Zur Homepage der Dissertation

Toxic effects of aromatic hydrocarbon mixtures isolated from crude oils to blue mussels (*Mytilus edulis*)

Toxische Wirkung von Gemischen aromatischer Kohlenwasserstoffe aus Erdölen auf Miesmuscheln (*Mytilus edulis*)

Von der

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Kurzfassung

Gaschromatographisch unaufgelöste komplexe Kohlenwasserstoffgemische (unresolved complex mixtures, UCMs) wurden in den letzten Jahren in beträchtlichen Mengen in Umweltproben verschiedener Kompartimente beobachtet, allerdings fand ihr möglicher Beitrag zu aufgetretenen Schäden bisher wenig Beachtung. Das liegt vor allem daran, dass Auftrennung und Analyse der Gemische methodisch schwierig sind, sodass es bezüglich der Toxizität der weitgehend unidentifizierten Einzelkomponenten keine gesicherten Erkenntisse gibt. Die Alternative, die Toxizität des gesamten Gemisches zu bestimmen, ist erst seit kurzem Gegenstand der Forschung. In der bisher einzigen Studie auf diesem Gebiet zeigte Smith (2002), dass ein unaufgelöstes monoaromatisches Kohlenwasserstoffgemisch, welches aus einem Nordseeöl (Gullfaks) isoliert worden war, narkotisch toxisch auf Miesmuscheln (*Mytilus edulis*) wirkte. Somit wurde gezeigt, dass UCMs ökotoxikologisch keineswegs irrelevant sind. Ausgehend von jener Studie wurden in der hier vorliegenden Arbeit UCMs von vier Ölen hinsichtlich ihrer Zusammensetzung (mittels *full scan*-GC-MS-Analyse) und ihrer narkotischen Toxizität für Miesmuscheln untersucht. Durch diese Kombination von chemischer Analytik und Biotest sollten die genannten ersten Ergebnisse von Smith (2002) innerhalb eines Kooperationsprojektes der Arbeitsgruppe Organische Geochemie an der Universität Oldenburg mit Partnern an der Universität Plymouth anhand weiterer Öle überprüft und der Einfluss der Ölzusammensetzung auf die Toxizität untersucht werden. Eine solche Verknüpfung der Methoden in der Ökotoxikologie wird seit einiger Zeit gefordert.

In der vorliegenden Arbeit wurden vier Erdöle verwendet, zwei aus der Monterey-Formation in Kalifornien und zwei aus dem Wiener Becken in Österreich. Jeweils eines der beiden Öle pro Gebiet wurde in der Lagerstätte mittelstark biodegradiert. Die Öle aus Kalifornien und Österreich unterscheiden sich in einigen Aspekten, die sich in der molekularen Zusammensetzung widerspiegeln, z.B. in ihrer Reife: Die beiden kalifornischen Öle haben ein mittleres Reifestadium erreicht, die österreichischen Öle hingegen sind reifer. Die kalifornischen Öle haben zudem einen höheren Gesamtschwefelgehalt.

Bei der Probenaufarbeitung wurde von jedem Öl die Gesamtaromatenfraktion mittels MPLC isoliert. Die Gesamtaromatenfraktion wurde anschließend mit Normalphasen-HPLC in die Unterfraktionen der monoaromatischen und polyaromatischen Kohlenwasserstoffe aufgetrennt. Bei diesen Schritten verdampfen die flüchtigen Verbindungen teilweise, sodass man generell C_{11+}-Fraktionen erhält.

Die drei Hauptfragestellungen dieser Studie bezogen sich auf die Auswirkungen der unterschiedlichen Zusammensetzung der aromatischen Kohlenwasserstofffraktionen auf die

narkotische Wirkung der Öle. Dazu wurden einerseits die monoaromatischen mit den gesamtaromatischen Fraktionen jedes Öls verglichen, andererseits wurden die Auswirkungen der Biodegradation innerhalb der beiden Öle eines Ölfeldes betrachtet. Der dritte Aspekt beschäftigte sich mit dem Einfluss von schwefelhaltigen Verbindungen auf die Öltoxizität.

Zur Untersuchung der narkotischen Wirkung wurden Miesmuscheln für 24 Stunden verschieden stark dotierten wässrigen Lösungen ausgesetzt. Die wässrigen Lösungen der Fraktionen der Kohlenwasserstoffe, die aus den zwei Ölen der Monterey-Formation isoliert wurden, verursachten einen Rückgang der Filteraktivität der exponierten Muscheln aus Cornwall und bewiesen so die narkotische Wirkung. Gesamtkonzentrationen der aromatischen Kohlenwasserstoffe im Muschelgewebe erreichten 100 µg/g Nassgewicht (entspricht ca. 900 µg/g Trockengewicht). Aus Feldstudien wurde von ähnlich hohen Konzentrationen in freilebenden Muscheln berichtet, die physiologische Beeinträchtigungen zeigten, z.B. gestörte Stoffwechselaktivität (Widdows et al., 1995). Demgegenüber zeigten die Miesmuscheln von der norddeutschen Küste, die Fraktionen der beiden Wiener-Becken-Öle und des biodegradierten Öls der Monterey-Formation ausgesetzt worden waren und ähnliche Gewebekonzentrationen angereichert hatten, keine eindeutige narkotische Wirkung. Grund hierfür ist vermutlich eine niedrigere Sensibilität dieser Miesmuscheln, welche durch vorangegangene Exposition gegenüber Xenobiotika in der Nordsee verursacht worden sein könnte. Aufgrund dieses Befundes konnte nicht abschließend geklärt werden, ob die Öle aus der Monterey-Formation stärker narkotisch toxisch wirken als die Öle aus dem Wiener Becken.

Die monoaromatischen und die gesamtaromatischen Kohlenwasserstofffraktionen der beiden kalifornischen Öle verursachten einen ähnlich starken narkotischen Effekt. Die Dosis-Wirkungs-Beziehungen zwischen narkotischem Effekt und Gewebekonzentrationen zeigten, dass die Fraktionen der monoaromatischen und der gesamtaromatischen Kohlenwasserstoffe der beiden Öle aus der Monterey-Formation über sehr ähnliche toxische Potentiale verfügen. Dies wird durch die TEC_{50}-Werte (die Gewebekonzentration, die benötigt wird, um einen toxischen Effekt von 50% zu bewirken) ausgedrückt, die exemplarisch für die Fraktionen des nicht biodegradierten Öls der Monterey-Formation ermittelt wurden und 116 µg/g für die monoaromatischen und 119 µg/g für die gesamtaromatischen Kohlenwasserstoffe betragen. Grund für die ähnliche Toxizität der C_{11+}-Fraktionen ist vermutlich, dass die akut toxischen niedermolekularen Aromaten wie z.B. Benzol in den verwendeten Fraktionen nicht vorhanden waren, was aber im Rahmen dieser Studie nicht vollständig geklärt werden konnte.

