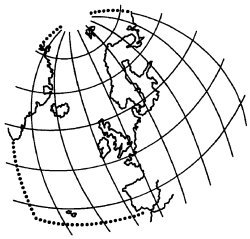
Hazardous Substances Series

OSPAR Practical Study 2005 on Whole Effluent Assessment



OSPAR Commission
2007

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

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

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contents

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Summary

Within OSPAR, it is generally recognised that Whole Effluent Assessment (WEA) has an added value compared with the traditional substance-by-substance approach, especially for complex effluents. By applying WEA to an effluent the PBT (Persistency, Bioaccumulation, Toxicity) criteria of an effluent can be determined in very few measurements, instead of measuring and determining the PBT criteria of all the known and unknown substances in an effluent.

Since 1999 an Intersessional Expert Group (IEG) within OSPAR has been set up to examine the value of WEA in support of the implementation of the OSPAR Hazardous Substance Strategy. In this IEG group a flowchart was recently developed so that the different WEA tests can be carried out in a flexible and cost-effective manner. To achieve this flexibility and cost effectiveness, the flowcharts contain some shortcuts. The rationale behind these shortcuts was tested in the WEA Practical Study 2005. The four shortcuts that were tested are:

1. Is the amount of Potentially Bioaccumulating Substances (PBS) a trigger for the presence of chronic toxicity in an effluent?
2. Can the organic carbon content of an effluent be used as a trigger for the presence of toxicity?
3. Can removal of organic carbon content in a persistency step be used as a trigger for toxicity removal?
4. What is the added value of the biodegradation test for effluents that already have been treated according to the Best Available Techniques (BAT)?

In total 25 effluents were selected by 8 participating Contracting Parties. Acute toxicity for bacteria, algae and crustaceans was measured in all effluents, next to a number of additional tests that were used by the parties, being both acute and chronic tests. Liability to bioaccumulate was measured in 21 effluents with the Solid Phase Micro Extraction (SPME) method. Besides, in some effluents liability to bioaccumulate was measured according the LLE (Liquid-Liquid Extraction) method to Persistency of toxicity was measured in 16 effluents using an “inherently biodegradable” test for indirect effluents or a “readily biodegradable” test for direct effluents. Besides, the organic carbon content (TOC/DOC) of the effluents was measured.

All parameters displayed a wide range of measured values, which allows a robust check on rationale behind the shortcuts within the flowcharts.

For the dataset of 25 effluents tested, the following conclusions can be drawn:

1. In general no strong relationship was found between liability to bioaccumulate and chronic toxicity in the effluents, although for some sectors (e.g. petrochemical sector) the correlation is stronger than for other sectors.
2. Although the effluents with a high organic load displayed higher toxicity for bacteria, algae and crustacean, no clear relationships could be distinguished between organic carbon content and acute toxicity within this dataset.
3. Furthermore, the results showed that organic carbon content removal was higher than toxicity removal after a biodegradation test. In some effluents within the dataset even an increase in toxicity was found after performing a biodegradation test. Further analysis showed that differences exist in the execution of the different types of biodegradation test between participating parties.
4. For the biologically treated effluents in this dataset, a biodegradation step had no added value, since hardly any decrease in toxicity took place.

In this document the facts and data of the practical study are presented. Further evaluation on for instance, technical aspects of the persistency step and its position in the flowcharts will take place in the IEG’s work of 2006/2007. The consequences with regard to the flowchart are incorporated in the WEA Guidance document.

1. Introduction

1.1 Whole Effluent Assessment in general

It is generally recognised that the substance-by-substance approach has some shortcomings. Results from chemical analysis of wastewater samples have shown that only a limited number of substances can be analysed, identified and/or quantified (Gerritsen et al., 2004). Besides, environmental data (P,B,T) are often lacking for a substantial part of the eventually identified substances in an effluent.

This is one of the reasons for the ongoing interest in the development and implementation of biological tests that can be applied to entire environmental samples, like effluents. These tests have already shown that the substances identified can only partly explain the measured adverse effects. This means that a large fraction of the adverse effects in effluents is caused by 'unknown' substances or by the mixture of substances.

Whole Effluent Assessment (WEA) can be defined as the assessment of effluents by using a range of biological methods (P,T) and chemical analyses (B) in order to reveal potential PBT effects. Since the "unchanged" effluent sample is tested, WEA increases the understanding of the combined effects of all known and unknown substances within effluents, especially in complex mixtures.

1.2 WEA in OSPAR

OSPAR's objective with regard to hazardous substances is to prevent pollution of the maritime area by continuously reducing discharges, emissions and losses of hazardous substances, with the ultimate aim of achieving concentrations in the marine environment near background values or close to zero. To achieve this objective OSPAR selects and prioritises substances based on their PBT criteria. These are the criteria that reflect the intrinsic hazardous properties of substances.

In 1997 it was concluded that WEA could be a very valuable addition to OSPAR's objectives on hazardous substances. In 1999 the OSPAR Point and Diffuse Sources working group set up an Intersessional Expert Group (IEG) to examine the value of WEA in support of the implementation of the OSPAR Hazardous Substance Strategy.

Application of WEA is regarded as having added value where the substance-by-substance approach cannot perform an adequate assessment. This will mostly be the case for effluents with a complex composition. When the processes result in 'simple' wastewater with a predictable chemical composition, chemical assessment may provide sufficient information to estimate the environmental impact.

However, for effluents, where for instance side-products are formed that will end-up in the effluent, or production processes are batch-wise, the composition of the waste water is less predictable and many unknown or unidentifiable substances may be present.

1.3 OSPAR Practical study 2005

As a result of earlier IEG workshops a basic flowchart for the application of WEA was proposed. The purpose of this flowchart is to use the different parameters within the WEA toolbox in a flexible and cost-effective manner within the Hazardous Substances Strategy of OSPAR. In order to be flexible and cost-effective, this flowchart contains some shortcuts (tiered approaches) where (cause and effect) relations between parameters may exist. Four different shortcuts were formulated during earlier IEG workshops and were tested in the Practical Study of 2005. The shortcuts and flow schemes are displayed in Figure 1.1.

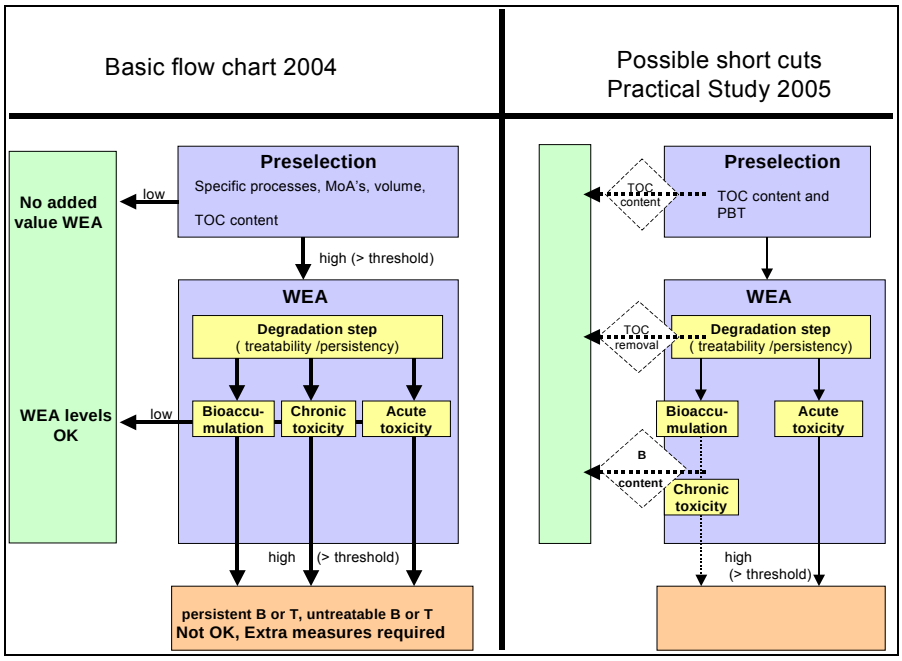

Figure 1.1. Flowchart for the application of WEA, including the shortcuts tested in this practical study

The purpose of the first shortcut, the preselection box is to facilitate the choice of effluents for which WEA has the most added value. The assessment of effluents, for which WEA will not have an added value will not pass through the (complete) flowchart, but will leave the flowchart in an earlier stage.

For these effluents a preselection box is developed in which a tentative assessment of the effluent is made. The outcome of this assessment will be used to determine whether WEA has an additional value for the selected effluent or not.

The other shortcuts are concerned with the choice of tests to be used within the flowchart. Robust relationships between tests or parameters may facilitate the application of WEA and make it more cost-effective.

The two main goals of the Practical study 2005 were:

- Testing the shortcuts in the flowcharts
- Gain more insight in the toolbox

In 2004, different parties within the WEA-IEG GROUP proposed several shortcuts. It was decided that the application of some of these shortcuts would be tested in practice on a selection of effluents within the OSPAR Practical study for WEA in 2005.

The shortcuts to be tested are:

1. Is there a relationship between the organic load of an effluent and the persistent toxic and potentially bioaccumulating load within the same effluent? High DOC/TOC levels in an effluent may be an indication for a complex effluent containing many different organic compounds. This could mean that more potentially bioaccumulating and toxic substances are present in the effluent. Then, high DOC/TOC levels might be a good preselection criterium. However, low levels of DOC/TOC are no guarantee that the (few) substances present are not potentially bioaccumulating or toxic, and may not be ignored, since they may represent the persistent substances after treatment.
2. Is there a relation between the DOC removal and removal of liability to bioaccumulate and toxicity in biodegradation tests? The rationale behind this is that when DOC removal during a biodegradation test is high, the risk that the liability to bioaccumulate and toxicity are still present in the effluent is low. This would indicate that there is no need to execute more toxicity and bioaccumulation tests after the persistency step.
3. Is there a relation between the liability to bioaccumulate and chronic toxicity? The rationale is that chronic toxicity is caused by (persistent) bioaccumulation substances.

4. Which biodegradation test is needed for which effluent? For industrial effluents that received a biological treatment, it can be assumed that all biodegradable substances successfully have been removed. In these cases a “ready Biodegradability” Test would be sufficient in order to assess the potential additional biodegradation in the receiving environment. For indirect effluents it is stated in the IPPC directive as well as in the Water Framework Directive that the effect of an MWTP should be taken into account when determining limit values. Therefore the flowchart for indirect effluents also takes the treatment in an MWTP into account. This will mean that the biodegradation step used for these effluents should simulate the conditions in an MWTP, after which tests for bioaccumulation and toxicity are applied. Test within this category are the “inherent biodegradability” tests, in particular the Zahn Wellens test.

Six Contracting Parties and two industrial organisations participated in this practical study: Netherlands, UK, Belgium, Germany, Portugal, Ireland, Concawe and Arkema. The programme was designed in cooperation with all participants, while the Netherlands had the overall coordination.

Each of the eight participants selected and tested approximately three effluents. Since the programme was designed for research objectives only, the names and locations of the plants concerned will not be made public.

Some of the preliminary results were already discussed during an OSPAR-IEG workshop in London on 28 and 29 November 2005. During this workshop consensus was found for most of the conclusions drawn in this report.

2. Materials and Methods

2.1 Selection of effluents

In total 25 effluents were selected by the participating parties. Each participant was free to contribute with effluents of his or her choice. The focus however should be on complex effluents, as this is the category of effluents for which the added value of WEA is the greatest.

As a consequence, samples should preferably originate from industry categories resulting in effluents from (partly) unknown and (highly) variable composition, like (petro)chemical, pharmaceutical industry and Municipal Wastewater Treatment Plants (MWTP). In addition, the effluents in the data set should contain a wide range of organic carbon content. This was necessary to be able to address some of the questions, raised by the flowcharts. Therefore the participant should measure the organic carbon content of the effluents. Preference was given for TOC measurement above DOC measurement. In Table 2.1, detailed information is given about the effluents that were selected.

2.2 Selection of tests

2.2.1 Toxicity

Just like for the effluents, participants were also free to choose their toxicity tests of choice. In this way, participants performed tests with which they were the most experienced. In all 25 effluents an acute Microtox test, algal test and a test with an invertebrate were performed. Besides that, some participants choose to conduct additional acute and chronic toxicity and/ or genotoxicity tests on their effluents. An overview of the tests that were conducted can found in Table 3.1.

2.2.2 Persistency

Participants were also free to choose their biodegradation test of choice. As agreed earlier by the IEG group, preference was given to an “inherent biodegradability” test (i.e. Zahn Wellens test) for indirect effluents that did not receive any biological treatment before testing. The rationale is that the “inherent-test” is supposed to simulate the biodegradation that may occur in biological treatment step. A “ready biodegradability” test (i.e. DOC die away test) was preferred for direct effluents, which received biological treatment before discharging. With this approach the rationale is that the “ready-test” is supposed to simulate the biodegradation that may additionally occur in the receiving environment.

With respect to the questions concerning the shortcuts within the proposed WEA flowcharts, it was necessary to measure the reduction of organic carbon content as well as toxicity and liability to bioaccumulate in the effluents after the biodegradation test.

2.2.3 Liability to Bioaccumulate

In the WEA Practical study of 2003 the SPME method (Solid Phase Micro Extraction) was used to measure Liability to Bioaccumulate. After the Practical study it was concluded by the IEG group that this method was not robust enough at that moment for implementation in the WEA strategy. Most important shortcomings were that the variation between replicates was too large and that the discriminating power of the method was too small at that moment.

It was therefore decided in 2004 to put up a ring test for both the SPME method and the LLE method (Liquid Liquid Extraction), after adjusted protocols for both methods had been developed (Leslie and Leonards, 2005 a,b). At the start of the WEA Practical study 2005, the bioaccumulation ring test hadn’t started yet. Therefore it was decided that both methods could be used, with an emphasis on the SPME method.

code	Origin	Treatment received	Receiving environment	Flow rate (m^3/day)	TOC (mg/L)	DOC (mg/L)
NI-1	Organic Fine Chemicals（OFC)	biological	Surface water	1350	28	18.7
NL2	OFC	biological	Surface water	1703		67.4
GeB	OFC	biological	Surface water	8000	51.8	48.7
GeD	paper	biological	Surface water	16000	46.1	39.0
GeC	chemical	biological	Surface water	1900	3	1.8
GeA	metal	physical/chemical	MWTP	700	61.5	56.3
Po-1	metal	no treatment	Surface water	3		14.1
Po-2	pharmacy	Physical/chemical	MWTP	183		180
Ir-1	chemical	no treatment	Marine environment	150	110	110
Ir-2	pharmacy	no treatment	MWTP	5	7875	4875
Be-1	MWTP	biological	Surface water	PM	48	52
UK1	chemical	No treatment	Marine environment	5446		1310
UK2	chemical	No treatment	Marine environment	3320		381
Ar-1	chemical	no treatment	Biological treatment	2220	180	
Ar-2	chemical	no treatment	Ozone treatment	350	522	
Ar-3	chemical	no treatment	Biological treatment	2000	2642	
Co1	refinery	biological	Surface water	6624		22.9
Co2	refinery	no treatment	MWTP	ND		222
Co3	refinery	biological	Marine environment	5040		8.2
Co4	refinery	physical/chemical	Marine environment	92160		7.8
Co5	refinery	biological	Marine environment	6480		12.6
Co6	petrochemical	biological	Surface water	25920		10.2
Co7	Refinery	biological	Surface water	ND		12.2
Co8	refinery	biological	Surface water	19152		12.7
Co9	refinery	biological	Marine environment	11520		10.6

Table 2.1. Details of the effluents in the Practical Programme

3. Results

3.1 Organic carbon content effluents

Figure 3.1 shows the organic carbon content of the effluents selected. The TOC content of the effluents displayed a wide range and varied between 3 and 7875 mg/L. Most of the direct effluents are biologically treated, some of them are not, and these are presented as shaded.
As can be seen the organic carbon content of effluents that are biologically treated is in general much lower than effluents that did not have any treatment at all, or only physical/chemical treatment.

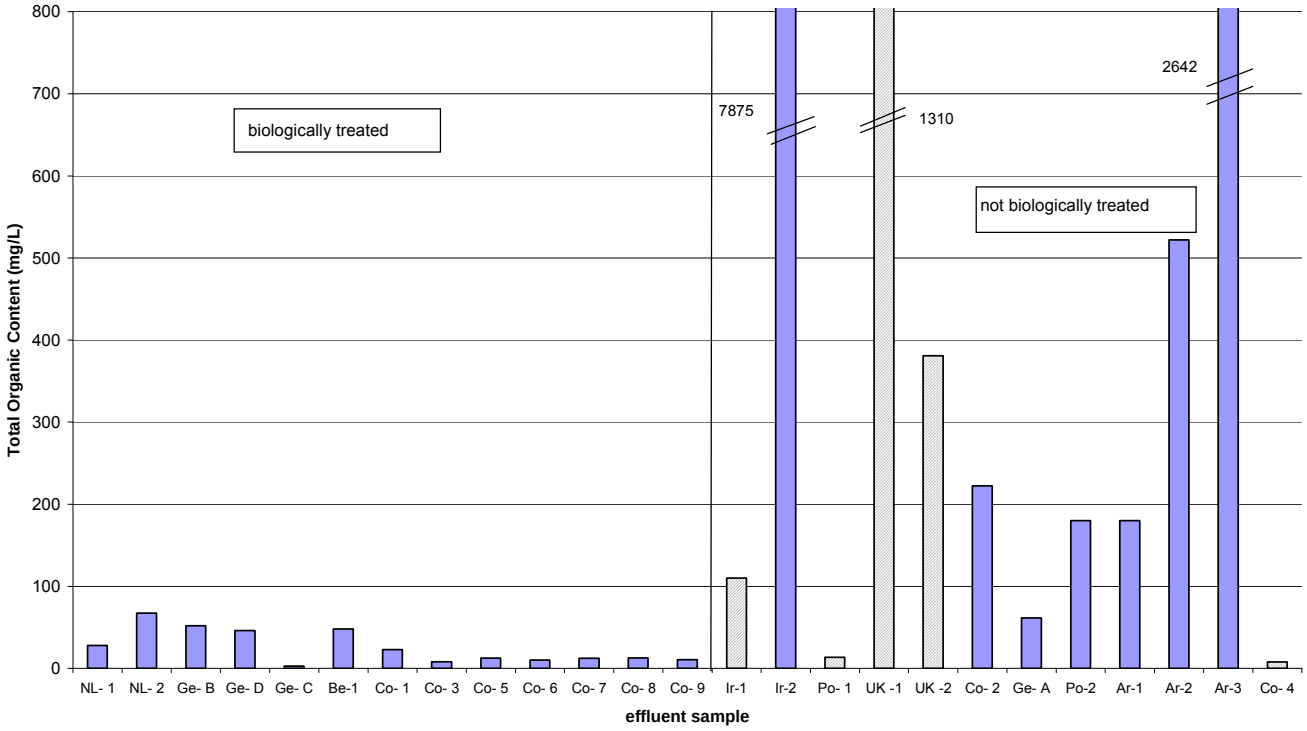

Figure 3.1: Organic carbon content of effluents. The shaded effluents are direct effluents that are not biologically treated.

3.2 Acute toxicity and organic carbon content

Table 3.1 gives an overview of the different tests that were used before the biodegradation tests and the participants that used them in this Practical study. In total 111 toxicity tests were performed, 89 acute tests and 22 chronic tests. Besides that 8 genotoxicity tests were performed. As already mentioned, all effluents were tested with the Microtox test, an algal test and a test with an invertebrate. In most cases the crustacean was *Daphnia magna,* for a few effluents a salt-water species was chosen (*Acartia tonsa* or *Tisbe battagliai*), because of the salinity of the effluent and/or the receiving surface water. For the latter effluents also a saltwater algal species was used (*Skeletonema costatum)*, for the other effluents *Pseudokirchneriella subcapitata* was the most common species. Only Germany used another algal species, *Scenedesmus subspicatus.*

Test	Guideline	Number of tests	Participants	Endpoint
Acute				
Microtox (*Vibrio fischeri)*	ISO 11348-2	25	Nl, UK, Ir, Po, Be, Ge, Arkema, Concawe,	EC_{50} [1]
Crustacean *(Daphnia magna)*	ISO 6341	17	Nl, Ir, Po, Arkema, Be, Concawe	EC_{50}
Crustacean *(Daphnia magna)*	DIN 38412-30	4	Germany	LID and EC_{50}
Algae (*Pseudokirchneriella subcapitata*)	ISO 8692	17	Nl, UK, Ir, Po, Be, Concawe, Arkema	EC_{50}
Algae (*Scenedesmus subspicatus*)	DIN 38412-33	4	Germany	LID and EC_{50}
Fish acute (*Oncorhynchus mykiss*)	OECD 203	6	Belgium, Ireland	EC_{50}
Saltwater Crustacean (*Tisbe battagliai*)	ISO 14669	3	UK, Ireland	EC_{50}
Saltwater Algae (*Skeletonema costatum*)	ISO 10523	3	UK, Ireland	EC_{50}
Fish egg test, *Danio rerio*	DIN 38412-6	4	Germany	LID[3]
Lemna minor	OECD, 1998	2	Portugal	EC_{50}
Salt water Crustacean (*Acartia tonsa)*	ISO 14669	1	Concawe	EC_{50}
chronic				
Fish chronic *Danio rerio*	OECD 212	2	Netherlands	NOEC[2]
Daphnia magna chronic	OECD 211	10	Netherlands, Portugal, Concawe, Germany	NOEC
Rotifer *Brachyonus calycifloris*	ISO 20666	3	Arkema	EC_{10}
Oyster larvae (*Crassostrea gigas)*	Bequalm protocol (2001)	1	Concawe	NOEC
genotoxicity				
Ames	ISO 10993	4	Germany	LID[3]
umu-C	ISO 13829	4	Germany	LID[3]

Table 3.1. Overview of the toxicity tests used

[1]: besides the EC50 also other parameters were reported. However the EC50 could be reported for every effluent. [2] No observed Effect Concentration. [3]Lowest Ineffective Dilution

Figures 3.2-3.4 show the results of the acute toxicity tests run on the effluents for the three tests that were used by most of the participants, namely the Microtox, *Daphnia* and algal test. EC50 values are expressed as volume percentages of the original effluents and are plotted against the TOC content of the effluents. Presented in this way, these figures might illustrate whether relationships exist between the organic carbon content in the samples and the toxicity found in the tests.

Note that the TOC content in all figures is plotted on a log-scale for practical reasons. It should also be mentioned that most effects in the toxicity tests are presented as EC50 values, except for the German tests. For these tests the results are presented as LID values (Lowest Ineffective Dilution). In general, LID values are lower than EC50 values, as they exhibit a smaller effect. An overview of the results of all the tests performed can be found in Annex 1.

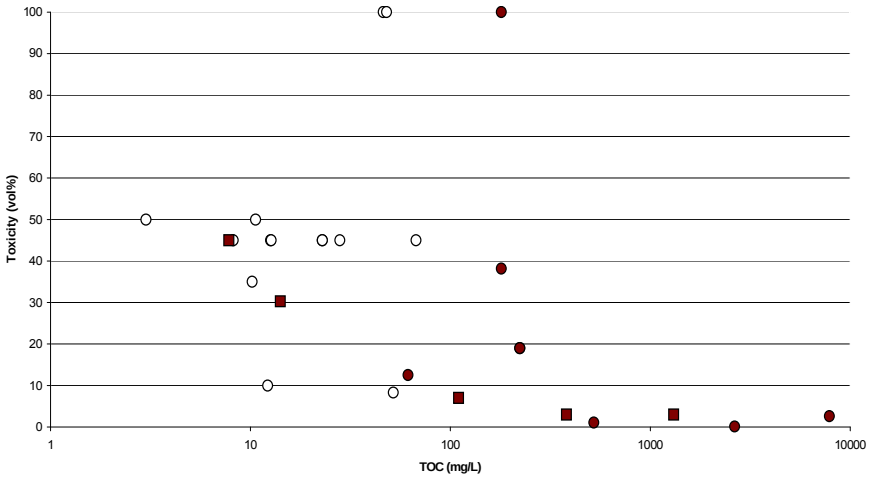

Figure 3.2. Toxicity of the effluents in the Microtox test, plotted against the TOC content of the effluents. The open dots are biologically treated effluents; the closed dots effluents that did not receive biological treatment. The closed squares represent direct effluents that were not biologically treated.

