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# Report


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# Development of biodegradation testing within a whole effluent assessment scheme: petrochemical application


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# Table of Contents

# Glossary

| | |
|---|---|
| COD | Chemical oxygen demand |
| DOC | Dissolved organic carbon |
| EC50 | The concentration of a compound which produces 50% of the maximum response |
| LC50 | The lethal concentration of a compound for 50% of animals exposed |
| LLE | Liquid-liquid extraction |
| NOEC | No effect concentration |
| PBS | Potentially Bioaccumulative Substances |
| SPME | Solid phase micro extraction |
| TDS | Total Dissolved Salts |
| TOC | Total organic carbon |
| WEA | Whole effluent assessment |

# Foreword

This study was performed for Concawe to obtain information on the development of an approach for assessing biodegradation of petrochemical effluents and the impact on assessing the toxicity and potential to bioaccumulate of the constituents. The project was guided by M. Comber (ExxonMobil) and G. Whale (Shell) and coordinated at RIVO by P. Leonards. This study was conducted in cooperation with J.F. Postma from Grontmij | AquaSense, who was responsible for conducting the degradation and ecotoxicological studies.

# Summary

Whole Effluent Assessments (WEA) are being investigated as a potential tool for controlling aqueous discharges and minimizing environmental impact. The key parameter in such assessments relates to the toxicity of the effluent to aquatic organisms. Increasingly concern over long-term toxicity and the potential for toxicity to persist after discharge requires investigation. Within a recent OSPAR WEA demonstration project, CONCAWE investigated how the toxicity and presence of potentially bioaccumulative substances altered when petrochemical effluents were subjected to biodegradation tests. Three petrochemical effluents were assessed, two freshwater and one saline. The parameters measured addressed the effluents content using SPME and liquid-liquid extraction (indicators of substances that potentially bioconcentrate), their toxicity to microorganisms, plants and invertebrates and a number of parameters characterizing an effluent, pH, conductivity and metals. The sub-samples of the effluent samples were then subjected to two different types of biodegradation tests, resembling either a ready style (DOC-die away) or an inherent style (Zahn-Wellens) test and the parameters re-measured during and after biodegradation. The objective being to develop an initial screen to prioritise effluents where additional information regarding persistence of constituents may be required. This study has shown that both the DOC-die away and the Zahn-Wellens test are suitable for this "persistence" screen. A high proportion of the potentially bioaccumulative substances (PBS) in these oil industry effluents is easily biodegradable. Biodegradation not only lowered the PBS concentration but also toxicity. Appropriate controls are required as some increases in toxic effect were observed after 4 hours. For those samples with PBS concentrations above the critical values toxicity was observed, a finding that is in line with the assumption that narcotic effects are responsible for the observed toxicity in refinery effluents.

Additionally to the three tested samples, further 6 petrochemical effluents were also assessed for their PBS content and toxicity in order to increase the database on these measurements and the relationship between PBS and toxicity. The results showed that the PBS concentrations in all samples were lower than the estimated benchmark of acute toxicity for algae, fish and crustacean, but two samples were above the critical values for chronic narcotic toxicity for *Daphnia magna*.

# 1. Introduction

OSPAR continue to actively develop Whole Effluent Assessment (WEA) approaches. These include an assessment of toxicity, potential for bioaccumulation and persistence. The advantage of using WEA is that the effluent can be assessed as a whole, and therefore, full chemical characterisation is not required. While OSPAR are developing their ideas, WEA is increasingly being seen as a tool within IPPC and potentially in support of the Water Framework Directive (WFD). It may also have potential for use within the development of risk assessment of facility effluents, overcoming the problem of addressing individual product groupings.

The current project focused on the development of two biodegradation approaches (persistency) of petrochemical effluents in combination with toxicity and bioaccumulation for the WEA assessment. Additionally, petrochemical effluents were tested for toxicity and the potency of constituents to bioaccumulate.

# 2. Objective

The objective of this study was to develop an approach for assessing biodegradation of petrochemical effluents and the impact on assessing the toxicity and potential to bioaccumulate of the constituents.

# 3. Outline of the project

The work plan consisted of the following stages:

1. CONCAWE selected a number of representative effluents (fresh water and marine) from refineries throughout the EU for this test programme.

2. Toxicity (T), bioaccumulation (B) and chemical parameters were determined in the selected effluents.

3. Three samples were selected for a degradation approach using two different degradation tests (Ready style and Zahn-Wellens style). After degradation the toxicity and bioaccumulation of the effluents were determined to study persistence.

For the degradation tests one low (COD) level freshwater, one high (COD) level freshwater and one marine effluent were tested. Both acute and chronic toxicity tests were undertaken using freshwater and marine species following the test methods descried in Section 4.3. Potential for bioaccumulation was assessed using both solid phase microextraction (SPME) and liquid-liquid extraction (EGOM LLE). A description of these methods together with an evaluation of their performance, based on a recent interlaboratory study undertaken for OSPAR, is provided by Leslie (2006).
In figure 1 an overview of the persistence/WEA approach for the three samples is presented. The toxicity and degradation studies were performed by Grontmij | AquaSense (Netherlands) and the bioaccumulation studies by RIVO (Netherlands).

Additionally, six other refinery effluents (five freshwater, and one saltwater) were tested using the WEA approach described to provide information on their toxicity, potential to bioaccumulate and chemical composition. However, no persistence assessment was undertaken for these six effluents.

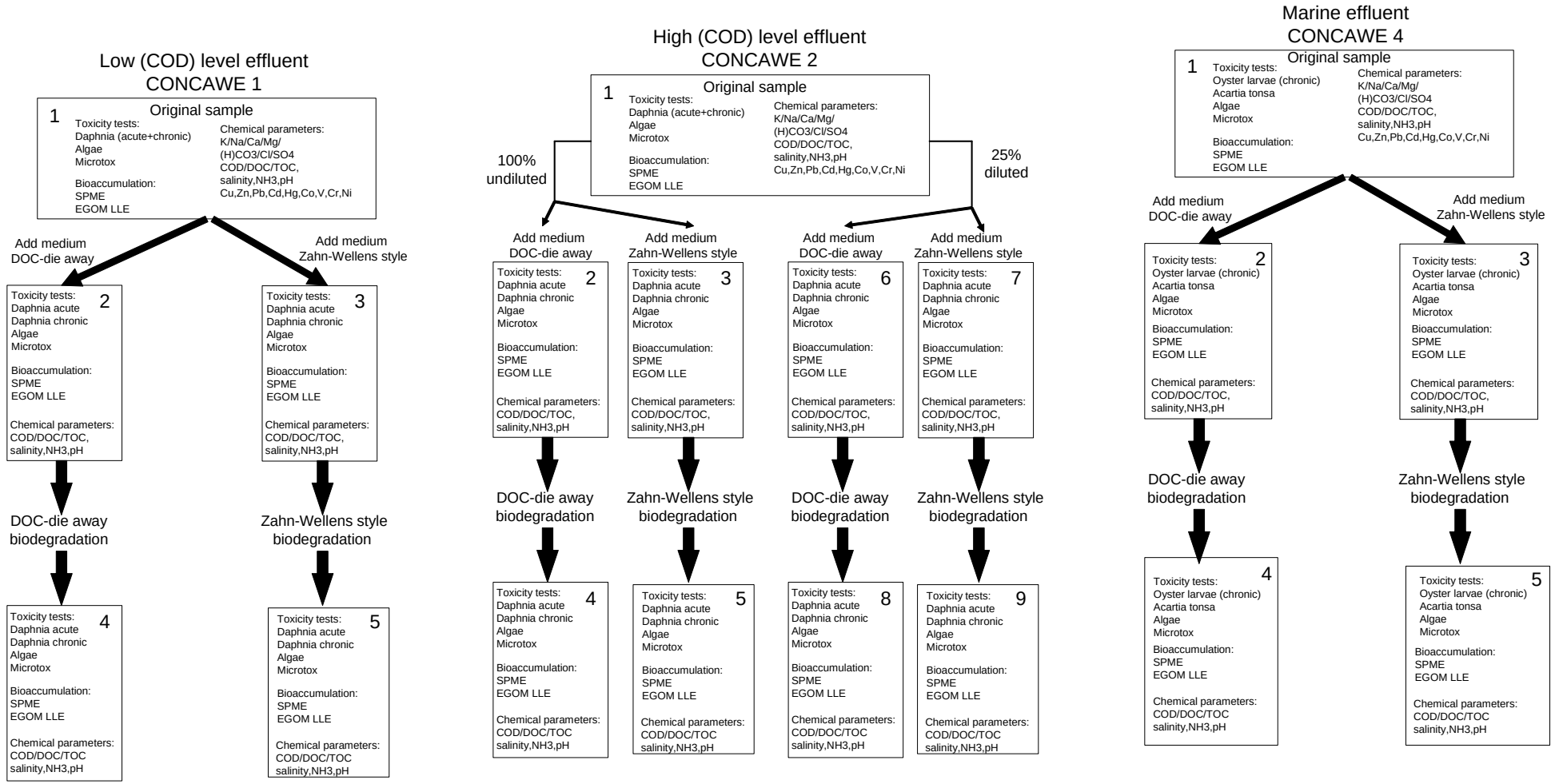


*Figure 1: Overview of sample treatment steps, toxicity, bioaccumulation and degradation tests for the three effluents that were selected for the persistence WEA assessment using two types of degradation approaches (DOC-die away and Zahn-Wellens). CONCAWE 1 a low (COD) level effluent, CONCAWE 2 a high (COD) level, and CONCAWE 4 a marine effluents.*

# 4. Materials and methods

## 4.1 Sampling

Sampling kits and a protocol for collection of effluents were prepared and sent to the facilities (RIVO). Each kit contained stainless steel container(s) and plastic bottles. An overview of the amount of each effluent that was needed for the WEA assessment studies is given in Table 1. The effluent samples were collected by refinery staff using the sampling containers and sampling protocol provided by RIVO. Samples were then dispatched to RIVO, which took 1 to several days. Two litres of the effluent from the stainless steel containers were used for the determination of the bioaccumulation parameters (SPME and EGOM LLE). An aliquot of the effluents was sent to Alcontrol (Netherlands) for analysis of metals (Cu, Zn, Pb, Cd, Hg, Co, V, Cr, Ni), COD, TOC, DOC, K, Na, Ca, Mg, (H)$CO_3$, Cl, $SO_4$. Salinity, $NH_3$ and pH were also determined. The remaining effluents in the stainless steel containers were sent to Grontmij | AquaSense to perform the toxicity and degradation tests. Samples were stored for the entire project at 4°C. After delivery of the samples various parameters (pH, $NH4^+$, conductivity, salinity, total dissolved salts (TDS), dissolved organic carbon (DOC)) were determined, table 2.

Table 1: Amounts of effluent needed to perform the WEA assessments.

| | **Amount (L) effluent needed** |
|---|---|
| **Persistence WEA assessment** | |
| Low (COD) level | 50 |
| High (COD) level | 75 |
| Marine effluent | 50 |
| | |
| Additional WEA assessement without P assessment, T and B only | |
| Freshwater 1 | 10 |
| Freshwater 2 | 10 |
| Freshwater 3 | 10 |
| Brackish effluent 1 | 10 |
| Brackish effluent 2 | 10 |
| Salt water effluent 1 | 10 |

Table 2: General characteristics of the original effluent samples, measured directly after delivery.

| **Sample code** | **LIMS nr. (RIVO)** | **Ecolims number (Aquasense)** | **Sampling date** | **pH** | **$NH_4^+$ (mg/l)** | **Conduct. (µS/mm)** | **Salinity (‰)** | **TDS[1] (mg/l)** | **DOC (mg/l)** |
|---|---|---|---|---|---|---|---|---|---|
| Concawe 1 | 2005/1480 | 335378 | nov 2005 | 7.3 | 10 | 793 | 4.9 | Ofl. | 22.9 |
| Concawe 2 | 2005/1481 | 335379 | 8/11/05 | 7.7 | <10 | 220 | 1.1 | Ofl. | 222 |
| Concawe 3 | 2005/1482 | 335380 | nov 2005 | 7.6 | <10 | 319 | 1.8 | Ofl. | 8.2 |
| Concawe 4 | 2005/1483 | 335381 | nov 2005 | 7.2 | <2.5 | 4200 | 30.2 | Ofl. | 7.8 |
| Concawe 5 | 2005/1484 | 335382 | 31/10/05 | 7.7 | <10 | 198 | 1.0 | Ofl. | 12.6 |
| Concawe 6 | 2005/1485 | 335383 | 28/11/05 | 7.5 | 13 | 278 | 1.5 | Ofl. | 10.2 |
| Concawe 7 | 2005/1486 | 335384 | 8/11/05 | 7.3 | <10 | 98 | 0.3 | 1054 | 12.2 |
| Concawe 8 | 2005/1487 | 335385 | nov 2005 | 7.6 | 10 | 94 | 0.3 | 1012 | 12.7 |
| Concawe 9 | 2005/1488 | 335386 | nov 2005 | 7.1 | 10 | 2000 | 13.4 | Ofl. | 10.6 |

[1]: TDS = Total Dissolved Salts. Ofl = Offline, samples should be diluted.

## 4.2 Degradation

The aim was to conduct ready and inherent biodegradation tests, which should be as similar as possible with respect to the techniques used, and the volume and dimension of test vessels etc. Therefore, for the ready biodegradation test the DOC-die Away test (ISO 7827/ OECD 301A), and for the inherent biodegradation the Zahn Wellens Test (ISO 9888/ OECD 302B) were used.

Both tests used the same technique for studying biodegradation (DOC measurements), have the same mineral medium and activated sludge as inoculum can be used for both tests. In both tests open glass vessels (10 l) were gently stirred and continuously aerated. The main difference between these tests is that a greater volume of activated sludge is used in the Zahn Wellens test.

Neither test is suitable for assessing biodegradation of volatile compounds. However, in assessments of waste water this aspect is not considered to be important because volatile compounds will be unlikely to persist in waste water as they will be removed in the sewage plant or by diffusion in surface water. The DOC-die Away test is primary used for chemical substances, but can be used for wastewater with a slight adaptation.

To study the impact of the addition of degradation medium (e.g. activated sludge, mineral medium) on the toxicity and bioaccumulation, effluents were sampled 4 hours after the addition of the medium. The DOC-die away degradation test was performed for 14 days, and the Zahn-Wellens for 28 days. An overview of the degradation test is given in Table 3.

Table 3: Overview of the biodegradation tests.

| **Sample** | **RIVO LIMS no.** | **Degradation Type** | **Degradation Time** |
|---|---|---|---|
| Concawe 1 | 2005/1480 | Original sample | 0 hours |
| Concawe 1 | 2005/1643 | DOC-die away | 4 hours |
| Concawe 1 | 2005/1651 | DOC-die away | 14 days |
| Concawe 1 | 2005/1644 | Zahn-Wellens | 4 hours |
| Concawe 1 | 2005/1652 | Zahn-Wellens | 28 days |
| | | | |
| Concawe 2 | 2005/1481 | Original sample | 0 hours |
| Concawe 2 | 2005/1645 | DOC-die away, 100% | 4 hours |
| Concawe 2 | 2005/1653 | DOC-die away, 100% | 14 days |
| Concawe 2 | 2005/1647 | Zahn-Wellens, 100% | 4 hours |
| Concawe 2 | 2005/1655 | Zahn-Wellens, 100% | 28 days |
| Concawe 2 | 2005/1648 | Zahn-Wellens, 25% | 4 hours |
| Concawe 2 | 2005/1656 | Zahn-Wellens, 25% | 28 days |
| Concawe 2 | 2005/1646 | DOC-die away, 25% diluted | 4 hours |
| Concawe 2 | 2005/1654 | DOC-die away, 25% diluted | 14 days |
| | | | |
| Concawe 4 | 2005/1483 | Original sample | 0 hours |
| Concawe 4 | 2005/1649 | DOC-die away | 4 hours |
| Concawe 4 | 2005/1657 | DOC-die away | 14 days |
| Concawe 4 | 2005/1650 | Zahn-Wellens | 4 hours |
| Concawe 4 | 2005/1658 | Zahn-Wellens | 28 days |

*4.2.1 DOC-die away*

The DOC die-away test is performed according to OECD 301A with some modifications to apply this test on waste water samples. The biodegradation of each waste water sample is assessed in 10 liter glass beakers, which were permanently aerated to provide oxygen as well as the necessary mixing. All tests were carried out in the dark at a temperature of 23 ± 2°C. Degradation is followed by DOC measurements at the start (t=0), after 4 hours and on weekly intervals thereafter. All tests were stopped after 14 days, afterwhich the degree of biodegradation is calculated by expressing the concentration of DOC removed as a percentage of the concentration initially present. Both controls and reference substances (NaAc; 40 mg C/l) were tested simultaneously (each in 2 liter glass beakers).

To each sample mineral salt medium was added according to OECD 301F, and activated sludge was used as an inoculum in a concentration of 30 mg dry matter/l. This sludge was obtained from a household, waste-water treatment plant in the eastern part of Amsterdam.

After 4 hours and after 14 days, the respective glass beaker was put aside and stored in a cold room (4 ±1°C). After settling overnight, the remaining water was sampled for both chemical analyses (SPME, LLE and metals) and bioassays. No further sludge removal was carried out. Possible toxic effects of the waste water samples on the inoculum were not assessed.

*Sample specific remarks:*
1) Concawe 2 was tested at a high and a low DOC content. In the high DOC sample the effluent was undiluted (100%), while the waste water in the low DOC content was diluted to 25% with demi-water. For both biodegradation tests, the same concentration of activated sludge (30 mg dry matter/l) was used.
2) Concawe 4 is a marine sample with a salinity of 30‰. The activated sludge was therefore acclimated to this salinity before the biodegradation tests were started. An adaptation scheme was used as presented in table 4. Separate controls and reference substances (NaAc; 40 mg C/l) were tested at the same salinity.

Table 4: Acclimation scheme used for the inoculum in Concawe 4. Furthermore, activity of the sludge was assessed on regular intervals.

| **Day** | **Salinity (‰)** | **TOC content** | | **Decrease** |
|---|---|---|---|---|
| | | **'Influent'** | **'Effluent'** | |
| 0 | 5 | | | |
| 3 | 7 | | | |
| 4 | 7 | 73.8 | 26.2 | 64.5 |
| 5 | 9 | | | |
| 7 | 12 | | | |
| 10 | 14 | | | |
| 12 | 17 | | | |
| 13 | 17 | 100 | 26.2 | 73.8 |
| 14 | 19 | | | |
| 17 | 22 | | | |
| 19 | 24 | | | |
| 20 | 24 | 95.1 | 24.9 | 73.8 |
| 21 | 26 | | | |
| 24 | 28 | | | |
| 27 | 28 | 93.7 | 27.9 | 70.2 |

### *4.2.2 Zahn-Wellens*

The Zahn-Wellens test was performed according to OECD 302B and aims to determine inherent biodegradability. A relatively large amount of activated sludge is therefore used. The biodegradation of each waste water sample was assessed in 10 liter glass beakers, which were permanent aerated and mechanically stirred. All tests were carried out in the dark at a temperature of 23 ±2°C. Degradation is followed by DOC measurements at the start (t=0), after 4 hours and on weekly intervals thereafter. All tests were stopped after 28 days, afterwhich the degree of biodegradation is calculated by expressing the concentration of DOC removed as a percentage of the concentration initially present. Both controls and reference substances (NaAc; 100 mg C/l) were tested simultaneously (each in 2 liter glass beakers).

To each sample mineral salt medium was added according to OECD 301F, and activated sludge was used as an inoculum in a concentration of 200 mg dry matter/l. This sludge was obtained from a household, waste-water treatment plant in the eastern part of Amsterdam.

After 4 hours and after 28 days, the respective glass beaker was put aside and stored in a cold room (4 ±1°C). After settling overnight, the remaining water was sampled for both chemical analyses (SPME, LLE and metals) and bioassays. No further sludge removal was carried out. Possible toxic effects of the waste water samples on the inoculum were not assessed.

*Sample specific remarks:*
1) Concawe 2 was tested neat (100%) and diluted, using demineralised water, by a factor of 4 (25%). In accordance to the the guidelines used, a different amount of activated sludge was used: 200 mg dry matter/l for the diluted and hence lower DOC content sample and 500 mg dry matter/l for the undiluted sample (which contained around 250 mg DOC/l).
2) Concawe 4 is a marine sample with a salinity of 30‰. The activated sludge was therefore acclimated to this salinity before the biodegradation tests were started. An adaptation scheme was used as presented in table 4. Separate controls and reference substances (NaAc; 100 mg C/l) were tested at the same salinity.

## 4.3 Toxicity tests

All toxicity tests were performed by Grontmij | AquaSense. For the freshwater effluents the following bioassays were carried out:

- Chronic toxicity to Daphna magna (16d test)
- Acute toxicity to Daphnia magna
- Acute toxicity to Pseudokirchneriella subcapitata (algae)
- Microtox test

For the marine effluents the following bioassays were carried out:

- Oyster larvae test
- Acute toxicity to Acartia tonsa
- Acute toxicity to Phaeodactylum tricornutum (algae)
- Microtox

### *4.3.1 Microtox*

The bioassays with the bacterium *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*) were conducted according to ISO 11348-3. The reduction in bioluminescence for this bacterium was assessed by measuring the light intensity (using Microtox® equipment) after 5, 15 and 30 minutes exposure to the following concentrations: 45, 22.5, 11.25 and 5.625 volume% for freshwater samples. For marine samples, the highest testconcentration is 50 vol%. All tests were conducted at 15°C, and in duplicate. $EC_{20}$- and $EC_{50}$-values were estimated, using the statistical programs belonging to the Microtox testsystem.