Die komplexe Zusammensetzung der Kohlenwasserstoffgemische verhindert eine Zuordnung der toxischen Effekte zu einzelnen Substanzklassen. Ein UCM besteht aus ca. 250.000 Substanzen (Hochrechnung von Sutton et al., 2005), die jeweils sehr ähnlich konzentriert vorliegen und so nur einen Bruchteil des Gemisches ausmachen, wie zum Beispiel die Summe von 17 quantifizierten Methyl- und Dimethylphenanthrenisomeren. Diese Summenkonzentration stellt ca. 1% des TEC_{50}-Wertes dar, der für Phenanthren ermittelt wurde (Donkin et al., 1989). Das bestätigt, dass die narkotische Wirkung nicht nur von einzelnen Substanzen, sondern von allen anwesenden Verbindungen verursacht wurde, wie es auch das additive Konzept zur Mischungstoxizität beschreibt (Grimme et al., 2000).

Der Einfluss von Unterschieden in der Zusammensetzung der Ölfraktionen aufgrund von Biodegradation in der Lagerstätte auf die Toxizität wurde anhand verschiedener aromatischer Substanzgruppen in Proben der Ölfraktionen, ihren wässrigen Lösungen und in Gewebeproben von exponierten Muscheln mittels GC-MS analysiert. Es stellte sich heraus, dass sich die Unterschiede in der Zusammensetzung der Öle (v.a. bei langkettigen Alkylbenzolen und -toluolen) nicht in den wässrigen Lösungen und den Gewebeproben widerspiegelten. Dies ist wahrscheinlich darauf zurückzuführen, dass die betreffenden Komponenten nur in sehr geringen Mengen wasserlöslich sind. Daher waren keine Unterschiede in der Toxizität zwischen den C_{11+}-Fraktionen der unterschiedlich stark biodegradierten Öle festzustellen.

Die Öle der Monterey-Formation weisen einen höheren Gehalt an schwefelhaltigen Verbindungen auf als die Öle aus dem Wiener Becken, sodass sich die Frage nach ihrer Bedeutung für die Öltoxizität stellte. Dibenzothiophen ist zum Beispiel stärker narkotisch toxisch als Phenanthren (Donkin et al., 1989), sodass die Hypothese aufgestellt wurde, dass das Vorhandensein schwefelhaltiger organischer Verbindungen die Toxizität eines Öls erhöht. Ein weiterer Aspekt in diesem Zusammenhang ist, dass schwefelhaltige Verbindungen eine höhere Wasserlöslichkeit haben als Kohlenwasserstoffe entsprechender Größe und Struktur (Beyer and Walter, 1988). Daher wurde untersucht, ob eine erhöhte Akkumulation schwefelhaltiger Verbindungen im Muschelgewebe relativ zur ursprünglichen Ölfraktion erfolgte. Als Modellsubstanzen für schwefelhaltige Verbindungen und Kohlenwasserstoffe ähnlicher Größe und Struktur wurden Methyldibenzothiophene und Methylphenanthrene ausgewählt. Die Analysen ergaben jedoch, dass keine bevorzugte Aufnahme von schwefelhaltigen Verbindungen erfolgte. Auch in den untersuchten wässrigen Lösungen befanden sich keine erhöhten Konzentrationen der Methyldibenzothiophene im Vergleich zu Methylphenanthrenen. So konnte gezeigt werden, dass sich die Schwefelverbindungen unter diesen Bedingungen nicht relativ anreichern. Allerdings kann

trotzdem angenommen werden, dass das Vorhandensein von Schwefelverbindungen die Toxizität eines Öls erhöht. Dafür spricht das für Dibenzothiophen nachgewiesene hohe narkotische Potential, welches nach dem Konzept der Mischungstoxizität bei narkotisch wirksamen Stoffen auf dessen Derivate übertragen werden kann (Hermens et al., 1984). Es existieren nur wenige Studien zur Toxizität von aliphatischen und aromatischen Schwefelverbindungen, sodass weitere Untersuchungen sinnvoll erscheinen.

Zusammenfassend lässt sich sagen, dass UCMs zur Gesamttoxizität von Ölen beitragen und trotz methodischer Schwierigkeiten nicht vernachlässigt werden dürfen. Es zeigte sich, dass Unterschiede in der relativen Konzentration einzelner Isomere keinen messbaren Einfluss auf die narkotische Wirkung der Gesamttoxizität eines UCMs haben. Das bedeutet, dass unaufgelöste komplexe Kohlenwasserstoffgemische als Ganzes bei Monitoringprogrammen erfasst werden sollten, da sie ein erhebliches toxisches Potential besitzen. Da eine vollständige Trennung in Einzelkomponenten nicht realistisch scheint, ist außer der Erfassung der UCMs (zum Beispiel bei Ölaustritten) auch eine Vereinheitlichung der verwendeten Quantifizierungsmethode notwendig.

Abstract

Unresolved complex mixtures (UCMs) of aromatic hydrocarbons have been found in a wide range of environmental matrices at high concentrations. However, limited consideration has been given to the potential detrimental effects of the accumulation of these compounds in the marine environment. The only previous study showed that a monoaromatic UCM isolated from a Norwegian North Sea Gullfaks crude oil was narcotically toxic to blue mussels (*Mytilus edulis*) (Smith, 2002). That study provided first evidence that the compounds in the UCM are not toxicologically irrelevant but that they contribute to oil toxicity. However, UCMs are often disregarded because they elude analysis by routine chromatographic methods.

In the presented study, the narcotic toxicity of monoaromatic and total aromatic hydrocarbon fractions dominated by unresolved complex mixtures isolated from four crude oils towards blue mussels was investigated to provide further evidence whether the compounds in the UCM contribute to oil toxicity. Two oils used in this study come from the Santa Maria Basin (Monterey Formation, California). They are moderately mature oils and have high sulphur contents. The other two oils are from the Vienna basin (Austria); they are mature and lean in sulphur. Each oil pair consists of a non-biodegraded and a corresponding moderately in-reservoir biodegraded oil. One main objective of this study was to compare the narcotic toxicity of monoaromatic and total aromatic hydrocarbon subfractions. In order to achieve this, total aromatic hydrocarbon C_{15+} fractions were isolated from the oils via MPLC. They were further fractionated by applying normal-phase HPLC to gain monoaromatic hydrocarbon C_{15+} fractions.