The maximum percentage effluent that normally can be tested in de Microtox test is 45 vol%. This explains the large number of effect concentrations of 45 vol% in Figure 3.2; in these tests no effects were found in the highest concentrations tested. Due to an adapted protocol some participants were able to use higher test concentrations in the Microtox test, which explains the effect concentrations higher than 45 vol%.

The graph shows that no clear relationship exists between the organic carbon content of an effluent and effects in the Microtox test. However, it can be concluded that effluents with a TOC content >100 mg/l have a high probability to be toxic, and that toxicity in effluents with a TOC content <100 mg/l is not so common. On the other hand, some effluents with low organic carbon content show substantial toxicity in one or more tests. A quick analysis on the origin of these effluents cannot always explain these results.

A more striking observation is that, above all, the type of treatment of an effluent, biologically treated or not, appears to be more influential than the organic carbon content of the effluent. Effluents that did not receive biological treatment appear (closed dots) in general to be more toxic than biologically treated effluents (open dots).

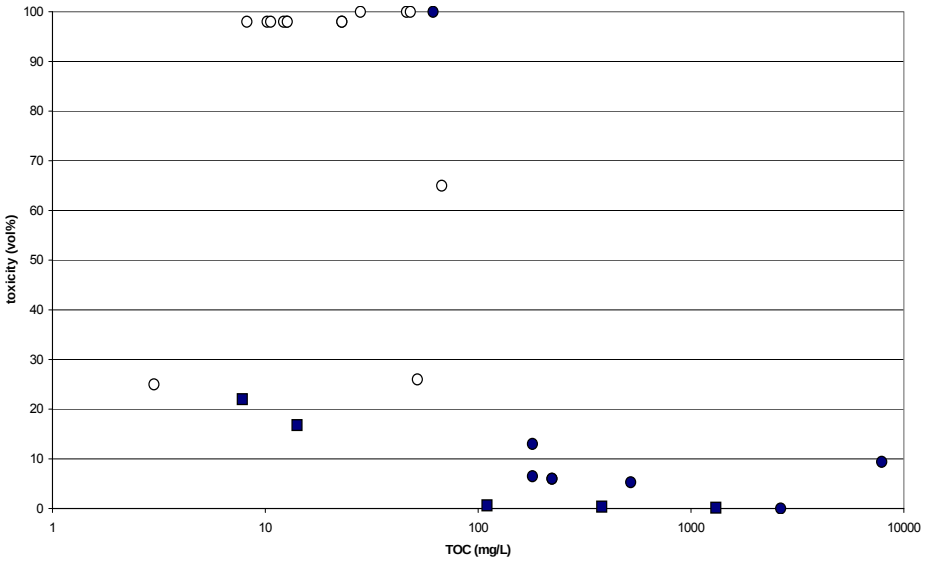

Figure 3.3. Toxicity of the effluents in the algal test, plotted against the TOC content of the effluents. The open circles are biologically treated effluents; the closed circles the effluents that did not receive biological treatment. The closed squares represent the direct effluents that did not receive biological treatment.

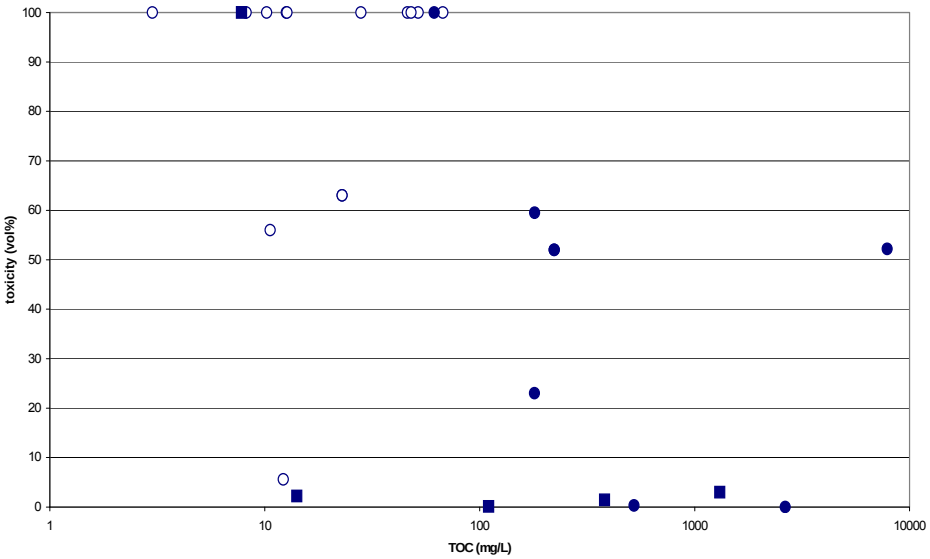

Figure 3.4. Toxicity of the effluents in the crustacean test, plotted against the TOC content of the effluents. The open dots are biologically treated effluents; the closed dots the effluents that did not receive biological treatment. The closed squares represent the direct effluents that were not biologically treated.

3.3 Liability to Bioaccumulate

In 22 effluents the liability to bioaccumulate was measured with the SPME method (Leslie and Leonards, 2005). Figure 3.4 shows the measured content of Potentially Bioaccumulating Substances (PBS) plotted against the TOC content of the effluents. Again, the TOC content is plotted on a log-scale. As can be seen, no strong correlation exists between these two parameters. The results of the LLE method are not shown, as the LLE method required all laboratories to measure very close to limits of detection, and in many cases, no PBS could be identified and quantified.

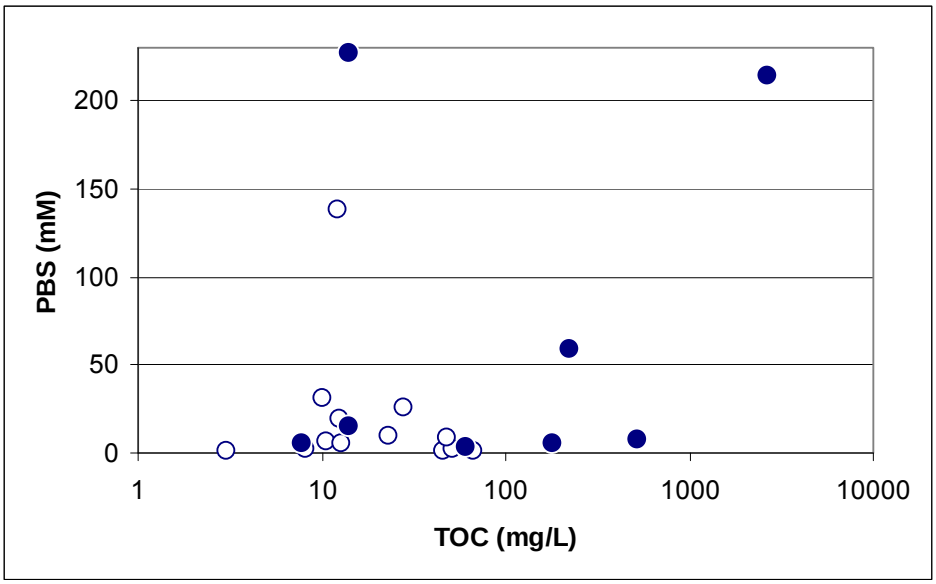

Figure 3.5. PBS content of the effluents plotted against the organic carbon content. The open dots are the biologically treated effluents; the closed dots are the effluents that did not receive biological treatment.

3.4 Persistency

Table 3.2 shows the details of the biodegradation tests that have been used by the different participants. In total, 21 biodegradation tests were performed on 16 effluents. In general, the applied methods were in line with the agreements made before the Practical study: a "readily biodegradable" test for direct effluents that were supposed to be treated according to BAT (Best Available Technique) and an "inherently biodegradable" test for indirect effluents that did not receive any biological treatment before testing. Only Portugal performed an "inherent-like" test on a direct effluent, as they did not have the possibilities to perform a "ready-like" test.

The UK performed two different "ready-like" tests with direct effluents. In the first method, the effluents were degraded in an active manner, by adding minerals and inoculum to the effluent (UK-a), in the second method biodegradation took place on a passive manner, just by aerating the sample with daily shaking (UK-b). Additionally, Concawe decided to perform both an "inherent-like" (referred to as Co-b) and a "ready-like" test (Co-a) on three of their effluents, one was biologically treated (Co1), two were not (Co2 and Co4). Co1 and Co4 were direct effluents, Co2 was an indirect effluent.

Participant	Test used	inoculum	Mineral medium added?	Effluent diluted
Direct effluents				
UK-a	OECD 301E	Secondary effluent of an STP	yes	Yes, Depending on TOC
UK-b	Novel Passive Biodegradation test	None	No	Yes, according to concentration range
Netherlands	OECD301E	Reference surface water	Only if TOC<20 mg/l	1:1
Germany	OECD 301 A	Effluent of an STP	Yes	10% inoculum, 90% effluent
Portugal	Zahn Wellens (ISO 9888)	Activated sludge (0.5 g/L)	yes	Yes, depending on TOC
Concawe	OECD 301E	Reference surface water	yes	Both, Co-2 was incubated 100 and 25%
Indirect effluents				
Germany	Zahn Wellens (OECD 302B, DIN EN 29888)	Activated sludge (1 g/L)	Yes, according to DIN EN 29888	Yes, depending on the concentration of TOC and activated sludge
Portugal	Zahn Wellens (ISO 9888)	Activated sludge (0.5 g/L)	yes	Yes, depending on TOC
Arkema	Zahn Wellens (OECD 302B)	Activated sludge	?	Yes, depending on TOC
Concawe	Zahn Wellens	Activated sludge	yes	Both, Co-2 was incubated 100 and 25%

Table 3.2. Details of the biodegradation tests used by the various participants

The differences in test details between the different "inherently biodegradable" test versions, which were used, are not large. However, between the different "readily biodegradable" test versions, substantial differences exist in the origin of an inoculum, the dilution and the minerals added.

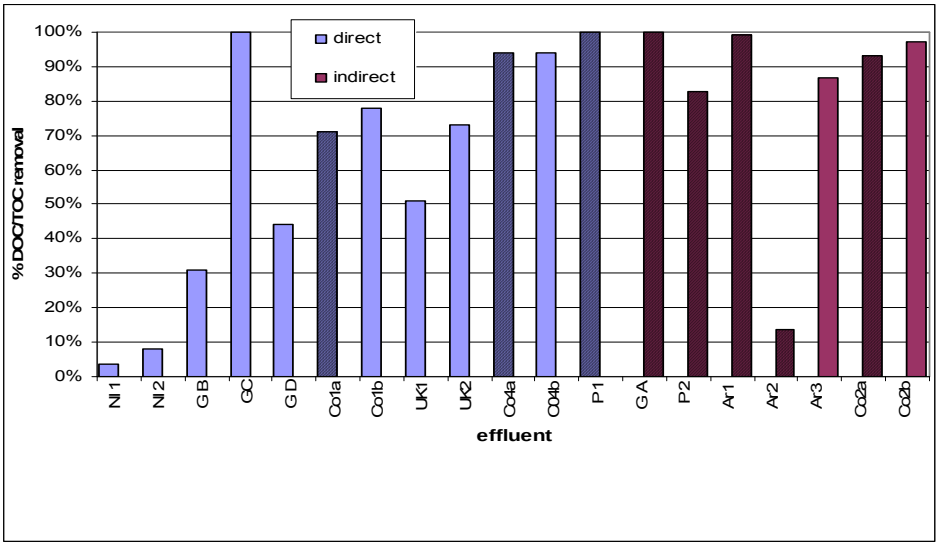

Figure 3.6. Organic carbon content removal in the effluents after a biodegradation test

Figure 3.6 shows the relative DOC reduction (in percentages) that was established during the biodegradation tests. The shaded bars represent the effluents that were treated with an inherent-like test; the blank bars are tested with a ready-like test. Effluents UK1, UK2, P1 and Co4 are direct effluents that don't receive any biological treatment.

In general, DOC reduction during the biodegradation tests is high, except for the Dutch effluents and effluent Ar-2. Effluent Ar-2 will normally pass through an ozone treatment before discharging, a treatment that is more aggressive than biological treatment. This explains the low DOC removal in the "inherent" like test, which is probably not aggressive enough to biodegrade this effluent.

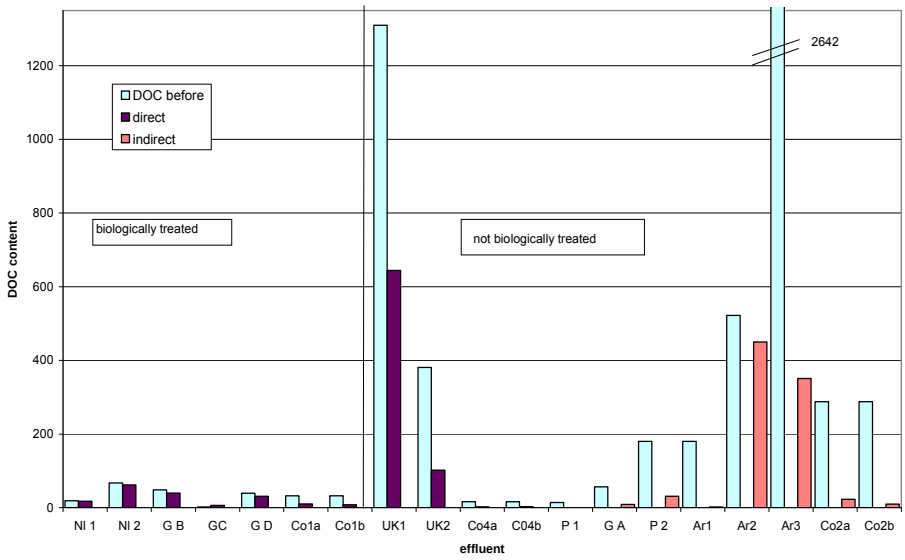

Figure 3.7. DOC content before and after a biodegradation test

Figure 3.7 shows the absolute DOC removal during a biodegradation test. From this figure it is clear that the DOC reduction in the effluents that already received biological treatment was lower than in the effluents that did not receive any biological treatment. Based on the three Concawe samples, which were tested with both test methods, the magnitude of DOC reduction does not seem to depend on the test method used, but on the pre-treatment of the effluent; i.e. biologically treated or not.

Figures 3.8-3.10 show the toxicity removal after the biodegradation steps for the most common tests, i.e. the Microtox, algal and crustacean test. The results are expressed in Toxic Units (TU's). Toxic Units are expressed as 100 vol%/EC50.

The results for the potential to bioaccumulate after a biodegradation test are not taken into account in this report, as the results are too scarce to draw conclusions.

Except for UK1, all effluents showed no or moderate toxicity reduction in the Microtox test as a consequence of the biodegradation step (Figure 3.8). Effluent UK-2 even showed an increased toxicity in both tests, although this increase was much larger in the active than in the passive degradation test.

These minor toxicity reductions are in contradiction with the TOC reductions after these tests, which were, especially for the not biologically treated effluents, substantial.

For the algal test, toxicity reduction was larger than in the Microtox test, especially for the effluents that did not receive any biological treatment (Figure 3.9). The more remarkable were the results for the UK effluents. Both effluents showed an increased toxicity in the active degradation test. The passive degradation test was not completed for these effluents. This increased toxicity, although slightly, was also shown in effluent GD and CO1b.

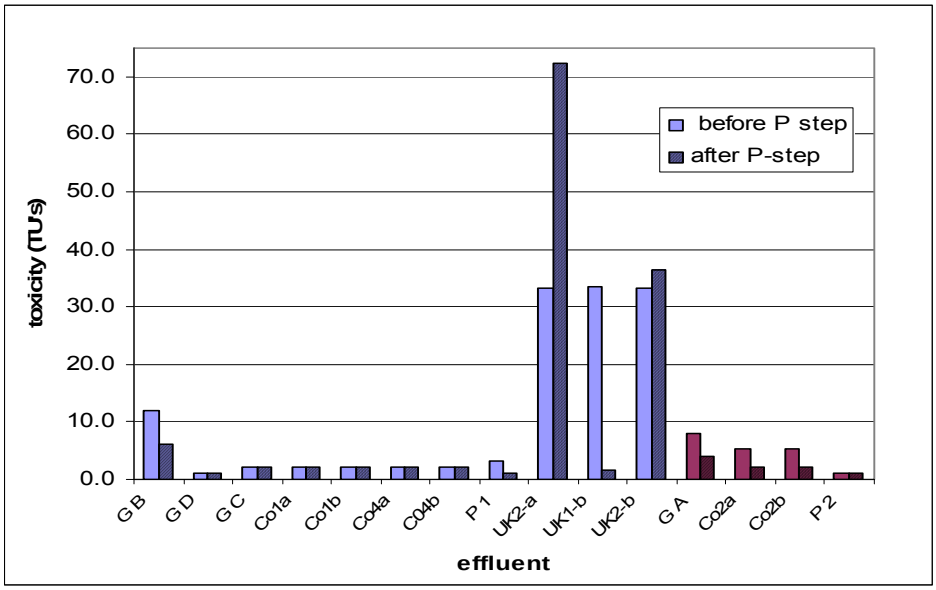

Figure 3.8. Toxicity removal in effluents after degradation step in the Microtox test. Direct effluents are indicated blue; indirect effluents are indicated red.

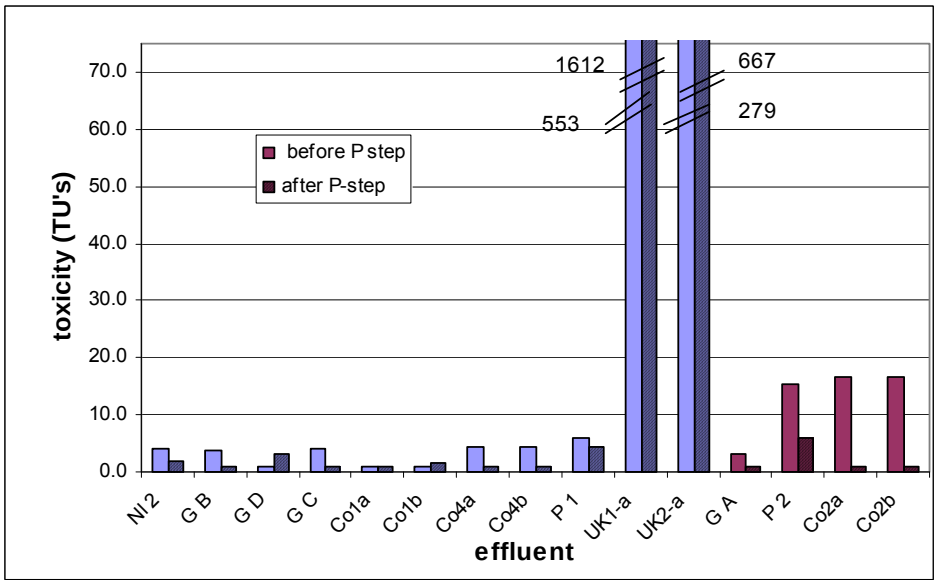

Figure 3.9. Toxicity removal in effluents after degradation step in algal test. Direct effluents are indicated blue; indirect effluents are indicated red.

Enhanced toxicity in the active degradation test for both UK effluents was also observed in the crustacean test (Figure 3.10). In the passive degradation test toxicity was reduced for these two effluents. For all the other effluents, toxicity was already low before the degradation test, except for effluent P1, for which the toxicity was substantially reduced.

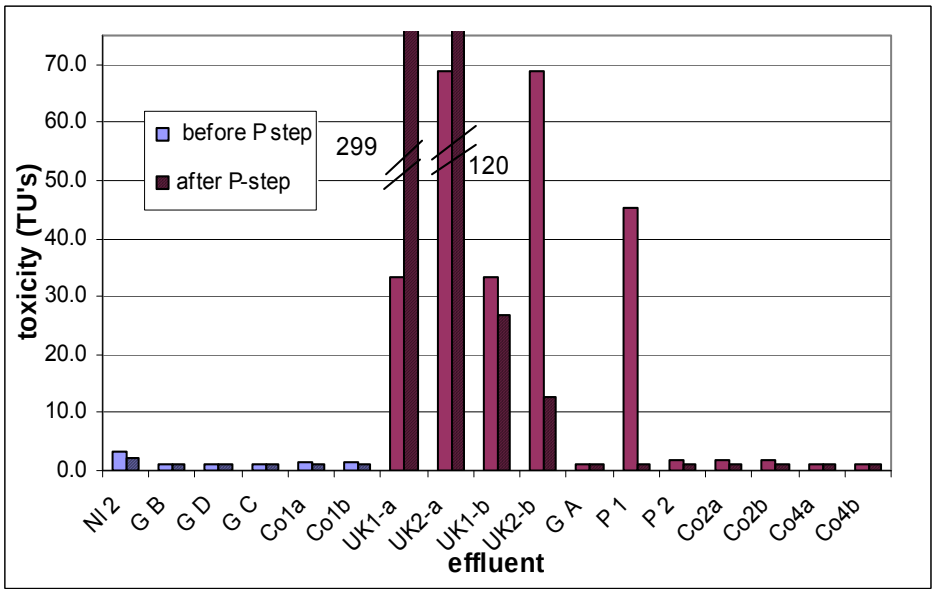

Figure 3.10. Toxicity removal in effluents after degradation step in the crustacean test. Direct effluents are indicated blue; indirect effluents are indicated red.

Figure 3.11 displays the result for the additional toxicity tests that have been performed by the different parties, as well before as after the biodegradation test. This data set includes the following tests: the zebrafish Early Life Stage test for the Dutch effluents, the zebrafish egg test for the German effluents, the *Daphnia magna* chronic test for the Concawe effluents Co1 and Co2, the oyster larvae test for effluent Co4, the *Lemna minor* test for the Portuguese effluents, and the *Brachyonus calycifloris* test for the Arkema effluents. Please note that the bars of effluent Ar-2 and Ar-3 are reduced 1000 respectively 100 times, because of the high toxicity found in these effluents.
In almost all effluents the toxicity was reduced after the biodegradation test, except for effluent GD, Ar1, and Co4, in which toxicity was increased. For GD this phenomenon was observed earlier in the algal test.

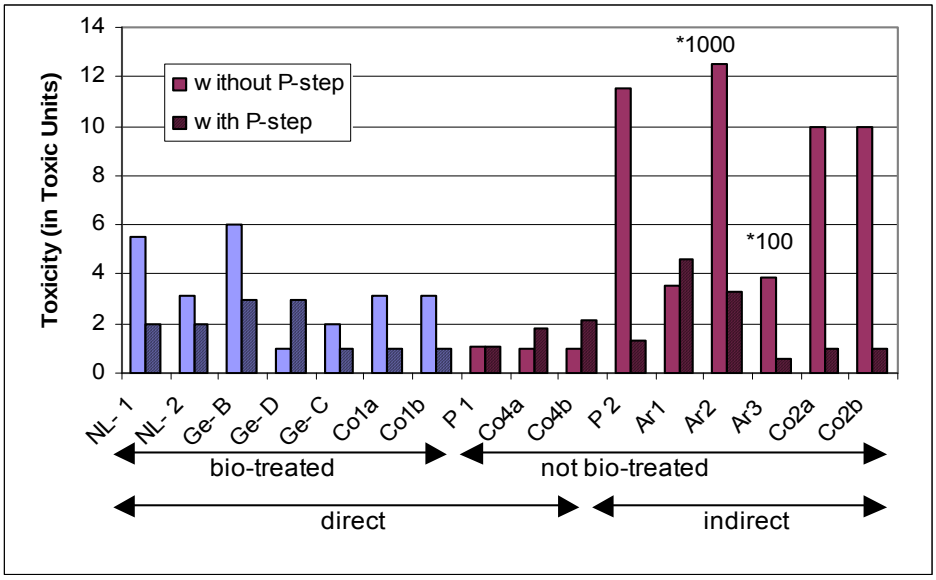

Figure 3.11. Toxicity reduction in other test (see text)

3.5 Liability to bioaccumulate versus chronic toxicity

Figure 3.12 displays the liability to bioaccumulate plotted against the chronic toxicity. Data were available for 15 effluents. For most effluents, chronic toxicity was measured in the chronic *Daphnia magna* test, except for the Arkema influents, which used *Brachyonus calycifloris* and effluent C04 that used the oyster larvae as test species.

