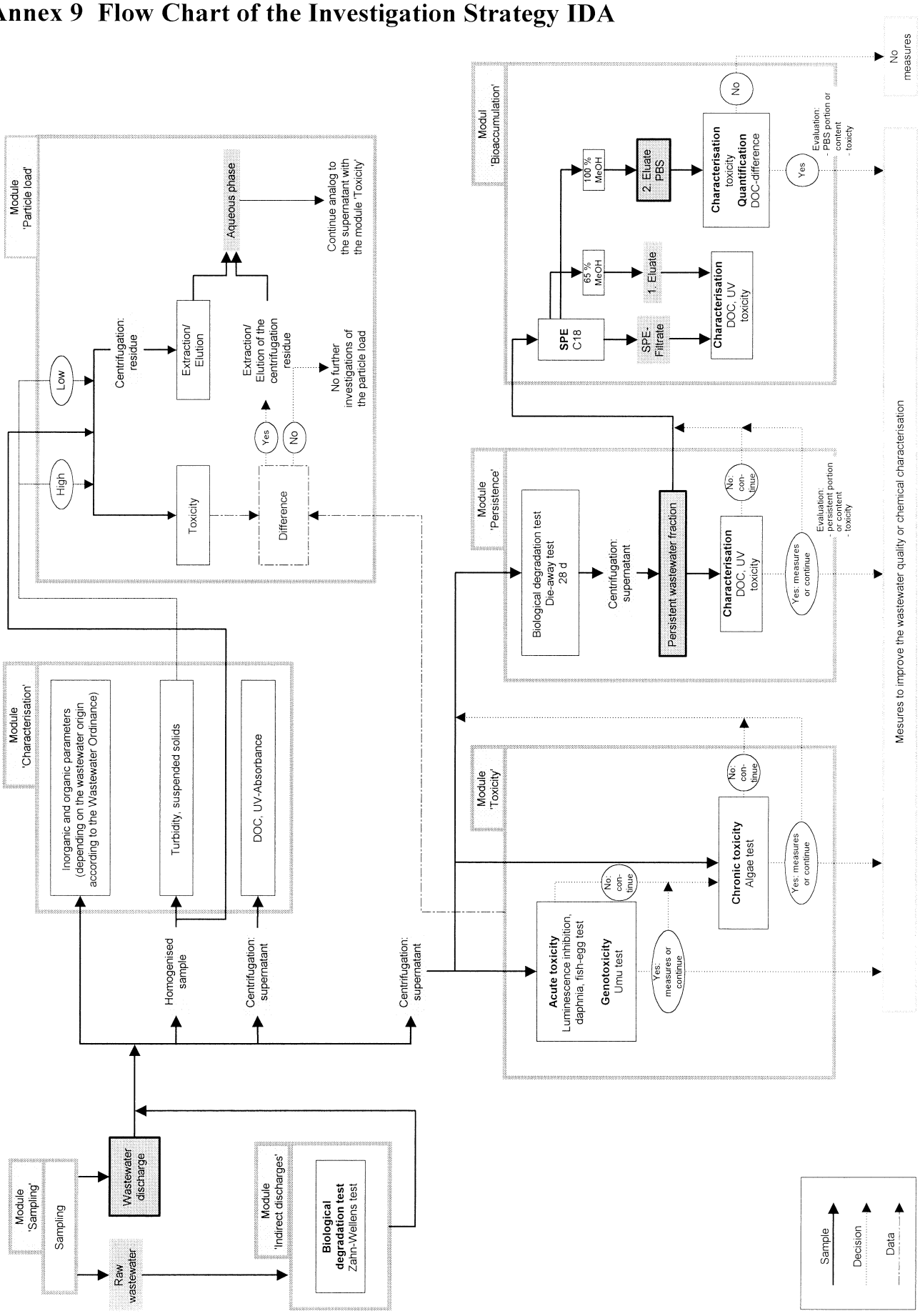



**Annex Fig. 10:** HPLC-Chromatograms of the two eluates of the sample A before the degradation test (above) and after the test (below) after the SPE with two-step elution (230 nm). Eluate 1: 68 % MeOH, Eluate 2: 100 % MeOH




**Annex Fig. 11:** HPLC-Chromatograms of the two eluates of the sample C before the degradation test (above) and after the test (below) after the SPE with two-step elution (230 nm). Eluate 1: 68 % MeOH, Eluate 2: 100 % MeOH

# Annex 9 Flow Chart of the Investigation Strategy IDA




**Annex Fig. 12** Detailed flow chart of the investigation strategy IDA.