The $EC_{20}$- and $EC_{50}$-values are defined as the concentrations, which (after a certain exposure period) reduce the bioluminescence with 20 or 50% in comparison with the control (which is also used as the solution to dilute the samples). The lowest of the three at different time intervals estimated values is used to indicate the toxicity of the wastewater samples.

Before the start of the experiments several physical and chemical parameters were measured in the undiluted sample, such as oxygen content, pH, ammonium and conductivity.

### *4.3.2 Algae: Phaeodactylum tricornutum and Pseudokirchneriella subcapitata*

Bioassays with the algae were performed according to ISO 8692 for the freshwater samples using Pseudokirchneriella subcapitata and ISO 10253 for the marine samples) using Phaeodactylum tricornutum. An exponentially growing pre-culture was used as an inoculum for the test. This pre-culture was started 3 days before the beginning of the test. Growth medium was prepared by dissolving nutrient stocks in milli-Q or artificial sea water, as defined in the guidelines. The growth medium was inoculated with a small volume of algae suspension in order to maintain exponential growth until the start of the test.

All tests were started with an inoculation concentration of $0.5x10^4$ cells/ml. The tests were performed in an incubator, continuously shaking at 200 rpm. Cell densities were measured by means of fluorescence at t=0, 48 and 72 hours using a plate-reader (670 nm). Based on the cell densities at the different time intervals, growth rates (μ) were calculated for each test concentration and replicate.

**NB**. Concawe 9 was tested with a marine algae at a salinity of 12 ‰. An additional control with the same salinity was also performed.

### *4.3.3 Daphnia magna- acute*

Acute toxicity tests were performed according to ISO 6341, in which immobility of the organisms after 24 and 48 hours is assessed. Organisms were considered immobile, if they did not respond within 15 seconds to a gentle agitation of the test vial.

All tests were performed at 20 ± 2 °C and a light regime of 16:8 h light:dark. Tests were started with juveniles <24 hours old. The test volume was 50 ml for each vial. Each concentration was tested in 4 replicates and with 5 individuals per replicate. Organisms were not fed during the exposure.

### *4.3.4 Daphnia magna- chronic*

The chronic bioassay with *Daphnia magna* is performed according to OECD 202, with some modifications. The duration of the test was shortened to around 16 days, as all tests were ended as soon as the control organisms had produced three different broods. All tests were started with individuals less than 24 hours old and obtained from adults of approximately 3 weeks old.

During the test, all daphnids were fed daily with an increasing amount of algae suspension (*Scenedesmus* with yeast suspension[1] (10g / 75 mL) in response to their anticipated growth, i.e. 200 µl/test vessel from day 0 to 2, 300 µl/test vessel from day 3 to 5 and 400 µl/test vessel from day 6 and onwards.

The medium was renewed twice a week. During the test, several physical and chemical parameters were measured in both the old as well as the fresh medium. The survival and reproduction was scored every day, while the juveniles are counted and removed three times a week. With these data

[1] Ratio algae : yeast suspension = 100 : 1

a 'cohort life-table' was prepared for every test concentration. This table summarises the number of juveniles per female during the test. From this 'cohort life-table' the average intrinsic population growth rate ($r_m$) is calculated for every test concentration as a measure for reproduction. Especially the moment of first reproduction (first brood) and the number of juveniles per brood are determinant. The average $r_m$-values per concentration have been statistically evaluated.

### *4.3.5 Acartia tonsa*

Acute toxicity tests were performed according to ISO 14669, in which immobility of the organisms after 24 and 48 hours is assessed. Organisms were considered immobile, if they did not respond within 10 seconds to a gentle agitation of the test vial. Test organisms were obtained from the Guernsey Sea Farms, Guernsey. Natural seawater (± 32 ‰) was used as a blank and dilution medium.

**NB**. For Concawe 9 a different procedure was used. This sample has a salinity around 12 ‰, which is too high for most freshwater tests but too low for several marine tests. The salinity of the sample was therefore increased up to 20‰, using artificial sea salt. An additional control with the same salinity was also prepared. The test volume was 25 ml for each vial. Each concentration was tested in 4 replicates and with 5 individuals per replicate. The test temperature was 20 ± 2°C. Organisms were not fed during the exposure.

### *4.3.6 Oyster: Crassostrea gigas*

Tests were performed as described in the ASTM guideline E724. Less than 4 hours after fertilization, bioassays are started when the embryos are in the 2-, 4- or 8-cell stages. Adult oysters were obtained from Guernsey Sea Farms, Guernsey. Normally 2-4 males and 2-4 females were selected. These conditioned oysters were stimulated to spawn by a temperature shock (18 – 25 – 18 – 25°C). If animals did not spawn, gametes were stripped from the gonads. Gametes (eggs and sperm cells) were collected in glass vessels. Solutions containing eggs were filtered and washed to remove excess of gonad tissue. After counting, 2 ml of sperm cells was added to a solution containing about 4000 eggs per ml. Each test vessel (20 ml) was inoculated with approximately 300 eggs. Three additional samples were directly fixed, by the addition of buffered formalin, and used to assess the fertilization success.

In the beginning of the test fertilized eggs will settle down. Within a few hours however, free-swimming spherical shaped larvae (the so-called trochophore larvae) develop. After 12 to 4 8 hours veliger larvae develop from the trochophore larvae. In this larval stage an active swimming organ and a shell is developed. Normal developing veliger larvae form an oval shaped shell with a straight edge, the so-called D-larvae. Natural seawater (32‰) was normally used as a control and dilution medium.

**NB**. For Concawe 9 a different procedure was used. This sample has a salinity around 12 ‰, which is too high for most freshwater tests but too low for several marine tests. The salinity of the sample was therefore increased up to 20‰, using artificial sea salt. An additional control with the same salinity was also prepared.

After 48 hours tests were ended and the embryos "fixed" by the addition of a few drops of buffered formalin (37%, pH 7). The number of normally developed, malformed and retarded (i.e. delayed in their development) larvae were determined using a microscope.

Normally, tests are only considered valid if >70% of the fertilized embryos have developed into live larvae with completely developed shells. However, in the months November and December it is often hard to get the usual high percentage of normal development. It is often observed that an increased amount of fertilized embryos stop their development in a very early stage (within a few hours). As an additional criterion, attention was therefore paid to the percentage of normal

development based upon the number of larvae found at the end of the experiment. If this values is >80% tests are considered valid.

The percentage of death, malformed and retarded larvae is corrected for the blank seawater by the formula of Abbott (ASTM guideline, E 724):

$$E = \frac{\frac{100 * (I - D_{test})}{I} - \frac{100 * (I - D_{ctr})}{I}}{100 - \frac{100 * (I - D_{ctr})}{I}}$$

In this formula E is the fraction of embryos that did not result in live larvae with completely developed shells adjusted for the controls, I is the number of embryos at the start of the test and $D_{ctr}$ and $D_{test}$ are the number of normally developed larvae in the control and the test concentrations. If E is 0, there is no effect compared to the control, if E is 100 there is a maximum affect, i.e. all larvae were death, malformed or retarded.
Statistical analyses were based on the percentage normal developed larvae. Validity criteria are based on the percentage of death, malformed and retarded larvae as calculated using the Abbott formula.

## 4.4 Bioaccumulation

Potentially bioaccumulation substances (PBS) in the effluents were determined by RIVO using two different approaches. The first method is a partitioning-based methodology using biomimetic solid phase microextraction (SPME), and the second method is based on liquid-liquid extraction (EGOM LLE). Both methods were recently evaluated in an interlaboratory study for OSPAR (Leslie, 2006).

### *4.4.1 SPME*

PBS concentrations were determined according the protocol used in the OSPAR interlaboratory study (Leslie and Leonards, 2005a). Briefly, SPME fibers were exposed to 250 ml of effluents, with agitation, for 24 h in a closed glass bottle. A 100 µm PDMS (poly(dimethylsiloxane)) fiber was used. Per effluent sample, SPME fiber measurements were performed in triplicate. After 24 hours exposure the fibers were removed from the effluent solution and dried with a tissue and directly injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID). A DB-1 (210 m x 0.25 mm x 0.1 µm) GC column was used. For quantification 2,3-dimethylnaphthalene (40 mg/l) was used as external standard. The temperature programme of the GC was fast to sum the peaks as much as possible. The total peak area of the chromatogram was integrated (between C9 and C38) and the molar concentration was calculated.

### *4.4.2 EGOM LLE*

PBS concentrations were determined according to the protocol used in the OSPAR interlaboratory study (Leslie and Leonards, 2005b) which was based on the 'EGOM' LLE method that was developed in Sweden by Adolfsson-Erici and Wahlberg (1992) and Hynning (1996) and measure the 'extractable gas-chromatographic organic matter' in an effluent sample. The effluent (300 ml) was acidified with 6M HCL (4 ml) to pH<2. This sample was extracted twice with cyclohexane in a separation funnel. Following an adjustment of the effluent to pH 10 with NaOH (2.5 M, 4 ml), the effluent was extracted twice with cyclohexane. The four extracts were combined and evaporated, and dried with sodium sulphate. The final extract was injected in a GC-FID using a splitt/splitless injector, and a DB-1 (10 m x 0.25 mm x 0.1 µm) GC column was used. An external standard (2,3 dimethylnaphthalene) was used for quantification. The total peak area of the chromatogram was integrated (C9 to C38).

# 5. Results and discussion

In the following sections the results of the degradation experiments (5.1), toxicity (5.2), bioaccumulation (5.3), metals (5.4), and the changes in toxicity and bioaccumulation after degradation (5.5) are discussed. The focus in these sections is on the individual effluents. Finally, an evaluation of all results is provided in section 5.6.

## 5.1 Degradation

The results of the biodegradation studies are presented in table 6, in which both the DOC-measurements as well as the percentage biodegradation are illustrated. The tests performed with NaAc as reference substance demonstrate that the activity of the inoculum was sufficient. Within a week all biodegradation percentages were well above 90%. Furthermore, measurements after 4 hours illustrate (as expected) a generally higher rate of biodegradation in the Zahn-Wellens test as compared to the DOC-die away test.

A similar increase in initial biodegradation rates was observed in the inherent tests was for the samples Concawe 1 and 2. Once again this difference tends to disappear during the tests. The marine test with Concawe 4 was the only sample in which such a difference between the DOC-die away and the Zahn-Wellens was not noted. This might partly be caused by the fact that DOC-measurements in marine samples with these, rather low, DOC contents tend to be more variable as compared to measurements in a freshwater system (see for example measurements after 4 hours and 21 days).

All three samples tested showed a high percentage of biodegradation with values above 90% at the end of the tests for Concawe 2 and 4. The percentage biodegradation in Concawe 1 was somewhat lower. Most of the biodegradation in this sample took place within the first week (around 70%). Thereafter only a low increase was noted, up to 78% after 4 weeks in the Zahn-Wellens test.

Table 6: Results of the biodegradation tests, including DOC measurements in blanc and references.

| | | DOC-measurements (mg/l) | | | | Biodegradation (%, including correction for DOC in blancs) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Time of sampling | DOC-die away test | | Zahn-Wellens test | | DOC-die away test | | Zahn-Wellens test | |
| | | Repl1 or undiluted | Repl 2 or 25% dilution | Repl1 or undiluted | 25% dilution | Undiluted | 25% dilution | Undiluted | 25% dilution |
| **Blanc -fresh** | T = 0 hrs. | 0.5 | 1.1 | 0.5 | | | | | |
| | T = 4 hrs. | 0.2 | 0.4 | 0.7 | | | | | |
| | T = 7 days | 0.6 | 1.9 | 2.1 | | | | | |
| | T = 14 days | 2.4 | 2.2 | 1.6 | | | | | |
| | T = 21 days | | | 1.6 | | | | | |
| | T = 25 days | | | 2.1 | | | | | |
| | T = 28 days | | | 1.6 | | | | | |
| | **avg.** | **1.2** | | **1.5** | | | | | |
| **Blanc -marine** | T = 0 hrs. | 1.1 | | 1.1 | | | | | |
| | T = 4 hrs. | 1.2 | | 1.4 | | | | | |
| | T = 7 days | 1.6 | | 1.9 | | | | | |
| | T = 14 days | 1.0 | | 1.9 | | | | | |
| | T = 21 days | | | 2.9 | | | | | |
| | T = 25 days | | | 1.9 | | | | | |
| | T = 28 days | | | 2.0 | | | | | |
| | **avg.** | **1.2** | | **1.9** | | | | | |
| **Reference -fresh** | T = 0 hrs. | 39.4 | 96 | 91.5 | | | | | |
| | T = 4 hrs. | 35.9 | 92 | 56.5 | | 9.2 | 4.2 | 38.9 | |
| | T = 7 days | 0.9 | 3.1 | 2.7 | | 100.7 | 98.0 | 98.6 | |
| | T = 14 days | 2.1 | 1.8 | 2.7 | | 97.5 | 99.3 | 98.6 | |
| | T = 21 days | | | 2.6 | | | | 98.7 | |
| | T = 25 days | | | 3.2 | | | | 98.1 | |
| | T = 28 days | | | 2.6 | | | | 98.7 | |
| **Reference -marine** | T = 0 hrs. | 41.2 | | 93.8 | | | | | |
| | T = 4 hrs. | 40.0 | | 75.3 | | 3.0 | | 20.0 | |
| | T = 7 days | 1.5 | | 3.6 | | 99.2 | | 97.7 | |
| | T = 14 days | 1.2 | | 3.5 | | 99.9 | | 97.8 | |
| | T = 21 days | | | 11.7 | | | | 88.9 | |
| | T = 25 days | | | 4.4 | | | | 96.8 | |
| | T = 28 days | | | 4.6 | | | | 96.6 | |
| **Concawe 1** (335378) | T = 0 hrs. | 32.4 | | 32.4 | | | | | |
| | T = 4 hrs. | 30.4 | | 21.5 | | 6.4 | | 35.2 | |
| | T = 7 days | 10.7 | | 10.9 | | 69.5 | | 69.5 | |
| | T = 14 days | 10.1 | | 10.4 | | 71.4 | | 71.1 | |
| | T = 21 days | | | 9.0 | | | | 75.7 | |
| | T = 25 days | | | 8.4 | | | | 77.6 | |
| | T = 28 days | | | 8.2 | | | | 78.2 | |
| | | **undiluted** | **25% dilution** | **undiluted** | **25% dilution** | **undiluted** | **25% dilution** | **undiluted** | **25% dilution** |
| **Concawe 2** (335379) | T = 0 hrs. | 288 | 74.3 | 288 | 74.7 | | | | |
| | T = 4 hrs. | 215 | 55 | 197 | 49.2 | 25.4 | 26.4 | 31.8 | 34.8 |
| | T = 7 days | 50.1 | 10.7 | 18.7 | 3.6 | 82.9 | 87.0 | 94.0 | 97.1 |
| | T = 14 days | 22.7 | 3.8 | 10.1 | 3.5 | 92.5 | 96.4 | 97.0 | 97.2 |
| | T = 21 days | | | 9.5 | 3.5 | | | 97.2 | 97.2 |
| | T = 25 days | | | 9.8 | 4.0 | | | 97.1 | 96.5 |
| | T = 28 days | | | 9.9 | 3.9 | | | 97.1 | 96.7 |
| **Concawe 4** (335381) | T = 0 hrs. | 16.5 | | 16.5 | | | | | |
| | T = 4 hrs. | 10.0 | | 17.2 | | 42.6 | | -4.8 | |
| | T = 7 days | 2.4 | | 2.8 | | 92.3 | | 93.7 | |
| | T = 14 days | 2.2 | | 2.8 | | 93.6 | | 93.7 | |
| | T = 21 days | | | 5.0 | | | | 78.6 | |
| | T = 25 days | | | 2.7 | | | | 94.3 | |
| | T = 28 days | | | 2.8 | | | | 93.7 | |

## 5.2 Toxicity

An overview of the toxicity results of the original effluents, the effluents after addition of the degradation medium (t = 4 hrs), and after degradation for Concawe 1 are shown in Table 7, for Concawe 2 in table 8, and for Concawe 4 in table 9. The toxicity data for the remaining samples, Concawe 3, 5, 6, 7, 8 and 9, which were not subjected to a persistence approach are given in table 10.

In the next sections information on confounding factors and special remarks of the effluents are provided.

### *5.2.1 Confounding factors*

Several confounding factors were measured for each bioassay at the beginning, during and at the end of the assay. Each of these measurements is compared with criteria. If the criteria were exceeded, this factor might have contributed to observed negative effects. If this is noted in undiluted samples, this parameter has also been measured in the diluted samples, up to the test concentration which fulfilled the criteria. In some case, criteria were exceeded but no toxic effects were noted or $EC_{50}$-values are estimated at such a dilution level for which this parameter did not play a role.

Beneath, an overview of the confounding factors is presented for each bioassay and sample combination if this factor might have affected the results.

Concawe 1, conductivity in acute *Daphnia* assay

All undiluted samples tested (approx. 750-800 µS/mm) exceeded the criteria for conductivity (<650 µS/mm). The mortality as observed in the undiluted samples might therefore be (at least partly) been caused by the conductivity of the sample. In the first dilution (32 vol%) such a possible effect of conductivity iwould no longer be anymore. Furthermore, no toxicity was observed in any of the tests performed during or after biodegradation (in both the DOC-die away and the Zahn-Wellens test). Since the undiluted samples in these tests also exceeded the criteria for the conductivity, it seems likely that the acute toxicity for daphnids in the freshly sampled waste water was at least partly caused by some other constituents.

Concawe 1, conductivity in algae test

Again conductivity plays a role in the undiluted sample. The effect on the algal growth rate in the original effluent could potentially be partly attributed to its conductivity (approx. 800 µS/mm, while the criteria for the algae is <400 µS/mm). The conductivity is however the same in all the samples (before, during and after biodegradation). It is therefore unlikely that the differences in toxicity observed were solely due to the conductivity.

Concawe 1, 2, 3, 5 and 6, conductivity in the chronic daphnid test

The criteria set for the chronic *Daphnia* assay is 185 µS/mm. This is based on tests with pure NaCl solutions. In everyday practice, several samples have been tested exceeding this criterion, with no adverse effects noted. This might be explained by differences in the ionic composition and a better balance with for example sodium and potassium. However, since this is not known in detail, the criterion (<185 µS/mm) is still used, although it will probably be conservative.

As noted before**, Concawe 1** has a conductivity around 800 µS/mm. This conductivity falls to values around the 300 µS/mm in the 32 vol% treatment and around 200 µS/mm in the 18 vol% dilution. As shown in table 7, toxic effects were only observed in the undiluted samples (both the original sample, the 4 hours DOC sample and the 14 days DOC). It seems therefore likely that the conductivity of these samples has at least partly influenced the reported toxic effects. However, no toxicity was found in the Zahn-Wellens samples (both the 4 hrs and the 28 days), although these samples had similar conductivity values.

With a conductivity of 220 µS/mm, **Concawe 2** also exceeded the criterion in the undiluted sample. However, acute mortality of 90% was observed within two days exposure to the undiluted sample. Over such a test duration the criterion for the acute daphnid tests (<650 µS/mm) would be more applicable andon this basis it would be reasonable to assume that this high mortality was not caused by the conductivity.

**Concawe 3** has a conductivity around 300 µS/mm. This conductivity falls to values around the 150 µS/mm in the 32 vol% treatment. However, as shown in table 10, no toxic effects were demonstrated, even in the undiluted sample.

**Concawe 5**, with a conductivity around 200 µS/mm, exceeds the criterion for conductivity only slightly. A strong effect of this confounding factor is therefore not to be expected and is supported in the fact no toxic effects were observed.

**Concawe 6**, with a conductivity around 280 µS/mm, exceeds the criterion in the undiluted sample. Based upon expert judgment it seems however unlikely that the complete mortality observed in this treatment was solely due to its conductivity. An increased mortality was also observed in the 32 vol%, where the criterion for conductivity was not exceeded. It is remarkable that no significant effects on reproduction were demonstrated in this treatment, although mortality at the end of the test had increased to 60%. This is explained by an increased size of the first brood: on average 21 juveniles as compared to 17 for the controls. This increase could have been a response to stress.

Concawe 2, oxygen saturation in acute *Daphnia* assay

All acute daphnid assays performed at t=0 or after 4 hours biodegradation failed in maintaining a proper oxygen saturation (tests were not aerated). In the undiluted samples oxygen air saturation values (ASV) at the end of the test ranged between 8-11 % (criteria >20%). This also occurs in the samples which had been diluted by 4 times before testing (25% treatment samples). In general no problems with the oxygen saturation were observed in the 18 vol% and lower treatments (for the undiluted tests) or from 32 vol% and lower in the 25% treatment. It is therefore likely that mortality in the highest concentrations is influenced by the low oxygen saturation values. As expected, no problems with the oxygen saturation were observed in the tests performed after 14 or 28 days of biodegradation.