Although the data are pretty scarce and scattered, it could be stated that effluents with a relative high PBS content, according to the SPME method, also exhibit high chronic toxicity. An exception to the rule is effluent Ar2, which has a high toxicity and a low PBS content.

It is well known from literature that logKow and (acute) toxicity are strongly correlated for narcotic chemicals. Focussing on the effluents that are supposed to contain a substantial part of narcotic chemicals (petrochemicals and refineries, closed dots) the relation gets clearer, although the dataset becomes even smaller to draw firm conclusions.

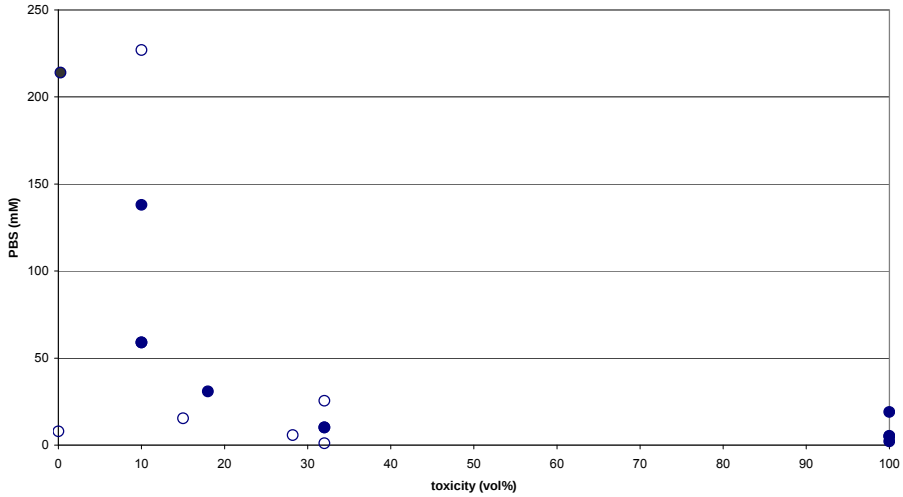

Figure 3.12. Liability to bioaccumulate versus chronic toxicity in 14 effluents

Discussion and conclusions

Effluents
Large differences in both toxicity results, liability to bioaccumulate as in organic carbon content were found between participating parties. An important advantage of these large differences is that the shortcuts in the flow schemes could be tested under a wide range of variables.

Toxicity tests
In total a number of 173 toxicity tests were performed in this practical programme, the majority of them being acute tests. Participants have a preference for acute tests, which is logical from a cost effective point of view. However, this preference hampers within this practical programme one of the questions which was addressed, i.e. the relationship between liability to bioaccumulate and chronic toxicity.
For acute toxicity, three tests (Microtox, algae and crustaceans) are generally accepted, deemed to be robust and therefore applied by all of the participants. All the other tests, acute or chronic, are not so commonly applied, as can be seen in Table 3.1.

Acute toxicity versus organic carbon content
For the three 'standard' tests the data set was large enough to analyse possible relationships between DOC content and toxicity found in the effluents. For this dataset, the toxicity that was observed largely depended on the treatment the effluents had received. In general the effluents that did already receive biological treatment before testing displayed lower toxicity than the effluents that were not biologically treated. As the biologically treated effluents in general also had a lower organic carbon content, it can be stated that in general a positive relationship exists between organic carbon content and toxicity for the three 'standard' tests. For all three tests effluents present in the dataset did not follow this relationship. Metal analyses, the most likely remaining cause for toxicity in these effluents could not always explain these "exceptions".

It can be concluded that the organic carbon content of an effluent may be an indicator for toxicity present in the effluents, and therefore for effluents of possible concern. However, as the dataset shows, effluents with low organic carbon content may also display high toxicity. Therefore, the organic carbon content of an effluent should not be used as a separate "stop-or-go' parameter, but should always be used together with e.g. available specific information about the processes and substances used on the site the effluent originates from.

Organic Carbon Content removal versus toxicity removal after biodegradation test
As expected, the DOC removal after the biodegradation test also merely depended on the treatment already received. For effluents that were not biologically treated before testing, DOC removal was in general larger than for biologically treated effluents. Although being a small dataset (3 effluents out of 25), the effluents which were tested with both a ready-like and an inherent-like test showed that the kind of test is not decisive with respect to DOC removal.

Results of the toxicity tests after the biodegradation test showed that no strong relationship exists between DOC removal and toxicity removal after a biodegradation test. In general it can be stated that toxicity removal is smaller, compared to DOC removal.

For biologically treated effluents, a biodegradation test did not result in decrease of toxicity.

Type of biodegradation test
The results with respect to toxicity removal are puzzling, as in some cases toxicity is increased after a biodegradation step. During the IEG workshop held in November 2005, it was speculated that adding the mineral medium by start of the degradation test might cause toxicity. For some effluents, this might be the case. However, for some effluents this cannot be the case, as no minerals are added (e.g. UK-2b).

Only the type of biodegradation test was an issue in this Practical study. The way these tests were performed was left open to the participants. Overall, it can be said that the way in which the biodegradation tests are performed, differ according to the participating party. A thorough investigation of the differences between these tests should be carried out within the OSPAR-WEA programme for 2006/2007. The results of this Practical study should be used as input or starting point for this investigation.

Biodegradation tests are internationally standardised for single substances. In these tests, the degradation of the parent compound and TOC content is evaluated, which is a relative straightforward process. However, when biodegradation tests on complex mixtures like effluents and surface waters are performed other processes are also taken into account. These processes may involve the formation of more toxic metabolites and/or confounding factors like the formation of ammonium, which may eventually increase the toxicity during the biodegradation test. This increase in toxicity will not be measured when performing the substance by substance approach for these tests. Although an increase in toxicity after the biodegradation test was sometimes shown in this practical program, it was not proven that this was caused by the formation of toxic metabolites or confounding factors. This could be the subject of further research within this field of tests.

Liability to bioaccumulate versus chronic toxicity
In this dataset there was a tendency for a positive relationship between these two parameters, although exceptions were also present. In general, it can be said that the dataset was too small to draw firm conclusions.

Especially within this relationship, the processes involved on the site should be taken into account. For instance, for industry sectors that produce merely organic chemicals with a non-specific mode of action, like refineries, this relationship could be stronger than for other sectors. From literature it is known that relationship exists between acute toxicity and hydrophobicity for compounds with a nonpolar narcotic mode of action, for example originating from gasoline blending processes (Mc Grath et al., 2005). There is also a constant relationship between hydrophobicity and chronic toxicity for this class of compounds, although the data set is smaller than for the relationship between acute toxicity and hydrophobicity (Di Toro et al., 2000). However, for other sites, like within the sector of Organic Fine Chemicals, compounds with specific modes of action may be present in the effluent. In these cases no strong relationship may be found between chronic toxicity and liability to bioaccumulate.

General
In conclusion it can be stated that the practical programme 2005 has given us much more insight in the methodology of Whole Effluent Assessment, especially in the relationship between organic carbon content and toxicity found in effluents, and the shortcomings of biodegradation test within the WEA toolbox. These biodegradation tests will be subject of investigation for the forthcoming year(s).

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Annexes

1. Overview of the results
2. Dutch contribution to the OSPAR Practical study 2005 on Whole Effluent Assessment
3. Results of the "Practical WEA Study" in Germany
4. Contribution to the WEA Practical Study 2005 Report of Portugal
5. Persistence Assessment within OSPAR WEA – UK report 2005
6. Practical <Whole Effluent Assessment> study on 23 Arkema wastewater samples

Annex 1: Overview of the results

Biologically treated effluents		Flow (m^3/day)	TOC - Pstep	DOC -Pstep	TOC/ DOC -Pstep	TOC/ DOC %	bac	alg	Crust.	Fish (egg)	In-verte-brate	SPME	Umu	Ames	persis tency	bac	alg	crust	Fish (egg)	lemna	In-vertebr ate	Umu	Ames	SPME
NL- 1	OFC	1350	28	18.7	18	4%	45	100	100	18	32	25.45	-	-	ready				50					
NL- 2	OFC	1703		67.4	61.9	8%	45	65	100	32	32	1.02	-	-	ready		50		50		50			
G-B	OFC	8000	51.8	45.9	39.8	31%	8.3	25	100	16.6		2.42	neg.	pos.	ready	16.6	100	100	33.3			neg	pos	2.45
G D	paper	16000	46.1	39.0	31.2	44%	100	100	100	100		1.27	neg.	neg.	ready	100	33	100	33.3		100	neg	neg	1,62
G C	chemical	1900	3	1.8	6.4	-	50	25	100	50		1.2	neg.	neg.	ready	50	100	100	100			neg	neg	2.66
Be-1	STP	PM	48	52		-	100	100	100	>100		8.4	neg.	-	-									
Co- 1	refinery	6624		22.9		71%	45	98	63	-	32	10.1	-	-	ready	45	98	100			100			
Co- 1	refinery	6624		22.9		78%	45	98	63	-	32	10.1	-	-	inher ent	45	64	100			100			3
Co- 3	refinery	5040		8.2		-	45	98	100	-	100	2.1	-	-	-									2.1
Co- 5	refinery	6480		12.6		-	45	98	100	-	100	19	-	-	-									
Co- 6	petrochemic al	25920		10.2		-	35	98	100	-	18	30.8	-	-	-									
Co- 7	?	ND		12.2		-	10	98	5.6	-	10	138	-	-	-									
Co- 8	?	19152		12.7		-	45	98	100	-	100	5	-	-	-									
Co- 9	?	11520		10.6		-	50	98	56	-		6.9	-	-	-									

Not biologically treated		Flow (m^3/day)	TOC -Pstep	DOC -Pstep	TOC/ DOC -Pstep	TOC/ DOC %	bac	alg	Crust.	Fish (egg)	lemna	Chronic Daphnia	SPME	UMU	Ames	Persis-tency	bac	alg	crust	Fish (egg)	lemna	Inverte brate	UMU	Ames	SPME
Ge- A	metal	700	61.5	56.3	9	85%	12.5	33	100	50	-		3.12	neg.	neg.	inherent	25	100	100	100			neg	neg	1.45
Po- 1	metal	3		14.1	0	100%	30.3	16.8	2.2	-	>90	10	227	-	-	inherent	100	22.5	90		90				
Po-2	pharmacy	183		180.1	31.3	83%	100	6.5	59.5	-	8.7	15	15.4	-	-	inherent	100	16.9	85		77.5				
Ir-1	chemical	150	110	110	-	-	7	0.62	0.12	4.2	-	43		-	-	-									
Ir-2	pharmacy	5	7875	4875	-	-	2.6	9.4	52.2	>32	-	0.61		-	-	-									
UK -1	chemical	5446		1310	644.5	51%	2.99	0.181	3	-	-	0.22		-	-	ready	45	0.062	0.335						
UK -2	chemical	3320		381	101.7	73%	3.02	0.359	1.45	-	-	0.46		-	-	ready	1.38	0.15	0.832						
Ar-1	chemical	2220	180		1.62	99%	38.2	13	23	67	-	28.2	5.8	-	-	inherent						21.6			
Ar-2	chemical	350	522		450	14%	1.1	5.3	0.33	7.7	-	0.008	7.85	-	-	inherent						0.03			
Ar-3	chemical	2000	2642		351	87%	0.13	0.009	0.01	1	-	0.26	214	-	-	inherent						1.8			
Co- 2	refinery	ND		222.4		93%	19	6	52	-	-	10	59	-	-	ready	45	93	45			100			0.5
Co- 2	refinery	ND		222.4		97%	19	6	52	-	-	10	59	-	-	inherent	45	92	100			100			1.6
Co- 4	refinery	92160		7.8		94%	45	22	100	-	-	100	5.3	-	-	ready	45	98	100			56			1.5
Co- 4	refinery	92160		7.8		94%	45	22	100	-	-	100	5.3	-	-	inherent	45	98	100			46			1.6

Annex 2: Dutch contribution to the OSPAR Practical study 2005 on Whole Effluent Assessment

by Erwin Roex

1. Introduction

As a contribution to the OSPAR Practical study 2005 on Whole Effluent Assessment, two effluents originating from the sector "Organic Fine Chemicals' were sampled. As plants from these sectors produce complex effluents with varying and unknown composition, WEA has an added value for these effluents.

Effluent NL-1 originates a producer from flavours and fragrances, Effluent NL-2 originates from a plant that produces anti-infectives and food specialities. Both effluents received a biological treatment on the site before discharging. Effluent NL-1 discharges to a lake, NL-2 into the marine environment.

2. Material and Methods

Effluents
Effluent NL-1 was sampled on the 1-8-2005, effluent NL-2 was sampled on 7-9-2005. Both samples were transported to the contract lab in a cooled van as soon as possible. Details about the two effluents at the sampling date are given in Table 2.1.

Effluent	Flow rate [m^3/d]	TOC [mg/L]	DOC [mg/L]	COD [mg/L]	SS [mg/L]
NL-1	1350	28	18.7	134	28
NL-2	1703	-	67.4	-	-

Table 2.1. Details of sampled effluents

Toxicity tests
Both effluents were tested with three acute and two chronic toxicity tests. A short description of these tests is given below.

Microtox® test
Toxicity test with the bacterium *Vibrio fischeri* was executed according to ISO-guideline 11348-3 (1998). In this test the natural light production (bioluminescence) of this bacterium determined with a spectrophotometer. This bioluminescence is closely coupled to the metabolic activity. Possible reduction of bioluminescence is determined in four dilutions of effluent: 45, 22.5, 11.25 and 6.25 vol.% effluent after 5, 15 and 30 minutes of exposure. EC20 and EC50 values are determined in duplicate treatments with the software belong to the Microtox® testsystem.

Algae test
The effluents were tested with the green algae *Pseudokirchneriela subcapitata* according to ISO-guideline8692 (1998). Growth inhibition (μ) was determined after 72 h of exposure to 5 dilutions of effluent. The EC_{50} was determined according to Dunnett's test and Maximum Likelyhood Probit Method. The test was conducted in a 48-wells polystyrene plate, after filtration over a 0.45μm filter. The test volume was 1 ml. The cells were counted with a fluorescence microplate reader.

Daphnia magna test
The acute toxicity test with the water flea *Daphnia magna* was performed according to ISO guideline 6341 (1996). Effect parameter (EC50) was immobility. Test duration was 46 hours. The test was executed in 5 dilutions of effluent with ISO medium. Every treatment had four replicates, every replicate containing 5 individuals per test container. The organisms were <24 hours old at the start of the test. Animals were not fed during the test. The EC_{50} was determined according to Dunnett's test and Maximum Likelyhood Probit Method.

Early Life Stage test with *Danio rerio*
The chronic toxicity was tested with the Early Life Stage (ELS) test, using zebrafish (*Danio rerio*) as a test organism. The test was performed according to the shortened RIZA version of OECD protocol 210

(1992) with an alternative length of 8 days. Effect parameters are survival, hatching and abnormalities, expressed as NOEC.

Fertilized zebrafish eggs and hatched larvae are exposed to 5 dilutions of effluent. Per concentration three replicates are used, each containing 25 eggs, 4 hours old, at the start of the experiment. After 24 hours the number of fertilized eggs is determined. The test medium is refreshed three times a week and dead eggs and /or larvae are removed. Fish are not fed during the test. The test temperature is 25±2°C.

Chronic test with *Daphnia magna*
The chronic test with the water flea *Daphnia magna* was performed according to OECD guideline 211 (1998). Effect parameters are survival and reproduction, expressed as NOEC. Organisms are exposed to 5 dilutions of effluent, each dilution containing 10 separate replicates with one individual (<24 hours old). The dilution medium is Elendt medium. Two-three times per week the test medium is renewed and both adults and offspring were counted. After renewal, animals were fed with unicellular algae. The test was terminated when the control adults had hatched three times.

Liability to bioaccumulate
Both effluents were tested on the amount of Potentially Bioaccumulating Substances (PBS) with the Solid Phase Micro Extraction (SPME) method according to the protocol of Leslie and Leonards (2005). Results were referred to the external standard 2,3 dimethylnaphtalene.

Persistency
Since both effluents are direct effluents that are directly discharged into the surface water both effluents were further biodegraded in a modified “DOC-die away” test, according to OECD guideline 301E.

The effluents were diluted 1:1 with surface water, which is supposed to be relative clean. This surface water serves as an inoculum for eventual further biodegradation. This mixture is incubated in the dark at a temperature of 15°C. During this incubation the sample is aerated continuously to stimulate biodegradation. At day 0, 3, 7, 14, 21, and 28 DOD levels are measured in the mixtures. Three control treatment run along with the sample:

1. a treatment with only surface water to correct for the DOC content of the surface water (blank)
2. a treatment with surface water/effluent mixture and a reference substance to check the activity of the mixture (inhibition control)
3. a treatment with surface water and a reference substance to check the activity of the inoculum (reference)

As a reference, sodium acetate is used.

After 28 days the biodegradation test was terminated, and the test(s) that were the most sensitive before the biodegradation test were tested again, thus assuming that the effluent/surface water mixture would only lose toxicity, and not gain toxicity during the biodegradation test.

Effect concentrations after the biodegradation test were corrected for the dilution with surface water.

3. Results

Toxicity
Table 3.1 displays the result for the toxicity tests performed on both effluents. Results are expressed as volume percentages effluent. As can be seen, the acute toxicity of both effluents was relative low. Chronic toxicity was moderate for both effluents. Based on these results, it was decided to conduct the zebrafish ELS test for NL-1 and the algal test, chronic *Daphnia magna* test and the zebrafish ELS test for NL-2 after the biodegradation test.

	Acute toxicity			Chronic toxicity	
Effluent	Microtox [EC50]	Algal test [EC50µ]	Daphnia [EC50]	Daphnia [NOEC]	Zebrafish [NOEC]
NL-1	>45	>98	>100	32	18
NL-2	>45	65 (56-80)	>100	32	32

Liability to bioaccumulate

Table 3.1. Toxicity in effluents

The PBS values were 24.45 nM for effluent NL-1 and 1.02 nM for effluent NL-2. The value for NL-1 is relative high for Dutch standards.

Persistency
Figure 3.1 shows the results of the DOC measurements during the test.

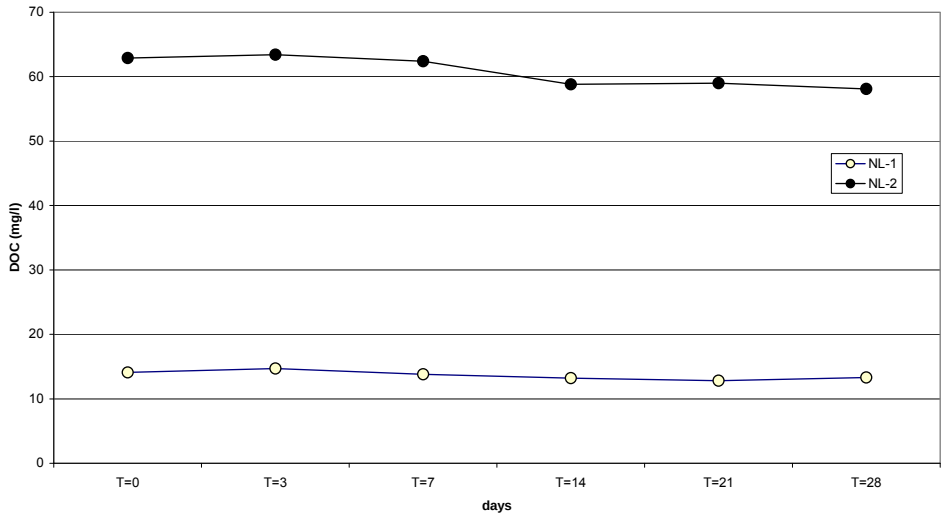

Figure 3.1. DOC removal during biodegradation test.

The DOC values are corrected for the DOC measurements in the blank controls As can be seen, the DOC reduction in both effluents is low. DOC reduction in the inhibition control was 94.8% for effluent NL-1 and 95.9% for effluent NL-2, both measured after 14 days. This indicates that the effluent/surface water mixture was able to biodegrade substances. However, the DOC, still present in the effluents after being biologically treated appeared to be non-degradable and persistent.

Table 3.2 presents the results of the toxicity tests that were performed after the biodegradation test.

Table 3.2. Toxicity of the effluents after the biodegradation test

effluent	Algal test [EC50]	Chronic Daphnia test [NOEC]	Zebrafish ELS test [NOEC]
NL-1	-	-	>50
NL-2	>50	>50	>50

These results might suggest that all the toxicity have disappeared after the persistency step. However, one should bear in mind that the effluents are diluted 1:1 in the biodegradation test, which causes already a “reduction of toxicity” with 50%. Therefore, the highest concentration tested is 50% effluent, which is close to the original effect concentrations before the degradation test. As for a few tests, with NOEC values of 32 vol. %, only effects were found in the highest concentration of the original effluent, i.e. 100%. This concentration cannot even be tested after degradation.

In conclusion, it can be stated that this method of measuring persistency is not suitable for effluents with low or moderate persistency.

Annex 3: Results of the "Practical WEA Study" in Germany

by Stefan Gartiser, Sven Oeking, Yvonne Ziser and Dr. Christoph Hafner,
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Supported by
Dr. Albrecht Paschke, UFZ-Centre for Environmental Research Leipzig

Contents

1. Participants

The investigations carried out within the OSPAR Whole Effluent Practical Study were sponsored by the German Environmental Agency as part of the project "Applicability of bioassays for the controlling of waste water discharges within OSPAR's strategy on hazardous substances (R & D Project No FKZ 205 44 324/01 from May 01, 2005, to April 30, 2007).

Ecotoxicity, genotoxicity and *(bio)degradability* tests were performed in the laboratory of the Hydrotox GmbH (Dr. Christoph Hafner, Sven Oeking). *Bioaccumulation tests* were done at the UFZ-Centre for Environmental Research Leipzig (by Dr. Albrecht Paschke).

2. Description of the samples

In total, four wastewater samples were investigated before and after a biodegradation step, of which one is indirectly discharged and three directly.

a. Metalworking industry, indirect discharges

The sample comes from the south-west part of Germany. The company produces around 400 000 automobiles each year with 35 000 employees. The production comprises the carriage, the pressing of sheet metal, the finishing and the assembling of the cars. The water cycle within production is almost closed. While about 1 million m^3 of water is used per day, only about 600-800 m^3 per day are discharged to a municipal wastewater treatment plant.

Before discharging, the wastewater from technical processes passes through a central chemical/physical treatment plant within the factory. The treatment steps consist of a mixing basin for neutralisation of pH, the addition of flocculation additives, a precipitator for elimination of particles, and a gravel filter. Besides the wastewater from the production, about 2500 m^3 per day of sanitary wastewater are discharged to the municipal treatment plant, but this is not considered within this study. A 24-h mixed sample was taken on June 30th, 2005.

The municipal treatment plant has a capacity of 250 000 inhabitant equivalents and purifies around 33 000 m^3/d (dry weather discharge). It consists of several primary sedimentation basins and trickling filters with a downstream de-nitrification. Elimination efficiency is around 90% for COD and phosphorus, and 70% for nitrogen.