The short-term exposure (24 hours) of blue mussels to aqueous solutions of the hydrocarbon fractions showed a similarly strong narcotic toxicity of the monoaromatic and total aromatic hydrocarbon fractions of the two Monterey Formation oils towards blue mussels collected on the Cornish coast (U.K.). Cause-effect relationships were obtained for total tissue concentrations. After exposure to the aromatic compounds of all oils, the mussels had accumulated tissue concentrations of up to 100 µg/g wet weight (approximately 900 µg/g dry weight), which is similar to body burdens in wild mussel populations, previously shown to exhibit reduced health (Widdows et al., 1995). The exposure of blue mussels collected from the German North Sea coast to fractions of the two Vienna basin oils and to the degraded Monterey Formation oils suggested that the German mussels were less sensitive than the Cornish ones. The mussels´ different sensitivities prevented a conclusive answer whether the Monterey Formation oils have a higher toxic potential than the Vienna basin oils.

A compound-specific attribution of the toxic effect observed for the Monterey Formation oils was not possible. The main reason for this is that thousands of compounds are present in each fraction, each representing only a small proportion of the total tissue concentration,

as illustrated by for example, the summed concentrations of 17 methyl- and dimethylphenanthrene isomers. This emphasises that the whole mixture of aromatic compounds combined exerts the toxic effect observed, as stated by the mixture toxicity concept of Concentration Addition.

Another main objective of this work was to examine the impact of compositional changes due to in-reservoir biodegradation on the toxicity of the oils. GC-MS analysis of several compound classes revealed that most compositional differences between the total aromatic hydrocarbon fractions of the undegraded and the degraded oils were not transferred into the tissue of exposed mussels. This is due to the fact that the respective compounds (e.g. long-chain alkylbenzenes and alkyltoluenes) are poorly water soluble and thus apparently were not available for uptake. Overall, compositional differences due to the level of biodegradation did not significantly determine the toxicity of the C_{11+} fraction as a whole.

A major difference in the composition of the Monterey Formation oils and the Vienna basin oils are the sulphur contents. Thus, the question arose whether sulphur-containing compounds influence oil toxicity. Since they are more water soluble compared to hydrocarbons of an analogous structure, they might be favoured in uptake. Since dibenzothiophene is a known narcotic toxicant for blue mussels (Donkin et al., 1989), accumulation of these organo-sulphur compounds may enhance the toxicity of a sulphur-rich compared to a sulphur-lean oil. Analysis of methyldibenzothiophenes and methylphenanthrenes, which were chosen as model compounds in this study, in the original oil fractions, their aqueous solutions and tissue extracts of exposed mussels showed that the sulphur-containing compounds were not preferentially accumulated by the mussels. However, further research on the toxicity of sulphur-containing compounds and their contribution to oil toxicity is necessary.

Overall, the results of this study on the composition and the mixture toxicity of aromatic hydrocarbon fractions provide further evidence of the toxic potential of UCMs. In addition to the well-established toxicants such as parent PAHs, there are many alkylated aromatic compounds present in UCMs of crude oils which contribute to their toxicity. Although they are methodically difficult to access, they should not be neglected in research since they are toxicologically relevant. Results from this present study suggest that the compounds in the UCM should be considered in routine environmental monitoring, especially in oil spill scenarios. For this it is crucial to establish a feasible method of investigation including for example full scan GC-MS analysis, as is sometimes already done. In addition, a standardised quantification scheme for UCMs is needed.

Contents

Abbreviations

ANOVA	analysis of variance
ARO	total aromatic hydrocarbon fraction
BB	body burden
BTEX	benzene, toluene, ethylbenzene and xylene
DAD	diode array detector
DBT	dibenzothiophene
DMDBT	dimethyldibenzothiophene
DMN	dimethylnaphthalene
DMP	dimethylphenanthrene
dw	dry weight
EC_x	concentration required to induce x% toxic effect
EPA	Environment Protection Agency
EtN	ethylnaphthalene
EtP	ethylphenanthrene
FR	feeding rate
GC	gas chromatography
GC-FID	gas chromatography with a flame ionisation detector
GC-MS	gas chromatography coupled with mass spectrometry
HPLC	high performance liquid chromatography
ISTD	internal standard
K_{ow}	octanol-water coefficient
M	Monterey Formation oil
m/z	mass:charge ratio
MA	monoaromatic hydrocarbon fraction
MDBT	methyldibenzothiophene
MP	methylphenanthrene
MPI-1	methylphenanthrene index 1
MPLC	medium pressure liquid chromatography
N	naphthalene
NSO compounds	compound classes containing nitrogen, sulphur and/or oxygen atoms
OMV	Österreichische Mineralölvereinigung (Austrian oil association)
OSPAR	Oslo Paris Commission

P	phenanthrene
PAH	polycyclic aromatic hydrocarbons
Ply	toxicity experiments 1 and 2 performed in the University of Plymouth in November and December 2003 (this study)
PM level	Biodegradation level according to Peters and Moldowan (1993)
pri/*n*-C_{17}	pristane/*n*-heptadecane ratio
Pri/Phy	pristane (C_{19}) and phytane (C_{20}) ratio
PropN	propylnaphthalene
RIC	reconstructed ion chromatogram
sd	standard deviation
se	standard error
SFG	Scope For Growth
TEC_{50}	tissue concentration required to induce 50% toxic effect
TIE	toxicity identification evaluation
TMN	trimethylnaphthalene
TMP	trimethylphenanthrene
UCM	unresolved complex mixture
V	Vienna basin oil
WAF	water accommodated fraction
WEC_{50}	water concentration required to induce 50% toxic effect
Whv-I	toxicity experiments 3 and 4 performed in the Terramare Research Centre in March 2004 (this study)
Whv-II	toxicity experiments 5 and 6 performed in the Terramare Research Centre in November 2004 (this study)
WSF	water soluble fraction
ww	wet weight

Figure captions

(abbreviated)

Table legends

(abbreviated)

1 Introduction

1.1 Toxicity assessment of crude oil in the marine environment

Each time crude oil is spilled into the environment it can cause severe damage to the respective ecosystem. The massive oil spills from tanker accidents, which make the headlines, are neither the only, nor the major releases of crude oil, discharged continuously into the marine environment. In addition, the birds and mammals immediately dying due to oiling are not the only victims of oil spills (Fent, 1998). Rather, oil constituents dissolved in the water column and oil residues, which may remain in the ecosystem for years, can exert more subtle and slower, but nevertheless relevant, damage towards marine organisms (Peterson et al., 2003).

In addition to oil compounds, many other potentially toxic compounds such as pesticides and industrial chemicals have been introduced to the environment during the last decades. Consequently, many detrimental effects have been reported in various ecosystems. They range from impaired health of individual organisms to the extinction of species and reduced biodiversity in an ecosystem (Fent, 1998). However, the observed detrimental effects are usually influenced by many factors so that it is often not possible to attribute a direct cause to a detected damage. Establishing cause-effect-relationships in a complex system is very difficult. This is especially the case if contamination has taken place over years to culminate in an apparent damage of a whole ecosystem.