Concawe 2, oxygen saturation in the chronic daphnid assay

Both the freshly tested sample as well as the samples originating from the biodegradation tests frequently suffered from a decreased oxygen saturationof the test media. Before biodegradation, oxygen saturation in the undiluted sample decreased frequently down to 10-15%, which is below the criterion of >30%. This increased oxygen consumption was also observed in the 32 vol% treatment (range of minimum values 15-30%) and even in the 10 and 18 vol% (range of minimum values 20-35). To limit the influence of this increased oxygen consumption, all test concentration and individual replicates, were daily shaken (after temporarily removal of the daphnid). It seems unlikely that this oxygen saturation caused the high mortality as observed in the higher test concentrations. However, it might have influenced the effects on reproductive output in some of the lower concentrations.

The same applies more or less to the samples taken during biodegradation (DOC and Zahn-Wellens after 4 hours), although oxygen depletion tended to be somewhat diminished. As to be expected, the oxygen depletion in the diluted samples was again lower, as compared to the undiluted samples. However, also in the 25% diluted samples, oxygen saturation falls below the criterion in the 'undiluted, 100 vol%' sample as well as (sometimes) in the 32 vol% treatment. It is therefore concluded that this frequently lowering of oxygen saturation values might have influenced the effects on reproduction of the daphnids.
The samples tested after biodegradation (DOC after 14 days and Zahn-Wellens after 28

days) did not show an increased oxygen consumption and oxygen saturation values stayed above the criterion.

Concawe 8, nitrite in chronic daphnid assay

While no detectable nitrite was observed at the beginning of the tests, somewhat increased values (between 2 – 5 mg/l) were observed in the undiluted samples from day 7 onwards. Since nitrite is already toxic in very low concentrations, this might effect the reproduction in undiluted samples. However, no toxic effects were observed.

### *5.2.2 Special remarks concerning results*

**Concawe 2**

**Note**: Test results obtained with the four times diluted sample are not corrected for this dilution.

*Acute and chronic Daphnia assay*

This sample caused some strange effects on daphnids. First of all, the freshly sampled and tested waste water caused most organisms to float on the water surface, even in strongly diluted samples. This effect commonly referred to as "surface trapping" often occurs in Daphnia tests with oil products (G. Whale personnel communication). It was therefore hard to judge whether mortality was caused by toxicants or by this physical effect of the sample. These tests were therefore repeated, while cetyl-alcohol was added. Small amounts of this substance were placed on the water surface and by lowering the 'strength' (surface tension) of the water surface this increased the potential for daphnids to escape from the surface layer. Only the results of these latter tests using cetyl-alcohol are reported.
Secondly, several strange dose-effect relations were noted. Especially in the 4 times diluted sample treated with both the DOC-die away and the Zahn-Wellens test. In these cases the undiluted sample (which is in fact 25 volume % of the original sample!) did not cause any negative effects, while increased immobility was observed at other dilutions (especially the 18 or 32 vol%).

**Concawe 5** *chronic daphnid assay*

Significant effects on the $r_m$-value were observed in the 10 vol% treatment, in which the $r_m$-value was 11% lower as compared to the control. However, no such effects were observed in higher test concentrations. Even in the undiluted sample no toxic effects were demonstrated. The NOEC value is therefore set at 100 vol%.

**Concawe 7** *Algal test*

The effect in the undiluted sample is only a 4% reduction in growth rate. However, due to the small variation within this test, this reduction was still statistically significant.

*Acute and chronic Daphnia assay*

In especially the undiluted samples, daphnids started to float on the water-surface within a day. It was therefore hard to judge whether mortality was caused by toxicants or by this physical effect of the sample. The procedure to minimize surface trapping (as previously descriped for Concawe sample 2) was therefore used. As such, the tests were t repeated, with the addition of small amounts of cetyl-alcohol to lower surface tension. However, since toxic effects were also observed at concentrations were organisms did not start to float it was concluded that the could not be attributed to physical effects alone.

Table 7: Overview of the toxicity tests performed with **Concawe 1** (Ecolims nr. 335378) before and after biodegradation. Shaded results show a toxic effect.

| **Concawe 1** | | **Microtox** | | | **Algae** | | | **Daphnia - acute** | | **Daphnia - chronic** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | **Mortality** | | **Reproduction** | | |
| | | $EC_{20}$ (vol %) | $EC_{50}$ (vol %) | NOEC (vol %) | $EC_{50}$ (vol %) | Effect in 100 vol% | NOEC (vol %) | $LC_{50}$ (vol %) | Mortality in 100 vol % | NOEC (vol %) | $LC_{50}$ (vol %) | NOEC (vol %) | $EC_{50}$ (vol %) | Reduction $R_m$ in100 vol% (%) |
| Original sample | | 15 (14–16) | > 45 | 49 | >98 | 11 | 32 | 63 (55–71) | 85 | 32 | > 100 | 32 | > 100 | 42 |
| DOC-die away | T=4 hr. | 10 (9-11) | 34 (29-41) | 49 | >98 | 46 | 100 | > 100 | 15 | 32 | > 100 | 32 | 71 (57–83) | 84 |
| | T=14 days | > 45 | > 45 | 49 | >98 | 49 | 100 | > 100 | 5 | 100 | >100 | 32 | >100 | 14 |
| Zahn-Wellens | T=4 hr. | 10 (9–11) | 37 (34–40) | 12 | 51 (48–55) | 96 | 100 | > 100 | 5 | 100 | > 100 | 100 | >100 | 5 |
| | T=28 days | > 45 | > 45 | 25 | 64 (53–100) | 73 | 100 | > 100 | 5 | 100 | > 100 | 100 | >100 | 0 |

Table 8: Overview of the toxicity tests performed with **Concawe 2** (Ecolims nr. 335379) before and after biodegradation. Shaded results show a toxic effect.

| **Concawe 2** | | **Microtox** | | | **Algae** | | | **Daphnia - acute** | | **Daphnia - chronic** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | **Mortality** | | **Reproduction** | | |
| | | $EC_{20}$ (vol %) | $EC_{50}$ (vol %) | NOEC (vol %) | $EC_{50}$ (vol %) | Effect in 100 vol% | NOEC (vol %) | $LC_{50}$ (vol %) | Mortality in 100 vol % | NOEC (vol %) | $LC_{50}$ (vol %) | NOEC (vol %) | $EC_{50}$ (vol %) | Reduction $R_m$ in100 vol% (%) |
| Original sample | | < 5.6 | 19 (17–20) | <6.1 | 10[2] (9–11) | 75[2] | 32[1] | 52[1] (46–58) | 100 | 18 | 24 | 10 | 22 (20–23) | 100 |
| **Without dilution** | | | | | | | | | | | | | | |
| DOC-die away | T=4 hr. | 17 (14–21) | > 45 | 12.2 | 20 (18–23) | 78 | 32 | 45 (38–53) | 100 | 18 | 32 | 18 | 50 (44-55) | 100 |
| | T=14 days | 44 (30–65) | > 45 | 49 | 93 | 53 | 100 | >100 | 15 | 100 | >100 | 100 | >100 | 0 |
| Zahn-Wellens | T=4 hr. | 32 (26–39) | > 45 | 12.2 | 29 (26–38) | 68 | 32 | 71 (50–102) | 65 | 32 | 51 | 32 | 60 (57-63) | 100 |
| | T=28 days | > 45 | > 45 | 49 | 92 | 95 | 100 | > 100 | 5 | 100 | >100 | 18 | >100 | 8 |
| **25% dilution** (data not corrected for dilution!) | | | | | | | | | | | | | | |
| DOC-die away | T=4 hr. | > 45 | > 45 | 24.5 | 63 (58–73) | 91 | 100[2] | > 100[2] | 0 | 32 | 61 | 32 | 69 (64-73) | 83 |
| | T=14 days | > 45 | > 45 | 49 | 95 | 50 | 100[2] | >100[2] | 10 | 100 | >100 | 32 | >100 | 21 |
| Zahn-Wellens | T=4 hr. | > 45 | > 45 | 24.5 | 76 (61–91) | 75 | 100[2] | > 100[2] | 5 | 32 | 77 | 32 | 72 (61-91) | 78 |
| | T=28 days | > 45 | > 45 | 49 | 80 | 96 | 100[2] | > 100[2] | 5 | 18 | 26 (23-30) | 5.6 | 29 (27-32) | 65 |

[1]: animals floating on the surface
[2]: unclear dose-effect relation

Table 9: Overview of the toxicity tests performed with **Concawe 4** (Ecolims nr. 335381) before and after biodegradation. Shaded results show a toxic effect.

| **Concawe 4** | | **Microtox** | | **Algae** | | | ***Acartia tonsa*** | | | **Oyster larvae** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $EC_{20}$ (vol %) | $EC_{50}$ (vol %) | NOEC (vol %) | $EC_{50}$ (vol %) | Effect in 100 vol% | NOEC (vol %) | $LC_{50}$ (vol %) | Mortality in undiluted sample (%) | NOEC (vol %) | $LC_{50}$ (vol %) |
| Original sample | | > 45 | > 45 | 98 | >98 | 0 | 100 | > 100 | 5 | 100[1] | >100 |
| DOC-die away | T=4 hr. | > 45 | > 45 | 25 | 22 | 51 | 100 | > 100 | 13 | 32 | 57 (45 – 69) |
| | T=14 days | > 45 | > 45 | 98 | >98 | 0 | 100 | >100 | 20 | 32 | 56 (56-56) |
| Zahn-Wellens | T=4 hr. | > 45 | > 45 | 6[1] | >98[1] | 0[1] | 32 | >100 | 38 | 10 | 17 (16 – 18) |
| | T=28 days | > 45 | > 45 | 49 | >98 | 16 | 100 | > 100 | 23 | 10 | 46 (39-64) |

[1]: unclear dose-effect relation, with significant effects at lower test concentrations

Table 10: Overview of the results of the toxicity tests performed with the other samples without the biodegradation study. Shaded results show a toxic effect.

| | | **Microtox** | | **Algae** | | | ***Acute crustacean*** | | ***Chronic Crustacea*** | | | | **Oysterlarvae** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **Type water** | **$EC_{20}$** (vol %) | **$EC_{50}$** (vol %) | **NOEC** (vol %) | **$EC_{50}$** (vol %) | Effect in 100 vol% | **NOEC** (vol %) | **$LC_{50}$** (vol %) | **NOEC** (vol %) | **$LC_{50}$** (vol %) | **NOEC** reproduction (vol %) | **$EC_{50}$** reproduction (vol %) | **NOEC** (vol %) | **$LC_{50}$** (vol %) |
| Concawe 3 | Fresh | > 45 | > 45 | 98 | >98 | 0 | 100 | > 100 | 100 | >100 | 100 | >100 | | |
| Concawe 5 | Fresh | 31 (21 – 45) | > 45 | 49 | >98 | 19 | 100 | > 100 | 100 | >100 | 100[2] | >100 | | |
| Concawe 6 | Fresh | 11 (10 – 12) | 35 (31 – 40) | 98 | >98 | 0 | 100 | > 100[3] | 18 | 31 | 32 | 62 (54 – 68) | | |
| Concawe 7 | Fresh | < 5.6 | 10 (10 – 11) | 49 | >98 | 4 | < 5.6[1] | < 5.6[1] | 10 | 15 | 10 | 15 (14 – 15) | | |
| Concawe 8 | Fresh | > 45 | > 45 | 49 | >98 | 30 | 100 | > 100 | 100 | >100 | 100 | >100 | | |
| Concawe 9 | Marine | > 50 | > 50 | 25 | >98 | 9 | 32 | 56 (38 – 81) | | | | | <5.6 | 16 (8 – 27) |

[1]: animals floating on surface
[2]: unclear dose-effect relation, with significant effects at lower test concentrations
[3]: increased (but not statistical significant) mortality in undiluted sample (35%)

## 5.3 Bioaccumulation

An overview of the average PBS concentrations, determined using both SPME and LLE ,of the Concawe effluents 1, 2 and 4 before and after degradation are shown in table 11. The average PBS concentrations for Concawe effluents 3, 5, 6, 7, 8 and 9 without degradation are shown in Table 12. The individual data and the chromatograms are shown in appendix 3. The average SPME-PBS concentrations in the original samples ranged between 1.5 and 138 mM with RSDs from 7% to 34%. The LLE approach seems to have higher limits of detection than the SPME method, and is therefore less sensitive. A linear correlation in PBS concentrations between LLE and SPME was observed (figure 2).

The peak patterns of the effluents between the facilities showed large differences (Appendix 3). All effluents have a large number of peaks, and large differences in concentrations of specific compounds between facilities were found.

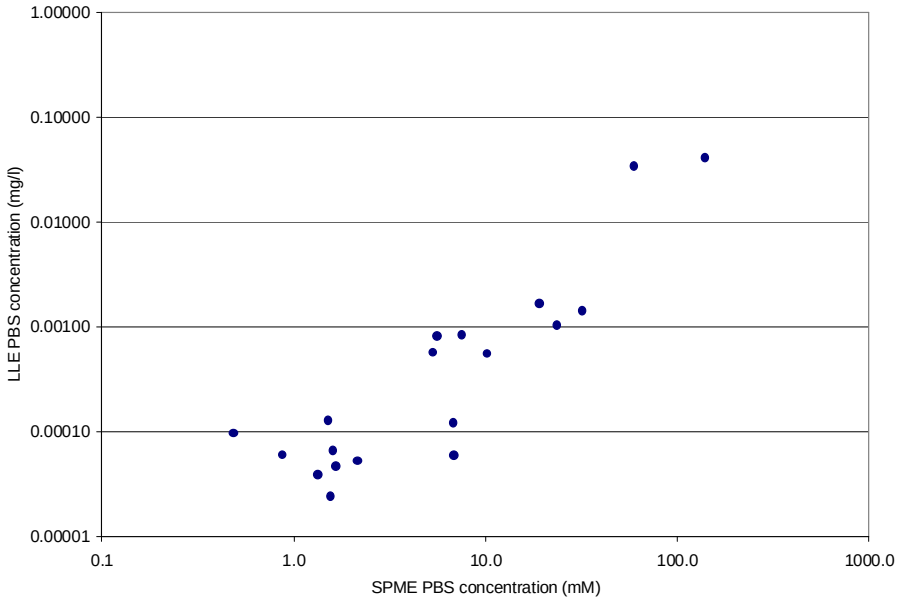


*Figure 2: PBS concentrations of effluents (including all data from the degradation studies) for SPME and LLE.*

For Concawe 2 and Concawe 7 the PBS concentrations are rather high, 59 mM and 138 mM, respectively. The critical PBS concentrations, (i.e. total molar concentration in the fiber, Cfiber) for acute narcotic effects for fish, algae and zooplankton range between 42 and 77 mM (Table 13). The estimated critical level for chronic narcotic effects with *Daphnia magna* is 8 mM. It is predicted that acute narcotic effects can be expected for Concawe 2 and Concawe 7 for i) trout (Concawe 7 only), ii) *Daphnia magna* (both effluents), and iii) for algae (both effluents). This is in agreement with the acute toxicity data for Daphnia magna, which showed that Concawe 7 was the effluent with the highest toxicity (NOEC and $LC_{50}$ <5.6 volume %).

Table 13: Critical benchmarks (acute and chronic) of Cfiber for trout, algae and zooplankton. Chronic narcotic effect was estimated using the ratio of acute-to-chronic (ACR) effects of 1/5 (Di Toro et al., 2000*).

| Endpoint | Critical Cfiber (mM) | Reference |
|---|---|---|
| Acute narcotic effect trout | 77 | Parkerton et al., 2001 |
| Acute narcotic effect algae | 57 | Parkerton et al., 2001 |
| Acute narcotic effect *Dapnia magna* | 42 | Parkerton et al., 2001 |
| Chronic narcotic effect *Daphnia magna* | 8 | Estimated based on ACR of 1/5* |

The PBS concentrations in five effluents (Concawe 1, 2, 5, 6, 7) were higher than the estimated benchmark of chronic narcotic toxicity for *Daphnia magna*, which indicates that negative effects can be expected (see Figure 3). These data are in agreement with the chronic Daphnia magna data (NOEC). The correlations between bioaccumulation and narcotic toxicity will be further illustrated in section 5.6. PBS concentrations for Concawe 3, 4, 8 and 9 are below the critical levels.

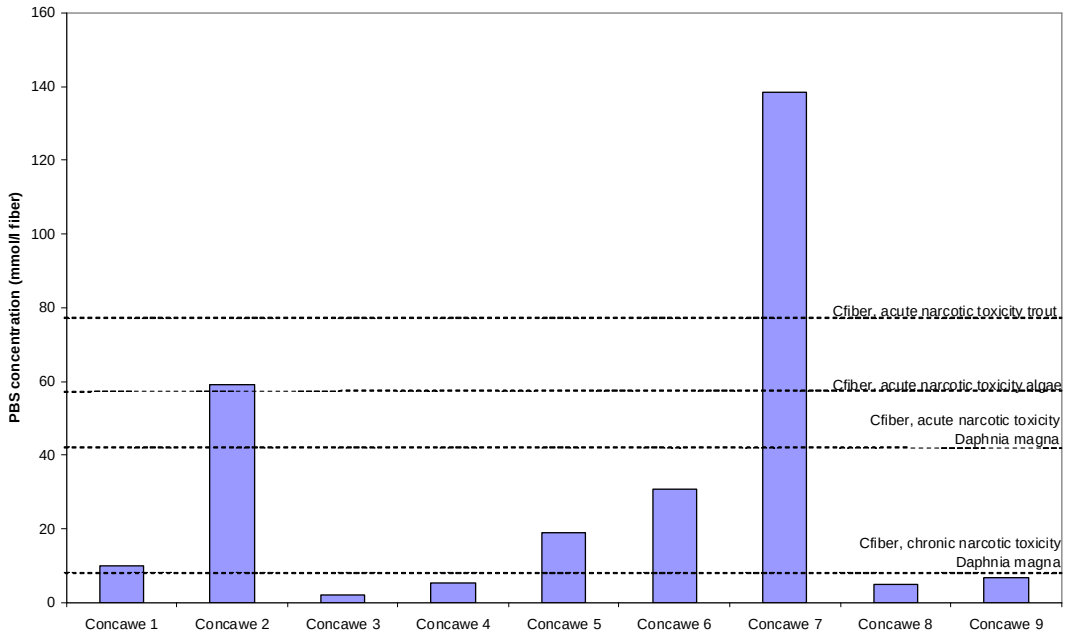


*Figure 3: Overview of PBS concentrations (mM) of the effluents and the critical benchmarks from Table 13.*

Table 11: PBS concentrations (mmol/l fiber) of the effluents that were subjected to the degradation determined by SPME and LLE. Average concentrations (n=3), and relative standard deviation (RSD) are shown. Raw data are provided in appendix 1 .

| **Sample** | **RIVO LIMS no.** | **Degradation** Type | **Time** | **SPME mmol/L fiber** | **SPME RSD (%)** | **LLE mg/l** | **LLE RSD (%)** |
|---|---|---|---|---|---|---|---|
| Concawe 1 | 2005/1480 | Original sample | 0 hours | 10 | 7 | 0.00056 | 26 |
| Concawe 1 | 2005/1643 | Ready Style | 4 hours | 1.5 | 23 | 0.00013 | 29 |
| Concawe 1 | 2005/1651 | Ready Style | 14 days | 3.0 | 21 | <LOD | |
| Concawe 1 | 2005/1644 | Zahn-Wellens | 4 hours | 6.8 | 10 | 0.000060 | 46 |
| Concawe 1 | 2005/1652 | Zahn-Wellens | 28 days | 2.1 | 10 | 0.000053 | 29 |
| | | | | | | | |
| Concawe 2 | 2005/1481 | Original sample | 0 hours | 59 | 23 | 0.035 | 35 |
| Concawe 2 | 2005/1645 | Ready Style, 100% | 4 hours | 32 | 5 | 0.0014 | 16 |
| Concawe 2 | 2005/1653 | Ready Style, 100% | 14 days | 0.5 | 17 | 0.00010 | 83 |
| Concawe 2 | 2005/1646 | Ready Style, 25% diluted* | 4 hours | 7.5 | 25 | 0.00085 | 20 |
| Concawe 2 | 2005/1654 | Ready Style, 25% diluted* | 14 days | 0.9 | 40 | 0.000061 | 20 |
| Concawe 2 | 2005/1647 | Zahn-Wellens, 100% | 4 hours | 23 | 15 | 0.0011 | 21 |
| Concawe 2 | 2005/1655 | Zahn-Wellens, 100% | 28 days | 1.6 | 25 | 0.000067 | 38 |
| Concawe 2 | 2005/1648 | Zahn-Wellens, 25%* | 4 hours | 5.5 | 20 | 0.00082 | 8 |
| Concawe 2 | 2005/1656 | Zahn-Wellens, 25%* | 28 days | 1.3 | 18 | 0.000039 | 11 |
| | | | | | | | |
| Concawe 4 | 2005/1483 | Original sample | 0 hours | 5.3 | 11 | 0.0006 | 22 |
| Concawe 4 | 2005/1649 | Ready Style | 4 hours | 6.7 | 4 | 0.00012 | 78 |
| Concawe 4 | 2005/1657 | Ready Style | 14 days | 1.5 | 3 | 0.000025 | 70 |
| Concawe 4 | 2005/1650 | Zahn-Wellens | 4 hours | 2.3 | 11 | | |
| Concawe 4 | 2005/1658 | Zahn-Wellens | 28 days | 1.6 | 14 | 0.000047 | 71 |

* Results are not corrected for the dilution.