COD	mg/L	259	Aluminium	mg/L	range <0.8 – 3.3
			Cadmium	mg/L	<0.05
TOC	mg/L	61.5 (this sample)	Copper-ion	mg/L	<0.05
DOC	mg/L	56.3 (this sample)	Chromium-ion	mg/L	<0.05
NH_4-N	mg/L	2.5 (this sample)	Ferrous	mg/L	0.63
NO_2-N	mg/L	range 4-9	Lead	mg/L	<0.05
NO_3-N	mg/L	0.1 (this sample)	Nickel	mg/L	<0.05
PO_4-P	mg/L	< 3.0	Zinc	mg/L	<0.05
conductivity	mS/cm	7.1 (this sample)	pH	8,1	
AOX	mg/L	range 0.23-0.36	Sulphate	mg/L	range 291-367
Mineral oil	mg/L	<0.13	Fluoride	mg/L	range 18-27
Suspended solids	mg/L	42	Cyanide	mg/L	<0.005

Table 1. Chemical analysis of sample A

b. Speciality chemical industry, direct discharges after treatment

The factory is situated in the south-west of Germany and produces specialities like dyes and pigments for paper and inkjet printing, polymers and varnishes. Additionally, optical brighteners and antimicrobials are produced. In total around 350 different chemicals are synthesised batch-wise, while there are only a few continuous processes. Wastewater from batch processes with known recalcitrant COD (from Zahn-Wellens test results) are nanofiltrated or extracted and then passed to the central treatment plant, the concentrates being burnt. The central treatment plant consists of a neutralisation stage, a flocculation/precipitation stage, a primary sedimentation basin and an activated sludge aeration basin. In the treatment plant sanitary wastewater from the factory, and municipal wastewater from the local township, which are fed separately, are also clarified. The combined treatment of industrial and municipal wastewater has advantages for the supply of nutrients. Around 4 million m^3 of wastewater are treated per year, half of it belonging to the factory and half to the township. More than 90% of the TOC

load and 99% of the total AOX load before treatment can be attributed to the industrial wastewater. The efficiency of the biological treatment plant (inlet-outlet of the activated sludge basin) is about 80%.

A 24 h mixed sample was drawn from the outlet of the final clarifier of the treatment plant on June 22nd, 2005. During the sampling day about 8000 m^3/day were discharged into a river (river flow about 1000 m^3/sec). The wastewater contains a high concentration of salts, especially chlorides and sulphates (around 1-3 g/l each).

The wastewater discharge permit considers also ecotoxicity tests with algae, Daphnia and *Vibrio fischeri* bacteria according to Annex 22 of the German wastewater ordinance.

COD	mg/L	247 (this sample)	AOX	mg/L	range 0.75 - 1.5
TOC	mg/L	51.8 (this sample) range 75 - 100	Conductivity	mS/cm	9.78 (this sample)
DOC		48.7 (this sample)	pH		8.1 (this sample)
NO_2-N	mg/L	0.06 (this sample)	PO_4-P (total)	mg/L	0.38 (this sample)
NO_3-N	mg/L	1.86 (this sample)	Sulphate	mg/L	1740 (this sample) range 1000 - 3000
NH_4-N	mg/L	1.6 (this sample)	Cobalt	mg/L	range 0.02 – 0.07
Chloride	mg/L	1998 (this sample) range 1000 - 3000	Chromium	mg/L	range 0.05 – 0.22
Bromide	mg/L	24.3 (this sample)	Copper	mg/L	range 0.08 - 0.35
Jodide	mg/L	1.36 (this sample)			

Table 2. Chemical analysis of sample B

c. Chemical industry, directly discharged after treatment

The factory is situated in the south of Germany and produces predominantly inorganic special chemicals. **For confidentiality reasons the more detailed description of product patterns is still being discussed with the company and will be integrated in the report later**. Depending on their particular origin, wastewater partial streams are passed through different chemical/physical or a biological treatment plants. Phosphoric acid, ammonium/urea, acetic acid and alcohols have to be added from external sources for the biological treatment process. The 24-h mixed sample was taken on June 16th, 2005 from the outlet of the biological treatment plant, where about 1900 m^3/d wastewater are purified. COD concentration (mean) is about 300 mg/l in the inlet and 15 mg/l in the outlet, respectively.

COD	mg/L	14.6 (this sample)	AOX (filtrated)	mg/L	0.05 (this sample)
BOD	mg/L	2.0 (mean)	pH		8.0 (/this sample
TOC	mg/L	3.0 (his sample)	Conductivity	mS/cm	1.126 (this sample)
DOC	mg/L	1.8 (this sample)	Chloride	mg/L	250 (mean)
NO_3-N	mg/L	1.2 (this sample)	Mercury	mg/m^3	0.11 (mean)
NH_4-N	mg/L	0.2 (this sample)	Titanium	mg/m^3	50 (mean)
PO_4-P (total)	mg/L	0.49 (this sample)	Zirconium	mg/m^3	28 (mean)

*) Company has been asked to provide further data on chemical analysis

Table 3. Chemical analysis of sample C *)

d. Paper industry, direct discharges after treatment

The paper mill is situated in the south-west part of Germany and produces coated papers for higher quality applications. The cellulose resources were supplied by ground wood pulp and ready-to-use cellulose pulp. Recycling paper is not used. The ground wood pulp is bleached with sodium hydroxide and hydrogen peroxide. The wastewater is passed to a biological treatment plant belonging to the factory, where municipal wastewater from township is also purified. The combined treatment of paper and municipal wastewater has advantages in the supply of nitrogen and phosphorus as nutrients, which also have to be added from other external sources (urea and phosphoric acid). The hydraulic load from the paper mill is about 12 000 m^3/d, the municipal wastewater adds up to 4000 m^3/d, but only up to 10% of the total COD load. The treatment plant consists of a mixing basin, where iron chloride is added for flocculation and precipitation, and several activated sludge basins and biological trickling filters. A 24-h mixed sample was drawn on June 22nd, 2005 from the outlet of the final clarifier basin.

The results of chemical analysis (performed with the sample and complemented with typical values from other samples) are given in Table 4.

COD	mg/L	132 (this sample) 77-165 (range)	AOX	mg/L	0.03 (typical value)
BOD	mg/L	4 (this sample)	conductivity	mS/cm	1.453
TOC	mg/L	46.1 (this sample)	pH		8.1
DOC	mg/L	39.0 (this sample)	PO_4-P (total)	mg/L	0.67 (this sample)
NO_2-N	mg/L	0.11 (his sample)	SO4	mg/L	range 268 - 300
NO_3-N	mg/L	1.5 (this sample) range 0.7 – 3.4	filtratable dry solids	mg/L	range 4 - 22
NH_4-N	mg/L	1.18 (this sample) range 0.1 – 2.6			

*) Company has been asked to provide further data on chemical analysis

Table 4. Chemical analysis of sample D *)

3. Test methods

According to the WEA concept, the PBT-criteria (**p**ersistency, **b**ioaccumulation and **t**oxicity) should be assessed. The toxicity should be tested by acute as well as chronic tests, the option for determining genotoxic effects was given as additional further tests. The overall test concept consisted in coupling the effect-based tests with biodegradation tests (see Figure 1).

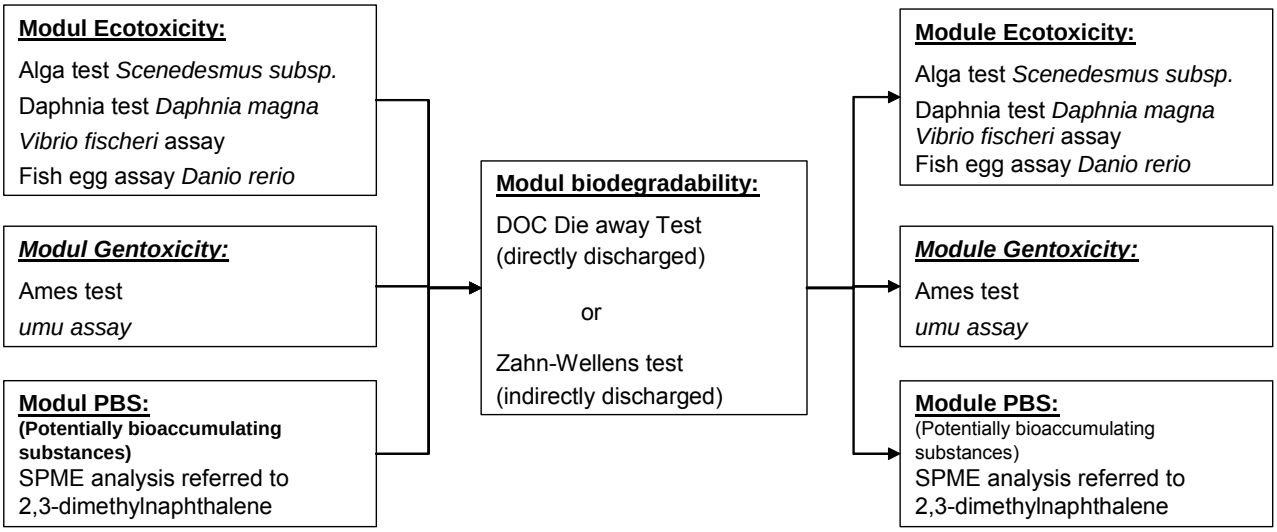


Figure 1. Practical WEA study test concept

3.1 Acute ecotoxicity

Daphnia test according to DIN 38412-30

The acute toxic effect of wastewater on *Daphnia magna* STRAUS (Crustacea, clone 5 of the German Federal Health Agency) was determined. The value measured is the dilution factor LID_D beyond which no acute toxicity for Daphnia is detected within 24 h. The LID_D-value corresponds to the least dilution factor by which a wastewater sample must be diluted in order for 90% of the Daphnia to maintain their ability to swim. The test was prolonged to 48 h in order to comply with OECD 202, and EC50 values were calculated after log-probit analysis if possible. The pH-value of the sample was adjusted with hydrochloric acid or sodium hydroxide solution to 7.0 +/- 0.2. No other pre-treatment was performed.

The sensitivity of the breeding strain is tested regularly with potassium dichromate.

Daphnia magna reproduction test following OECD 211

The chronic toxicity of wastewater on the reproductive output of *Daphnia magna* is not a standard procedure in the wastewater evaluation in Germany. The pH of the wastewater was adjusted to pH 7.0 +/-0.2. At the start of the test 10 female Daphnia aged less than 24 hours were exposed individually to a dilution series of wastewater in Elendt M4 medium (1:1, 1:2, 1:4 and 1:8). Daphnia were fed daily with living algae cells (*Scenedesmus subspicatus*, ration level between 0.1 and 0.2 mg C/Daphnia/d). The living offspring of each Daphnia was determined on a daily basis, the wastewater and Elendt M4 medium were renewed twice a week. The offspring produced during 21 days was calculated and the NOEC was determined by variance analysis (ANOVA with Dunnett's test with SPSS program SigmaStat). Due to the considerable effort (around 25 hours needed for each test), up to now only one sample was analysed (Effluent from paper industry after biological treatment in the DOC die away assay).

Fluorescent bacteria test according to DIN 38412-34 and EN ISO 11348-2

The toxicity of wastewater contaminants is detected for marine bacteria of the species *Vibrio fischeri*, which show a natural light production (bioluminescence) that is closely coupled with their metabolic activity. The test is performed with the LUMIS-tox system of the company Dr. Lange, Düsseldorf. The lyophilized bacteria of the strain *Vibrio fischeri* NRRL-B-11177 were obtained from the same company (LCK 482). The wastewater samples were tested without further pre-treatment after salinizing with sufficient sodium chloride to give a 2% solution and adjusting the pH-value to 7.0 +/- 0.2. The test result

is given as the least stepwise dilution (LID-value), for which the light emission is inhibited less than 20%.

Simultaneously with each series, potassium dichromate (4 mg/l) was tested as a reference substance. According to the Analytical Quality Assurance bulletin (AQS) of the German Working Group of the Federal States on water issues (LAWA), algal growth should be inhibited by 20-80% with 4 mg/l potassium dichromate.

Zebrafish embryo assay according to DIN 38412-6

The short term embryo assay with fish eggs of *Danio rerio* has replaced the acute fish toxicity test with *Leuciscus idus* in the wastewater evaluation for animal protection considerations. The test is classified as a suborganism test because the central nervous system of fish embryos is not fully developed. The fish were cultivated at 26 °C and 16 : 8 h light : dark cycle. They were daily fed with TetraMIN® flakes and additionally at least two times per week with newly hatched brine shrimps (*Artemia* sp.) The fertilised eggs were collected in a rectangular glass spawning box, covered by a stainless steel mesh and artificial plants, and were separated manually from unfertilised eggs using an inverted microscope. The eggs were incubated over 48 h, which covers the time from the blastula to the stage with fully developed blood circulation. For doing this, 10 fertilised eggs for each concentration are exposed in 24-well cell culture plates (2 ml each). Additionally, at least 10 eggs are tested with 3,4 dichloroaniline (3.7 mg/L) as positive control and 10 eggs as negative control. After 48 h the development of the embryos (heart beat, somites and tail differentiation) is observed microscopically at 25-40 fold magnification. The test result is given as the least dilution factor (LID-value), for which 90% of the eggs show no damage.

3.2 Chronic ecotoxicity

Algae test according to DIN 38412-33

The chronic inhibitory effect of wastewater samples on the growth of *Scenedesmus subspicatus*, a planktonic fresh-water alga, was determined. For this purpose, a dilution series of the water sample was made, without any further preparation, but adding an algal nutrient solution inoculated with a defined algal suspension (corresponding to 10^4 cells/ml) and incubating under defined light and temperature conditions. After 72 h, the number of cells was determined microscopically as a measure for the biomass. The result given is the least dilution step (LID_A-value), after which the measured inhibitory effect on biomass production is less than 20%. EC50 values were calculated after log-probit analysis if possible. Simultaneously with each series potassium dichromate (0.5 mg/l) was tested as a reference substance. According to the Analytical Quality Assurance bulletin (AQS) of the German Working Group of the Federal States on water issues (LAWA) alga growth should be inhibited by 30-80% with 0.5 mg/l potassium dichromate.

3.3 Biodegradability

Zahn-Wellens test according to OECD 302 B and DIN EN 29888

The COD- and DOC-elimination of the wastewater sample A, which is discharged to a municipal treatment plant, was determined using the Zahn-Wellens test with activated sludge (1 g/L d.s.) as inoculum. The wastewater sample was supplemented with an inorganic nutrient solution according to DIN EN 29888. All vessels were continuously stirred and aerated with an aquarium pump. The pH was adjusted to pH 7-8 each working day. After treatment for 7 days the activated sludge was allowed to settle for about 1 h and the supernatant was decanted.

DOC Die away assay according to OECD 301 A

The "DOC die away assay" was performed with the wastewater samples directly discharged to surface water following OECD 301 A. The outflow of a final clarifier of a municipal treatment plant, additionally filtered through a coarse sand filter with the addition of organic flocculation aid chemicals, was used as inoculum. The inoculum concentration was 10% of total volume, which is the upper limit allowed by OECD 301 A and corresponds to the mean dilution factor of municipal wastewater in surface water of Germany. DOC analysis was performed with a total carbon analyser TOC-5000A, Shimadzu Deutschland, Duisburg. All vessels were continuously stirred and aerated with an aquarium pump. The pH was adjusted at least two times per week. Test duration was 14 days. After treatment the activated sludge was allowed to settle for about 1 h and the supernatant was decanted.

3.4 Genotoxicity

Ames test according to DIN 38415-4 following ISO 16240

The Ames test is a bacterial mutagenicity test with *Salmonella typhimurium*. The Salmonella-bacterial strains used are deficient mutants, which are unable to grow in histidine-free medium. These histidine-requiring mutants can back-mutate (reversion) and then they are able to form colonies on minimal-agar plates. Each of the Salmonella-strains has a specific spontaneous back-mutation rate. The number of back-mutated bacteria (revertants) above this level provides a measure of the mutagenic potential of a substance or a sample. Certain mutagens in higher organisms are first activated by being metabolized (promutagens) or become inactivated metabolically. Therefore, the needed enzymes are added to the bacterial system in the form of rat liver extract S9 (Moltox Co.). The test version used is based on a simplified version of the OECD-Guideline 471 with the test strains TA98 and TA100. The strain TA98 detects frameshift mutagens; strain TA100 in contrast is for base pair substitution mutagens (point mutations). The water samples were sterilized over a membrane filter (0.2 µm). Up to 1 ml of wastewater per Petri dish could be added. A sample is then classified as mutagenic according to DIN 38415-4 if in one of the strains with or without S9 an induction difference compared to the control (solvent alone) of 80 (for TA100) or 20 revertants (for TA98) is induced and a dose-effect relationship is found. The LID-value corresponds to the last dilution step at which the induction difference established for that strain is not exceeded. Since the wastewater sample in the test is diluted by a factor of 3 with medium/inoculum, the lowest possible LID_{EA}-value = 3 (non-mutagenic). The number of revertants of the negative controls for TA100 should be in the range of 80-180 and for TA98 in the range of 15-40 revertants per plate.

Umu assay according to DIN 38415-3 following ISO 13829

The umu test is a genotoxicity test with the bio-technologically modified bacterial *Salmonella typhimurium* strain TA1535/pSK1002. The bacteria are exposed to various concentrations of the wastewater samples. Here gene toxins induce the so-called umuC-gene, which belongs to the SOS-repair system of the cell and which acts to prevent damage to bacterial genetic material. Through the coupling of the umuC-gene promotor with the lacZ-gene for ß-galactosidase, the activation of the umuC-gene can be indirectly measured spectrophotometrically at 420 nm through the formation of a coloured product from the ß-galactosidase substrate o-nitrophenyl-galactopyranoside (ONPG). The induction rate (IR) corresponds to the increase of the extinction at 420 nm relative to the negative control. In calculating the induction rates, one must take growth and its inhibition into account by normalizing by the growth factor, which is determined turbidimetrically from the optical density at 600 nm. An inhibition of bacterial growth is expressed as a reduced growth factor compared to the controls. For growth factors below 0.5 (50% growth inhibition) the results are not evaluated. The result given is the smallest dilution step (LID_{EU}-value) at which an induction rate < 1.5 is measured. If a different induction rate is seen upon addition of S9, the higher of the two values is taken (=LID_{EU}-value).

3.5 Bioaccumulation test

The potentially bioaccumulating substances (PBS) were determined by solid phase microextraction (SPME) according to the protocol of Leslie and Leonards (2005).[1] 250 mL wastewater was exposed to glass quartz glass fibres coated with 100 µm Polydimethylsiloxan (PDMS) (Supelco, Bellafonte, CA, USA) and continuously mixed at 500 rpm over 24 h. Gas chromatographic analysis was performed using a CP 9001 (Chrompack, Frankfurt) with FI-detector and an OPTIMA-1 column (Macherey-Nagel, Düren Germany). All data are normalised to the reference compound 2,3-dimethylnaphthalene and expressed as mmol/L DMN equivalents. The adsorption capacity of different fibres was compared by extraction of n-octanol from aquatic solution with SPME. Additionally, two blank values from two PE bottles filled with distilled water (one new, one used before) were determined according to the same procedure. Both the samples and the blanks were stored at -20°C in 500 mL PE-bottles before testing. The data are presented without subtracting the blank values.

In addition to the SPME extraction, an alternative liquid-liquid extraction method was performed according to the protocol of Leslie and Leonards (2005).[2] 300 ml of the effluent sample was acidified with 6 M HCl (4 mL) to pH<2 and extracted twice with 30 mL cyclohexane for 2 h each. Following this, the pH of the effluent sample was adjusted to >10 with 2.5 M NaOH and extracted twice with 30 mL cyclohexane as before. The four extracts are combined, concentrated and dried in a rotary evaporation with Na_2SO_4, further concentrated in a nitrogen steam to a volume of 1.5-2 mL, and measured by GC-

[1] H.A.Leslie, P.E.G. Leonards, Protocol – Determination of potentially bioaccumulatable substances (PBS) in whole effluents using biomimetic solid-phase microextraction (SPME), OSPA-IEG on WEA Interlaboratory Study 2005, RIVO, NL.

[2] H.A.Leslie, P.E.G. Leonards, Protocol – Determination of potentially bioaccumulating substances (PBS) in whole effluents using the 'EGOM' Liquid-Liquid Extraction. OSPA-IEG on WEA Interlaboratory Study 2005, RIVO, NL

FID. Results were referred to the external standard 2,3- dimethylnaphthalene. Nevertheless, no PBS were detected in the concentrated liquid-liquid extracts and therefore no data are shown. Currently the ten-fold more concentrated liquid extracts are being analysed with GC-MS. Data will be included in the report.

4. Results

4.1 Expression of results

The testing strategy for wastewater evaluation in Germany consists in the application of adapted screening test standards based on those used for chemical assessment. Therein fewer replicates are used and the "lowest ineffective dilution factor" (LID) is given as test result as specified in the informative annex of EN ISO 5667-16, in order to meet the criterion of cost effectiveness. The LID is the reciprocal value of the volume fraction of wastewater at which only effects not exceeding the test-specific variability have been observed (Daphnia and fish eggs, 10% mortality; alga/bacteria, 20% inhibition). As a first assumption, the NOEC (% wastewater) corresponds therefore to the reciprocal value of the LID. Additionally, the EC10 [3] and EC50 values are calculated if there are sufficient data pairs between 0 and 100% effects, as suggested by the IEG organisation committee. The EC50 was calculated with the ToxRat-program from probit-analysis using linear maximum likelihood regression. The NOEC calculation was performed by Student's t-test for homogeneous variance with Bonferroni adjustment after Cochran's test procedure on variance homogeneity using the ToxRat-program and with the SigmaStat program applying one way ANOVA with multiple comparisons (Dunnett's method). The DIN standards for wastewater evaluation with the alga/Daphnia and luminescent bacteria tests require only two replicates, thus further statistical analysis remains uncertain. Therefore the test design was partly extended to three replicates in order to meet the requirements discussed.

4.2 Evaluation of the different effluents

a. Metalworking industry, indirectly discharged

A COD-and DOC-elimination above 96% in the Zahn-Wellens test after 7 days shows that the wastewater is treatable in municipal wastewater treatment plants. With LID-values between 2 and 8 in the algae, Daphnia, *Vibrio fischeri* and fish egg tests, a moderate ecotoxicity is detected, which is completely or considerably reduced in the biological treatment. The *Vibrio fischeri* assay results showed that this is the most sensitive test. No genotoxicity/mutagenicity was detected in the umu test and Ames test. The potential bioaccumulating substances (PBS) as measured by SPME-analysis were scarcely two-fold of the corresponding blank values.

The discharge of wastewater from the metalworking industry is regulated in Annex 40 of the wastewater ordinance. Here, among others, the parameters documented in Table 5 are given. Hereby, the requirements for the total effluent at the pipe are only set for directly discharged effluents.

Requirements of total effluent at the pipe			**Requirements before mixture of different effluents**		
COD	mg/L	100-400	AOX	mg/L	1
Phosphorus total	mg/L	2	Cyanide	mg/L	0.2-1
NH_4-N	mg/L	20-100	Sulphide	mg/L	1
NO_2-N	mg/L	5	Arsenic	mg/L	0.1
Hydrocarbons	mg/L	10	Cadmium	mg/L	0.1-0.2
Fluoride	mg/L	20-50	Cobalt	mg/L	1
Aluminium	mg/L	2-3	Copper	mg/L	0.5
Iron	mg/L	3	Chromium	mg/L	0.5
Fish egg toxicity	LID	2	Chromium (VI)	mg/L	0.1
			Lead	mg/L	0.5
			Mercury	mg/L	0.05
			Nickel	mg/L	0.5
			Selenium	mg/L	1
			Silver	mg/L	0.1
			Tin	mg/L	2
			Zinc	mg/L	2
			Chlorine	mg/L	0.5

Table 5. Requirements for wastewater from metalworking industry (depending on production area)

[3] Data will be evaluated statistically further on. In this draft report not all data are considered.

b. Speciality chemical industry, direct discharges after treatment

The company has been selected because of its production spectrum of high concern, such as biocides. The results of the ecotoxicity tests with algae, Daphnia and *Vibrio fischeri* showed only moderate ecotoxicity within the corresponding permit limits (measured LID values 1-12), but the fish egg test clearly exceeded the limit value (LID 6 measured, LID 2 demanded). In this context, it must be noted, that the fish egg test recently replaced the acute fish toxicity test for animal protection reasons but that still there are only few comparative data available. Formerly, the effluent complied with the acute fish toxicity limit of LID 2. Within the biological treatment according to the OECD die away assay, a considerably part of the COD and DOC was removed (59% and 31%, resp., after 14 days). After the biological treatment, no algal toxicity was measured, but the *Vibrio fischeri* assay and fish egg test still presented effects (although reduced). While the umu assay showed no genotoxicity, the Ames test was clearly positive in strain TA98 + S9 up to a dilution factor (LID) of 12 before and after the biological treatment.

The concentration of potentially bioaccumulating substances was within the range of the corresponding blank value. The discharge of wastewater from the chemical/pharmaceutical industry is regulated in Annex 22 of the wastewater ordinance. Here, among others, the parameters documented in Table 6 are given.