Consequently, hundreds of substances, which were usually applied individually to selected species, were tested for their (eco)toxicity in the laboratory in the past three decades. Many constituents of crude oils were tested this way (e.g. Betton, 1994). These toxicity data are an important information base but – isolated as they are – they often cannot explain the biological response of a whole ecosystem. This is particularly true when a mixture of toxicants is present in a given environment, which is usually the case. Chemical field studies have determined an array of compounds, which range from a few to several hundred in an environmental sample (e.g. Hellou et al., 2002; Thomas et al., 2002; Biselli et al., 2005). The behaviour of a substance in a mixture may differ substantially from its behaviour when present alone. In addition, it is important to bear in mind that toxicity is not a specific property of a substance since it is species-dependent.

However, it does not seem sensible (for ethical and economic reasons) to re-examine the huge number of single substances tested for toxicity during the last 30 years for their behaviour in various mixtures. It is crucial to understand that the establishment of cause-effect-relationships is a manifold process. On the one hand, single substance data are needed to classify the toxic potency of a substance. On the other hand, mixtures of compounds are environmentally more relevant to actually predict and model the state of

contamination of a given ecosystem (Grimme et al., 2000). Furthermore, field studies are needed to determine which mixtures are environmentally relevant, which implies the call to include more chemicals into standard monitoring programs but also to complement chemical analysis with biological effect measurements (e.g. Widdows et al., 1995; Thomas et al., 2002; Kammann et al., 2005).

The field data have to be interlinked with laboratory results to sucessfully understand and predict ecosystem health, to decide upon regulations and laws concerning synthesis, transport, use and disposal of chemicals and to choose remediation steps. These steps are usually embodied in risk assessment procedures.

In general, crude oils should be regarded as sources of toxic compounds (Rice et al., 1977). However, ecotoxicological studies relating to marine ecosystems with whole oils have shown that there are two groups of compounds which are effective toxicants, namely the low-molecular-weight fraction and the aromatic compounds. For marine ecosystems, the low-molecular-weight fraction has only limited environmental relevance since these compounds usually evaporate within hours (e.g. Fent, 1998; Short et al., 2003). The aromatic compounds, on the other hand, are another issue.

The aromatic compounds are a very complex group. Analogous to the aliphatic hydrocarbons in a crude oil, many of these compounds cannot be separated or resolved from each other by routine chromatographic methods and together they form the so-called unresolved complex mixture (UCM). The UCM of a whole oil has been estimated to contain maybe 250,000 substances (Sutton et al., 2005). At present the individual nature of all the compounds comprising the unresolved complex mixture is not known. Besides their unidentified chemical nature, the ecotoxic potentials of many compounds are unknown. Thus, these mixtures in the crude oils may present a considerable (eco)toxic threat.

Smith (2002) found out that the monoaromatic hydrocarbon fraction isolated from a North Sea crude oil was toxic to blue mussels. In complex mixtures as these, toxic effects can often be attributed to compound classes rather than individual substances. Therefore, it seems a valuable approach to analyse groups of aromatic hydrocarbons when working with crude oils or oil-contaminated samples.

1.2 Objectives of the present study

This thesis is the result of a three-year cooperative project of the Organic Geochemistry Group at the University of Oldenburg, Germany, and the Petroleum and Environmental Geochemistry Group at the University of Plymouth, UK. This study “Investigating the “hump“: analysis of structure and toxicity of unresolved complex mixtures in crude oils” was linked to a previous study at the University of Plymouth about a monoaromatic unresolved complex mixture (UCM) isolated from a North Sea crude oil (Smith, 2002).

The starting point of the present study was to ascertain the potential ecotoxic threat that aromatic UCMs of crude oils pose to the marine environment, as found by Smith (2002). For this purpose, the aromatic fractions of four crude oils were tested with the same bioassay, using blue mussels (*Mytilus edulis*), which are wide-spread marine organisms. Filter feeding organisms such as blue mussels are known to accumulate contaminants from the water and high tissue concentrations of various xenobiotics have often been reported in field studies (e.g. Widdows et al., 1995; Hellou et al., 2002; Bakker et al., 2005).

Once toxic effects are recognised, it is of special interest to determine which substance or group of substances is causing this effect. Establishing these cause-effect-relationships, however, is very intricate, in particular when a complex mixture of largely unidentified compounds is present. The focus of this study was therefore to apply several complex aromatic mixtures of different compositions to the test organism *Mytilus edulis* in order to find out whether there are compounds or compound groups which are more effective toxicants than others or whether the overall toxic effect is the same. The aromatic mixtures used in this study differ from each other in the following respects. Of the four oils used, two have the same origin each (Monterey Formation and Vienna basin, respectively). The pairs from the two given petroleum provinces differ in their maturity and their sulphur content. The two oils of each pair are dissimilar regarding their level of biodegradation, which also has an impact on the composition of the oils. The total aromatic hydrocarbon fractions of all four oils were further separated to gain isolated monoaromatic mixtures of less complex composition. Thus, eight mixtures of aromatic compounds of different composition were investigated with respect to three main objectives – do the compositional differences due to varying maturity, level of biodegradation and origin of the oils determine the toxicity of the aromatic hydrocarbon fractions?

Chapters 2 and 3 of this work present the current state of the scientific background as well as methodical details. In Chapter 4, the toxic effects of the different aromatic hydrocarbon mixtures are described and discussed. In Chapter 5, the composition of the original oil fractions is investigated. Chapter 6 deals with the aromatic compounds in several aqueous solutions of the oil fractions and in tissue samples of exposed mussels. In Chapter 7, the results from Chapters 4-6 are linked and the overall conclusions of this study are drawn.

2 Aromatic hydrocarbon mixtures from crude oils – their environmental relevance, ecotoxicity and composition

In this chapter, aromatic hydrocarbons are discussed from different perspectives, starting with their occurrence in the marine environment. Subsequently, the focus will be laid on the fate of aromatic hydrocarbons in the marine environment (2.1). Special attention is paid to the toxic effects of hydrocarbons to the blue mussel *Mytilus edulis*, which was the test organism used in the bioassay applied in this study (2.2). Afterwards, the two concepts for describing toxic effects of mixtures are presented briefly (2.3). In Section 2.4, the composition of crude oils in general and of aromatic hydrocarbon fractions and unresolved complex mixtures in particular are portrayed. Section 2.5 deals with the factors that influence the solubilisation of poorly water-soluble hydrocarbons.

2.1 The fate of petroleum hydrocarbons in the marine environment

Hydrocarbons are common contaminants in the environment worldwide (Fent, 1998) and their occurrence has been documented frequently. When oil is discharged into the marine environment, it usually enters the water first and the volatile compounds evaporate, whereas the soluble compounds dissolve. The less soluble constituents of crude oil are bound to particles and end up in the sediments. Via the food chain, aromatic compounds can be taken up by organisms. Some microorganisms can biodegrade oil compounds, but many organisms accumulate them, which can influence their physiological and biochemical processes.