Table 12: PBS concentrations (mmol/l fiber) determined by SPME and LLE for the effluents without the degradation study. Average concentrations (n=3), and relative standard deviation (RSD) are shown. Raw data are provided in appendix 2.

| **Sample** | **RIVO** | **Degradation** | **Time** | **SPME** | **SPME** | **LLE** | **LLE** |
|---|---|---|---|---|---|---|---|
| | **LIMS no.** | Type | | **mmol/L fiber** | **RSD (%)** | **mg/l** | **RSD (%)** |
| Concawe 3 | 2005/1482 | Original sample | 0 hours | 2.1 | 24 | <LOD | |
| Concawe 4 | 2005/1483 | Original sample | 0 hours | 5.3 | 11 | 0.0006 | 22 |
| Concawe 5 | 2005/1484 | Original sample | 0 hours | 19 | 18 | 0.0017 | 16 |
| Concawe 6 | 2005/1485 | Original sample | 0 hours | 31 | 13 | | |
| Concawe 7 | 2005/1486 | Original sample | 0 hours | 138 | 14 | 0.041 | 10 |
| Concawe 8 | 2005/1487 | Original sample | 0 hours | 5.0 | 34 | <LOD | |
| Concawe 9 | 2005/1488 | Original sample | 0 hours | 6.9 | 17 | <LOD | |

## 5.4 Metals

Metal concentrations of the original samples are shown in table 14. In all samples concentrations of metals were below or very close to the limit of detection, which varied between the metals (<0.05 to <100 mg/l), except for cobalt and nickel. Concawe 2 had relative high levels of cobalt and nickel, 120 µg/l and 270 µg/l respectively. Nickel was also present in Concawe 1 (29 µg/l), and vanadium was found in Concawe 3 and Concawe 6, 8.4 µg/l and 5.9 µg/l, respectively. Chromium was present Concawe 1, 2 and 5 (1.6, 4.1, 3.9 µg/l, respectively).

Table 14: Levels of metals and other parameters in effluent samples.

| | **Effluent** | **Concawe 1** | **Concawe 2** | **Concawe 3** | **Concawe 4** | **Concawe 5** | **Concawe 6** | **Concawe 7** | **Concawe 8** | **Concawe 9** |
|---|---|---|---|---|---|---|---|---|---|---|
| | **RIVO LIMS no.** | **2005/ 1480** | **2005/ 1481** | **2005/ 1482** | **2005/ 1483** | **2005/ 1484** | **2005/ 1485** | **2005/ 1486** | **2005/ 1487** | **2005/ 1488** |
| Metals | | | | | | | | | | |
| Cd | µg/l | <0.4 | <0.4 | <0.4 | <4 | <0,4 | <0,4 | <0.4 | <0.4 | <4 |
| Cr | µg/l | 1.6 | 4.1 | <1 | <10 * | 3.9 | <1 | <1 | <1 | <10 * |
| Co | µg/l | <5 | 120 | <5 | <50 * | <5 | <5 | <5 | <5 | <50 * |
| Cu | µg/l | <5 | 5.3 | <5 | <50 * | <5 | <5 | <5 | <5 | <50 * |
| Hg | µg/l | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 |
| Pb | µg/l | <10 | 15 | <10 | <100 * | <10 | <10 | <10 | <10 | <100 * |
| Ni | µg/l | 29 | 270 | <10 | <100 * | <10 | <10 | <10 | <10 | <100 * |
| V | µg/l | <5 | <5 | 8.4 | <50 * | <5 | 5.9 | <5 | <5 | <50 * |
| Zn | µg/l | <20 | 200 | <20 | <200* | 86 | 42 | 150 | 310 | <200* |
| Ca | µg/l | 107000 | 35000 | 72000 | 337000 | 43000 | 204000 | 83000 | 58000 | 175000 |
| Mg | µg/l | 140000 | 5500 | 21000 | 1100000 | 7000 | 18000 | 13000 | 18000 | 360000 |
| Na | mg/l | 1400000 | 370000 | 540000 | 8300000 | 440000 | 470000 | 86000 | 110000 | 2800000 |
| K | mg/l | 75000 | 9000 | 22000 | 450000 | 3700 | 13000 | 26000 | 8200 | 180000 |
| Ca/mg ratio | | 0.8 | 6.4 | 3.4 | 0.3 | 6.1 | 11.3 | 6.4 | 3.2 | 0.5 |
| Na/K ratio | | 19 | 41 | 25 | 18 | 119 | 36 | 3 | 13 | 16 |
| Sulphate | mg/l | 350 | 210 | 630 | 2400 | 910 | 1400 | 72 | 160 | 1000 |

Limit of detection was higher due to interfering matrix compounds.

## 5.5 Changes in toxicity and bioaccumulation during degradation

In this section the changes in toxicity and bioaccumulation during the degradation experiments are discussed.

**Concawe 1**

After 4 hours a general increase in toxicity was observed for both the DOC-die away and the Zahn-Wellens test, since 5 out of 8 toxicity tests showed an increasing effect. Bioaccumulation (PBS concentration) did not increase after 4 hours, but decreased in the DOC-die away test. On the contrary, the Zahn-Wellens test showed a slight decrease in PBS concentration only. Interesting to note is the change in peak profile in the chromatogram between the original sample and the sample after addition of the degradation medium (t=4 hr), which is observed for both the DOC-die away and Zahn-Wellens test (figure 4).

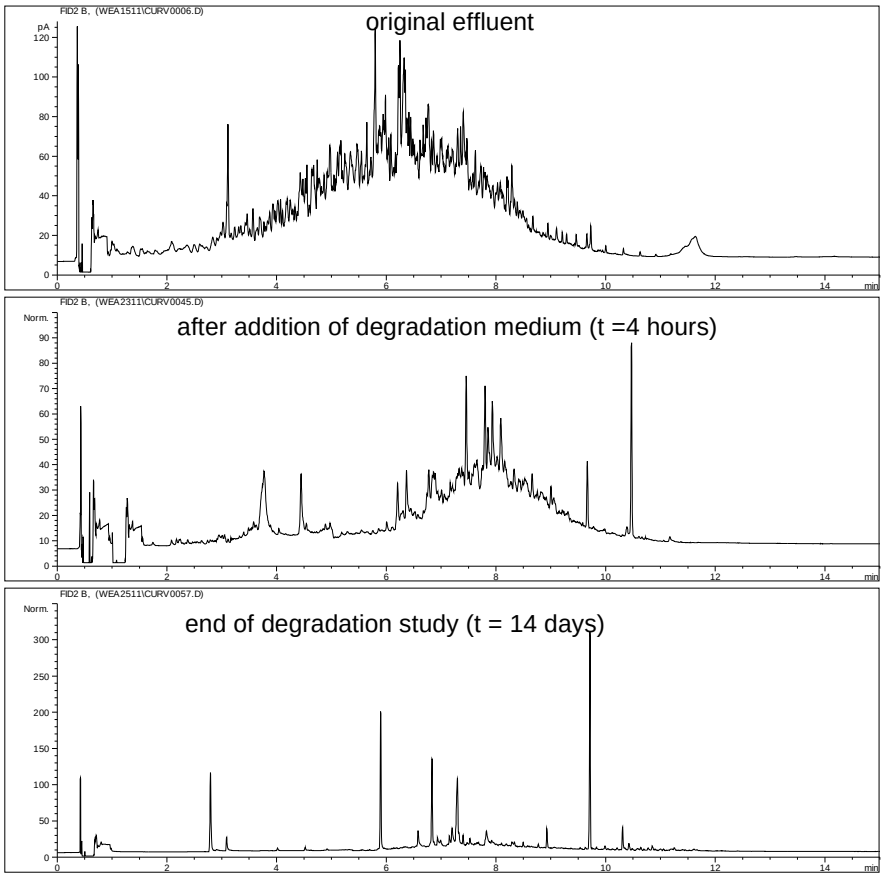


*Figure 4: SPME GC-FID chromatogram of the original effluent (Concawe 1), the chromatogram of the effluent after addition of the degradation medium (t = 4 hours), and after the DOC-die away degradation study (t = 14 days).*

At the end of the biodegradation studies the toxicity increase had changed into a decrease, as the toxicity in 6 out of 8 tests was lower compared to the tests performed at t=0. Only in the algae tests an increased effect was maintained. Bioaccumulation also decreased for both degradation approaches at the end of the degradation studies (figure 5), and the peak profile changed from a large hump of peaks to some individual peaks.

Furthermore, there were no consistent differences in toxicity between the DOC-die away and the Zahn-Wellens test. The toxicity in the Zahn-Wellens test was lower for the chronic *Daphnia* assay, while the opposite holds true for the algae tests. No differences were observed for the Microtox and the acute *Daphnia* test.

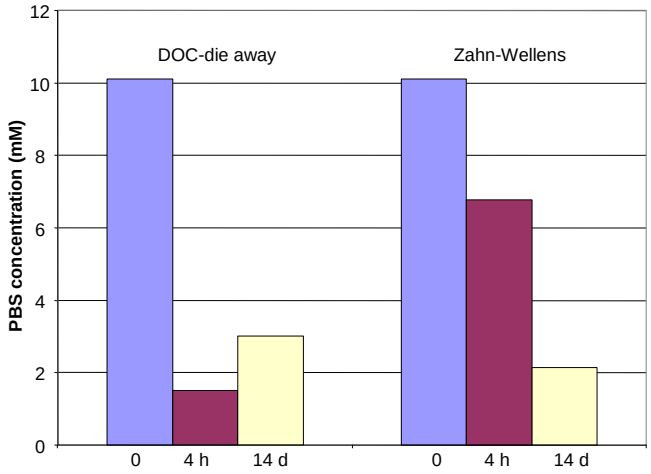


*Figure 5: Bioaccumulation (SPME PBS concentration) of Concawe 1 before (0), after addition of the medium (4 hr.) and after degradation (14 d or 28 d).*

**Concawe 2**

The toxicity tests performed with the undiluted samples showed very consistent results, since a decrease in toxicity and bioaccumulation (figure 6) was observed for almost all tests performed during or after biodegradation. Furthermore the toxic effects and bioaccumulation demonstrated at the end of the biodegradation studies were lower as compared to the samples tested after 4 hours. No strong differences were observed between the DOC-die away and the Zahn-Wellens tests for both toxicity and bioaccumulation.

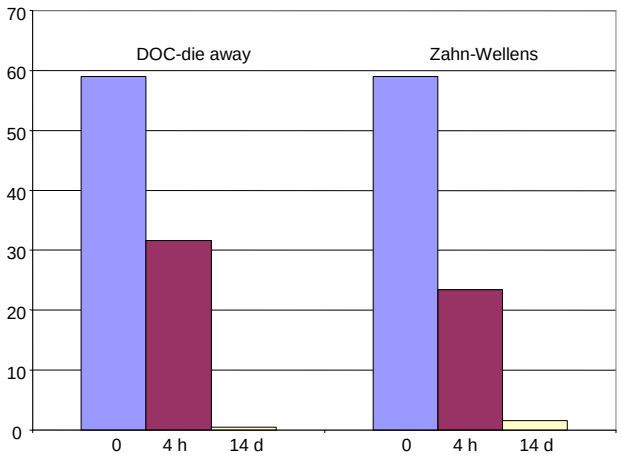


*Figure 6: Bioaccumulation (SPME PBS concentration) of Concawe 2 before (0), after addition of the medium (4 hr.) and after degradation (14 d or 28 d).*

However, the results of both the algae tests and the chronic daphnia assays show remarkable differences between the diluted and the undiluted samples, since an increase in toxicity was observed for the diluted samples tested with both the DOC-die away and the Zahn-Wellens test. $EC_{50}$-values for the algae varied for example between 63 and 95 vol%, which is (after correction for the dilution) 16 – 24 vol% of the original sample. The same applies to the chronic Daphnia, were in 3 out of the 4 tests $EC_{50}$-values could be calculated. This means that these $EC_{50}$-values were all <25vol% of the original sample.
The diluted effluent showed a remarkable difference in peak profile compared to the undiluted effluent (figure 7). Especially, peaks between 2 and 3 minutes present in the undiluted effluent are not present in the 25% diluted sample. Possibly these compounds are

volatile and were evaporated during the process of dilution and homogenization or were removed by the addition of the degradation medium.

Bioaccumulation decreased in the 25% dilution effluent during or after the degradation for both degradation studies (figure 8). The decrease in PBS concentration between the 4 hour sample and the sample after degradation for both the DOC-die away and Zahn-Wellens studies was not as steep as observed for the undiluted sample, which illustrates that dilution of an effluent has an effect on rate of degradation.

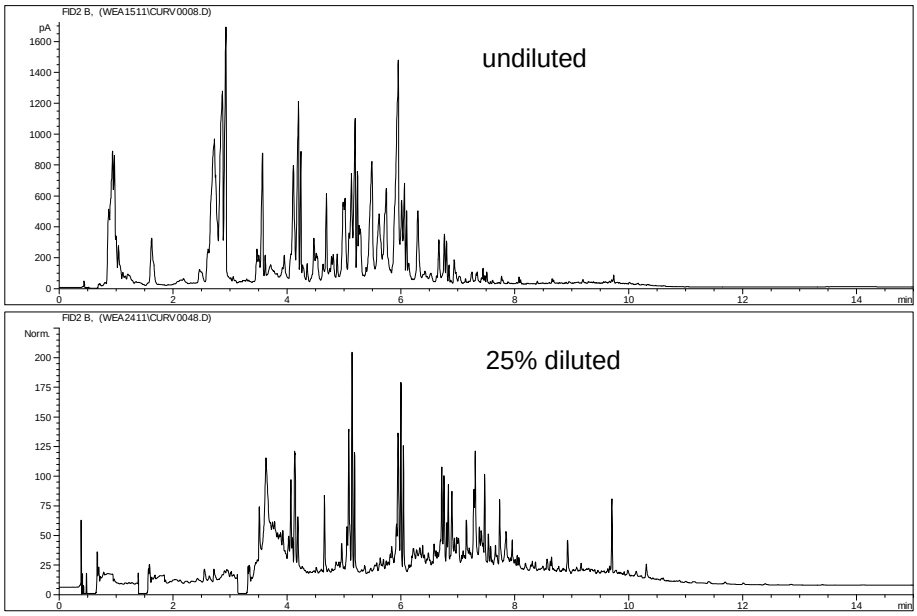


*Figure 7: SPME GC-FID chromatogram of the undiluted (original Concawe 2 effluent) and 25% diluted effluent (t=4 hr) after addition of the DOC-die away degradation medium.*

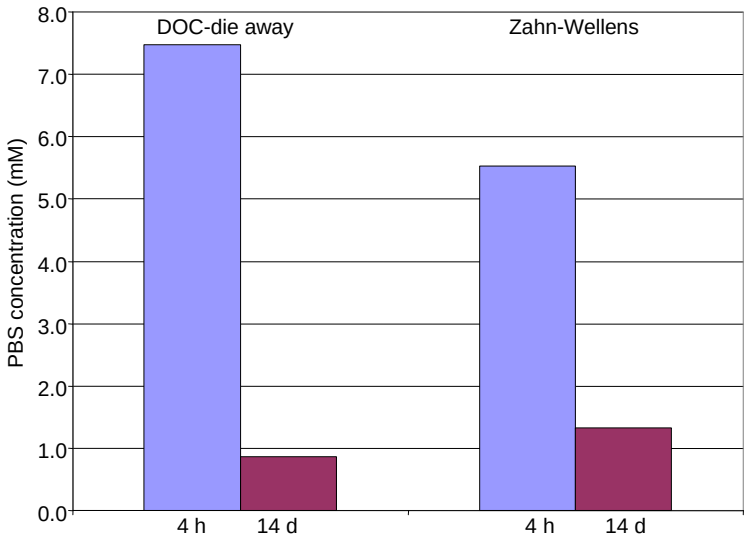


*Figure 8: Bioaccumulation (SPME PBS concentration) of Concawe 2 25% dilution, after addition of the medium (4 hr.) and after degradation (14 d or 28 d).*

**Concawe 4**

Biodegradation tended to increase the toxicity, especially after 4 hours where in 5 out of 8 toxicity tests an increase was observed. At the end of the biodegradation study such an increase was still observed. The frequency was however somewhat decreased since in 3 out of 8 tests an increase in toxicity as compared to t=0 was observed. Bioaccumulation also tended to increase for the DOC-die away study after addition of after 4 hours, and the end of the degradation the PBS concentration decreased (figure 9). The Zahn-Wellens study, in contrast to the DOC-die away study, showed a decrease after 4 hours of degradation, and a slight decrease after the end of the study.

Differences between the DOC-die away and the Zahn-Wellens test were especially noted for the oyster larvae where effects in the Zahn-Wellens test were generally more severe.

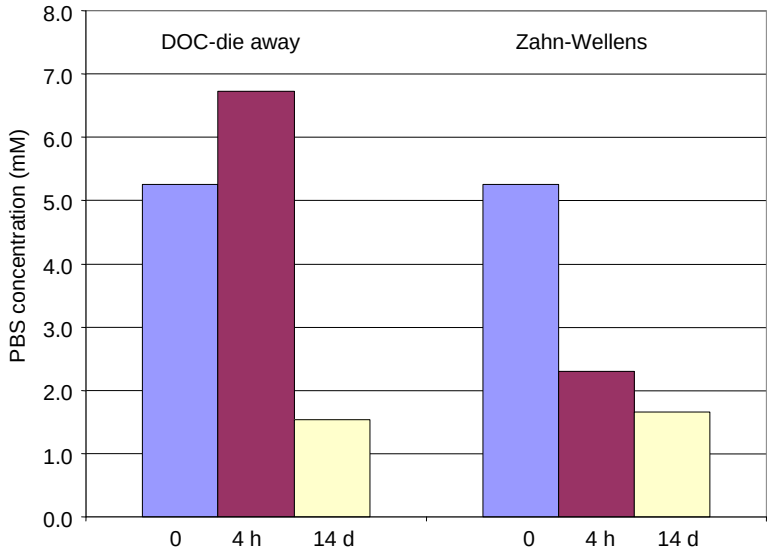


*Figure 9 Bioaccumulation (SPME PBS concentration) of Concawe 4 of the original effluent, after addition of the medium (4 hr.) and after degradation (14 d or 28 d).*

## 5.6 Evaluation of all results

To study the correlation between toxicity and bioaccumulation scatter plots (figure 10) were made between toxicity (expressed in toxic units) and bioaccumulation (SPME PBS concentration). Good correlations between SPME and microtox (plot A, $R^2 = 0.76$), and SPME and chronic Daphnia magna NOEC (plot D, $R^2 = 0.86$) were observed. This indicates that narcotic toxicity could have played a role in the toxicity for Microtox and chronic Dapnia magna bioassays. The relative high levels of cobalt and nickel in Concawe 2 are may be responsible for the higher toxicity expected from the SPME measurements narcotic toxicity), as can been seen from figure 9, plot A: Concawe 2 deviates from the regression line, higher toxicity was found than based on SPME measurements.