Requirements of total effluent at the pipe			**Requirements before mixture of different effluents**		
COD	mg/L	75 (or >90% elimination)	AOX	mg/L	0.3-8 mg/L depending on production
Phosphorus total	mg/L	2	Copper	mg/L	0.1-0.5
NH_4-N+NO_3-N+ NO_2-N	mg/L	5 0	Chromium	mg/L	0.05-0.5
Fish egg toxicity	LID	2	Mercury	mg/L	0.001-0.05
Daphnia toxicity	LID	8	Cadmium	mg/L	0.005-0.2
Alga toxicity	LID	16	Nickel	mg/L	0.05-0.5
Bacteria toxicity	LID	32	Lead	mg/L	0.05-0.5
Genotoxicity umu	LID	1.5 (no genotoxicity)	Zinc	mg/L	0.2-2
			Purgeable halogenated hydrocarbons	mg/L	10
			TOD-load of different wastewater parts must only be mixed if the elimination in the Zahn-Wellens test is >80%		

Table 6. Requirements for wastewater from chemical/pharmaceutical industry

The results show clearly the added value of WEA for a hazard assessment of the effluent. A TIE approach has been started in order to identify the sources of the fish egg toxicity as well as the elevated algae toxicity observed formerly (but not in the sample tested within the practical study). There is a first suspicion, that the biocide production might be the cause of both the varying algal toxicity and the observed fish egg toxicity. Next to the biocide produced, which belongs to the class of polychloro-phenoxy phenols and has a very high algal toxicity (NOEC is in the 0.001 ppm range), intermediate products are removed from the wastewater by adsorption on carbon granulate. However additional testing of the partial stream from biocide production after treatment in the Zahn-Wellens test did not confirm that suspicion. The biocide production is also not responsible for the mutagenicity observed in the Ames test, which was also confirmed by testing the partial stream separately before and after performing the Zahn-Wellens test (data not shown). The high amount of salts probably has no decisive influence on fish egg toxicity (EC10 of Chloride is about 4 g/l and of sulphate is 9.6 g/l)[4] [5]. Further tests will be performed. The investigation is therefore not finished.

[4] Anonym. 2001. Validierungsdokument Nr. 25 für DIN 38415 T6 "Bestimmung der nicht akut giftigen Wirkung von Abwasser auf die Entwicklung von Fischeiern über Verdünnungstufen". Hauptauschuss I "Deutsche Einheitsverfahren" der Wasserchemischen Gesellschaft in der GdCH und Arbeitsausschusses I.3 "Wasseruntersuchungen" im Normenausschuss Wasserwesen (NAW) im DIN (http://www.gdch.de/strukturen/fg/wasser/publikat/vali/vd.htm).

[5] According to the wastewater ordinance, high salt amounts are considered with a salt correction factor. This correction factor determines that the LID allowed arises plus 1 for each 3 g/L of chloride and sulphate.

c. Chemical industry, direct discharges after treatment

The ecotoxicity of the sample as measured in the algae, Daphnia, *Vibrio fischeri* and fish egg tests was low (with LID-values between 1 and 4) and was further reduced during the biological treatment. No genotoxicity or mutagenicity effects were detected in the umu and Ames assay, but evaluation of the Ames assay was hindered due to toxic effects to bacteria. Due to the very low concentration of organics (3 mg/l TOC), the determination of DOC- and COD-elimination in the DOC die away test was not suitable. The concentration of potentially bioaccumulating substances were within the range of the corresponding blank value.

The wastewater permits follow Annex 22 of the wastewater ordinance (see sample B).

d. Paper industry, direct discharges after treatment

There was no ecotoxicity detected in the algae, Daphnia, *Vibrio fischeri* and fish egg tests at all.

In the algae and fish egg tests after biological treatments, slightly elevated values were measured. Only the *Daphnia magna* reproduction test showed a slight chronic effect after the biological treatment. The NOEC was at 25 vol. % of wastewater (CI 95%), the EC50 was clearly above 100 vol. %. No genotoxicity or mutagenicity effects were detected. The concentration of potentially bioaccumulating substances was within the range of the corresponding blank value.

Within the biological treatment and according to the OECD die away assay, a considerable part of the COD and DOC was removed (58% and 44% resp., after 14 days). The discharge of wastewater from the paper and cardboard industry is regulated in Annex 28 of the wastewater ordinance. Here, among others, the parameters documented in Table 7 are given.

Requirements of total effluent at the pipe			**Requirements before mixture of different effluents**		
COD	kg/t	3-5 (load depending on production)	AOX	g/t	60-100 (load depending on production)
BOD	mg/L	50			
Phosphorus total	mg/L	2			
NH_4-N+NO_3-N+ NO_2-N	mg/L	10			

Table 7. Requirements for wastewater from paper and cardboard industry (depending on production area)

4.3 Statistical analysis

The detailed statistical analysis has not been finished completely (NOEC for bacteria is missing). The EC50 calculation revealed additional useful information but applicability was often limited by the low observed ecotoxicity.

For example the LID of the *Vibrio fisheri* assay for “sample A” was 8 before and 4 after the degradation test, thus the NOEC as calculated from LID was 12.5 and 25 vol. %. While the corresponding EC10-toxicity values where in the same range of 7-8 vol%, the EC50-toxicity revealed that the concentration-effect curve was flatter after degradation (EC50 before degradation, 45%; after degradation, 334%). The question arises whether NOEC or EC50 values above 100 vol. % of the wastewater really give suitable information.

Only few tests could be evaluated with ANOVA; the NOEC (95% conf. interval) corresponded quite well with the NOEC calculated from the reciprocal LID-values. The EC10 was lower than the corresponding NOEC calculated from the LID, but that is not surprising, as the LID is derived from the <20% inhibition effect for algae and bacteria.

The confidence limits of the EC10 and EC50 were within a useful range where the evaluation was possible. Samples with low toxicity, for which usually data for less than 3 concentrations were available, were not evaluated.

A more sophisticated statistical analysis requires only minor additional effort for toxic samples, where sufficient concentrations can be evaluated. For samples with only minor toxicity no added value of detailed statistics was observed and considerably additional effort in testing more concentrations is needed.

Appendix to Annex 3

Tab. 1: DOC-measurement Zahn-Wellens test OECD 302 B

	OSPAR A Metalworking industry		sodium acetate *)	
	Test vessel	Abiotic control	Reference	Blank
TOC original sample	61,5	61,5	14680	
Total volume in vessels [ml]	4000	1600	1600	
Wastewater volume [ml]	3130	1257	40	
DOC calculated at start	48	48	367	
0 h	47	51	367	8
3 h	54	51	362	9
1 d	33	48	32	7
2 d	25	47	33	16
3 d	15	40	41	15
7 d	9	33	54	20

*) Sodium acetate 50 g/l

Tab. 2: DOC-elimination Zahn-Wellens test [%]

	OSPAR A Metalworking industry		sodium acetate *)
	Test vessel	Abiotic control	Reference
0 h	19	-6	2
3 h	5	-6	4
1 d	45	0	93
2 d	81	3	95
3 d	101	18	93
7 d	123	33	91

Tab. 3: COD-measurement Zahn-Wellens test OECD 302 B

	OSPAR A Metalworking industry		sodium acetate *)	
	Test vessel	Abiotic control	Reference	Blank
COD original sample	259	259		
COD sample after filtration	238	238		
Total volume in vessels [ml]	4000	1600	1600	
Wastewater volume [ml]	3130	1257	40	
COD calculated at start	203	203		
0 h	165	169	929	17
3 h	161	165	927	17
1 d	103	143	76	21
2 d	70	137	30	18
3 d	44	121	33	19
5 d	31	101	34	23
7 d	28	95	25	19

*) Sodium acetate 50 g/l

Tab. 4: COD-elimination Zahn-Wellens test [%]

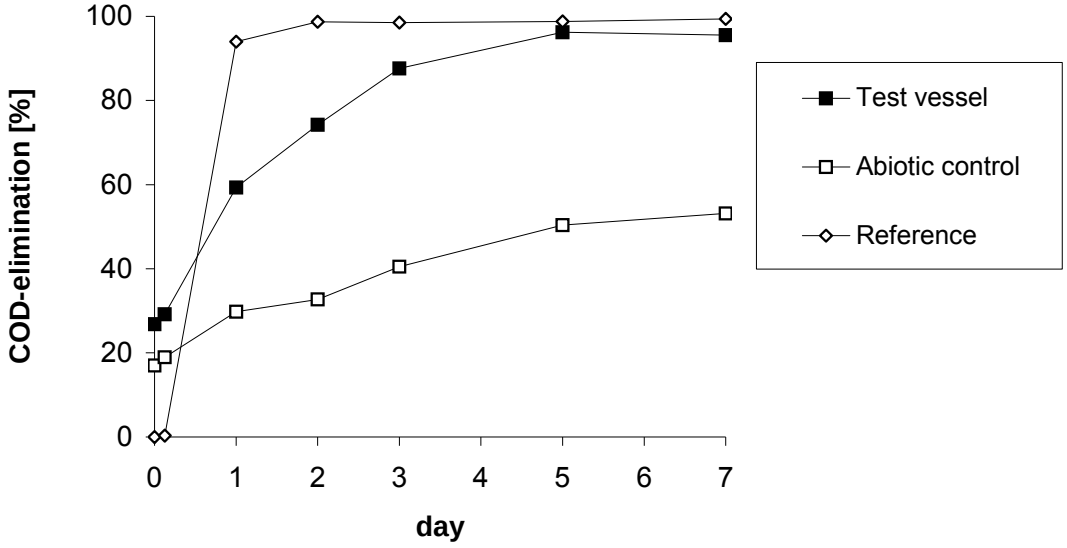
	OSPAR A Metalworking industry		sodium acetate *)
	Test vessel	Abiotic control	Reference
0 h	27	17	0
3 h	29	19	0
1 d	59	30	94
2 d	74	33	99
3 d	88	41	99
5 d	96	50	99
7 d	96	53	99

Tab. 5: pH-values

	OSPAR A Metalworking industry		sodium acetate *)	
	Test vessel	Abiotic control	Reference	Blank
0	6.1+OH	7.5	7.3	7.4
3 h	6.7+OH	7.6	7.4	7.3
1	7.0+OH	7.6	8.7+OH	7.2
2	7.0	7.6	8.7+OH	7.2
3	7.1	7.7	8.8+OH	7.2
5	7.2	7.9	8.9+OH	7.2
7	7.2	7.8	8.8	7.2

*) OH: pH-adjustment with NaOH on pH 7.0 - 8.0
*) H: pH-adjustment with HCl on pH 7.0 - 8.0

Figure 2: COD-elimination OSPAR A
Metalworking industry 30.06.05



Tab. 6: DOC-measurement DOC Die away assay OECD 301 A

Ospar sample	B	C	D	sodium benzoate *)	
	Test vessel	Test vessel	Test vessel	Reference	Blank
TOC original sample	51,8	3,0	46,1	5830	
Total volume in vessels [ml]	4000	4000	4000	1600	
Wastewater volume [ml]	3548	3548	3548	5,5	
DOC calculated at start	45,9	2,7	40,9	20,0	
3 h	47,9	11,8	33,2	23,0	2,6
1 d	45,3	4,9	36,5	21,2	4,3
3 d	47,8	5,8	39,8	7,8	5,3
5 d	43,5	9,0	29,1	5,2	6,5
7 d	44,2	4,4	33,7	6,6	5,4
11 d	49,1	5,1	32,3	10,7	11,0
14 d	39,8	6,4	31,2	7,3	8,2

*) sodium benzoate 10 g/l

Tab. 7: DOC-elimination DOC Die away assay [%]

Ospar sample	B	C	D	sodium benzoate *)
	Test vessel	Test vessel	Test vessel	Reference
3 h	2	-244	25	-2
1 d	11	78	21	16
3 d	7	79	16	87
5 d	19	7	45	107
7 d	15	135	31	94
11 d	17	321	48	102
14 d	31	167	44	104

Tab. 8: pH-values

Ospar sample	B	C	D	sodium benzoate *)	
	Test vessel	Test vessel	Test vessel	Reference	Blank
0	7.8	7.9 + H	8.0 + H	7.4	7.4
1	8.1 + H	8.3 + H	8.4 + H	7.5	7.5
3	8.0 + H	8.3 + H	8.4 + H	7,7	7.6
5	7.9	8.1 + H	8.2 + H	7.6	7.5
7	8.0 + H	8.1 + H	8.1 + H	7,7	7.5
10	8.0 + H	8.1 + H	8.1 + H	7.7	7.6
14	7.9	8.0	8.0	7,7	7.7

*) OH: pH-adjustment with NaOH on pH 7.0 - 8.0
*) H: pH-adjustment with HCl on pH 7.0 - 8.0

Tab. 9: Summary of results

	Protocol	Organism	test duration	Pre-treatment samples	Results	A Metalworking industry, indirectly discharged		B Chemical industry, directly discharged		C Chemical industry, directly discharged		D Papermill, directly discharged	
					Biological Treatment	before	after	before	after	before	after	before	after
Acute toxicity													
Algae	DIN 38412-33	*Scenedesmus subspicatus*	72 h 72 h	none none	LID NOEC % ww EC50 % ww	3 33,3	1 100	4 25 26	1 100	4 25 22	1 100	1 100	3 33,3
Bacteria	DIN EN ISO 11348-2	*Vibrio fisheri*	30 min.		LID NOEC % ww EC50	8 12,5 45	4 25 345	12 8,3 68	6 16,6 130	2 50	2 50	1 100	1 100
Crustacea	DIN 38412-30	*Daphnia magna*	24 h / 48 h	sed.	LID NOEC % ww EC50 % ww	1 / 1 100 / 100	1 / 1 100 / 100	1 / 1 100 / 100	1 / >3 100 / > 33.3	1 / 1 100 / 100	1 / 1 100 / 100	1 / 1 100 / 100	1 / 1 100 / 100
Fish egg test (early life stage)	DIN 38415-6	*Danio rerio*	24 h / 48 h		LID NOEC % ww EC50 % ww	2 50	1 100	6 16,6 21,3	3 33,3	2 50	1 100	1 100	3 33,3
Genotoxicity													
Ames assay	DIN 38415-4	*Salmonella typhimurium TA98/TA100*	48 h	0,2 µm filtr.	LID NOEC % ww max. induction	3 (neg.) 1,0	3 (neg.) 1,1	12 8,3 4,7	12 8,3 5,4	3 (neg.) 1,0	3 (neg.) 0,7	3 (neg.) 1,1	3 (neg.) 1,0
umu assay	DIN 38415-3	*Sal. typhimurium* TA1535/pSK 1002	2 h	0,2 µm filtr.	LID NOEC % ww max. induction	1,5 (neg.) 1,0	1,5 (neg.) 1,1	1,5 (neg.) 1,4	1,5 (neg.) 1,0	1,5 (neg.) 1,0	1,5 (neg.) 1,1	1,5 (neg.) 1,0	1,5 (neg.) 1,1
Potential bioaccumulating substances													
SPME-analysis			Bank values: 1.90 ± 0.81 mmol/L DMN equivalent		mmol/L DMN equivalent	3.12 ± 0.54	1.45 ± 0.10	2.42 ± 1.10	2.45 ± 0.40	1.20 ± 0.06	2.66 ± 0.35	1.27 ± 0.40	1.62 ± 0.19
Biodegradability													
						test vessel	abiotic control	test vessel		test vessel		test vessel	
			original samples		COD [mg/l]	259		247		14,6		132	
			original samples		TOC [mg/l]	61,5		51,8		3,0		46,1	
Zahn-Wellens test	DIN EN 29888 mod.	Activated sludge 1 g/l d.s.	7 d	none	% COD-elimination	27 (0 h) 29 (3 h) 96 (7 d)	17 (0 h) 19 (3 h) 53 (7 d)						
					% DOC-elimination	19 (0 h) 5 (3 h) 123 (7 d)	-6 (0 h) 6 (3 h) 33 (7 d)						
Modified Die-away t	OECD 301 A mod.	outflow final clarifier after filter	14 d	none	% COD-elimination			45 (3 h) 47 (3 d) 55 (7 d) 59 (14 d)		not suitable due to low start concentration		25 (3 h) 28 (3 d) 45 (7 d) 58 (14 d)	
					% DOC-elimination			2 (3 h) 7 (3 d) 15 (7 d) 31 (14 d)				25 (3 h) 16 (3 d) 31 (7 d) 44 (14 d)	

LID: Lowest ineffective dilution, ww: wastewater, DMN: 2,3-dimethylnaphthalene, NOEC % ww = 100/LID [% wastewater]

Tab. 10: Detailed statistical evaluation Summary of results

Organism	**Results**	**A Metalworking industry, indirectly discharged**		**B Chemical industry, directly discharged**		**C Chemical industry, directly discharged**		**D Papermill, directly discharged**	
		before	**after**	**before**	**after**	**before**	**after**	**before**	**after**
Vibrio fisheri	LID	8	4	12	6	2	2	1	1
	NOEC % ww			will be calculated afterwards					
	NOEC (95% conf.) % ww	no ANOVA-analysis possible because only two replicates							
	EC10 % ww	8,3	7,4	5,0	8,5				
	EC10 (95% conf.) % ww	(7,3 .. 9,3)	(5,5 .. 9,2)	(4,3 .. 5,7)	(7,8 .. 9,2)				
	EC50 % ww	44,7	343,8	68,2	130				
	EC50 (95% conf.) % ww	(40,4 .. 50,5)	(214,8 .. 685)	(61,5 .. 76,8)	(115,8 .. 147,2)				
Daphnia magna	LID	1 / 3	1 / 1	1 / 1	1 / >3	1 / 1	1 / 1	1 / 1	1 / 1
	NOEC % ww	100 / 100	100 / 100	100 / 100	100 / > 33,3	100 / 100	100 / 100	100 / 100	100 / 100
	EC50 % ww	not calculable with ToxRat-program because at least 4 inhibiting concentrations must be evaluated							
Scenedesmus subspicatus	LID	3	1	4	1	4	1	1	3
	NOEC % ww	33	100	25	100	25	100	100	33,3
	NOEC (95% conf.) % ww			25		25			
	EC10 % ww			8,1		9,4			
	EC10 (95% conf.) % ww			-		(4,7 .. 12,8)			
	EC50 % ww			26,0		22,0			
	EC50 (95% conf.) % ww			-		(17,8 .. 27,2)			

ww: wastewater
LID: Lowest ineffective dilution
NOEC = 100/LID
NOEC (95% conf.): ToxRat-program, ANOVA-analysis with Bonferroni t-test after Holm
EC10 and EC50: ToxRat-program, probit-analysis using linear maximum likelihood regression

Annex 4: Contribution to the WEA Practical Study 2005 Report from Portugal

Contents

1. Introduction

In some countries, bioassays are used in the characterisation of wastewaters, in the establishment of criteria for industrial discharges and in studies of evaluation of the efficiency of WWTP. There is a gap in the Portuguese legislation in what concerns the ecotoxicological characterisation of wastewaters.

Ecotoxicological assays are only described in the legislation regarding chemical substances and limit values are given to allow a classification of such substances. The application of those techniques to wastewater control is not yet done. The recent transposition of Water Framework Directive to national law (Portuguese Water Law – L. nº 58/2005, DR nº 249/05 – série I-A) opens new perspectives in terms of future national, regional or sector regulations.

In this report, permit compliance was analyzed in what concerns physico-chemical parameters. For the industry discharging into the sewage system, limits used were from Municipality regulation (SMAS, 1993), and for the industry discharging into the receiving water, limits used were from Decret Law 236/98 (D.L. 236/98, DR nº 176/98 –série I-A).

2. Participants

Two Portuguese institutions participated in the OSPAR Whole Effluent Assessment Practical Study 2005: INETI (National Institute for Engineering, Technology and Innovation) and IA (Institute for the Environment).

Two ecotoxicity tests (Alga, *Lemna*) and biodegradability tests were performed at INETI laboratory (A. Picado, L. Silva, S. Paixão). Two ecotoxicity tests (Microtox, *Daphnia*) and bioaccumulation tests were performed at IA laboratory (F. Brito, M.A. Morbey, S. Leitão, P. Viana).

3. Description of the samples

Two complex wastewaters (Table I), one directly discharged and another indirectly discharged to the receiving waters were studied before and after a biodegradation step.

	Sampling	Sector	Discharge	Treatment	Flow (m3/day)
P1	May	Metal-Mechanical	Direct	No	3
P2	June	Chemical-Pharmaceutical	Indirect	Physico/Chemical	183

Table I – Information on Portuguese effluents tested under WEA Practical Study 2005

3.1 Metal-Mechanical industry

The company is located in Lisbon and Tagus valley area. The company activity is in the area of machinery production and surface treatment. About $3m^3$ untreated wastewater are discharged directly to the receiving waters. A point sample was taken on the 12th May 2005.

This effluent was not analysed during the OSPAR Demonstration Program on WEA (2003).
Some physico-chemical parameters exceeded permit limits (BOD_5, COD, TSS, Nitrate) once or twice. Metals present were: Al, Cr, Cu, Fe, Pb, and Zn; from these, concentrations of Cu, Fe and Zn exceeded the permit limits. This effluent showed to be very toxic to *Daphnia* and moderately toxic to other organisms.

3.2 Chemical-Pharmaceutical industry

The company is located in Lisbon and Tagus valley area. The company does complex chemical synthesis of active pharmaceutical ingredients. Before discharging, the wastewater passes through a physico-chemical treatment with the following steps: stripping, pH adjustment, aeration, decantation and final pH adjustment. About $183m^3$ wastewater are discharged to a municipal wastewater treatment plant. A composite sample was taken on the 2nd June 2005.

Some data from the OSPAR Demonstration Programme on WEA (2003) allow describing this effluent as low levels of PBS (6,5-6,8 mM), with 100 substances determined by GC/MS, of which 10 identified and explaining 25% of peak area. Wastewater toxicity could not be explained by identified substances toxicity. The added value of WEA in comparison to the SbS approach was seen.

Other historical data exist on physico-chemical and ecotoxicological parameters from the Ecoriver project. Some physico-chemical parameters exceeded permit limits (BOD5, COD, TSS). Metals present

were detected: Al, Fe, Pb, Ni and Zn; from these concentrations of Fe and Pb exceeded the permit limits. This effluent showed minor toxicity to different organisms.

4. Test methods

4.1 Ecotoxicity

To assess acute and chronic toxicity, four taxonomic groups (bacteria, algae, crustaceans and plants) were tested in order to obtain an idea of the effects on the aquatic ecosystem (Table IV).

	Organism	Species	Endpoint	Parameter	Method
Acute	Bacteria	Vibrio fischeri	Luminescence inhibition	EC_{50}-30min	Microbics (1994)
	Algae	*Pseudokirchneriella subcapitata*	Growth inhibition	EC_{50}-72h	ISO 8692 (2000)
	Crustacea	*Daphnia magna*	Inhibition of mobility	EC_{50}-48h	ISO 6341 (1996)
	Macrophyte	*Lemna minor*	Growth inhibition	EC_{50}-7d	ISO 20079 (2005)
Chronic	Crustacea	*Daphnia magna*	Inhibition of reproduction	NOEC-21d	ISO 10706 (2000)

Table IV – Toxicity tests performed on Portuguese samples under 2005 WEA practical study

Acute toxicity

Four acute toxicity tests (Table IV), Microtox, algae, *Daphnia* and *Lemna,* were performed on the two samples P1 and P2 before and after Persistence tests.

Microtox test: Toxicity was assessed by determining the inhibition of the luminescence of *Vibrio fischeri* (strain NRRL B-11177) exposed for 30 minutes (Microtox® Test, Microbics, Carlsbad, U.S.A.). The test was performed according to the basic test procedure as described in Microbics (1994) and using lyophilized bacteria (SDI, UK).

Algal test: Toxicity was assessed by determining the inhibition of growth of *Peudokirchneriella subcapitata* exposed for 72 hours according to ISO 8692 (2000). The algal test was miniaturized and performed in microtitration plates.