2.1.1 Distribution of hydrocarbons in the marine environment

An approximation of background levels for total petroleum hydrocarbons in different marine matrixes is given by Farrington and Meyer (1975). Typical levels of background concentration for total hydrocarbons in seawater range from 1-100 µg/L with higher amounts near industrial and other discharge areas. Deeper waters contain less hydrocarbons. Organisms are differently afflicted with 1-200 µg/g wet weight total body burdens while some organs may contain more. In sediments, a range from 1-100 µg/g dry weight is typical for the continental margins. Estuarine sediments may have higher loads of hydrocarbons due to river discharge. Sediments in coastal areas may contain up to 12400 µg/g dry weight (Farrington and Meyer, 1975). Oil-polluted seawater (without overlaying slick or emulsion) may contain up to 1000 µg/L and samples of organisms from coastal waters over 500 µg/g wet weight (Farrington and Meyer, 1975).

Often, total petroleum hydrocarbons are quantified without further fractionation or distinction. In contrast, certain compound classes (in particular polycyclic aromatic hydrocarbons) are sometimes used as indicators for total hydrocarbons. Much research has

been and is done regarding certain aromatic compounds, e.g. very low-molecular-weight compounds and polycyclic aromatic hydrocarbons (PAH). The low-molecular-weight compounds (benzene, toluene, ethylbenzene and xylene; also called BTEX) are not described here since they fairly quickly evaporate when spilled in the aquatic environment and are thus removed to the atmosphere after a short time. Furthermore, they cannot be quantitatively studied with the methods applied in this study due to their high volatility.

Each of these methods of quantification has its justification but also its limitations. On the one hand, it may be interesting to know the relation of aliphatic to aromatic hydrocarbons in a study which only measures the amounts of total hydrocarbons. On the other hand, a crude oil may lack large amounts of the compound class used for quantification, which renders the estimation of total hydrocarbons based on this value inadequate. Thus, data often cannot be compared directly. The following section will present field data and laboratory evidence from an assortment of studies dealing with total oil hydrocarbons or subfractions (unresolved complex mixtures are rarely mentioned).

In many studies, only the non-alkylated ("parent") PAHs are investigated (Figure 2-1), which are mainly the 16 EPA-PAHs (EPA = US Environmental Protection Agency). These are products of incomplete combustion, namely in industrial processes and traffic but they are also constituents of crude oil (Fent, 1998). Thus, they are almost ubiquitous nowadays. However, their concentration in crude oil varies and in most oils alkylated derivatives are more abundant than the parent compounds, owing to the vast amount of possible isomers with increasing chain length. Carls et al. (2001) studied PAHs in mussels (*Mytilus trossulus*) from Prince William Sound in Alaska years after the Exxon Valdez oil spill. Unfortunately, they do not provide a gas chromatogram of the extracts but the listed amounts of some alkylated and parent PAHs clearly show that most parent compounds are either not present in the samples or that the amount of parent compounds relative to alkylated ones is negligible in oil-contaminated samples. Thus, when dealing with crude oil contamination (or oil products), it is necessary to include not only the relatively few and low-concentrated parent PAHs but the whole array of alkylated aromatic hydrocarbons.

Estuarine sediments from the UK coast contained 42 µg/g dry weight for the sum of the 16 EPA-PAHs (Rogers, 2002), while sediments from the German Bight (North Sea) had low amounts of PAHs with a maximum of 1 µg/g dry weight although they were influenced by the discharge of the river Elbe (Biselli et al., 2005). Another study investigated parent and alkylated PAHs in North Sea sediments from the Fladen Ground northeast of Aberdeen (Russell et al., 2003), where extensive oil and gas production is undertaken. Total PAH content of the sediments was between 0.03 and 0.6 µg/g dry weight, which is low compared to the values reported by Rogers (2002). Total hydrocarbon content was also quantified, giving values of 5 to 61 µg/g dry weight (Russell et al., 2003).

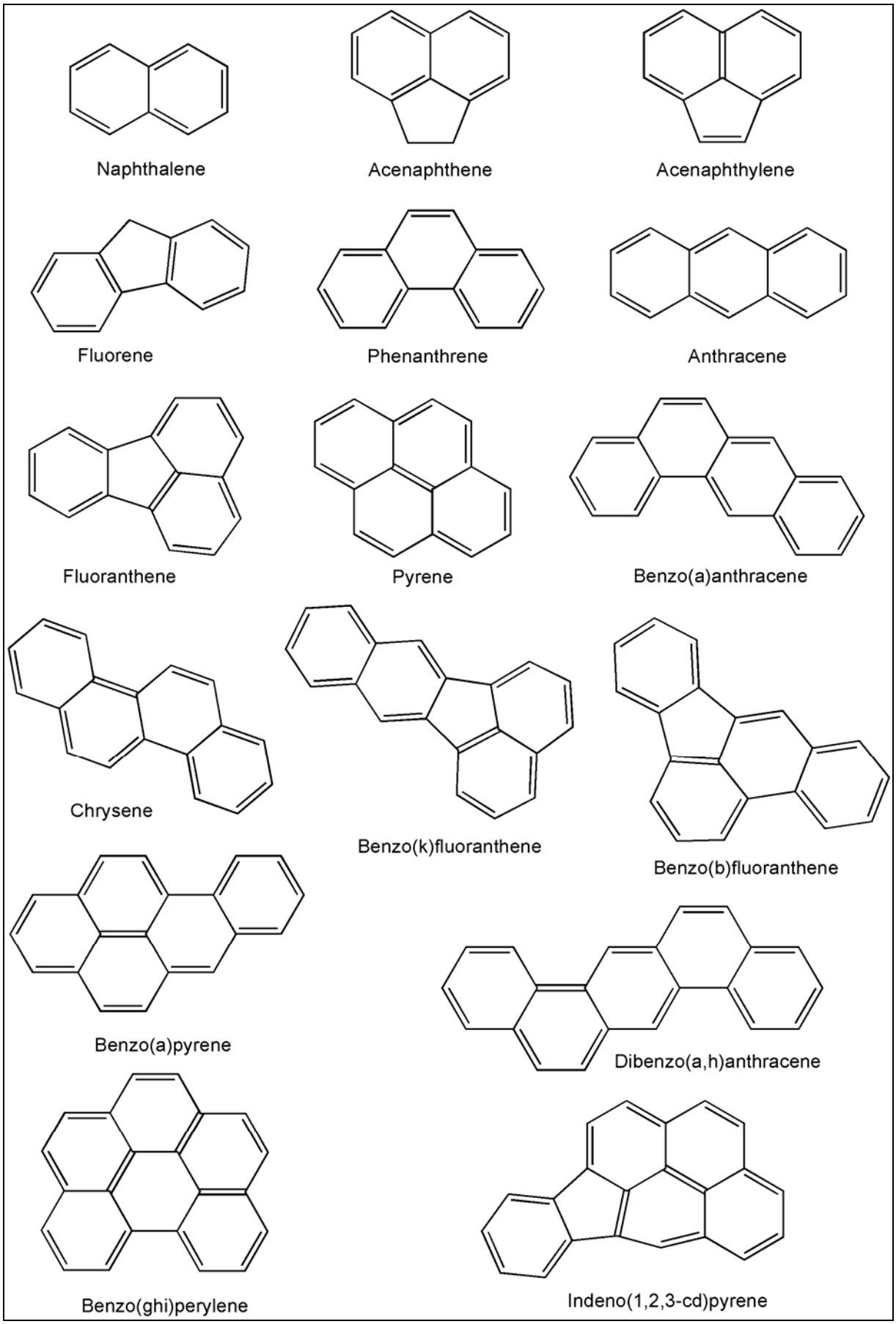


Figure 2-1 Structures and names of the 16 EPA-PAHs.