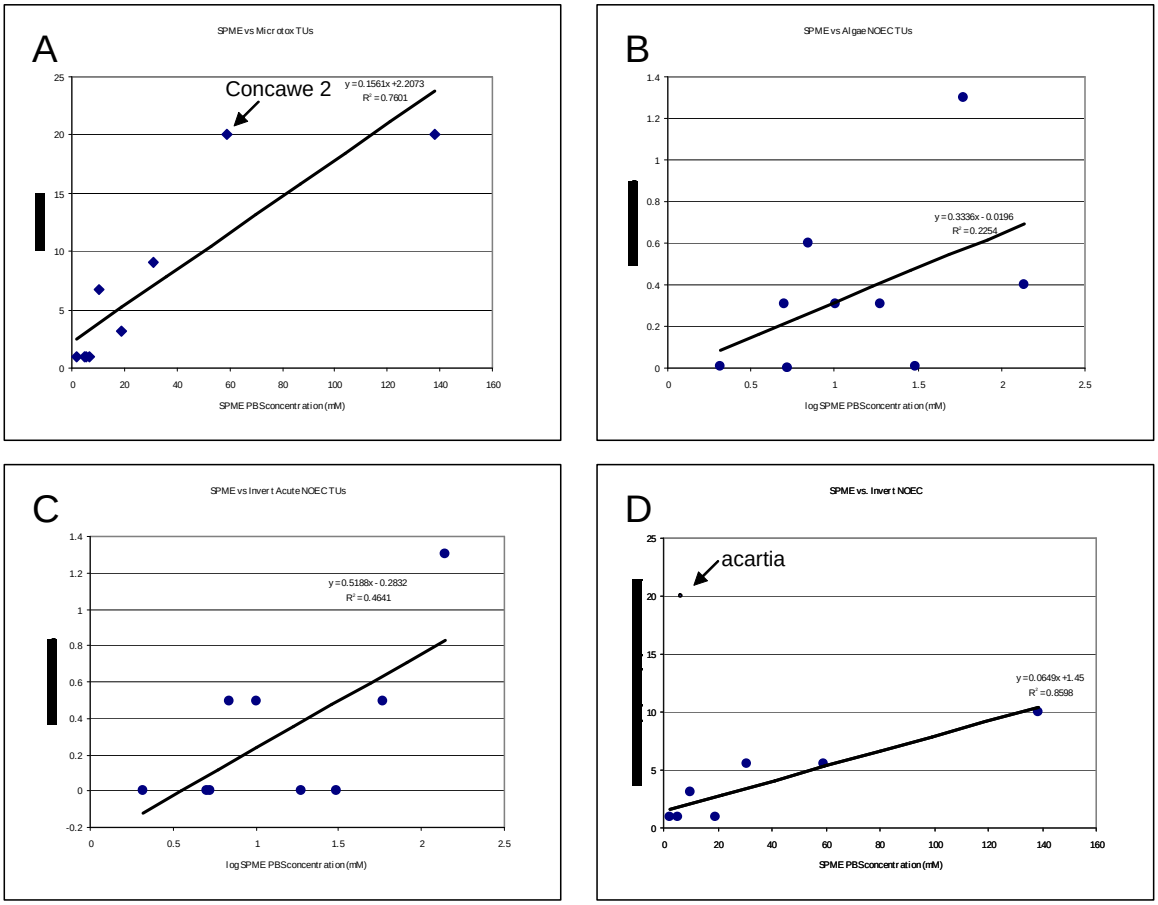


*Figure 10: Toxicity (in toxic units, TUs) vs. bioaccumulation (SPME) of the original samples. Linear regression fitting was used; data for plot B and C were logarithmic transformed. The Acartia data point in plot D was not included in the linear regression fitting, only Daphnia magna data was included. Invert: Invertebrates.*

# 6. Conclusions

- Both DOC-die away and Zahn-Wellens degradation approaches are suitable methods to study persistence of constituents in effluents.
- However, the consequences of the addition of the degradation medium, which was monitored shortly after the start of the experiment (t = 4 hours), on toxicity and bioaccumulation can not fully be understood. The results showed that changes in toxicity, bioaccumulation, and the constituent's pattern of the effluents were already observed after 4 hours. For some effluents the toxicity and bioaccumulation increased and for some effluents the toxicity and bioaccumulation decreased at t = 4 hours. Also the constituent's pattern and level changed. A more detailed study is needed to explain the affects and consequences of the degradation medium in relation to toxicity and bioaccumulation.
- In general, the toxicity and bioaccumulation decreased at the end of degradation studies (14 to 28 days) for both degradation approaches.
- A large proportion of the PBS in these oil industry effluents is readily biodegradable.
- Biodegradation not only lowered the PBS concentration but also toxicity. Appropriate controls are required as some increases in toxic effect were observed after 4 hours and in the saline sample.
- Toxicity was observed for all samples with PBS concentrations above the critical values indicating that narcotic effects are responsible for the observed toxicity.

# 7. References

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Leslie, H.A. and P.E.G. Leonards. 2005b. Protocol. Determination of potentially bioaccumulating substances (PBS) in whole effluents using the 'EGOM' Liquid-Liquid Extraction Method OSPAR Intercessional Expert Group (IEG) on Whole Effluent Assessment (WEA), Interlaboratory Study. RIVO, IJmuiden, The Netherlands.

Parkerton, T., D. Letinske, Febbi, C.Dzamba, M. Connely, D. Winklemann and D. Peterson. 2001. Biomimetic extraction as a cost-effective analytical tool for determining the aquatic toxicity hazard of complex petroleum products. Proceedings SETAC, Madrid, Spain, May 7-10.

**Appendix 1:** PBS concentrations (mmol/l fiber) of the effluents determined by SPME. Average concentrations (AVG), standard deviation (SD), and relative standard deviation (RSD) are shown.

**SPME results (mmol/l) in effluents**

| Sample | RIVO LIMS no. | Degradation Type | Time | #1 mmol/L fiber | #2 mmol/L fiber | #3 mmol/L fiber | AVG mmol/L fiber | SD | RSD(%) |
|---|---|---|---|---|---|---|---|---|---|
| Concawe 1 | 2005/1480 | Original sample | 0 hours | 9.3 | 10.3 | 10.7 | **10.1** | 0.7 | 7 |
| Concawe 1 | 2005/1643 | Ready Style | 4 hours | 1.3 | 1.7 | | **1.5** | 0.3 | 23 |
| Concawe 1 | 2005/1651 | Ready Style | 14 days | 3.3 | 3.4 | 2.3 | **3.0** | 0.6 | 21 |
| Concawe 1 | 2005/1644 | Zahn-Wellens | 4 hours | 6.0 | 7.2 | 7.2 | **6.8** | 0.7 | 10 |
| Concawe 1 | 2005/1652 | Zahn-Wellens | 28 days | 2.4 | 1.9 | 2.1 | **2.1** | 0.2 | 10 |
| | | | | | | | | | |
| Concawe 2 | 2005/1481 | Original sample | 0 hours | 55 | 48 | 74 | **59** | 13.8 | 23 |
| Concawe 2 | 2005/1645 | Ready Style, 100% | 4 hours | 32.6 | 29.9 | 32.5 | **31.6** | 1.6 | 5 |
| Concawe 2 | 2005/1653 | Ready Style, 100% | 14 days | 0.49 | 0.39 | 0.56 | **0.5** | 0.08 | 17 |
| Concawe 2 | 2005/1646 | Ready Style, 25% diluted | 4 hours | 9.3 | 7.6 | 5.5 | **7.5** | 1.9 | 25 |
| Concawe 2 | 2005/1654 | Ready Style, 25% diluted | 14 days | 0.9 | 0.5 | 1.2 | **0.9** | 0.3 | 40 |
| Concawe 2 | 2005/1647 | Zahn-Wellens, 100% | 4 hours | 26.0 | 24.9 | 19.3 | **23.4** | 3.6 | 15 |
| Concawe 2 | 2005/1655 | Zahn-Wellens, 100% | 28 days | 2.0 | 1.6 | 1.2 | **1.6** | 0.4 | 25 |
| Concawe 2 | 2005/1648 | Zahn-Wellens, 25% | 4 hours | 6.4 | 4.3 | 5.9 | **5.5** | 1.1 | 20 |
| Concawe 2 | 2005/1656 | Zahn-Wellens, 25% | 28 days | 1.6 | 1.1 | 1.2 | **1.3** | 0.2 | 18 |
| | | | | | | | | | |
| Concawe 4 | 2005/1483 | Original sample | 0 hours | 5.2 | 4.7 | 5.8 | **5.3** | 0.6 | 11 |
| Concawe 4 | 2005/1649 | Ready Style | 4 hours | 7.0 | 6.5 | 6.6 | **6.7** | 0.3 | 4 |
| Concawe 4 | 2005/1657 | Ready Style | 14 days | 1.5 | 1.5 | 1.6 | **1.5** | 0.04 | 3 |
| Concawe 4 | 2005/1650 | Zahn-Wellens | 4 hours | 2.5 | 2.0 | 2.4 | **2.3** | 0.3 | 11 |
| Concawe 4 | 2005/1658 | Zahn-Wellens | 28 days | 1.4 | 1.9 | 1.7 | **1.6** | 0.23 | 14 |
| | | | | | | | | | |
| Concawe 3 | 2005/1482 | Original sample | 0 hours | 2.4 | 1.5 | 2.4 | **2.1** | 0.5 | 24 |
| Concawe 4 | 2005/1483 | Original sample | 0 hours | 5.2 | 4.7 | 5.8 | **5.3** | 0.6 | 11 |
| Concawe 5 | 2005/1484 | Original sample | 0 hours | 18.3 | 22.7 | 15.9 | **19.0** | 3.4 | 18 |
| Concawe 6 | 2005/1485 | Original sample | 0 hours | 28.0 | 33.6 | | **30.8** | 3.9 | 13 |
| Concawe 7 | 2005/1486 | Original sample | 0 hours | 156 | 118 | 141 | **138** | 18.7 | 14 |
| Concawe 8 | 2005/1487 | Original sample | 0 hours | 3.7 | 7.0 | 4.4 | **5.0** | 1.7 | 34 |
| Concawe 9 | 2005/1488 | Original sample | 0 hours | 6.0 | 6.4 | 8.3 | **6.9** | 1.2 | 17 |

**Appendix 2:** PBS concentrations (mmol/l fiber) of the effluents determined by LLE. Average concentrations (AVG), standard deviation (SD), and relative standard deviation (RSD) are shown.

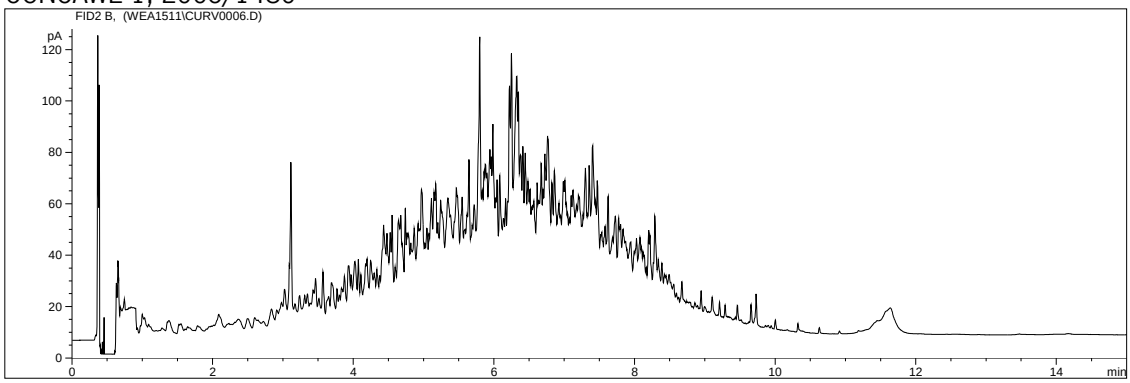
**LLE results (mmol/l) in effluents**

| Sample | RIVO LIMS no. | Degradation Type | Time | #1 mg/l | #2 mg/l | #3 mg/l | AVG mg/l | SD | RSD(%) |
|---|---|---|---|---|---|---|---|---|---|
| Concawe 1 | 2005/1480 | Original sample | 0 hours | 0.00065 | 0.00064 | 0.00039 | **0.00056** | 0.00015 | 26 |
| Concawe 1 | 2005/1643 | Ready Style | 4 hours | 0.000156 | 0.000145 | 0.000087 | **0.00013** | 0.000037 | 29 |
| Concawe 1 | 2005/1651 | Ready Style | 14 days | <LOD | <LOD | <LOD | **<LOD** | | |
| Concawe 1 | 2005/1644 | Zahn-Wellens | 4 hours | 0.000051 | 0.000038 | 0.000092 | **0.000060** | 0.000028 | 46 |
| Concawe 1 | 2005/1652 | Zahn-Wellens | 28 days | 0.000035 | 0.000061 | 0.000064 | **0.000053** | 0.000016 | 29 |
| | | | | | | | | | |
| Concawe 2 | 2005/1481 | Original sample | 0 hours | 0.047 | 0.023 | 0.034 | **0.035** | 0.012 | 35 |
| Concawe 2 | 2005/1645 | Ready Style, 100% | 4 hours | 0.0016 | 0.0012 | 0.0015 | **0.0014** | 0.00023 | 16 |
| Concawe 2 | 2005/1653 | Ready Style, 100% | 14 days | 0.000155 | 0.000041 | | **0.00010** | 0.000081 | 83 |
| Concawe 2 | 2005/1646 | Ready Style, 25% diluted | 4 hours | 0.00072 | 0.0010 | 0.00078 | **0.00085** | 0.00017 | 20 |
| Concawe 2 | 2005/1654 | Ready Style, 25% diluted | 14 days | 0.000051 | 0.000059 | 0.000074 | **0.000061** | 0.000012 | 20 |
| Concawe 2 | 2005/1647 | Zahn-Wellens, 100% | 4 hours | 0.00112 | 0.0012 | 0.00081 | **0.0011** | 0.00022 | 21 |
| Concawe 2 | 2005/1655 | Zahn-Wellens, 100% | 28 days | 0.000039 | 0.000073 | 0.000089 | **0.000067** | 0.000025 | 38 |
| Concawe 2 | 2005/1648 | Zahn-Wellens, 25% | 4 hours | 0.00089 | 0.00082 | 0.00076 | **0.00082** | 0.000064 | 8 |
| Concawe 2 | 2005/1656 | Zahn-Wellens, 25% | 28 days | 0.000043 | 0.000041 | 0.000035 | **0.000039** | 0.000004 | 11 |
| | | | | | | | | | |
| Concawe 4 | 2005/1483 | Original sample | 0 hours | 0.0007 | 0.0005 | 0.0006 | **0.0006** | 0.0001 | 22 |
| Concawe 4 | 2005/1649 | Ready Style | 4 hours | 0.000233 | 0.000074 | 0.000061 | **0.00012** | 0.000096 | 78 |
| Concawe 4 | 2005/1657 | Ready Style | 14 days | 0.000012 | 0.000018 | 0.000044 | **0.000025** | 0.000017 | 70 |
| Concawe 4 | 2005/1650 | Zahn-Wellens | 4 hours | | | | | | |
| Concawe 4 | 2005/1658 | Zahn-Wellens | 28 days | 0.000085 | 0.000035 | 0.000021 | **0.000047** | 0.000034 | 71 |
| | | | | | | | | | |
| Concawe 3 | 2005/1482 | Original sample | 0 hours | <LOD | <LOD | <LOD | **<LOD** | | |
| Concawe 4 | 2005/1483 | Original sample | 0 hours | 0.0007 | 0.0005 | 0.0006 | **0.0006** | 0.0001 | 22 |
| Concawe 5 | 2005/1484 | Original sample | 0 hours | 0.0014 | 0.0019 | 0.0018 | **0.0017** | 0.0003 | 16 |
| Concawe 6 | 2005/1485 | Original sample | 0 hours | | | | | | |
| Concawe 7 | 2005/1486 | Original sample | 0 hours | 0.037 | 0.046 | 0.040 | **0.041** | 0.0042 | 10 |
| Concawe 8 | 2005/1487 | Original sample | 0 hours | <LOD | <LOD | <LOD | **<LOD** | | |

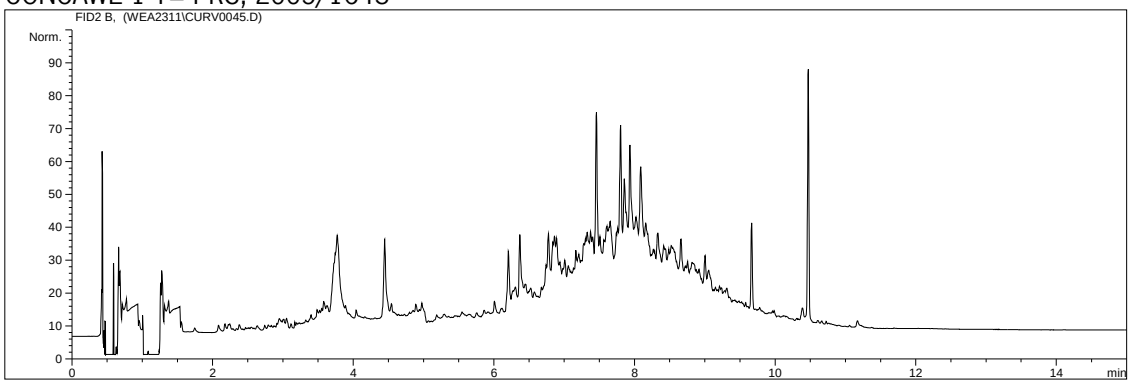
**Appendix 3:** SPME chromatograms of effluents