Daphnia test: Crustacean acute toxicity was assessed by determining the inhibition of the mobility of *Daphnia magna* (clone IRCHA-5) exposed for 48 hours according to EN ISO 6341 (1996). Juveniles for testing were obtained from cultures maintained in the laboratory.

Lemna test: Plant toxicity was assessed by determining the growth inhibition of *Lemna minor* (clone ST) exposed for 7 days, according to ISO 20079 (2005). Plants for testing were obtained from cultures maintained in the laboratory. Two growth parameters, total number of fronds and total frond area, were quantified by an image analysis system – Scanalyzer (LemnaTec, Würselen, Germany).

The sensitivity of the testing organisms is regularly tested with reference substances, phenol for Microtox, potassium dichromate for *Daphnia* and algae and 3,5- dichlorophenol for *Lemna*.

Chronic toxicity

One chronic toxicity test was performed on the two samples P1 and P2 before persistence tests. Crustacean chronic toxicity was assessed by determining the inhibition of reproduction of *Daphnia magna* (clone IRCHA-5) exposed for 21 days, according to ISO 10706 (2000). Juveniles for testing were obtained from cultures maintained in the laboratory.

At the start of the test 10 female Daphnia aged less than 24 hours were exposed individually to a dilution series of wastewater in Elendt M4 medium. Daphnia were fed daily with *Chlorellla vulgaris* living cells. The media were renewed every other day and living offspring of each female determined on the same basis. The offspring produced during 21 days was calculated and results are presented in NOEC-21 d, the No Observed Effect Concentration, the highest concentration for which reproduction is not significantly different from the control. NOEC calculation was done using Mann-Whitney test.

4.2 Persistence

Both samples were tested with the same method, chosen according to discharge/treatment characteristics: P1 directly discharging but with no treatment and P2 indirectly discharging after physico-chemical treatment.

The Zahn-Wellens test was performed for the two samples and for diethylene glycol, a reference compound, as described in the ISO Standard 9888 (1999). The activated sludge samples used as inoculum were collected from the aeration tank of a wastewater treatment plant, treating predominantly domestic sewage. The concentration of activated sludge samples was 2-4 g/l suspended solids and for the assays the inoculum concentration was adjusted to 0.5 g/l of suspended solids in the final mixtures. The assays were carried out in 2 l Erlenmeyer flasks with a final volume of 500 ml of test mixture (mineral test medium + test sample + inoculum), at 25 ºC in a rotary shaker at 150 rpm. Each sample was added to obtain a DOC concentration of 50 mg/l to 400 mg/l in the final mixture. A blank without the sample was also set up in parallel.

The biodegradation process was followed measuring the dissolved organic carbon (DOC) through the test period, usually 28 days or until a plateau is reached. This biodegradability test is considered valid if the % biodegradation for the reference compound is higher than 70% on the 14th day (ISO, 1999). The sample tested is considered inherently biodegradable if more than 70% DOC is removed during the 28 days.

4.3 Bioaccumulation

The total amount of Potentially Bioaccumulating Substances (PBS) was determined by solid phase microextraction (SPME) and Flame Ionization Detector (FID) according to the protocol (OSPAR-IEG, 2003). This determination was only done for the two effluents before Persistence tests.

5. Results

Results of WEA tests are presented in Table V.

		P1 Metal		P2 Pharma	
Test		Before P	After P	Before P	After P
Zahn-Wellens	DOC (mg/l)	14,1	0	180	31,3
	DOC-elimination (%)	-	100	-	83
Bacteria	EC50-30min (%)	30	>100	>100	>100
Algae	EC50-72h (%)	17	23	6,5	17
Crustacea	EC50-48h (%)	2,2	>90	60	85
	NOEC-21d (%)	10	-	15	-
Lemna	EC50-7d (%)	>90	>90	8,7	78
SPME	PBS (mM)	227	-	15,4	-

Table V. Results for Persistence, Toxicity and Bioaccumulation of the effluents studied under OSPAR/WEA practical study 2005.

5.1 Metal-Mechanical industry

A DOC-elimination of 100% in the Zahn-Wellens test after 7 days shows that the wastewater is treatable by biological processes or in the WWTP. The PBS measured before persistence test are high.

The sample showed to be toxic to *Daphnia*, minor toxic to bacteria and alga and not toxic to *Lemna* before P step. After P step, minor toxicity to alga persists and the sample is no longer toxic to bacteria and *Daphnia* (Figure 1).

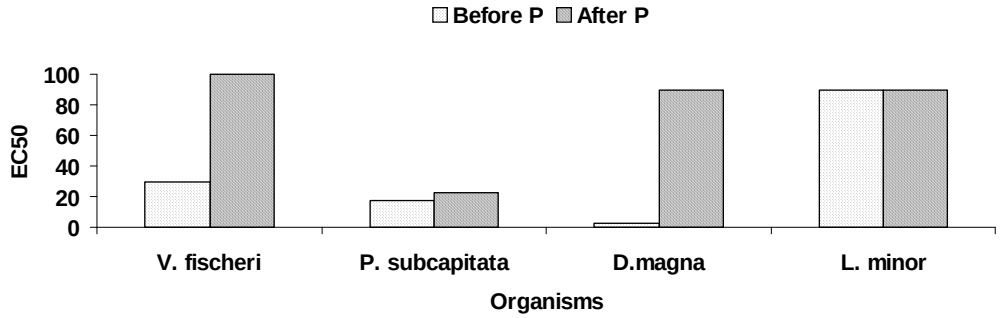


Figure 1. EC_{50} values for *V. fischeri*, *P. subcapitata*, *D. magna* and *L. minor* tests with P1 (Metal-Mechanical sector wastewater), before and after persistence test.

Sample P1 proved also to be chronically toxic to *Daphnia*.

From historical data we know that the wastewater is not compliant with chemical permit requirements, and has significant acute toxicity to *Daphnia*. Some persistent acute toxicity can be seen from the above results, probably related to non biodegradable compounds responsible for high PBS determined.

5.2. Chemical-Pharmaceutical industry

A DOC-elimination of 83% in the Zahn-Wellens test was reached in the plateau phase after 14 days showing that the wastewater is inherently biodegradable. The PBS measured before persistence test are low.

The sample showed to be toxic to alga and *Lemna*, minor toxic to *Daphnia* and not toxic to bacteria before P step. After P step, minor toxicity to alga, Daphnia and *Lemna* persist (Figure 2).

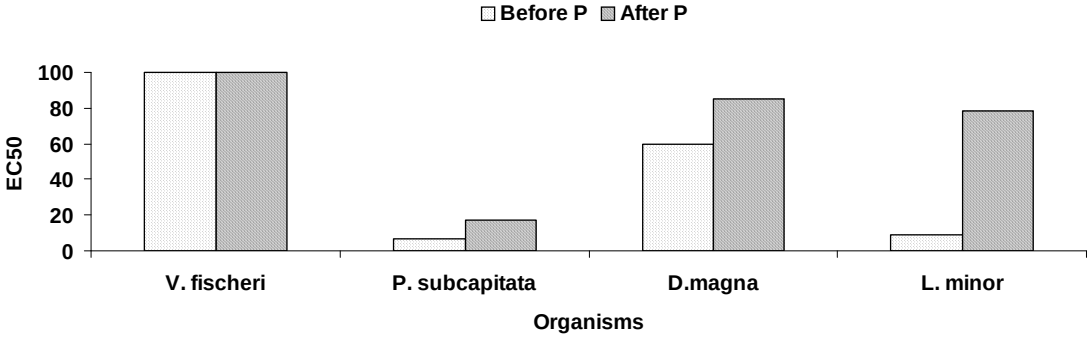


Figure 2. EC_{50} values for *V. fischeri*, *P. subcapitata*, *D. magna* and *L. minor* tests with P2 (Chemical-Pharmaceutical sector wastewater), before and after persistence test.

Sample P1 proved also to be chronically toxic to *Daphnia*.

From historical data we know that the wastewater is not compliant with chemical permit requirements, and has significant acute toxicity to alga and *Lemna.* Some persistent acute toxicity can be seen from the above results.

6. Discussion

The analysis of the results obtained for P1 (Metal-mechanical industry) and P2 (Chemical-pharmaceutical industry) wastewaters allow concluding that a biological treatment before the discharge to the receiving system or the biological treatment in the WWTP can reduce the acute toxicity of both wastewaters. There seems to be some relationship between DOC removal and toxicity removal.
No direct relation can be seen between organic carbon content and the level of acute toxicity. Between chronic toxicity and bioaccumulation there seems to be no relation, in what concerns these wastewaters.

There was no possibility to do the studies of Persistence of Chronic Toxicity and Persistence of Bioaccumulation for these wastewaters, but this is considered most relevant for future developments.

Annex 5: Persistence Assessment within OSPAR WEA – UK report 2005

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Introduction

The Science Group of the Environment Agency for England and Wales represents the UK Government by participating in the OSPAR intersessional expert group for Whole Effluent Assessment (WEA) of point source discharges. The overall aims of this group include the development of a guidance document (that would present flowcharts as a management tool for effluents entering the marine environment) and a toolbox of test methods (that would characterise effluents in terms of environmental persistence, potential to bioaccumulate and toxicity). A "learning by doing" approach was adopted by the participants in an attempt to identify the interrelationships inherent in these three characteristics (e.g. how the toxicity of an effluent persists).

The EA Science Group took the lead in the assessment of the toxicity persistence. This was done by assessing toxicity before and after two test effluents have been given the opportunity to degrade. This opportunity was to be provided in two ways:

1. A standard 'ready biodegradability' test (OECD 301 E) in which an inoculant is added to the sample and maintained in an artificial mineral media; and
2. A novel passive biodegradability test developed at EA Science Group (modified OECD 306) in which no inoculant is added to the sample and the test is conducted in natural seawater.

Many effluents in the U.K. receive no treatment prior to entry into marine waters and, whilst the first method is standardised, the second method (described in detail below) simulates this situation more specifically. Through the addition of an inoculant and the purposeful manipulation of the testing medium, the first test was considered to represent an 'active' form of biodegradation. In contrast, the second method was regarded as a 'passive' form of biodegradation due to the absence of an inoculant and an artificial testing medium.

Persistence was examined in these effluents through an assessment of toxicity toward marine species (*Tisbe battagliai*, *Skeletonema costatum* and *Vibrio fischeri* (Microtox®)) before and after each biodegradation test. The loss of total organic carbon (TOC) content of the effluents was measured during the standard test (OECD 301 E) and compared to the loss of toxicity.

Practical work aims

1. To assess the toxicity of 2 test effluents toward *Tisbe battagliai*, *Skeletonema costatum* and the Microtox® test method;
2. To biodegrade these effluents using one standard (active) and one novel (passive) method;
3. To reassess the toxicity of these effluents post-biodegradation.

Practical study 2005

1) Method of selection of effluents

The EA Science Group maintain excellent working relationships with members of the UK chemicals industry and 2 complex effluents (discharged direct to the marine environment) were donated to the EA Science Group for research purposes. These were labelled UK1 and UK2 on receipt and were sampled from sites that produce an average daily flow rate of 5446 and 3320 m^3 effluent respectively. Previous experience with these effluents indicated that they were acutely toxic to laboratory test organisms.

2) Materials and methods used

2.1. Test effluents

Eight litres of both effluents were sampled using 1 litre glass Duran bottles and transported, on ice, to the EA Science Group. All replicates of each effluent were pooled and divided into 250 ml and 1 litre plastic bottles before being frozen and maintained until use below -80°C.

2.2. Test organisms

2.2.1) Tisbe battagliai
Laboratory cultures of *Tisbe battagliai* are maintained in natural filtered (0.2 μm) seawater at the EA Science Group (originally from Brixham Environmental Laboratory, Brixham, Devon, U.K.). Cultures are held at 20 ± 2°C under moderate illumination (16 hours of approximately 1000 lux per day). Adult *T. battagliai* in breeding stocks are isolated (using meshes of differing sizes) and allowed to produce neonates over a 24-hour period before being isolated again. Following 5 days culture, the isolated neonates (now juveniles) are ready for use in toxicity tests.

2.2.2) Skeletonema costatum
Laboratory cultures of *Skeletonema costatum* are maintained in a low EDTA artificial media (ISO 10253:1998) at the EA Science Group (originally from SAMS Research Services, Dunbeg, Oban, U.K.). A pre-test culture is inoculated 5 days prior to testing and maintained at 21 ± 2°C under constant illumination (*circa*. 10,000 lux) and orbitally shaken (~100 rpm).

2.2.3) Vibrio fischeri *(Microtox reagent)*
Freeze-dried *Vibrio fischeri* were purchased from SDI Europe (Wokingham, Berkshire, RG41 5TU, U.K.) and stored at -20 ± 1°C.

2.2.4) Inoculant
The inoculant used in the standardised active biodegradation test was derived from the secondary effluent of a sewage treatment plant and was donated by the Environment Agency's National Laboratory Service (Waterlooville, Hampshire, PO7 7XX, U.K.).

2.3. Test methods

2.3.1) Tisbe battagliai *acute toxicity test*
The *T. battagliai* acute toxicity experiments were carried out in a controlled temperature room subject to 20 ± 2°C under moderate illumination (16 hours of *circa.* 1000 lux light per day) in polystyrene cell culture plates (Fisher Scientific U.K., Loughborough, LE11 5RG, U.K.). Five-day old juveniles were transferred between culture and test vessels using a narrow-bore glass Pasteur pipette. Five individuals were allotted to each 5mL test vessel (containing 2.5mL of test solution) with 4 replicate test vessels were used for each test concentration, at 2.2, 4.6, 10, 22 and 46% effluent (v/v), whereas the control group consisted of six replicates. Animals were not fed during the 48-hour duration of the test. Water quality characteristics (salinity, pH and dissolved oxygen) were measured at the beginning and end of the test. Mortality was the only endpoint used in this test and animals were considered dead when they did not display any (external or internal) movement for a 15-second period. The number of dead animals was noted for each replicate 48 ± 2 hours after the start of the test.

2.3.2) Tisbe battagliai *chronic toxicity test*
The *T. battagliai* chronic toxicity experiments were carried out in identical environmental conditions to that of the acute toxicity test but at 0.046, 0.1, 0.22, 0.46, 1.0, 2.2 and 4.6% effluent (v/v). Ten (5-day old) juveniles were allotted to control and test vessels. Animals were not fed during the first 48 hours of the test and fed every second day thereafter. The feed consisted of algal concentrates (of *Nannochloropsis oculata* and *Rhinomonas reticulata*) at concentrations prepared to provide 3250 μg organic Carbon /L. The number of gravid females developing within each test concentration was recorded and compared between treatments. The number of neonates produced in the first brood of each gravid female was also compared between different replicates and test concentrations, as was the mean number of offspring produced per female, over the duration of the 16-day test.

2.3.3) Skeletonema costatum *3 day growth inhibition test*
The *S. costatum* inhibition of growth test was maintained at 21 ± 2°C under constant illumination (*circa*. 10,000 lux) and shaken conditions (~100 rpm). The algae were cultured in a low EDTA artificial media (ISO 10253:1998). The constituent stocks A, B and C are described in Table 1 and were added at 15, 0.5 and 1ml/L of sterile reverse osmosis grade water. Stock solutions were then sterilised by passing through a (0.2 μm) membrane filter.

Stock A	mg/L	Stock B	mg/L	Stock C g/L	
$FeCl_3.6H_2O$	5.3	Thiamin hydrochloride	50	$NaNO_3$	50
$MnCl_2.4H_2O$	144	Biotin	0.01	K_3PO_4	3
$ZnSO_4.7H_2O$	4.4	Vitamin B12	0.1	$Na_2SiO_3.5H_2O$	14.9
$CuSO_4.5H_2O$	0.157				
$CoCl_2.6H_2O$	0.404				
H_3BO_3	1140				
Na_2EDTA	6.67				

Table 1. Nutrients used in the preparation of stock solutions A, B and C.

Following a microscopic determination of cell numbers, control and test solutions were inoculated with 2,500 ± 250 cells/ml. The dilution water used was filtered sterilised natural seawater. The initial and final cell density was examined by fluorescence using a Tecan Ultra Evolution Plate Reader (Tecan, Theale, Reading, RG7 5AH, U.K.).

2.3.4) Microtox ®

Vials of freeze-dried *Vibrio fischeri* were used as a source of bioluminescence following treatment with a saline reconstitution solution. A Microtox ® Model 500 Analyser was used in conjunction with an IBM compatible PC to perform and analyse these experiments. Microtox ® saline diluent is used to dilute the samples for use in the active biodegradation test. The samples intended for 'passive' degradation were diluted with the natural seawater sampled from Hayling Island.

2.3.5) Standard Active Biodegradation Test (OECD 301E)

The effluent samples were filtered using Whatman 0.2μm filter and analysed for the Dissolved Organic Carbon (DOC). A mineral medium was used to accommodate the diluted effluent sample and inoculant. The four stocks used to prepare this medium were dissolved in 'reverse osmosis' grade water and made up to 1 litre and consisted of:

Stock	Chemical	Concentration (g/L)
1.	KH_2PO_4	8.5
	K_2HPO_4	21.75
	$Na_2HPO_4.2H_2O$	33.4
	NH_4Cl	0.5
2.	$CaCl_2$	27.5
3.	$MgSO_4.7H_2O$	22.5
4.	$FeCl_3.6H_2O$	0.25

The mineral medium used in this test used stocks 1, 2, 3 and 4 at 10, 1, 1 and 1 ml/L respectively. The Dissolved Organic Carbon (DOC) measurements of the filtered effluents, UK1 and UK2, were 1310 and 381 mg/L C respectively. As a result, the concentrations of effluents used in this test (UK1: 0.75%; UK2: 2.6%) were calculated to give a DOC content of 10mg/L (as specified in OECD 301E). This test used 1 Litre aliquots in sterile 2-Litre conical flasks plugged with sterile cotton wool and shaken (~100 rpm) in a darkened orbital incubator. The biodegradation of each effluent was monitored (in duplicate) alongside (2) inoculant-only blanks and one sterile filtered sample for each effluent.

2.3.6) Novel Passive Biodegradation Test

A twenty-Litre sample of natural seawater was taken at 11:00am on 03/10/2005 from Gunner Point on Hayling Island (GB grid reference SZ 689 991) and transported to the EA Science Group where it was chilled to 4°C and filtered through glass wool to remove coarse particles. The biodegradation of both unfiltered effluents was measured in terms of the change in their toxicity toward the three laboratory species. Both effluents were biodegraded for 21 days at 0.046, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10 and 22 % (v/v) before freezing at -84°C to ensure stability. Sample thawing took place immediately before use in the post-biodegradation toxicity tests.

2.4. Water quality analysis

Salinity, pH and dissolved oxygen (DO) meters were used to monitor water quality variables at intervals throughout all tests. An ammonia probe was used to measure the levels of total ammonia (NH_3 and NH_4^+) in the original effluent samples, the seawater, the mineral medium and the post-biodegradation samples from both biodegradation tests.

2.5. Data analysis

All analyses were carried out using Minitab version 5.0. Analysis of the dose-response relationship between effluent concentration and effect was made using the Maximum Likelihood- Weibull calculation. The median Effective Concentration (EC50) was recorded, with 95% confidence limits, where possible. A Mann-Whitney comparison of heterogeneous groups was performed on the reproductive characteristics from the *Tisbe battagliai* chronic toxicity test.

3) Results

3.1. *Tisbe battagliai* acute toxicity test

The control group of animals experienced 0.03% mortality in the initial toxicity test and the 48-hour EC50 for UK1 was 3.00% effluent (1.95 – 5.72%). Whilst the 48-hour EC50 for UK2 was 1.45% effluent, it was not possible to calculate confidence limits owing to heterogeneity of results. Following degradation using the active biodegradation test, the EC50 of UK1 was 0.335% effluent (without confidence limits) and that of UK2 was 0.832% (0.448 – 1.152%). Following treatment using the passive biodegradation test, the EC50 of UK1 and UK2 was 3.72% (1.70 – 4.61%) and 7.98% (4.72 – 10.60%) respectively.

3.2. *Tisbe battagliai* chronic toxicity test

The water quality observed throughout the test is described in Table 2 below. The effects of the effluents upon reproductive endpoints are summarised in Table 3. Briefly, UK1 at 0.46% effluent inhibited the number of offspring in first brood (P = 0.0052) and the number of offspring per reproducing female (P = 0.0428).

Sample		pH (min –max)	DO (%ASV)	Salinity (‰)
	Control	7.62 - 8.38	101.9 ± 4.0	35.7 ± 0.4
UK1	0.046	7.54 - 8.33	99.1 ± 4.5	35.8 ± 0.4
	0.1	7.56 - 8.37	98.1 ± 4.4	35.7 ± 0.4
	0.22	7.58 - 8.38	96.1 ± 3.6	35.5 ± 0.5
	0.46	7.62 - 8.4	97.6 ± 3.9	35.4 ± 0.5
	1	7.61 - 8.46	96.1 ± 3.6	35.6 ± 0.5
	2.2	7.69 - 8.48	94.2 ± 3.2	35.2 ± 0.5
	4.6	7.87 - 8.03	95.5 ± 1.4	34.4 ± 0.4
UK2	0.046	7.58 - 8.36	94.4 ± 4.0	35.7 ± 0.4
	0.1	7.57 - 8.35	95.1 ± 3.4	35.8 ± 0.5
	0.22	7.52 - 8.33	94.9 ± 4.0	35.6 ± 0.4
	0.46	7.51 - 8.35	93.7 ± 3.7	35.4 ± 0.4
	1	7.51 - 8.35	92.7 ± 4.3	35.1 ± 0.4
	2.2	7.57 - 8.3	95.0 ± 2.8	34.9 ± 0.3

Table 2. Water quality characteristics observed in the *Tisbe battagliai* chronic toxicity test. The minimum and maximum pH values are presented as well as the mean ± standard error dissolved oxygen and salinity values (n = 9 except for UK1 4.6% sample for which n = 5).

		UK1					UK2					
Control		0.046	0.1	0.22	0.46	1	0.046	0.1	0.22	0.46	1	2.2
No. of reproducing females	7.5 ± 1.3	5.5 ± 0.9	6.0 ± 0.4	8.0 ± 1.2	6.0 ± 1.7	0 ± 0	4.0 ± 1.2	6.0 ± 0.9	6.0 ± 2.3	5.0 ± 1.0	4.5 ± 1.2	* 1.0 ± 0.6
No. of offspring in first brood	35.5 ± 3.6	28.5 ± 5.6	* 26.1 ± 2.7	29.2 ± 1.9	* 23.3 ± 5.1	0 ± 0	34.1 ± 3.5	30.5 ± 5.0	30.2 ± 4.5	25.9 ± 3.0	* 25.0 ± 4.1	1.8 ± 8.6
No. of offspring per reproducing female	91.6 ± 12.5	96.1 ± 21.1	84.9 ± 16.6	105.6 ± 8.3	* 69.0 ± 10.1	0 ± 0	101.9 ± 42.6	122.3 ± 20.5	155.5 ± 40.0	73.3 ± 11.4	116.8 ± 21.6	37.0 ± 19.1

Table 3. The effects of the original effluent samples on *T. battagliai* reproductive parameters. Data presented as median values ± standard errors. Asterisks denote a statistically significant difference between the sample and the control group (n = 6 for the control and 4 for the exposure concentrations).

The difference between the values observed in the control group and those in the UK1 1% group were not suited to statistical analysis (owing to absence of sample variability). Similarly, too few values available for statistical analysis from the UK2 2.2% effluent group for comparison in both the number of offspring in first brood and of offspring per reproducing female. Nonetheless, the uppermost UK2 concentration (2.2% effluent produced fewer gravid females throughout the test than the control group (P = 0.0136). Although the size of the first brood was smaller in the 1% effluent group than in the control (P = 0.0294) it was not statistically so for the 2.2% effluent group (P = 0.1317). Although conducted, the results of the post-biodegradation *Tisbe battagliai* chronic toxicity test are not described here owing to anomalous results causing it to fail the quality standard examination of the laboratory.

3.3. *Skeletonema costatum* 3 day growth inhibition test

The 3 day EC50 for growth inhibition by UK1 was 0.181% effluent (0.145 – 0.242%), whilst the same value for UK2 was 0.359% effluent (0.335 – 0.372%). Following degradation using the active biodegradation test, the EC50 of UK1 was 0.062% effluent (0.048 – 0.198%) and that of UK2 was 0.150% (0.089 – 0.202%).