Some environmental concentrations for hydrocarbons in blue mussels, which were used as test organisms for the toxicity test used in this study, are shown in Table 2-1, with values ranging from no contamination for clean sites up to 5000 µg/g dry weight for heavily impacted sites.

Table 2-1 Concentrations of hydrocarbons in *Mytilus edulis* (Widdows and Donkin, 1992 and references therein, if not indicated otherwise).

Sampling location	Hydrocarbon fraction(s)	Concentration [µg/g dw]
Norwegian fjord	PAH (3-5 ring)	4-150
Shetland (oil terminal)	PAH (2-3 ring)	0.3-8
French coast	total PAH	0.1-300
UK coast (Widdows et al., 1995)	total hydrocarbons	28-434
	(of which 2-3 ring PAH)	(of which 0.2-55)
South Africa	total hydrocarbons	10-5000
USA, near Boston	aliphatic UCM and	5-300
	selected PAH	0.05-3.6
USA, Alaska	aliphatic + aromatic fractions	20-936
South-east Australia	aliphatic + aromatic fractions	40-1975

Page et al. (1987) analysed oysters affected by the *Amoco Cadiz* crude oil spill in France, 1978. Tissue extracts were dominated by a UCM, total aromatics attributed to 725 µg/g (not stated whether wet or dry weight). A contaminated surface sediment from the site contained 1930 µg/g total aromatics (not stated which reference weight). Heavily oiled intertidal sediments contaminated in the Gulf War 1991 were found to contain up to 30,000 µg/g (Sauer et al., 1993 in; Lee and Page, 1997).

Carls et al. (2001) studied persistence and weathering of Exxon Valdez oil in intertidal mussels (*Mytilus trossulus*) in Prince William Sound and along the Gulf of Alaska. Total petroleum hydrocarbons in sediments ranged from 62 µg/g wet weight for the control sites to 62,258 µg/g wet weight, corresponding to 0 to 523 µg/g dry weight total sedimentary PAHs (parent EPA-PAHs, some C_1-C_4 EPA-PAHs and C_0-C_3 dibenzothiophenes).

2.1.2 Uptake and elimination of hydrocarbons by marine organisms

When chemicals are dissolved in the water, they are bioavailable to organisms. For example, a continuous flow-through system experiment with a fuel oil showed that sediments and the two benthic organisms used (the worm *Glycera americana* and the mollusc *Crepidula sp*.) accumulated hydrocarbons from the aqueous phase. A pronounced unresolved complex mixture (UCM) was present in the gas chromatograms of the samples (Farrington et al., 1983).

Bioavailability and uptake of contaminants, followed by distribution within the body, often inhibits normal physiological responses of an organism.

For apolar compounds like hydrocarbons, the main uptake route is via passive diffusion due to a concentration gradient through the membrane and its phospholipid bilayer. The evolving equilibrium is influenced by a hydrocarbon´s degree of dissolving in the water (in this case hydrophobicity) and its tendency to partition into the lipid tissue (lipophilicity) (Fent, 1998). The extent to which a substance partitions from an aqueous to a lipid phase is expressed as the octanol-water coefficient K_{ow}, which acts as a measure for hydrophobicity. The higher the log K_{ow}, the more hydrophobic a compound is. For example, the log K_{ow} of toluene is 2.7, of propylbenzene 3.7 and of octylbenzene 6.7 (data from Donkin et al., 1991). In general, log K_{ow} increases with the degree of alkylation within a homologous series (Hellou et al., 2002). Apolar narcosis usually is caused by chemicals with a log K_{ow} value between 2 and 5.5 while chemicals with a log K_{ow} value <2.9 are polar narcotics whose toxic action differs (Donkin, 1994 and references therein). The values show that a clear distinction at the lower limit is not easy, demanding further information besides the log K_{ow} value. In addition, it has been suggested that this model approach is limited due to the higher complexity of a biological membrane system compared to phase partitioning between water and octanol (e.g. Zhang and Gobas, 1995).

Marine organisms, especially mussels, can accumulate high amounts of contaminants from seawater via filter-feeding, often by adsorption on the gill tissue. In addition, it is known that many organic compounds are sorbed on particles in the environment, especially those with a low aqueous solubility and a high hydrophobicity. Thus, it was suggested that different uptake routes exist for these differently available compounds. Shaw (1977) related the size of oil droplets or contaminated particles to the size of food taken up by marine organisms. In an exposure experiment, two differently-feeding marine organisms did not accumulate the same organic compounds, indicating separate uptake mechanisms for PAHs of the various sizes (Farrington et al., 1983). Baussant et al. (2001a) also found different distributions of PAHs from a dispersed crude oil in turbot and blue mussels, which reflected the different feeding behaviour of these two species. The dissolved compounds enter marine organisms through the gills while the higher-molecular-weight compounds are taken up with particles during feeding so that they enter the digestive system. Once inside the organism, organic contaminants tend to accumulate in tissue with high lipid content.

Several marine species possess mechanisms for detoxification or elimination of toxicants. For blue mussels, however, evidence for metabolism of organic contaminants is scarce. For example, extremely low metabolic rates of benzo[a]pyrene were measured (Stegeman, 1985). This leads to the assumption that in general passive diffusion is the more important elimination process in blue mussels (Livingstone and Pipe, 1992; Widdows and Donkin, 1992).

Studies concerning toxicant elimination in several species point towards two phases of toxicant elimination, suggesting two compartments of storage (e.g. Broman and Ganning, 1986). The longer the exposure period is, the slower is the elimination kinetics, probably due to gradual transport within the organisms to organelles and lipid stores (Livingstone and Pipe, 1992; Widdows and Donkin, 1992).