## CONCAWE 1 – DOC-die away study

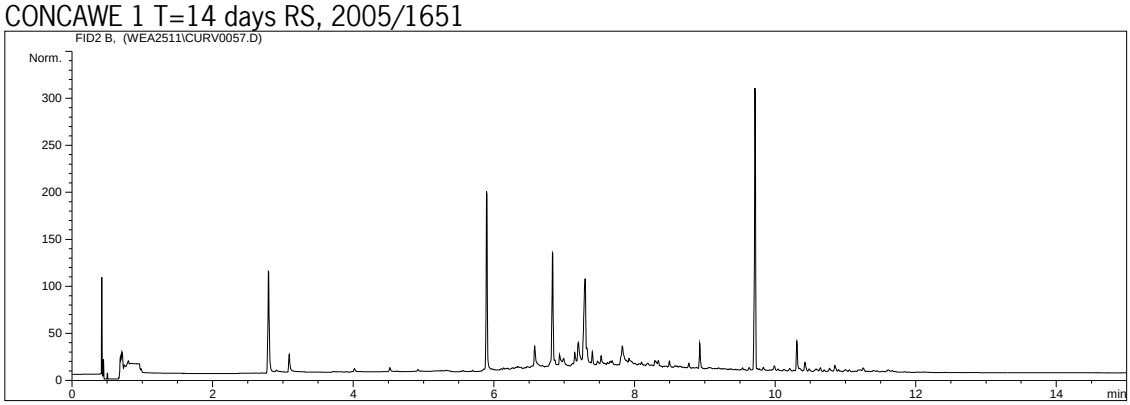
CONCAWE 1, 2005/1480




CONCAWE 1 T=4 RS, 2005/1643

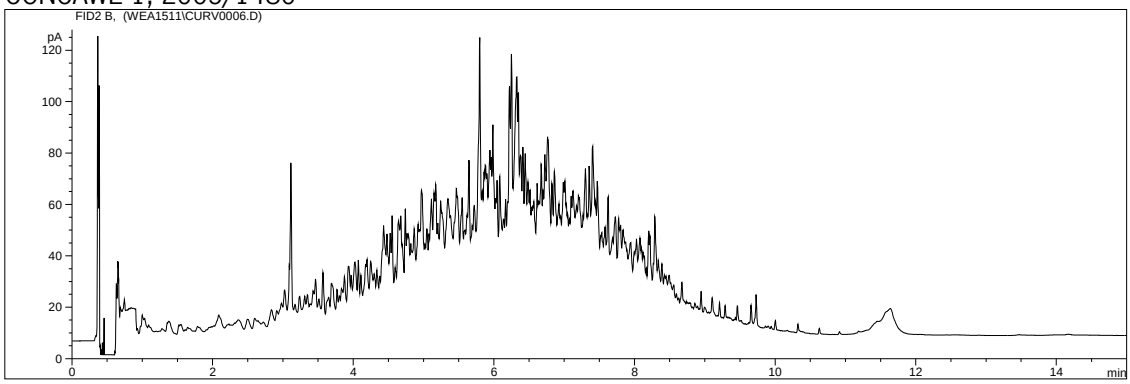



CONCAWE 1 T=14 days RS, 2005/1651

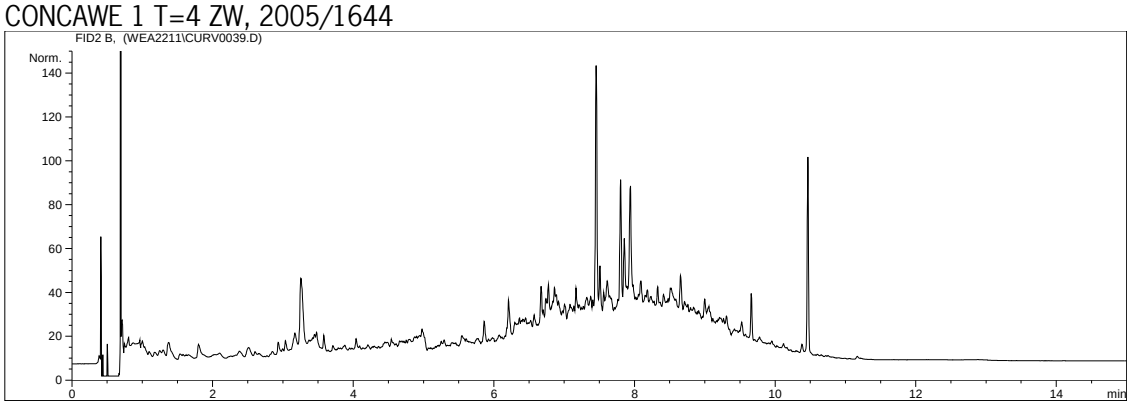


## CONCAWE 1 –Zahn-Wellens style study

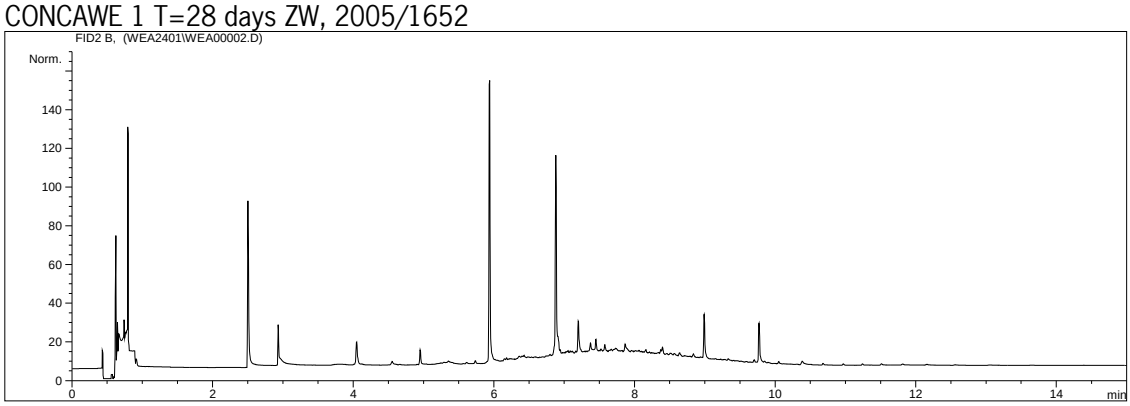
CONCAWE 1, 2005/1480




CONCAWE 1 T=4 ZW, 2005/1644

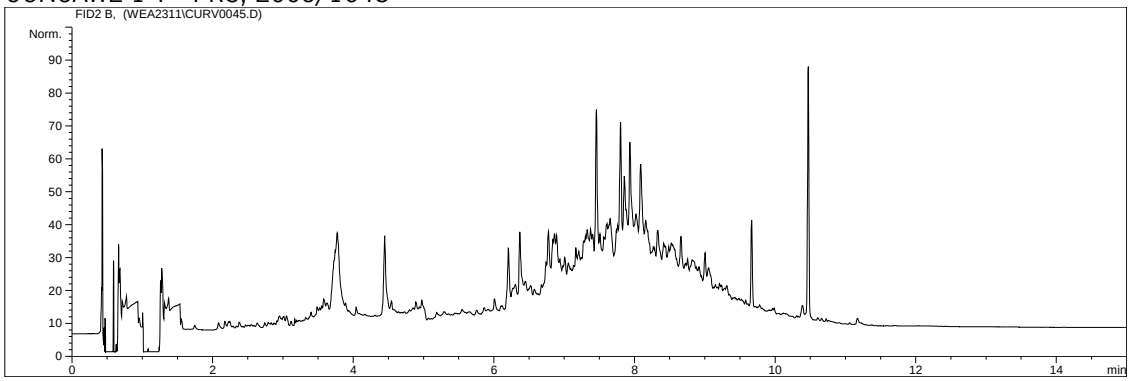



CONCAWE 1 T=28 days ZW, 2005/1652

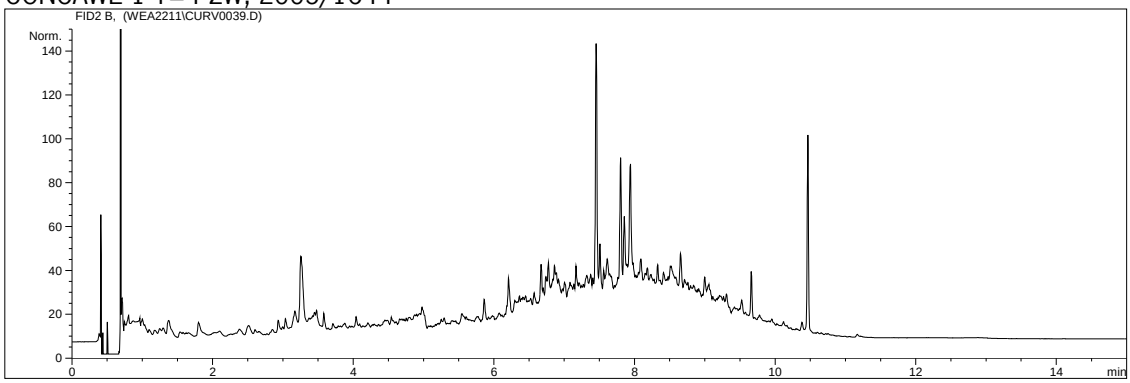


## CONCAWE 1 – DOC-die away study v Zahn-Wellens style study (4h and 14/28d)

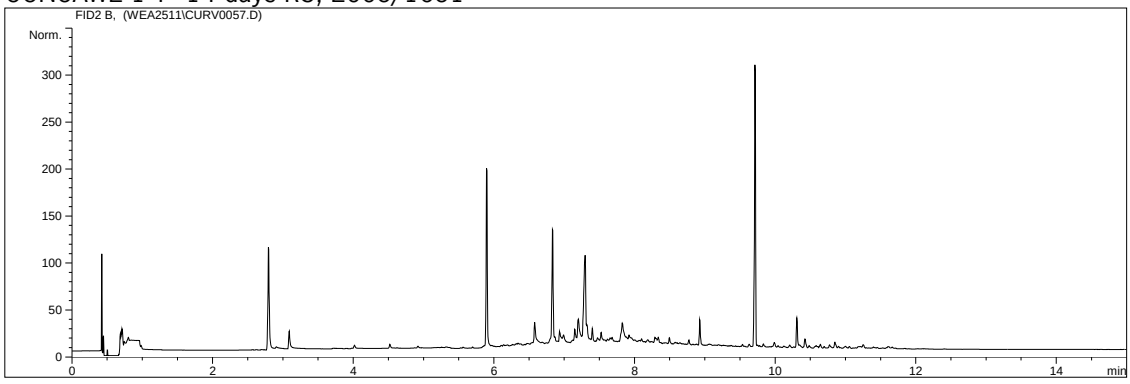
CONCAWE 1 T=4 RS, 2005/1643



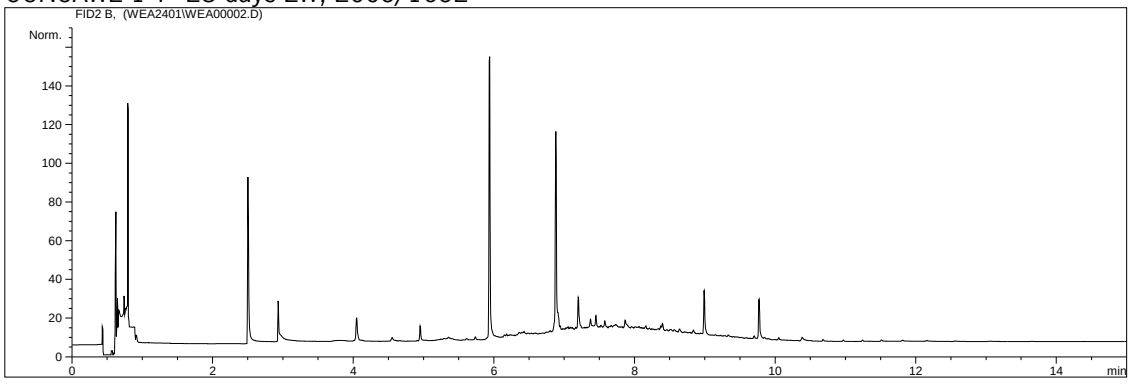

CONCAWE 1 T=4 ZW, 2005/1644




CONCAWE 1 T=14 days RS, 2005/1651

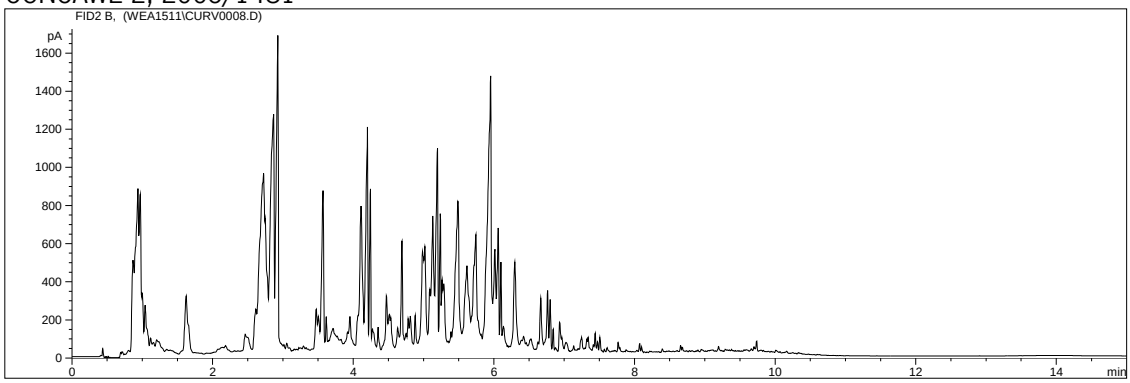



CONCAWE 1 T=28 days ZW, 2005/1652

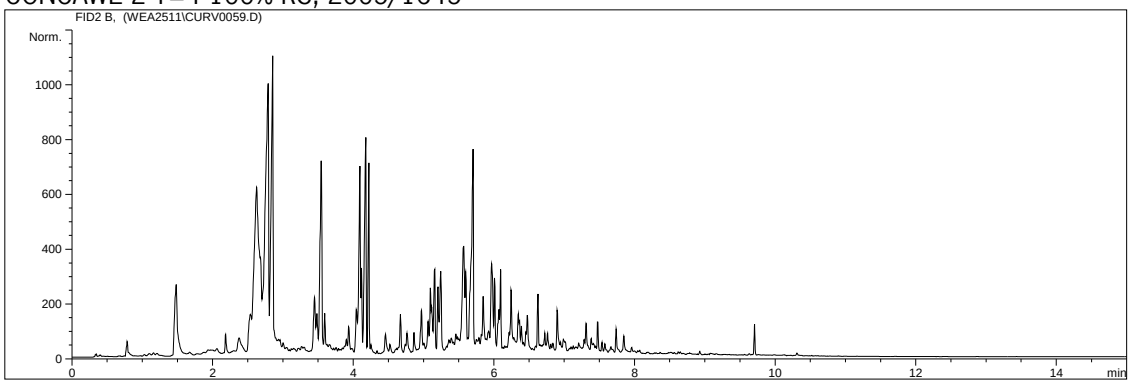


## CONCAWE 2 – DOC-die away study – 100%

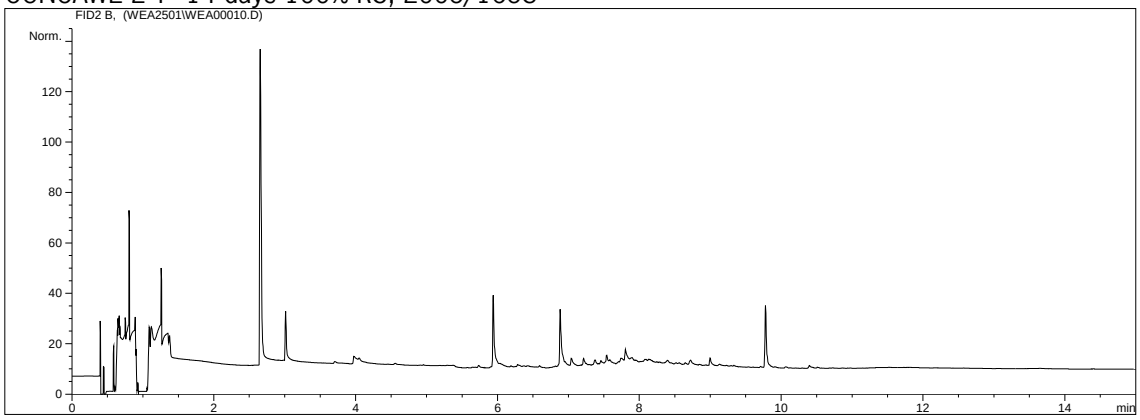
CONCAWE 2, 2005/1481




CONCAWE 2 T=4 100% RS, 2005/1645

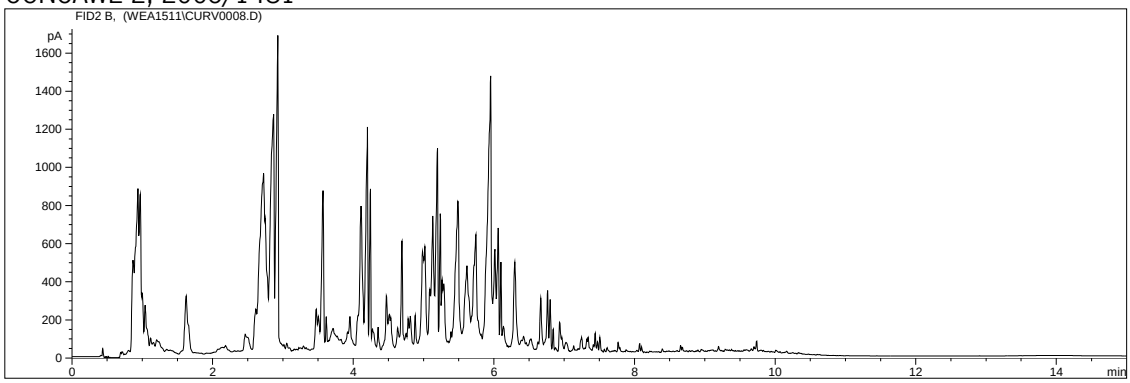



CONCAWE 2 T=14 days 100% RS, 2005/1653

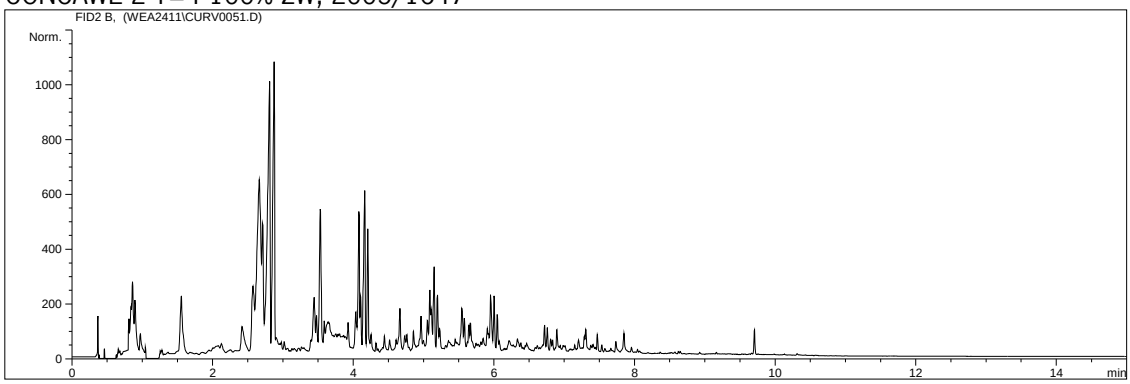


## CONCAWE 2 –Zahn-Wellens style study – 100%

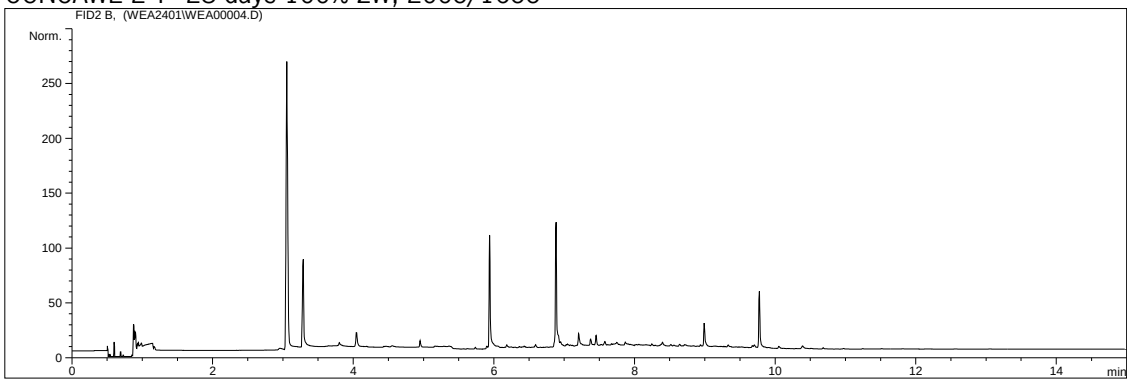
CONCAWE 2, 2005/1481




CONCAWE 2 T=4 100% ZW, 2005/1647

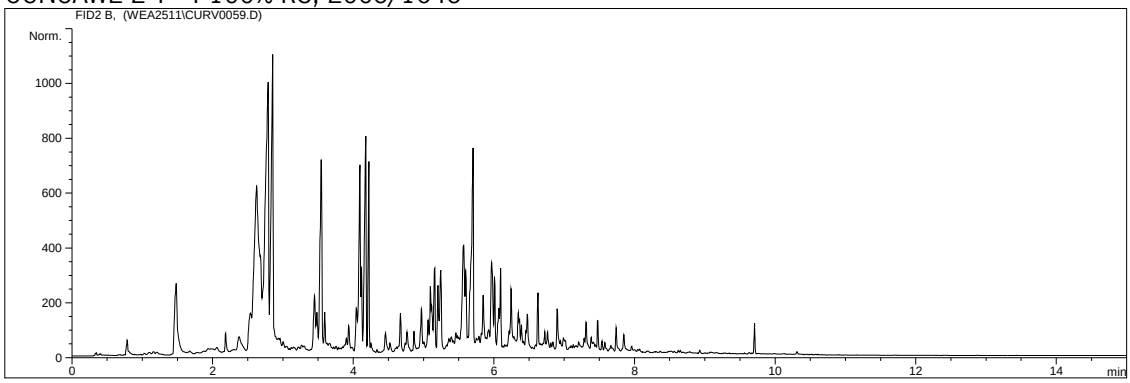



CONCAWE 2 T=28 days 100% ZW, 2005/1655

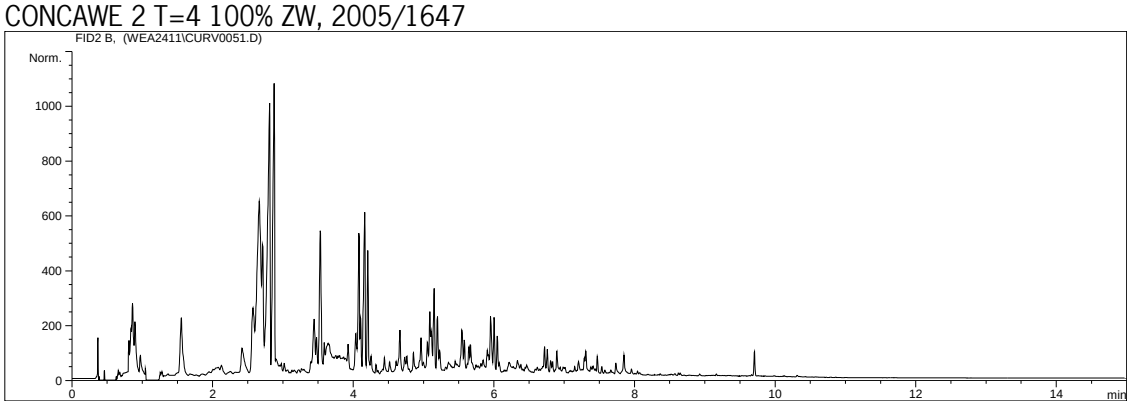