(a)

	Sample	pH	Salinity (‰)
	Control	8.06	34.2
UK1	0.046	8.02	34.2
	0.1	8.06	34.2
	0.22	8.05	34.2
	0.46	8.06	34.1
	1	8.04	34.0
	2.2	7.98	33.6
	4.6	7.94	32.8
UK2	0.046	8.02	34.2
	0.1	8.03	34.3
	0.22	8.03	34.3
	0.46	8.05	34.1
	1	8.04	34.1
	2.2	8.15	33.7
	4.6	8.27	33.0

(b)

	Sample	pH	Salinity (‰)
	Control	8.03	34.4
	Biodeg. solution	7.75	30.4
UK1	0.07	8.05	30.8
	0.14	7.93	31.1
	0.28	7.63	30.9
	0.56	7.89	30.5
UK2	0.06	8.16	31.4
	0.123	8.09	31.1
	0.245	7.87	31.0
	0.49	8.17	30.8
	0.975	7.72	30.9
	1.95	7.89	30.5

Table 4. Water quality characteristics (a) before and (b) after biodegradation using the active biodegradation test. Biodeg. solution refers to the mineral medium used in the active biodegradation test.

Although this toxicity test was not completed for the passive biodegradation test, a (75% strength) solution of the mineral medium used in the active biodegradation test caused a 93.7% inhibition of growth. The water quality characteristics of the control and test concentrations were examined and are described in Table 4 (a and b).

3.4. Microtox ® test system

The EC50 for UK1 was 2.99% effluent (2.81 – 3.18%) and the EC50 for UK2 was 3.02% effluent (2.98 – 3.12). It was not possible to calculate an EC50 of UK1 following degradation, in the active biodegradation test, as the maximum concentration of effluent available for testing after biodegradation was 0.75% effluent. This produced a 14.1% response. The EC50 of UK2 following active degradation was 1.38% effluent (1.30 – 1.48%). Following treatment using the passive biodegradation test protocol, the EC50 of UK1 and UK2 was 62.69% (8.53 – 460.50%) and 2.75% (1.62 – 4.67%) respectively.

3.5. Standard Active Biodegradation Test

The dissolved organic carbon (DOC) content decreased in both effluents after a lag phase of 2 days. The degree of biodegradation at the end of the 10-day examination window was 50.8% in UK1 and >95% in UK2 (the carbon was no longer detectable on or after day 5 of degradation). Using the criteria described in the OECD guideline for testing of chemicals, the effluent UK1 must be described as not readily biodegradable whereas the effluent UK2 may be considered biodegradable.

3.6. Novel Passive Biodegradation Test

The biodegradation examined in this test was measured using the toxicity tests described above and, as such, the results have been recorded elsewhere.

3.7. Water quality analysis

The water quality of the effluents was examined upon receipt (Table 6).

Sample	pH	Dissolved Oxygen (%)	Conductivity (mS/cm)	Total Ammonia (mM)
UK1	8.10	19.0	7.02	11.5
UK2	10.09	96.5	12.97	3.0

Table 6. Water quality characteristics of sample effluents.

Ammonia was not detected in the seawater sampled from Hayling Island (<0.1mM NH_3) but was observed in the biodegradation solution used in the active biodegradation test at 0.3mM NH_3. This was as a result of ammonia in one of the stock solutions (2.8mM NH_3). Ammonia was present in UK1 at 0.5mM NH_3 following biodegradation by the active biodegradation test. A concentration-dependant relationship was observed between the UK1 concentration in the passive biodegradation test and the concentration of ammonia measured at the end of the test (R^2 = 0.9969). A maximum value of 9.8mM NH_3 was observed in a 22% effluent sample. Ammonia was not detected in UK2 following biodegradation using either the active biodegradation test or the passive biodegradation test.

4. Evaluation, conclusions and recommendations

The complex effluents UK1 and UK2 were toxic to representative organisms of the marine environment at with worst-case EC50values of 0.181% and 0.359% effluent concentrations respectively. The *Skeletonema costatum* 3-day growth inhibition test was found to be the most sensitive test. Applying the active biodegradation test (OECD 301E) demonstrated that UK1 was not readily biodegradable whereas UK2 was readily biodegradable. Both effluents were more toxic to the *Skeletonema costatum* following biodegradation than before (EC50 values of 0.062 and 0.150 respectively) although this owed, at least in part, to the toxicity of the biodegradation medium. Biodegradation conducted in conditions simulating more closely the natural environment (passive biodegradation test) degraded the acute toxicity toward *T. battagliai* of UK1 slightly (EC50: 3.00 to 3.72% effluent) and UK2 more so (EC50: 1.45 to 7.98% effluent). The presence of ammonia in this test system was higher than in the active biodegradation test but did not appear induce a toxic response. The use of OECD 301E to examine the toxicity of biodegraded industrial samples toward biota may bias calculations of their toxicity towards overestimation. As a result, the removal of a mineral medium (which may add confounding factors to subsequent toxicity tests) from biodegradation studies is recommended. The benefit derived from the addition of an inoculant was also questionable as degradation of toxicity occurred in the passive biodegradation test without the addition of a bacterial seed. Further studies are recommended in which these factors, currently deemed to be necessary in biodegradation studies but seem confounding in subsequent toxicity tests, are elucidated and remediated.

Annex 6: Practical "Whole Effluent Assessment" Study on 3 Arkema wastewater samples

Lacq Research Center

Analysis Department

Ecotoxicology and Microbiology

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REPORT

PRACTICAL « WHOLE EFFLUENT ASSESSMENT" STUDY
ON 3 ARKEMA WASTEWATER SAMPLES

Report to the OSPAR Intersessional Expert Group(IEG)
on Whole Effluent Assessment

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Introduction

In the frame of the IEG OSPAR program on whole effluent assessment, having developed for a long time a strategy to how to handle this problem, ARKEMA has engaged herself to participate to this program. After discussion with the IEG members we have decided to sample effluent before the water treatment plant in order to develop the method in its totality (even if WWTP were present on each of the three chemical sites).

2) Description of wastewater samples

2.1 ARKEMA « A » Chemical Plant

The wastewater sample has been taken before the biological treatment unit. Date of sampling is 15/06/2005. The average flow for the industrial effluent at this point is 92.5 m^3/h (2220 m^3/d).

After the biological treatment, the effluent is mixed with 800 m^3/h of other effluents that are treated on the physico-chemical unit.

After treatment, the industrial effluent is discharged into a river where the average flow is 400 000 m^3/d, which corresponds to a dilution factor of 0.555 %.

The total sample volume was 23 litres. It has been cooled during transportation, and frozen for conservation during the study.

The sample was light orange to yellow, and showed turbidity. Table 1 below indicates physical-chemical characteristics:

Effluent	Aspect	pH	Suspended solids	Chemical Oxygen Demand (COD)	Dissolved Organic Carbon (DOC)	Total Nitrogen	Chloride ions
			mg/l	mg/l	mg/l	mg/l	mg/l
Effluent A 0015/05 Before biological treatment 15/06/05	Light orange-yellow colour Turbid	6.69	43	623	180	10.8	2150

Table 1. Physical-chemical characteristics of effluent A (GRL internal reference 0015/05)

2.2 ARKEMA « B » Chemical Plant

The wastewater sample has been taken before the ozone treatment unit. Date of sampling is 6/07/2005. The average flow for the industrial effluent at this point is 14.6 m^3/h (350 m^3/d).

After treatment, the industrial effluent is discharged into a river where the average flow is 121 000 m^3/d, which corresponds to a dilution factor of 0.29 %.

The total sample volume was 21 litres. It has been cooled during transportation, and frozen for conservation during the study.

The sample was dark brown, and showed low turbidity. Table 2 below indicates physical-chemical characteristics:

Effluent	Aspect	pH	Suspended solids	Chemical Oxygen Demand (COD)	Dissolved Organic Carbon (TOC)	Total Nitrogen	Chloride ions
			mg/l	mg/l	mg/l	mg/l	mg/l
Effluent B 0022/05 Before ozone treatment 6/07/05	Dark brown colour Low Turbidity	8.37	353	1567	522	251	8200

Table 2. Physical-chemical characteristics of effluent B (GRL internal reference 0022/05)

2.3 ARKEMA « C » Chemical Plant

The wastewater sample has been taken before the biological treatment unit. Date of sampling is 12/09/2005. The average flow for the industrial effluent at this point is 83 m^3/h (2000 m^3/d).

After treatment, the industrial effluent is discharged in a river where the average flow is 1.555 x 10^6 m^3/d, which corresponds to a dilution factor of 0.13%.

The total sample volume was 25 litres. It has been cooled during transportation, and frozen for conservation during the study.

The sample was orange, showed turbidity and some kind of film at surface. Table 3 below indicates physical-chemical characteristics:

Effluent	Aspect	pH	Suspended solids	Chemical Oxygen Demand (COD)	Dissolved Organic Carbon (TOC)	Total Nitrogen	Chloride ions
			mg/l	mg/l	mg/l	mg/l	mg/l
Effluent C 0034/05 Before biological treatment 12/09/05	Orange colour Turbidity Film on surface	7.16	654	8355	2642	1230	104

Table 3. Physical-chemical characteristics of effluent C (GRL internal reference 0034/05)

3) Test methods

In the whole effluent assessment method developed within ARKEMA company, two aspects are taken into account (see graph n°1). On one side acute toxicity is assessed and on the other side chronic toxicity through identification and quantification of PBT (Persistent and Bioaccumulable and Toxic) substances being present in the effluent.

For assessing acute toxicity, four acute short term standardized tests representative of different trophic levels have been chosen including algae (growth ISO 8692), daphnia (immobilization ISO 6341), fish (mortality ISO 7346) and bacteria (photoluminescence ISO 11348).

It is important to stress that results from these tests are expressed as a percentage of dilution. We have chosen to determine the EC10 (Effective dilution having 10% of effect) as a value close to a no-effect concentration. Among the results of the four trophic level, the most sensitive result is chosen as being equivalent to the dilution factor needed to have no effect on the ecosystem (the figure being called "**PNEC-like**"). Assessment of the extent of exposure of the receiving system to the effluent is made by calculating the ratio between the flow-rate of the effluent divided by the one of the receiving river (the annual low flow-rate is selected as a reasonable worst case). This ration is called "**PEC-like**"). By comparing PEC-like to PNEC-like, we predict if any effect has to be expected from the effluent when mixed in the river water (if the ratio between PNEC-like/PEC-like is less than one, we conclude to the absence of risk).

No extra safety factor should be added to the lowest EC10 for the following reason: we have often experienced effluents with no acute toxicity, i.e. EC10 not determined on pure sample. Adding a safety factor would mean requiring to dilute clean water!

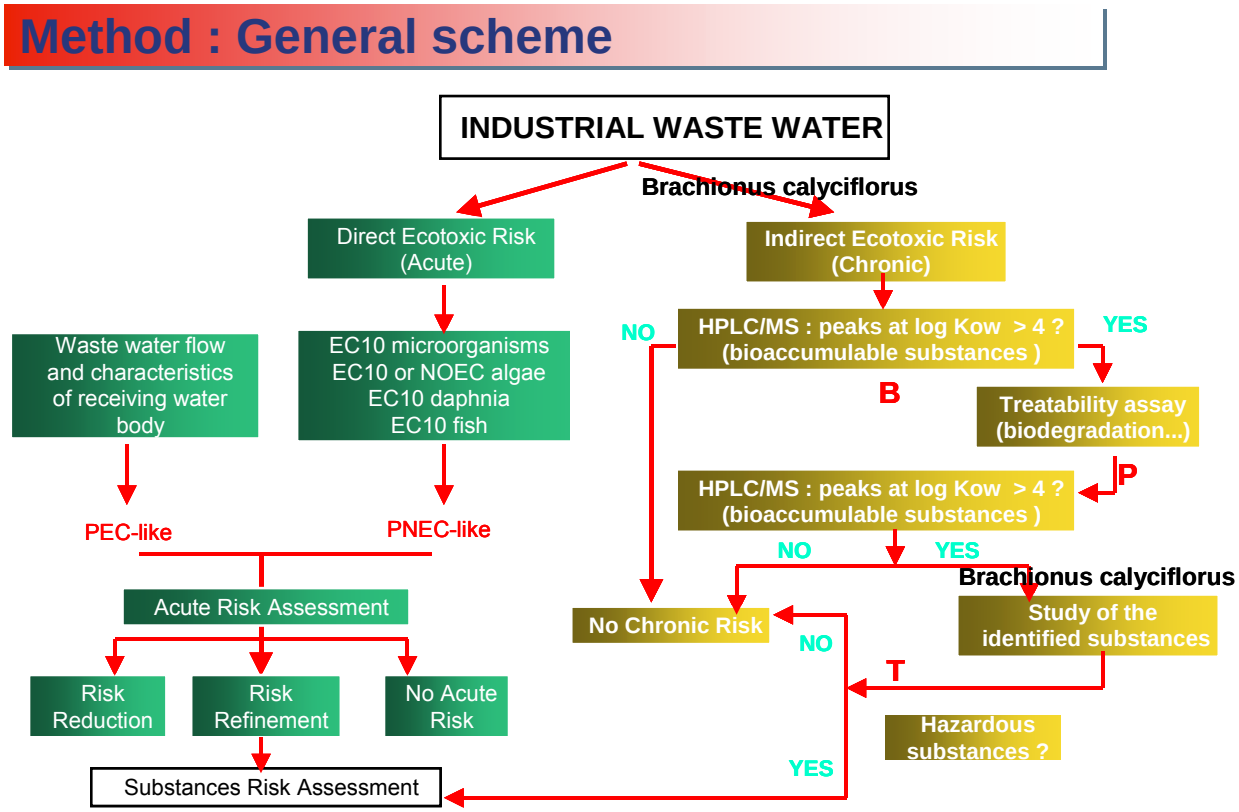
For the assessment of chronic toxicity, our target was to determine the presence of PBT substances within the effluent. First of all, a short duration chronic test is carried out on reproduction of *Brachionus calyciflorus* during 48h (ISO/CD 20666) to test the global chronic toxicity before water treatment.

The presence of bioaccumulable (BCF) substances within the effluent is done through analysis of lipophilic substances by Kow determination by the standard HPLC method (OECD 117). If peaks are observed on the chromatogram for retention times corresponding to logKow > 4, then we consider that the effluent contain some (or several, if several peaks) substance having the "B" property.

In the present inter-laboratory study, we have added the potentiality for substance to bind to SPME membrane.

The second tier consists in making a biodegradation test to evaluate if the substance(s) having a potential to bioaccumulate are biodegradable or not (assessment of criteria "P"). This is done by 1) carrying out an

inherent biodegradation test according to Zahn-Wellens (OECD 302B) and 2) repeating the Kow determination by HPLC on the treated effluent. An additional chronic test on *Brachionus* is made after the biodegradation test to check if any chronic toxicity remained after biodegradation treatment. If any bioaccumulative substance persists after biodegradation, this means it fulfils the criteria P and B. If so, an investigation is conducted (mass spectrometry) in order to identify this (or these) substance(s) and corresponding available ecotoxicity data for conclusion on its "T" properties.



3.1. Acute toxicity testing

<u>*Vibrio fischeri* testing according to ISO 11348</u>
Toxicity of wastewaters against bacteria is measured through the use of the bioluminescent bacteria *Vibrio fisheri*. The test is performed with the M500 analyser from Microbics, and freeze-dried bacteria.

<u>*Daphnia magna* testing according to ISO 6341and OECD Guideline 202</u>
Acute toxicity effect of wastewaters on *Daphnia magna* Straus was determined. The parameter measured is EC_{10} that corresponds to the dilution where 90 % of the Daphnia population remains active. Test duration is 48 h. No adjustment of pH was done.

<u>Algae testing according to ISO 8692 and OECD Guideline 201</u>
Toxicity of wastewaters was measured on *Pseudokirchneriella subcapitata* freshwater algae. In this case, the EC_{10} parameter corresponds to the effluent dilution giving an inhibition of growth rate of 10 %. Sometime algae test results could be also use for chronic toxicity.

<u>*Danio rerio* testing according to ISO 7346 and OECD Guideline 203</u>
Acute toxicity effect of effluents on zebra fish was determined. The parameter used is EC_{10} that corresponds to the dilution where 90 % of the fish population remains alive after 96 h. No adjustment of pH was used.

3.2 Chronic toxicity testing

Brachionus calyciflorus testing according to ISO/CD 20666
Chronic toxicity of wastewaters was also measured on *Brachionus calyciflorus*. The parameter used is the EC_{10} value which corresponds to the dilution for which the increase of offspring population is inhibited at a 10 % rate.

3.3 Biodegradability testing

Zahn-Wellens test according to OECD 302B
DOC elimination of the 3 wastewater samples was determined with the Zahn-Wellens EMPA test method, using sludge from the wastewater treatment unit from Abidos (F-64 France).

This test simulates the treatability of the effluents by biological domestic wastewater treatment units. Effluents A, B and C were properly diluted accordingly to the DOC content, in agreement with the corresponding guideline (DOC test concentration 50 – 400 mg/l).

3.4 Bioaccumulation
For the 3 effluents, potentially bioaccumulating substances (PBS) were evaluated either by solid phase microextraction (SPME), or by the Kow methodology.

Solid Phase Micro Extraction according to Leslie and Leonards (2005)

For each sample, 250 ml of effluent was exposed to 100 µm PDMS (Polydimethylsiloxane) coated fibre under stirring during 24 h. GC analysis was then performed using a 6890 Agilent chromatograph, using 2,3 dimethylnaphtalene* as a reference compound. Results were expressed as mmole of equivalent DMN per litre of fibre.
* DMN, [581-40-8], $C_{10}H_6(CH_3)_2$, MW 156.22

Partition coefficient Kow methodology
Potentially bioaccumulating substances that is substances with marked lipophilic character, show high values of octanol-water partition coefficient (Kow). The HPLC method used for Kow determination was the OECD Guideline 117, with an UV detection at 210 nm. Standard substance with a Kow of 4 correspond to a retention time of 4.77 min (see graphs on Annex).

4) Results
For each effluent, acute toxicity measurements were performed using bacteria, daphnia, algae and fish, and chronic toxicity values were obtained by *Brachionus* testing.

Treatability was evaluated by Zahn-Wellens inherent biodegradability measurement as decrease of dissolved organic carbon (DOC).

Potential bioaccumulation properties of effluent substances were evaluated either by SPME or by log Kow determination and GC-MS analysis.

4.1 ARKEMA « A » chemical plant wastewater

4.1.1 Acute toxicity
For this effluent, experimental data show a low toxicity. Algae test is the most sensitive as shown in the table below:

Effluent	Bacteria		Daphnia		Fish		Algae	
A 0015/05	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv
Before biological treatment 15/06/05	7.4	38.2	17	23	54	67	2.8	13

Table 4.1.1: Acute toxicity of effluent A (GRL internal reference 0015/05)

Compared to the 2.8 % EC_{10} of the most sensitive species, the flows ratio between effluent and river is 0.555 %, which corresponds to a PEC-like/PNEC-like ratio of 0.2. This ratio is lower than 1, showing no acute toxicity of this effluent.

4.1.2 Chronic toxicity

Effluent	Brachionus before Zahn-Wellens		Brachionus after Zahn-Wellens	
A 0015/05 Before biological treatment 15/06/05	EC_{10} % vv	EC_{20} % vv	EC_{10} % vv	EC_{20} % vv
	28.2	33	21.6	45.2

Table 4.1.2. Chronic toxicity of effluent A (GRL internal reference 0015/05)

The EC_{10} value for *Brachionus* shows that there is only a low chronic risk of this effluent. There is no dramatic change in chronic toxicity after biodegradation showing that the remaining toxicity is not due to biodegradable substance.

4.1.3 Bioaccumulation

Kow
For the A effluent, no peak was observed in the potentially bioaccumulable part of the chromatograms (logKow > 4 corresponding to a retention time of 4.77 min, see annex 4). The only result was a substance with a log Kow value less than 3.5. This shows that no bioaccumulable substance is detected in the test conditions. So this effluent is not fulfilling the “B” criteria.

SPME
Results are expressed as mmoles equivalent 2,3-dimethylnaphtalene per litre of fibre. The effluent A gave 5.8 mmoles eq. DMN/L of fibre when the blank gave 0.
In order to figure out this value, it corresponds to 2.3 µg eq. DMN/L of wastewater that is equivalent to 2.3 ppb.

4.1.4 Biodegradation
Log Kow value shows that there are no bioaccumulating substances in the conditions of the test. However, as the SPME determination was not 0, showing PBS not shown by Kow testing, a Zahn-Wellens biodegradation test was performed.

Prior to biodegradation, the effluent was diluted 2 times to obtain the test condition of a DOC content equal to 90 mg/l.

Zahn-Wellens biodegradation test showed that 92.6 % of the organic carbon (DOC) was eliminated in 7 days, and almost 100 % in 28 days. No more substance with a value of Kow more than 4 appeared after biodegradation confirming that that no persistant substances are present in the effluent, indeed the “P” criteria is not fulfilled.

After Zahn-Wellens biodegradation, *Brachionus* EC_{10} and EC_{20} dilution values were weakly affected by the treatment (see Table 4.1.2).

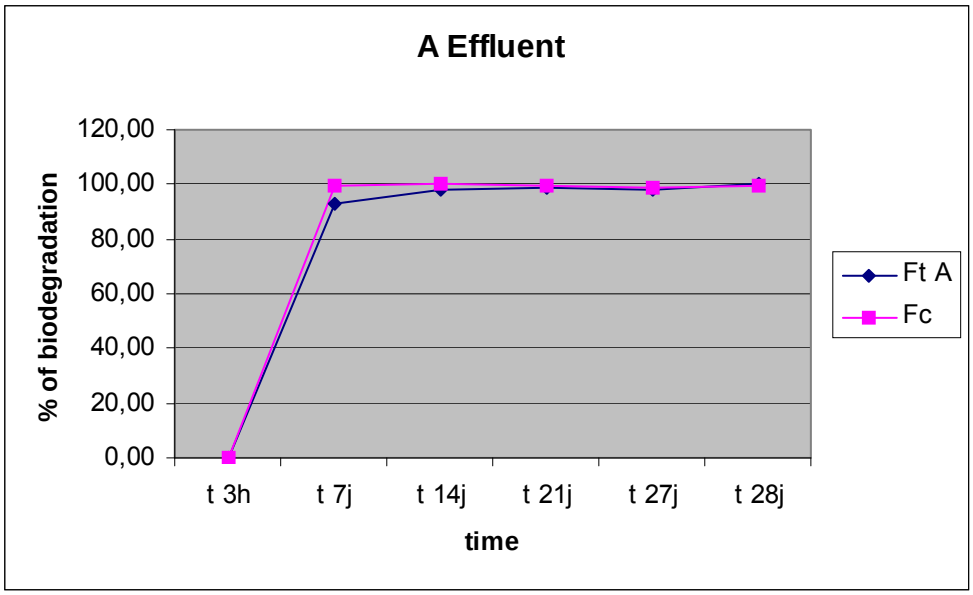


Figure 4.1.4. Zahn-Wellens biodegradation of A effluent (GRL internal reference 0015/05)

4.2.3 Conclusion for effluent "A"

In conclusion, in the effluent "A", sampled before waste water treatment plant, there is no acute toxicity as well as chronic one based on the PBT approach as we are not fullfiling the "P" and "B" criteria.

4.2. ARKEMA « B » chemical plant wastewater

4.2.1 Acute toxicity

For this effluent, experimental data show a low toxicity. *Daphnia* test is the most sensitive as shown in the table below:

Effluent	Bacteria		Daphnia		Fish		Algae	
B 0022/05 Before ozone treatment 6/07/05	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv
	0.1	1.1	0.05	0.33	6.2	7.7	0.6	5.3

Table 4.2.1. Acute toxicity of B effluent (GRL internal reference 0022/05)

Compared to the 0.05 % EC_{10} of the most sensitive species, the flows ratio between effluent and river is 0.29%, which corresponds to a PEC-like/PNEC-like ratio of 5.8.
This ratio is higher than 1, showing the presence of acute toxicity of the effluent if testing is done prior to waste water treatment plant.