2.2 Toxic effects of petroleum hydrocarbons on marine organisms

Anderson (1977) reviewed responses of marine organisms to sublethal exposures of aqueous solutions of various petroleum hydrocarbons. It is problematic to compare data from different studies, though, since the actual composition of the emerging toxicant solution is hardly ever analysed in detail. Thus, the focus of his review was put on naphthalenes (C_0-C_2). Increasing oil toxicity was attributed to rising concentrations of aromatic compounds in the emerging aqueous mixture. In addition, it was found that the alkylated naphthalenes are more toxic than the parent compound. This trend of increasing toxicity with higher alkylation has been reported repeatedly (e.g. Thomas et al., 2002). In general, hydrocarbons in the water column caused various toxic effects to marine organisms. For example, oysters (*Crassostrea virginica*) were slowed down in their normal adaptation to salinity changes in their intertidal habitat, growth of the polychaete *Neanthes arenaceodentata* larvae was reduced and the Longnose Killifish (*Fundulus similus*) revealed swimming abnormalities derived from loss of equilibrium leading to death (Anderson, 1977 and references therein). On the population level of an ecosystem, the presence of hydrocarbons (*n*-alkanes and a UCM) in the sediments from petroleum-contaminated sites in Todos os Santos Bay (Brazil) reduced the species diversity and density of communities of benthic organisms and induced community structure changes (Peso-Aguiar et al., 2000).

2.2.1 Narcosis as a universal mode of toxicity

Most hydrocarbons induce narcosis. Narcosis is defined as the nonspecific reversible disturbance of the functioning of the membranes caused by the accumulation of pollutants in the hydrophobic phases within the organism. There is no specific chemical interaction or a unique receptor involved. Since chemicals of different compound classes can induce narcosis, it is also called the “minimal level of toxicity” or “baseline toxicity” (van Wezel and Opperhuizen, 1995). The overall symptom in narcosed animals is an impairment of physiological responses, often related to a decrease in metabolic rate. In addition, activity and reactivity are slowed down. Animals usually can recover from narcosis when they are transferred to clean water. However, ongoing disturbance of membrane functions due to narcosis can ultimately lead to death (van Wezel and Opperhuizen, 1995).

In general, the adverse effects observed in narcosed animals can be due to various biochemical and physiological processes. Often, there are different explanations or models

applicable and the accuracy of interpretation decreases with increasing complexity of the observed biological endpoint. Even in one species, narcosis may be exerted through different molecular mechanisms (Schüürmann, 1998).

For blue mussels, which were used as test organism for the toxicity experiments in this study, narcosis particularly affects the feeding rate of the animals. This means that they filter less water, which contains their algal food. Thus, they do not take up sufficient food when the water is contaminated and their health starts to deteriorate (e.g. Donkin et al., 1989).

Possible biochemical mechanisms of narcosis

For apolar chemicals like hydrocarbons, hydrophobic interactions are the main cause for the disturbance of membrane functions. Membranes are important for many biological processes, e.g. they act as a barrier and transport system for ions and building blocks for cell constituents. They consist of a bilayer of various phospholipids, which are associated with proteins. The ion channels are surrounded by the lipids. There are numerous equilibria to control transport of molecules. These are probably very sensitive to changes, which can be caused by the presence of contaminants. Consequently, biological processes are disturbed (van Wezel and Opperhuizen, 1995). The mechanism for narcotic action has not been uncovered, yet, but it is certain that membranes are damaged in the process (Fent, 1998). From this follows that cell permeability, which is often high for hydrophobic compounds, is a crucial factor (Thomas et al., 1995).

Different hypotheses regarding the mechanism of narcosis have been formulated. The first one is called the Meyer-Overton rule and goes back to the beginning of the 20th century. It states that narcotic chemicals modify the physical state of phospholipid-related compounds in the cell membrane due to absorption (also called lipid theory). Later, Mullins modified the lipid theory to the “volume fraction theory”, implying that the volume fraction rather than the molar fraction of a substance in the membrane is relevant. In 1939, Ferguson formulated the body burden concept theory, saying that narcosis is caused by the physical presence of the chemical at the target site. Thus, the activity of the chemical at the point of action is more relevant than the exposure concentration (van Wezel and Opperhuizen, 1995 and references within).

Disturbance of the membrane structure by hydrocarbons

On a molecular level, there are several ways for chemicals to influence the membrane structure. On the one hand, van-der-Waals interactions of apolar compounds can disturb the bilayer structure of phospholipids (interdigitation), resulting in a restructuring of the membrane constituents. However, this seems only to occur at much higher concentrations than needed to induce narcosis (van Wezel and Opperhuizen, 1995 and references within).

Another mechanism which disturbs the structure of the membrane is the potential of narcotic chemicals to disrupt water-macromolecular interactions. Lipid membranes are assembled by the interaction with water molecules. For alcohols it was shown that the narcotic potency correlated to their hydrogen bond breaking activity (Chiou et al., 1990).

There is also increasing evidence that the most sensitive manifestations of narcosis can be related to specific regions of the nervous system and perhaps to certain proteins but the association between membrane and protein can be so intimate that an unequivocal mechanistic answer may remain elusive (Donkin, 1994 and references within).

Adverse interactions of xenobiotics with proteins

An alternative theory to the above-mentioned disturbance of membrane structure suggests that narcosis is the result of direct interaction between proteins and the xenobiotic chemical. This theory goes back to the 1970s. The mechanism for membrane function disruption is linked to the proteins and is broadly termed protein-lipid interaction. The inhibition of proteins can be exerted by both polar and apolar classes of compounds since proteins have polar and apolar structures (van Wezel and Opperhuizen, 1995). Several mechanisms of interaction are proposed.

Firstly, fluidity of the surrounding lipid channels determines the opening of the sodium channel. Thus, if fluidity of the membrane is affected by the presence of contaminants, sodium ion exchange can be disturbed. Enzymatic activity also depends on membrane fluidity. Changes in fluidity were found combined with swelling of the membrane due to the presence of aromatic hydrocarbons in addition to an increased passive flux of protons and altered enzyme activity (Sikkema et al., 1992). All hydrocarbons studied, which included BTEX, naphthalene, tetralin, biphenyl and phenanthrene, exerted this toxic effect, indicating that the structure of the hydrocarbons does not play a major role (Sikkema et al., 1994). Since these aromatic compounds all are constituents of crude oils, these findings show the importance of investigation of the whole aromatic fraction of a crude oil for narcotic potential.

Secondly, membrane thickness is altered in the presence of contaminants, which can cause changes in the electric potential, which again renders the sodium ion channels inactive, preventing the transmission of nerve impulses. For *n*- and *cyclo*-pentane it was shown that the minimum stimulus which was required for generating action potentials, fell to a low level but did not return to its control value (van Wezel and Opperhuizen, 1995 and references there). Thus, the continuous transmittance of action potentials led to an adaptation of the neuron or receptor receiving the potential. The adapted receptor was not responsive anymore to further stimuli, leading to the observed lethargy of narcoticised animals. Furthermore, xenobiotics can interfere with the assembly of membrane proteins from their subunits just by blocking the way and preventing diffusion of subunits to a specific site (van Wezel and Opperhuizen, 1995 and references there).