## CONCAWE 2 – DOC-die away study v Zahn-Wellens style study – 100% (4h and 14/28d)

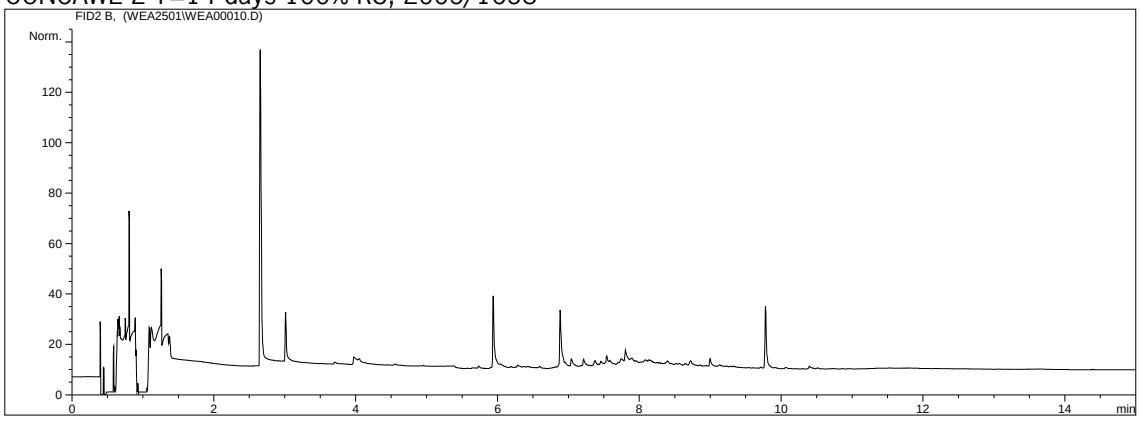
CONCAWE 2 T=4 100% RS, 2005/1645



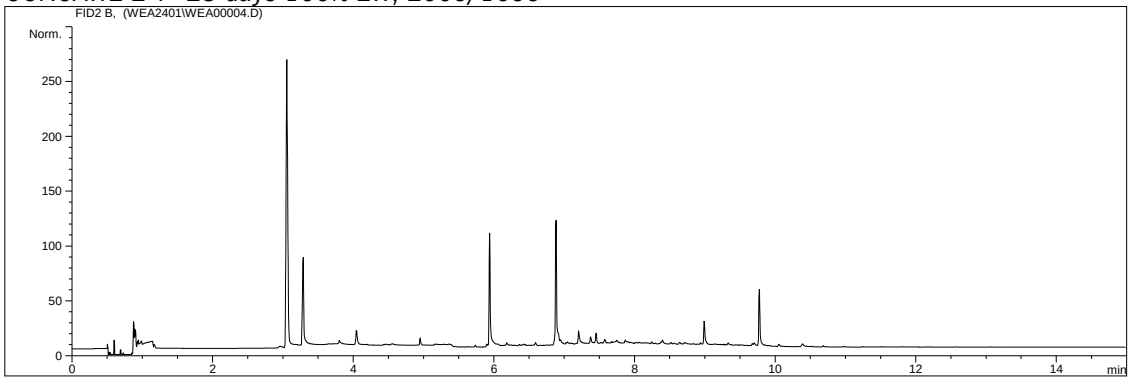

CONCAWE 2 T=4 100% ZW, 2005/1647




CONCAWE 2 T=14 days 100% RS, 2005/1653

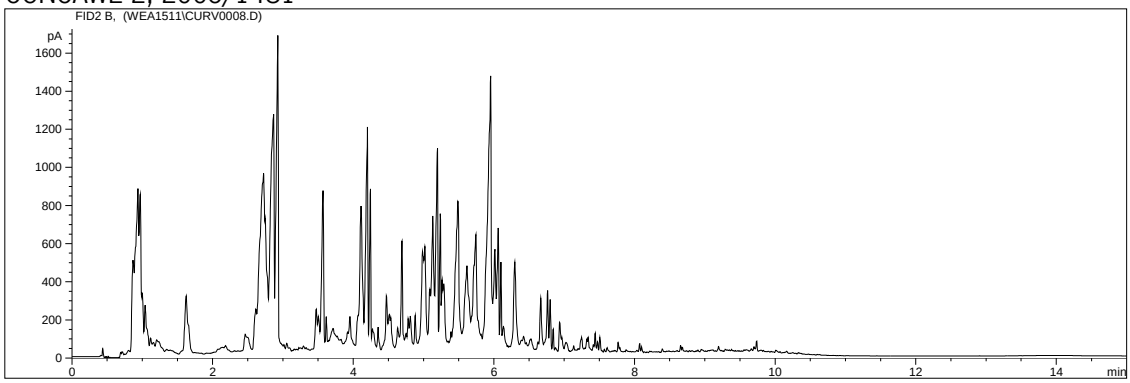



CONCAWE 2 T=28 days 100% ZW, 2005/1655

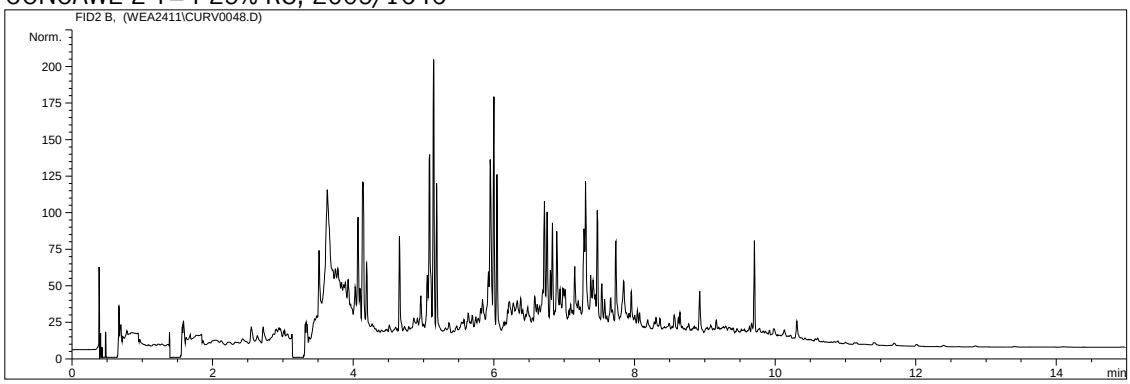


## CONCAWE 2 – DOC-die away study – 25%

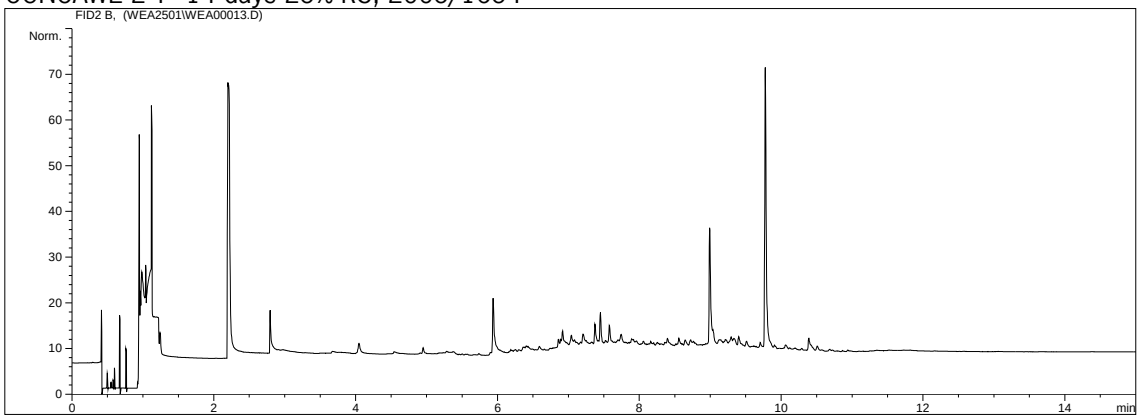
CONCAWE 2, 2005/1481




CONCAWE 2 T=4 25% RS, 2005/1646

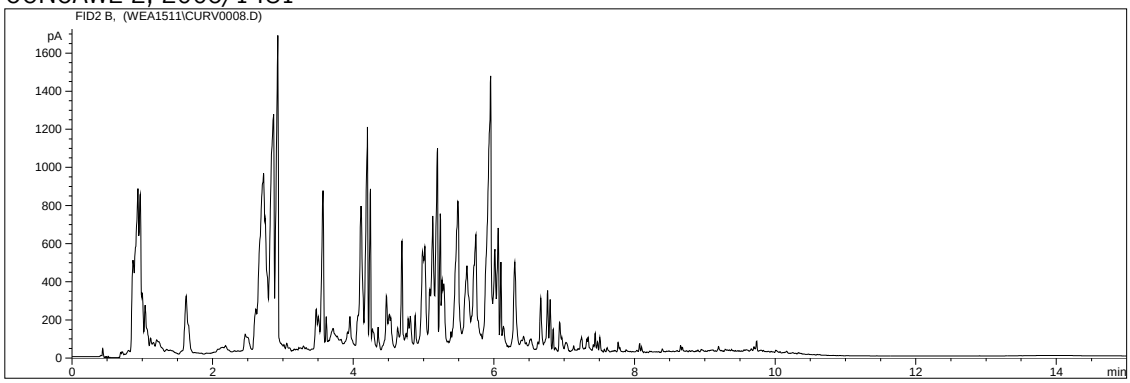



CONCAWE 2 T=14 days 25% RS, 2005/1654

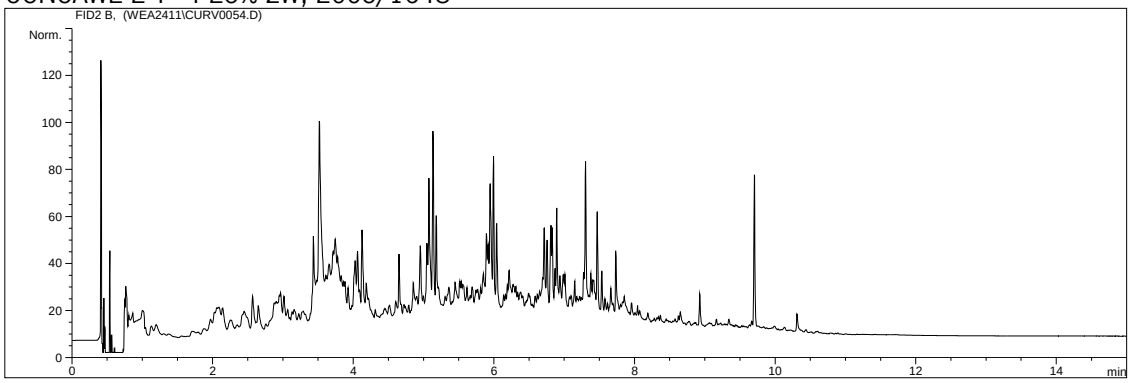


## CONCAWE 2 –Zahn-Wellens style study – 25%

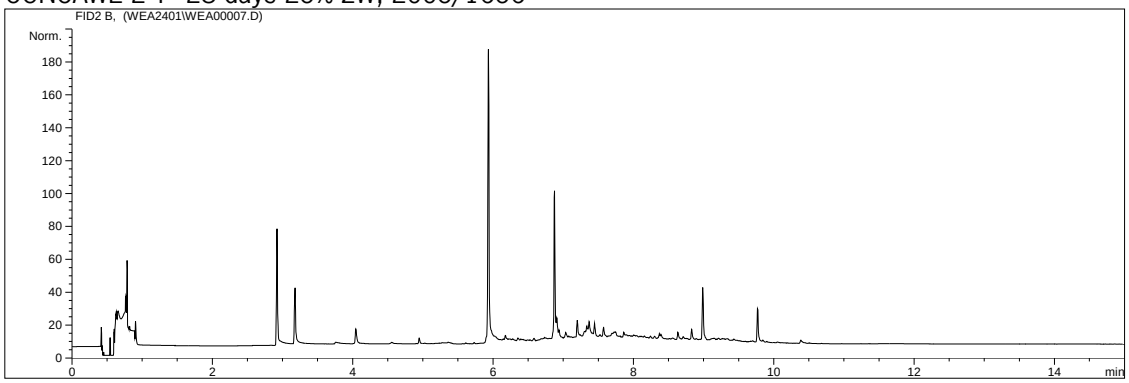
CONCAWE 2, 2005/1481




CONCAWE 2 T=4 25% ZW, 2005/1648

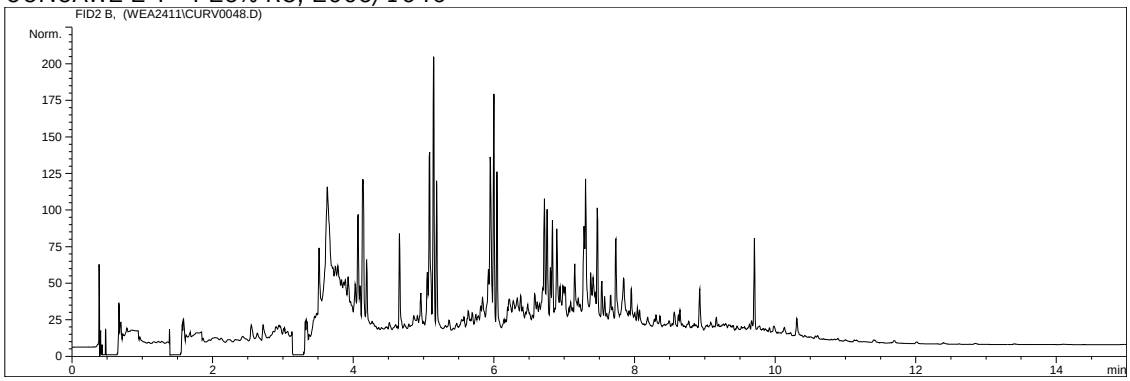



CONCAWE 2 T=28 days 25% ZW, 2005/1656

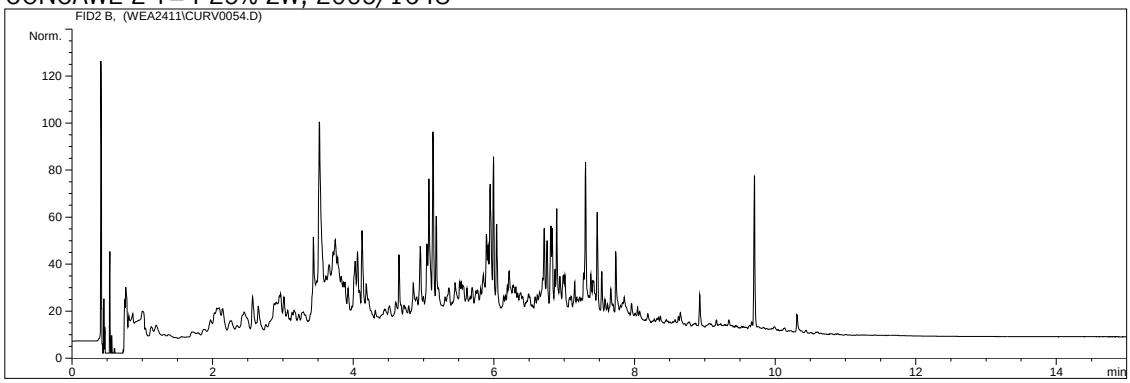


## CONCAWE 2 – DOC-die away study v Zahn-Wellens style study – 25% (4h and 14/28d)

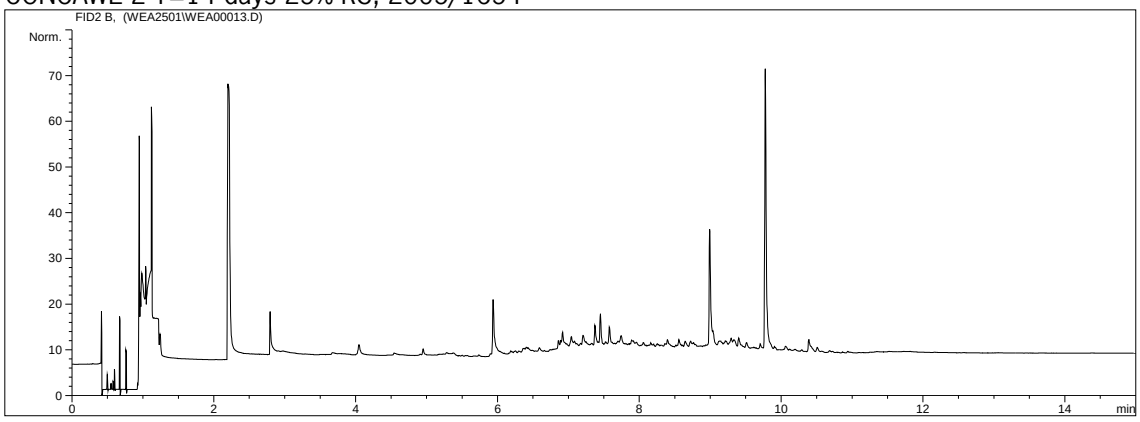
CONCAWE 2 T=4 25% RS, 2005/1646



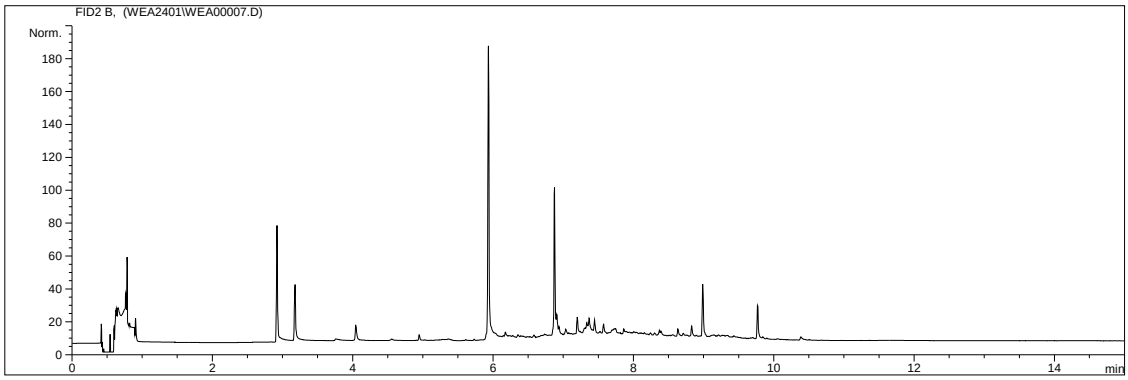

CONCAWE 2 T=4 25% ZW, 2005/1648




CONCAWE 2 T=14 days 25% RS, 2005/1654

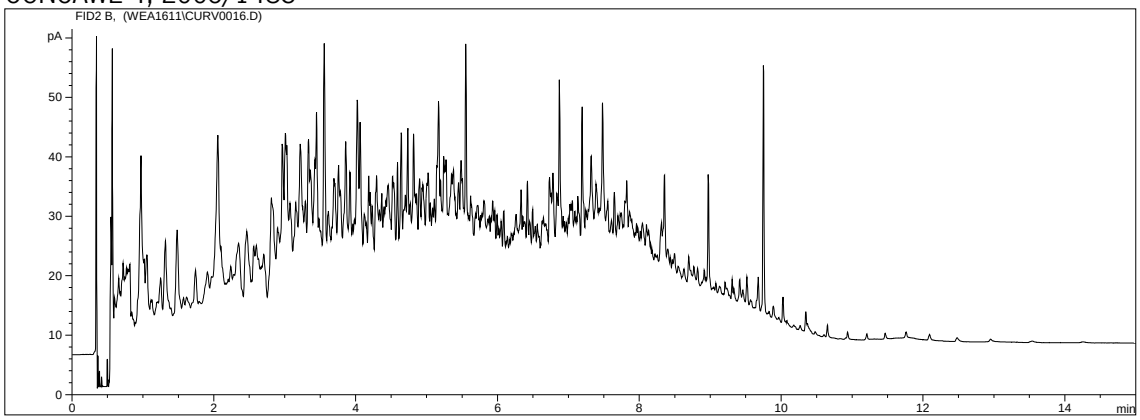



CONCAWE 2 T=28 days 25% ZW, 2005/1656

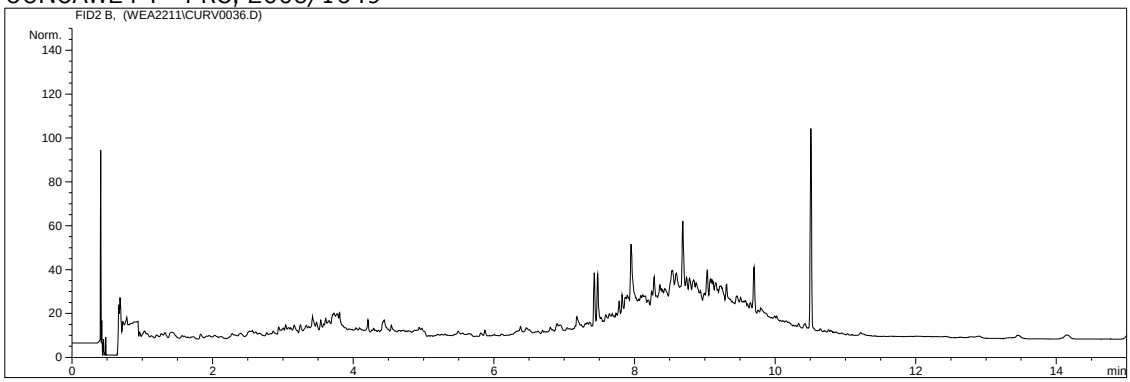

FID2 B, (WEA2401\WEA00007.D)
Norm.
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
min