4.2.2 Chronic toxicity

Effluent	Brachionus before Zahn-Wellens		Brachionus after Zahn-Wellens	
B 0022/05 Before ozone treatment 6/07/05	EC_{10} % vv	EC_{20} % vv	EC_{10} % vv	EC_{20} % vv
	0.008	0.036	0.03	0.17

Table 4.2.2. Chronic toxicity of B effluent (GRL internal reference 0022/05)

The 0.008% EC_{10} value for *Brachionus* shows a high chronic risk for this effluent which is reduced of about a factor of 4 after Zahn-Wallens biodegradation, showing the existence of a residual chronic toxicity.

4.2.3 Bioaccumulation

Kow
For the B effluent, the result was a global log Kow value less than 3.5. However, a small peak was observed with a Kow close to 4 (retention time 4.77 min) showing the presence of at least one putative compound fulfilling the "B" criteria. After 28 days Zahn-Wellens biodegradation, the same area of the curve was reduced by approximatively 60 % (see annex 4) showing the disappearance of the putative "B" substance which has been researched by GCMS analysis as described in the Annex 5.

SPME
Results are expressed as mmoles equivalent 2,3-dimethylnaphtalene per litre of fibre. The B effluent gave 7.85 mmoles eq. DMN/L of fibre when the blank gave 0.
In order to figure out this value, it corresponds to 3.2 µg eq. DMN/L of wastewater that is 3.2 ppb.

4.2.4 Biodegradation
As one substance with Kow more than 4 has been observed, we are doing a biodegradation test to check if the substance is persistent.

Prior to biodegradation, the effluent was diluted 2 times to reach a DOC content of 261 mg/l. For the Zahn-Wellens biodegradation test, only 6.3 % of the organic carbon (DOC) was eliminated in 7 days, and 13.7 % in 28 days.

After the biodegradation, the substance with a Kow >4 is reduced to 40% of its initial value showing the presence of a persistent substance, so the effluent is fulfilling the "B" criteria.

However, after biodegradation, *Brachionus* EC_{10} and EC_{20} dilution values were respectively increased to 0.03 and 0.17 % (see Table 4.2.2).

These results show that despite the low biodegradation level, the chronic toxicity was strongly decreased by the Zahn-Wellens treatment (4 fold).

As the effluent is fulfilling both "P" and "B" criteria, the next step consist to try and identify the responsible substance (see Annex 5). Based on the GCMS analysis several substances has been identified (see Annex 5) but not quantified, further investigations should be necessary to refine and identify quantitatively the responsible substance among the one presented in the table. After identification, it will be necessary to look for ecotoxicity data of this substance. In the frame of this program we did not have enough time to go further.

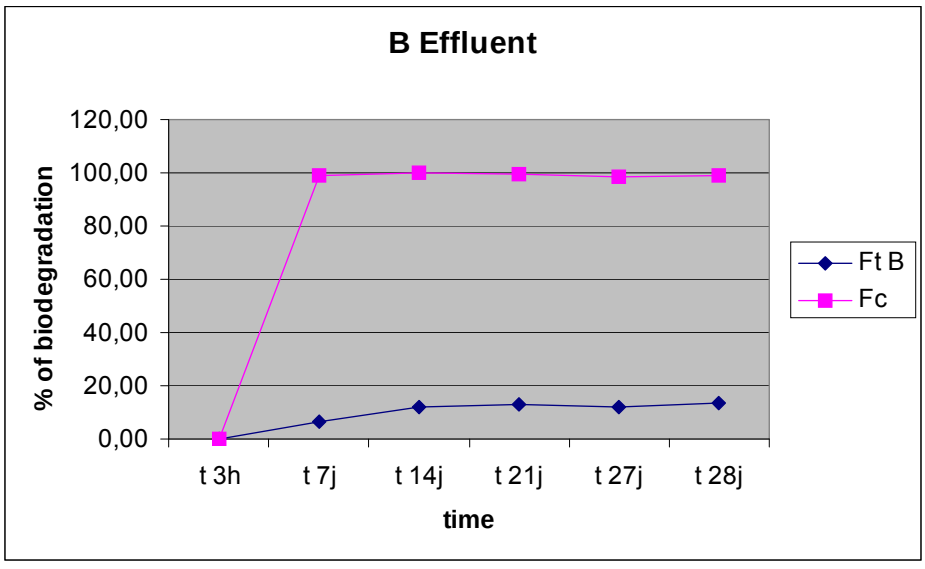


Figure 4.2.4. Zahn-Wellens biodegradation of B effluent (GRL internal reference 0022/05)

4.2.3 Conclusion for effluent « C »

This effluent shows an acute toxicity when sampled before waste water treatment plant. It also shows the presence of persistent and bioaccumulable substances which could be toxic.

4.3. ARKEMA « C » chemical plant wastewater

4.3.1 Acute toxicity

For this effluent, experimental data also shows a high toxicity. Algae test is the most sensitive as shown in the table below:

Effluent	Bacteria		Daphnia		Fish		Algae	
C 0034/05 Before biological treatment 12/09/05	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv	EC_{10} % vv	EC_{50} % vv
	0.01	0.13	0.004	0.01	0.64	1.0	0.003	0.009

Table 4.3.1. Acute toxicity of C effluent (GRL internal reference 0034/05)

Compared to the 0.003 % EC_{10} of the most sensitive species, the flows ratio between effluent and river is 0.13 %, which corresponds to a PEC-like/PNEC-like ratio of 43.

This ratio is much higher than 1, showing the high acute toxicity of the effluent when sampled before waste water treatment plant.

4.3.2 Chronic toxicity

Effluent	Brachionus before Zahn-Wellens		Brachionus after Zahn-Wellens	
C 0034/05 Before biological treatment 12/09/05	EC_{10} % vv	EC_{20} % vv	EC_{10} % vv	EC_{20} % vv
	0.26	0.30	1.8	8.7

Table 4.3.2. Chronic toxicity of C effluent (GRL internal reference 0034/05)

The 0.26% EC_{10} value for *Brachionus* shows a chronic risk for this effluent which is decreased by a factor of 7 after Zahn-Wellens biodegradation.

4.2.3 Bioaccumulation

Kow

For the C effluent, no peak was observed in the potentially bioaccumulable part (Kow >4, retention time 4.77 min) of the chromatograms (see annex 4), and all the results showed a log Kow value < 3.5. This shows that no bioaccumulable substance is detected in the conditions of the test and the “B” criteria is not fulfilled.

SPME

Results are expressed as mmoles equivalent 2,3-dimethylnaphtalene per litre of fibre. The C effluent gave 214.2 mmoles eq. DMN/L of fibre, which is near 30 times higher than the two others samples.

In order to figure out this value, it corresponds to 88 µg eq. DMN/L of wastewater that is 88 ppb.

4.3.4 Biodegradation

Global log Kow value shows that there are no bioaccumulating substances in the conditions of the test. However, as the SPME determination showed a pretty high value, indicating some potentially

bioaccumulating substances not shown by Kow measurement, a Zahn-Wellens biodegradation test was performed.

Prior to biodegradation, the effluent was diluted 6.6 times to reach a DOC content of 400 mg/l.

For the Zahn-Wellens biodegradation test, 85.9 % of the organic carbon (DOC) was eliminated in 7 days, and 86.7 % in 28 days and no more substance with a Kow more than 4 appeared. So the "P" criteria is not fulfilled.

After biodegradation, *Brachionus* EC_{10} and EC_{20} dilution values were respectively increased to 1.8 and 8.7 %. This decrease indicates that substances with chronic toxicity were degraded by the bacteria (see Table 4.3.2).

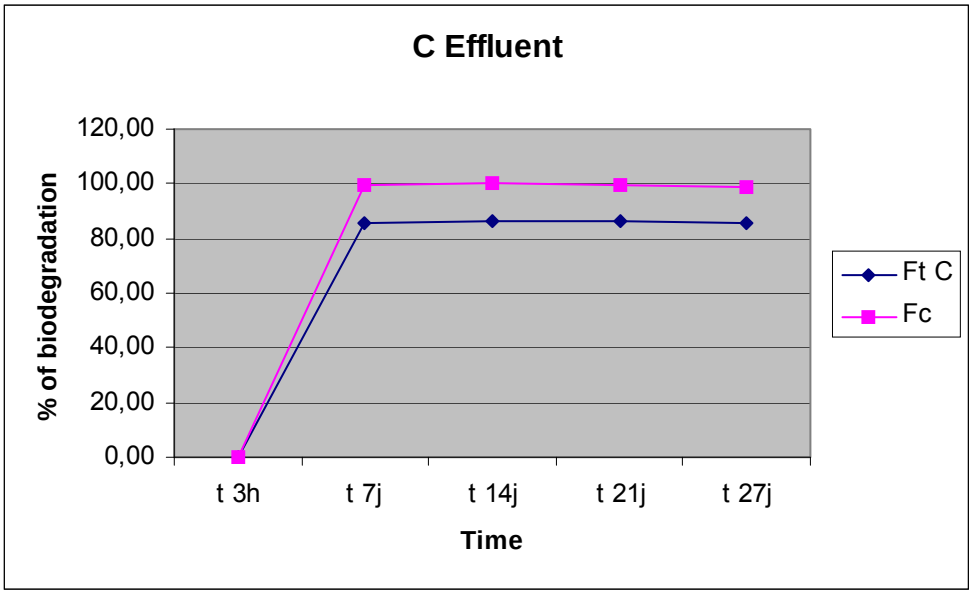


Figure 4.3.4. Zahn-Wellens biodegradation of C effluent (GRL internal reference 0034/05)

4.3.5 Conclusion

This effluent before waste water treatment plant was showing an acute toxicity but no chronic toxicity was observed.

5) Conclusion

For the three effluents coming from the chemical industry before waste water treatment plant, two of them have been found to have an acute toxicity and only one with a chronic toxicity fulfilling the "P" and "B" criteria and maybe the "T".

6) Appendices

6.1 Appendix 1: Table of results

6.2 Appendix 2: Zahn-Wellens Biodegradation

Appendix 1: Summary of results

	Physical-chemical characteristics							Acute and chronic toxicity – Bioaccumulation - Biodegradability							
Effluents	**Aspect**	**pH**	**MES mg/l**	**COD mg/l**	**TOC mg/l**	**Total nitrogen mg/l**	**Chloride ions mg/l**	**Daphnia CE10 %**	**Algae CE10 %**	**Fish CE10 %**	**µTox CE10 %**	**Brachionus Before Zahn-Wellens CE10 %**	**Kow OECD 117**	**SPME mmole eq. DMN per L of fibre**	**Zahn Wellens DOC decrease %**
A Effluent 0015/05 Before biological treatment 15/06/2005	Light orange-yellow colour Turbid	6.69	43	623	180	10,8	2150	17	2.8	54	7.37	28.2	No peak obser-ved	5.7 – 5.9	92.6 in 7 days 99.1 in 21 days
B Effluent 0022/05 Before ozone treatment 6/07/2005	Dark brown colour Low turbidity	8.37	353	1567	522	251	8200	0.052	0.61	6.2	0.1	0.008	Reduc-tion of 60 % after ZW	7.7 – 8.0	6.3 in 7 days 13.7 in 28 days
C Effluent 0034/05 Before biological treatment 12/09/2005	Orange colour Turbid Film on surface	7.16	654	8355	2642	1230	104	0.004	0.003	0.64	0.011	0.26	No peak obser-ved	213.3 – 215.1	85.9 in 7 days 86.7 in 28 days

Appendix 2: Zahn-Wellens

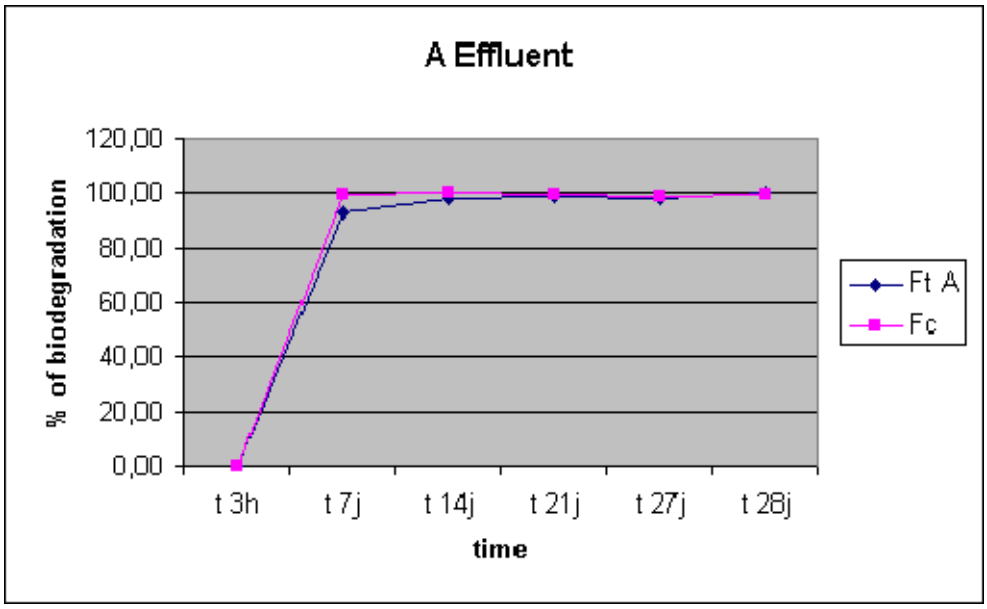
Effluent A biodegradation

DOC (mg/l)

	t 3h	t 7j	t 14j	t 21j	t 27j	t 28j
Fb	4	5,5	8,5	9,4	6,6	8,5
Ft A	87,5	12	10	10,2	8,1	5,5
Fc	405	8,5	8,5	11	12	11,6

% of biodegradation

	t 3h	t 7j	t 14j	t 21j	t 27j	t 28j
Ft A	0,00	92,57	98,29	99,09	98,28	100,00
Fc	0	99,25	100	99,6	98,65	99,23



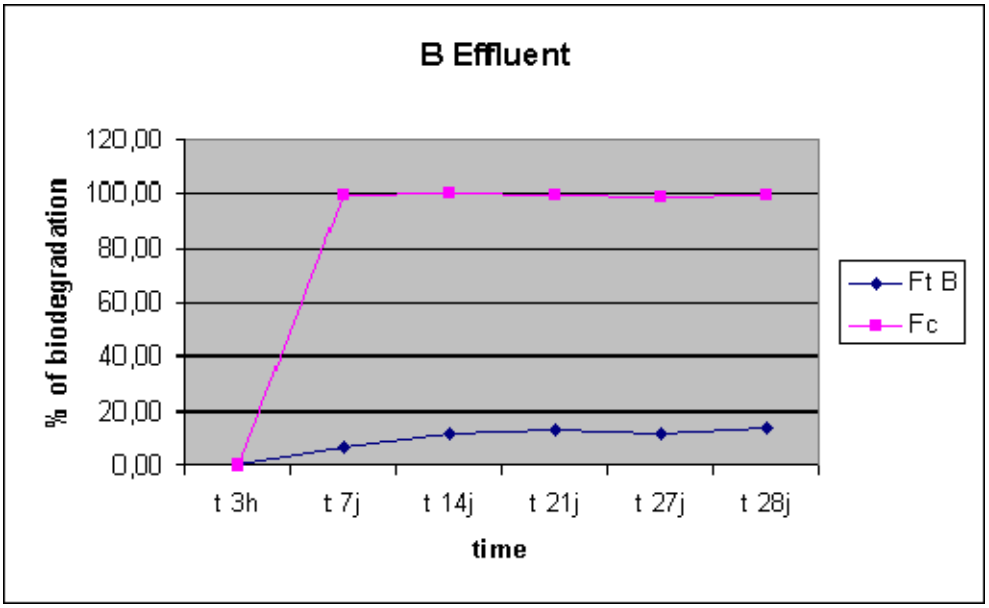
Effluent B biodegradation

DOC (mg/l)

	t 3h	t 7j	t 14j	t 21j	t 27j	t 28j
Fb	4	5,5	8,5	9,4	6,6	8,5
Ft B	233	220	210,33	208,5	208,5	206,2
Fc	405	8,5	8,5	11	12	11,6

% of biodegradation

	t 3h	t 7j	t 14j	t 21j	t 27j	t 28j
Ft B	0,00	6,33	11,86	13,06	11,83	13,67
Fc	0	99,25	100	99,6	98,65	99,23



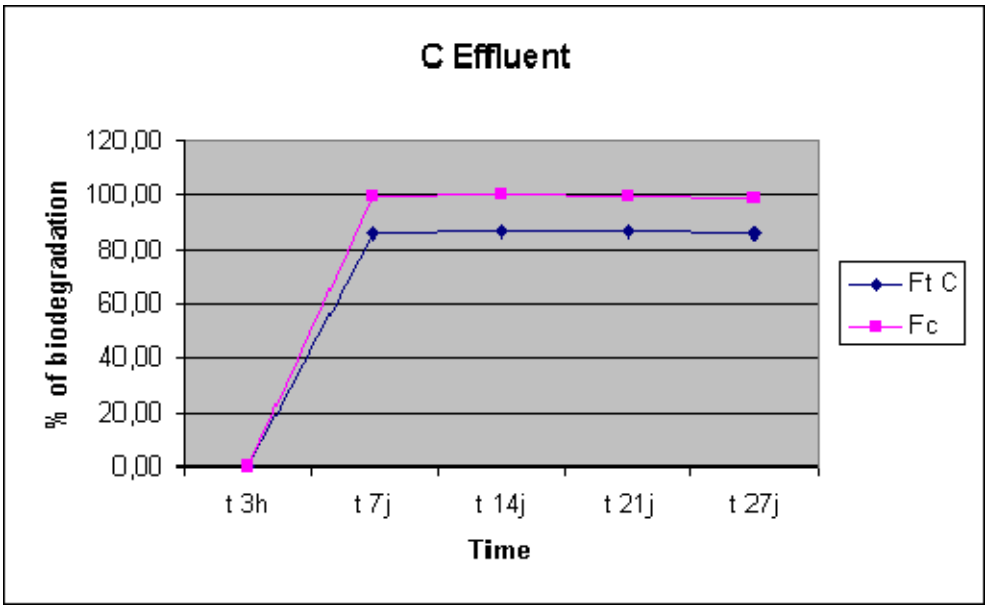
Effluent C biodegradation

DOC (mg/l)

	t 3h	t 7j	t 14j	t 21j	t 27j	t 28j
Fb	4	5,5	8,5	9,4	6,6	8,5
Ft C	350	54,3	56,6	56,6	57,3	54,6
Fc	405	8,5	8,5	11	12	11,6

% of biodegradation

	t 3h	t 7j	t 14j	t 21j	t 27j	t 28j
Ft C	0,00	85,90	86,10	86,36	85,35	86,68
Fc	0	99,25	100	99,6	98,65	99,23



Concawe original samples

Sample	Flow rate m3/min	Mean SPME mmol/L fiber	%RSD	Mean LLE mmol/l	% RSD	pH	NH4+	Cond uS/mm	Salinity ‰	TDS mg/l	DOC mg/l
Concawe 1	4.6	10.1	7	0.00056	26	7.3	10	793	4.9	*	22.9
Concawe 2	ND	59	23	0.035	35	7.7	<10	220	1.1	*	222
Concawe 3	3.5	2.1	24	<LOD		7.6	<10	319	1.8	*	8.2
Concawe 4	64	5.3	11	0.00058	22	7.2	<2.5	4200	30.2	*	7.8
Concawe 5	4.5	19.0	18	0.002	16	7.7	<10	198	1.0	*	12.6
Concawe 6	18	30.8	13	Not determined		7.5	13	278	1.5	*	10.2
Concawe 7	ND	138	14	0.041	10	7.3	<10	98	0.3	1054	12.2
Concawe 8	13.3	5.0	34	<LOD		7.6	10	94	0.3	1012	12.7
Concawe 9	8	6.9	17	<LOD		7.1	10	2000	13.4	*	10.6

* : sample needs dilution # : Acartia tonsa α : Oyster lavae

Sample	Microtox		Algae		Daphnia acute		Daphnia chronic - mortality		Daphnia chronic - repro	
	EC20 vol%	EC50 vol%	NOEC vol%	EC50 vol%	NOEC vol%	EC50 vol%	NOEC vol%	LC50 vol%	NOEC vol%	EC50 vol%
Concawe 1	15	>45	49	>98	32	63	32	>100	32	>100
Concawe 2	<5.6	19	<6.1	10	32	52	18	24	10	22
Concawe 3	>45	>45	98	>98	100	>100	100	>100	100	>100
Concawe 4	>45	>45	25	22	100#	>100#	100α	>100α	100α	>100α
Concawe 5	31	>45	49	>98	100	>100	100	>100	100	>100
Concawe 6	11	35	98	>98	100	>100	18	31	32	62
Concawe 7	<5.6	10	40	>98	<5.6	<5.6	10	15	10	15
Concawe 8	>45	>45	49	>98	100	>100	100	>100	100	>100
Concawe 9	>50	>50	25	>98	32	56	<5.6α	16α		

Concawe results after biodegradation

Sample	Degradation type	Degradation Time	SPME mmol/L fiber	LLE mg/L	%degradation (DOC removal)	Microtox		Algae		Daphnia acute		Daphnia chronic - mortality		Daphnia chronic - repro	
						EC20 vol%	EC50 vol%	NOEC vol%	EC50 vol%	NOEC vol%	EC50 vol%	NOEC vol%	LC50 vol%	NOEC vol%	EC50 vol%
Normal direct sample															
Concawe 1		0 hours	10.1	0.00056		15	>45	49	>98	32	63	32	>100	32	>100
Concawe 1 Ready Style		4 hours	1.5	0.00013	6.4	10	34	49	>98	100	>100	32	>100	32	71
Concawe 1 Ready Style		14 days	3		71.4	>45	>45	49	>98	100	>100	100	>100	32	>100

Concawe results after biodegradation														
Concawe 1 Zahn-Wellens	4 hours	6.8	0.00006	35.2	10	37	12	51	100	>100	100	>100	100	>100
Concawe 1 Zahn-Wellens	28 days	2.1	0.000053	78.2	>45	>45	25	64	100	>100	100	>100	100	>100
High level (non direct sample)														
Concawe 2	0 hours	59	0.035		<5.6	19	<6.1	10	32	52	18	24	10	22
Concawe 2 Ready Style, 100%	4 hours	31.6	0.0014	25.4	17	>45	12.2	20	32	45	18	32	18	50
Concawe 2 Ready Style, 100%	14 days	0.5	0.0001	92.5	44	>45	49	93	100	>100	100	>100	100	>100
Concawe 2 Ready Style, 25% diluted	4 hours	7.5	0.00085	26.4	>45	>45	24.5	63	100	>100	32	61	32	69
Concawe 2 Ready Style, 25% diluted	14 days	0.9	0.000061	96.4	>45	>45	49	95	100	>100	100	>100	32	>100
Concawe 2 Zahn-Wellens, 100%	4 hours	23.4	0.0011	31.8	32	>45	12.2	29	32	71	32	51	32	60
Concawe 2 Zahn-Wellens, 100%	28 days	1.6	0.000067	97.1	>45	>45	49	92	100	>100	100	>100	18	>100
Concawe 2 Zahn-Wellens, 25%	4 hours	5.5	0.00082	34.8	>45	>45	24.5	76	100	>100	32	77	32	72
Concawe 2 Zahn-Wellens, 25%	28 days	1.3	0.000039	96.7	>45	>45	49	80	100	>100	18	26	5.6	29
Marine sample														
Concawe 4	0 hours	5.3	0.00058		>45	>45	98	>98	100#	>100#	100α	>100α		
Concawe 4 Ready Style	4 hours	6.7	0.00012	42.6	>45	>45	25	22	100	>100	32α	57α		
Concawe 4 Ready Style	14 days	1.5	0.000025	93.6	>45	>45	98	>98	100	>100	32α	56α		
Concawe 4 Zahn-Wellens	4 hours	2.3		-4.8	>45	>45	6	>89	32	>100	10α	17α		
Concawe 4 Zahn-Wellens	28 days	1.6	0.000047	93.7	>45	>45	49	>98	100	>100	10α	46α		