## CONCAWE 4 – DOC-die away study

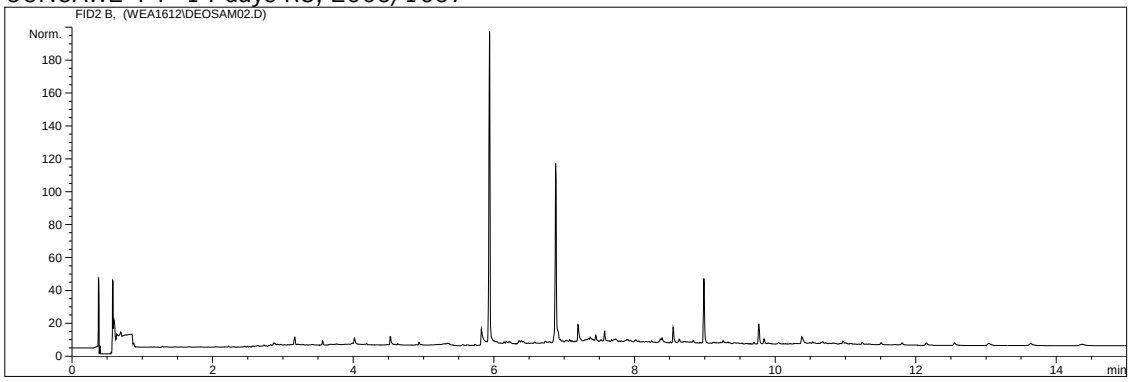
CONCAWE 4, 2005/1483




CONCAWE4 T=4 RS, 2005/1649

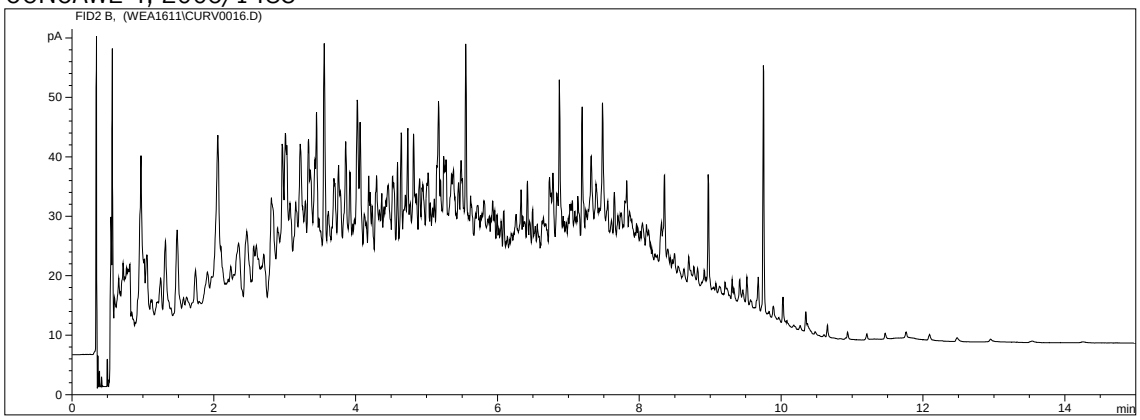



CONCAWE 4 T=14 days RS, 2005/1657

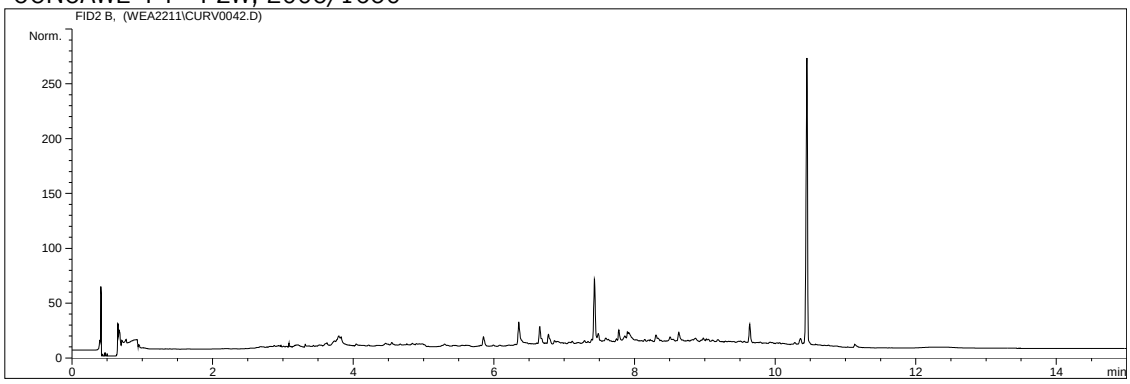


## CONCAWE 4 –Zahn-Wellens style study

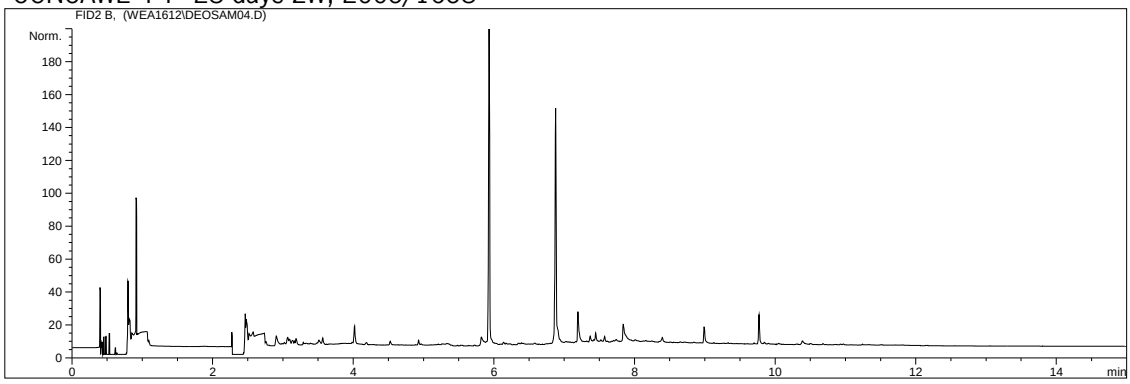
CONCAWE 4, 2005/1483




CONCAWE 4 T=4 ZW, 2005/1650

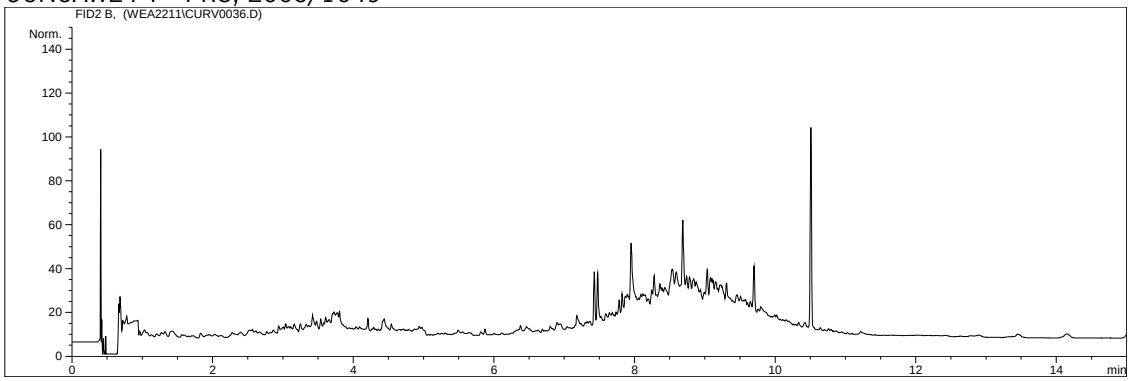



CONCAWE 4 T=28 days ZW, 2005/1658

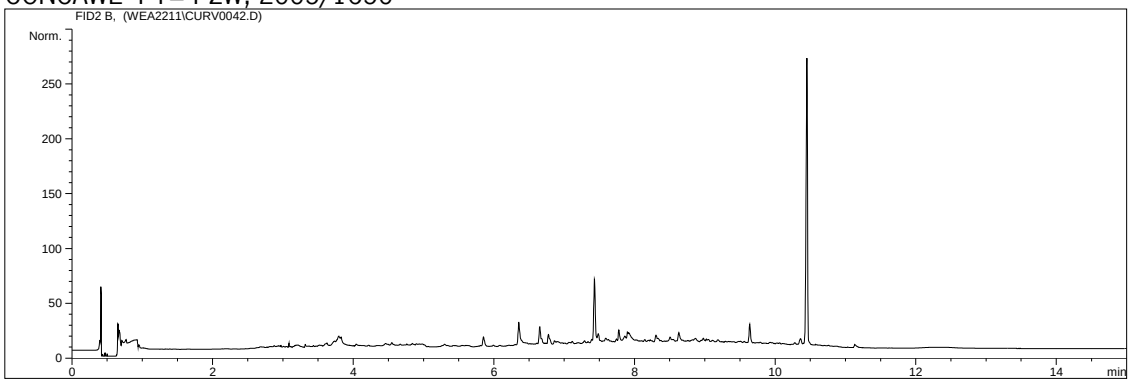


## CONCAWE 4 – DOC-die away study v Zahn-Wellens style study – (4h and 14/28d)

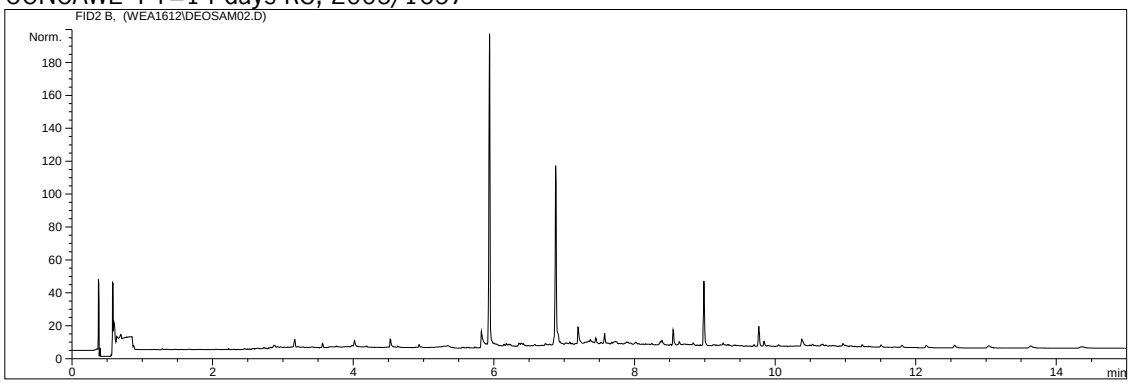
CONCAWE4 T=4 RS, 2005/1649



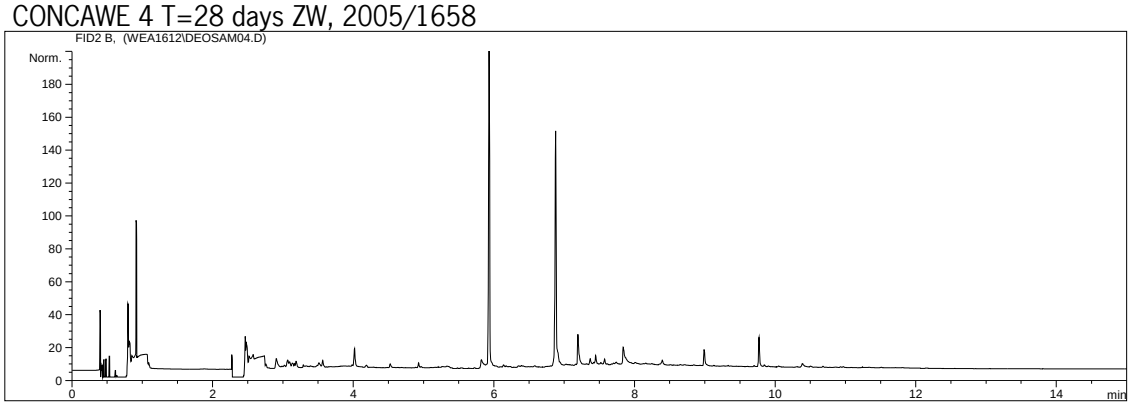

CONCAWE 4 T=4 ZW, 2005/1650




CONCAWE 4 T=14 days RS, 2005/1657

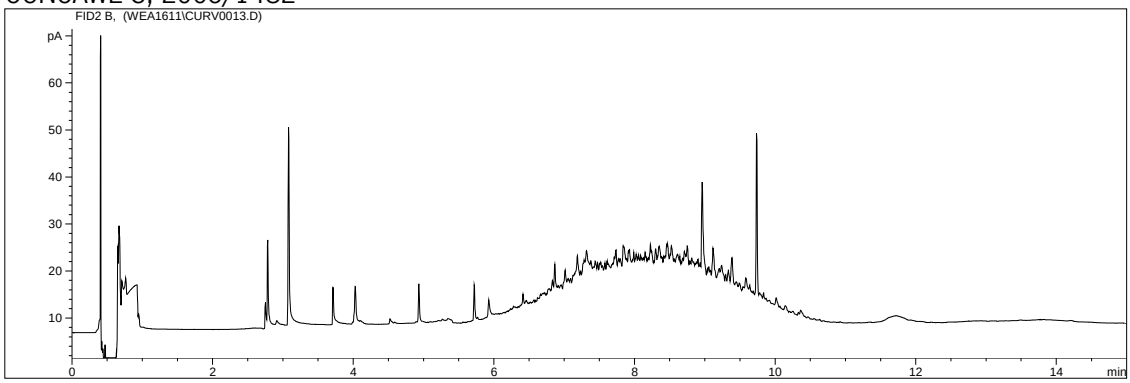



CONCAWE 4 T=28 days ZW, 2005/1658

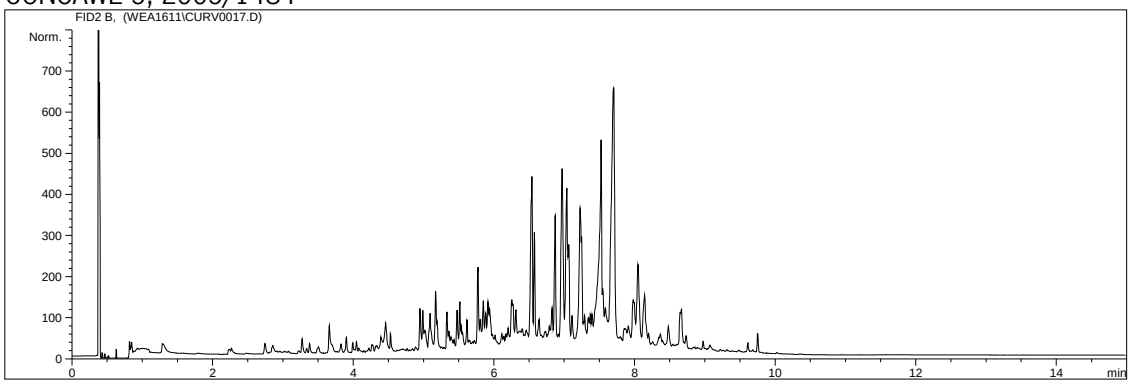


## CONCAWE 3, 5, 6, 7, 8, 9 – Original chromatograms + blank

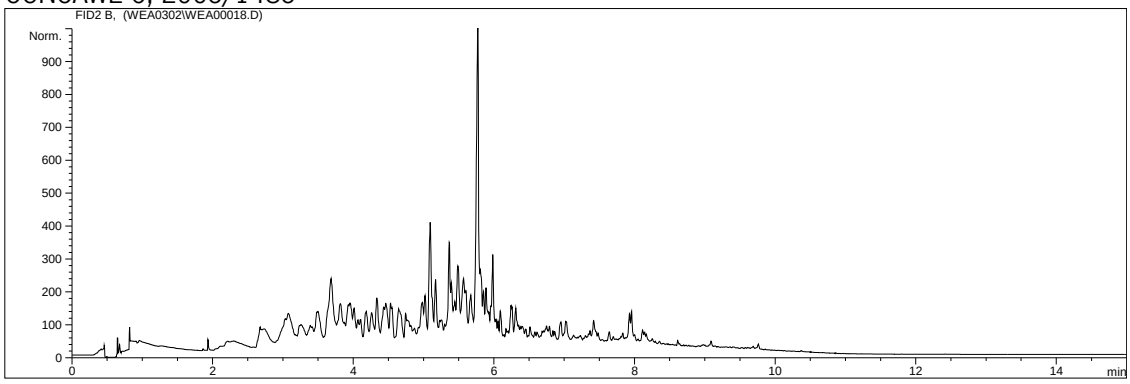
CONCAWE 3, 2005/1482



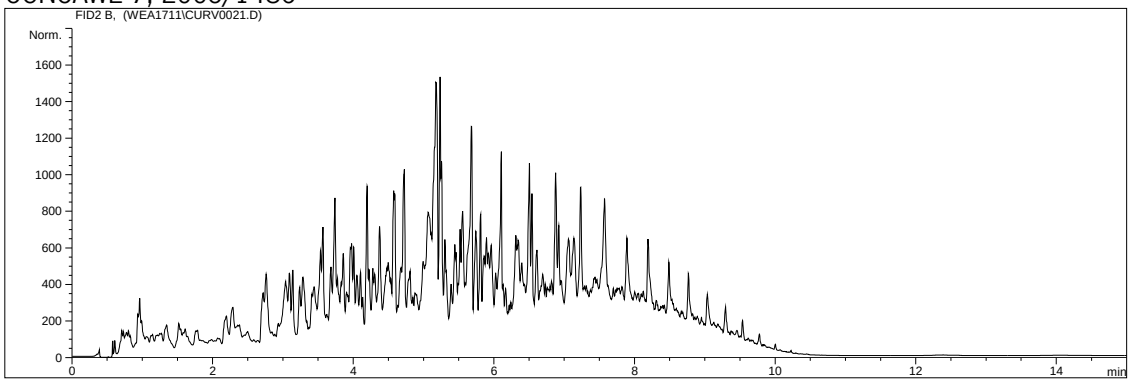

CONCAWE 5, 2005/1484



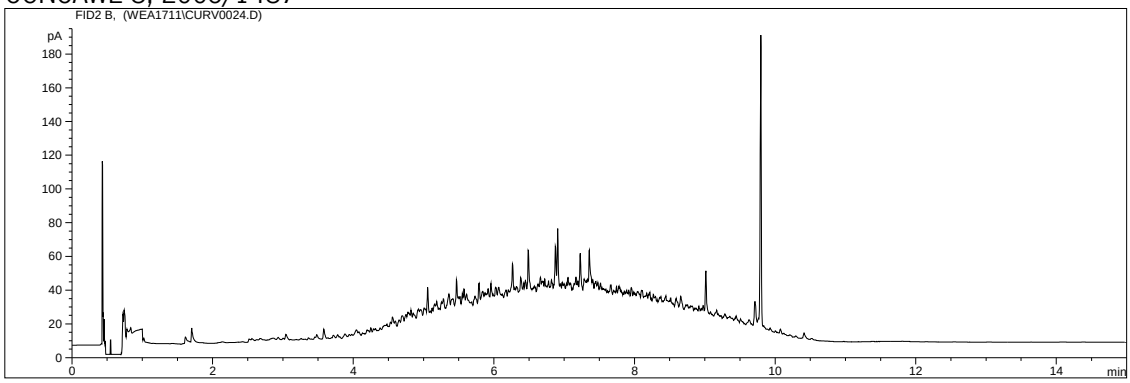

CONCAWE 6, 2005/1485



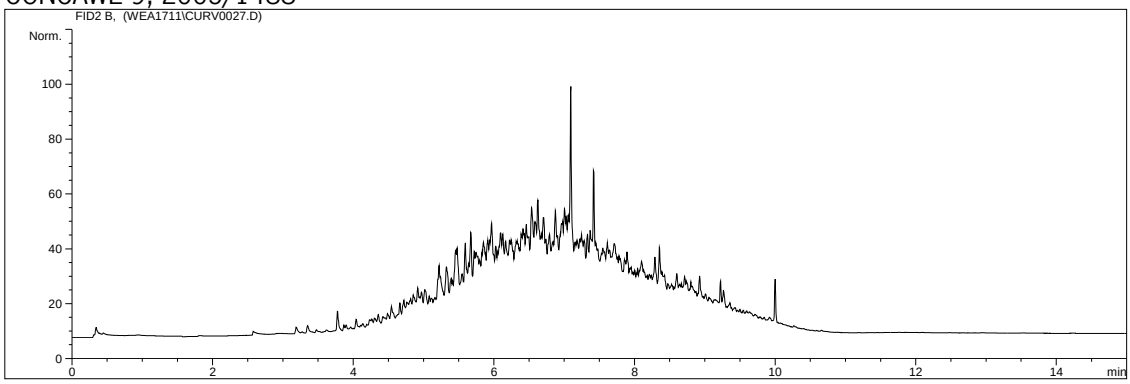

CONCAWE 7, 2005/1486



CONCAWE 8, 2005/1487




CONCAWE 9, 2005/1488



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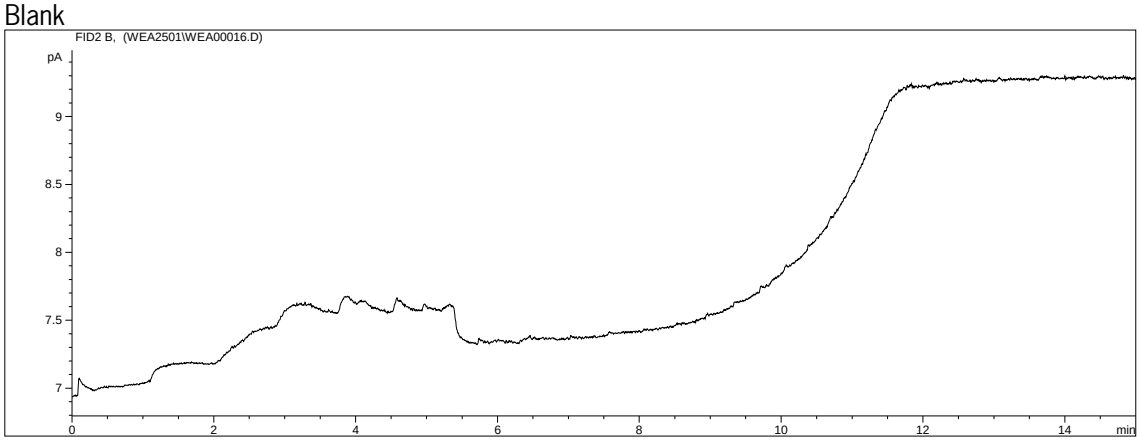
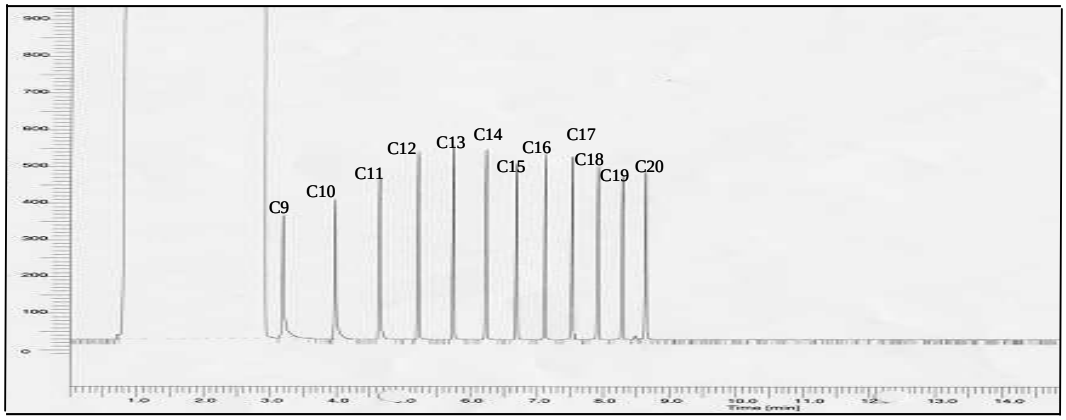



GC-FID chromatogram of a C8-C21 standard. Retention times of the standard can have small deviations from the retention times of the samples due to the different time period of measurements.



Approved by: drs. E. Jagtman

Signature: _____

Date: 11 January 2007