The "narcotic cutoff"

This theory of protein interaction can also explain an often observed phenomenon: the "narcotic cutoff". It refers to a loss of narcotic potency in a homologous series of hydrocarbons with increasing size (Chiou et al., 1990). The cutoff is often ascribed to reduced aqueous solubility of the high-molecular-weight molecules. However, it may also be the case that there is no room for the bigger molecules in the membrane and that the binding pocket of the protein has a finite functional volume, thus excluding big compounds from the site of action (van Wezel and Opperhuizen, 1995). Thus, when a chemical is too large to fit into the binding pocket of a protein, no narcotic effect is observed. There may be different possible sites of interaction, which may be an explanation for the huge variety of chemicals that can induce narcosis (Donkin, 1994 and references within).

It may also be the case that small compounds affect the membrane bilayer in a different way than the bigger molecules; for example in (xenobiotic) lipid-(membrane) lipid interactions. Changes in the fatty acid composition of phospholipids have been observed in microorganisms, in particular *cis*-to-*trans* conversion (Heipieper et al., 1996), which increases the ordering in the membrane as a reaction to disturbing xenobiotics (Sikkema et al., 1995 and references within).

Another reason for the "lacking" toxicity of long-chain alkanes might be that the presence of long-chain alkanes does not disturb the phospholipid structure as much as short chains do. Rather, the straight chain may interact with both fatty acid chains in the bilayer, thus increasing the degree of ordering of the membrane. This could also neutralise additional adverse effects caused by the presence of the alkane (Sikkema et al., 1995).

Summary of mechanisms of narcosis

In has been shown that in general the presence and interaction of lipophilic compounds in the phospholipid bilayer can cause dramatic changes in the structure of the membrane. Accumulation of such compounds leads to modifications in membrane fluidity, swelling of the membrane and changes in the protein interactions, resulting in disruptions of membrane functions (Sikkema et al., 1995). Sikkema et al. (1994; 1995) argue that the toxicity theories should be combined since hydrocarbons seem to exert both mechanisms of interaction with the membrane as well as interaction with proteins.

2.2.2 Specific toxicity of some petroleum hydrocarbons

Some petroleum hydrocarbons cause toxicity via a specific mechanistic pathway in addition to unspecific narcosis. Many of the 16 EPA-PAHs and/or their metabolic products are mutagenic and often carcinogenic (Fent, 1998). In addition to the specific toxicity at higher concentrations, parent and alkylated PAHs from low total concentrations in water (0.4 µg/L) can accumulate in fish eggs where they are taken up by embryos (exposure for

16 days). The evolving adult herrings showed abnormalities (Carls et al., 1999). In another study, pink salmon embryos were exposed to PAHs in aqueous solution (19 µg/L) for eight months through the larval stage. Then they were tagged, released into the environment and after 16 months, adults were re-examined. Survival was reduced in comparison to a control group (Heintz et al., 2000). These delayed lethal effects after long-term embryonic exposure are due to specific toxic mechanisms which affect the development of the embryo.

Most importantly, Thomas et al. (2002) found alkylnaphthalenes in a sediment extract fraction that was mutagenic, indicating that alkylderivatives can induce effects similar to those of the parent compounds. A fraction with a prominent aromatic UCM was found to be mutagenic, too (Thomas et al., 2002). These studies indicate that alkylated PAHs may also cause toxicity via specific mechanistic pathways.

2.2.3 Sublethal toxic effects of hydrocarbons on blue mussels (*Mytilus edulis*)

The presence of hydrocarbons, especially aromatic compounds, in organisms is known to be harmful to the health of an organism. Sublethal effects, i.e. an impaired health, on blue mussels are a sensitive parameter and were determined in the laboratory as well as in the field (Widdows and Donkin, 1992). Usually, the state of health of an organism is best reflected in the growth rate. However, this is not a good parameter when dealing with bivalves because growth is slow and cannot be easily observed due to their shells.

For mussels, a combination of physiological responses (feeding rate, food adsorption, respiration, excretion and production) is representative of the growth potential of the animal. This integration of parameters, especially the calculation of production from the difference in energy acquisition and energy loss, is called Scope For Growth (SFG). It represents the bioenergetic status of the organism (Widdows and Donkin, 1992).

Basically, a mussel has a high Scope For Growth when it has a positive energy balance (e.g. high feeding rate and food adsorption efficiency). This can only be achieved if all biochemical mechanisms are working correctly. Thus, a reduced SFG reflects an impaired health of the animal (e.g. a low feeding rate). The Scope For Growth-technique was shown to be a reliable indicator of general stress in mussels. In addition, it is much more sensitive than lethal responses (Widdows and Donkin, 1992).

It is known that nonspecific narcosis affects the ciliary feeding activity of mussels (Widdows and Donkin, 1992). This reduction in feeding rate is caused by reduced pumping of the lateral cilia of the gill. The cilia are under neuronal control (Paparo, 1972 in Smith, 2002), thus the underlying mechanism of feeding rate reduction is narcotic action (Donkin et al., 1989).

It has been shown that impaired health of blue mussels is best correlated to aromatic hydrocarbons among all contaminants investigated (Gilfillan et al., 1977; Widdows et al.,

1995). In contrast, aliphatic hydrocarbons did not cause high toxic effects although they were found to accumulate in mussels to a high extent.

Toxicity of aliphatic hydrocarbons

In laboratory studies with individual aliphatic hydrocarbons and blue mussels, low-molecular-weight alkanes induced toxic effects up to *n*-decane while *n*-undecane and longer-chain alkanes did not elicit a toxic response after 48 hours exposure, which was attributed to the low aqueous solubility on the one hand and a toxicity cutoff beginning at *n*-decane on the other hand (Donkin et al., 1989; Donkin et al., 1991). Wraige (1997) examined the toxicity of 4-propyl-octane with the same mussel bioassay (exposure time between 24 and 96 hours). This branched C_{11}-alkane has a much higher aqueous solubility than the straight-chain isomer. It turned out that 4-propyl-octane is indeed toxic to blue mussels, thus indicating that not the aliphatic hydrocarbons themselves are nontoxic but that the low solubility of the commonly applied *n*-alkanes prevents an observable effect. Since aliphatic fractions of crude oils contain many branched isomers, they might pose a threat in the environment. However, an aliphatic UCM from a lubricating oil did not induce a toxic effect in *Mytilus edulis* after 24 hours exposure. Water concentrations or body burdens were not determined. The nominal aqueous concentration was 1000 µg/L (Thomas et al., 1995) but the actual aqueous and tissue concentrations may have been lower than anticipated. Oxidation of the UCM prior to the bioassay resulted in enhanced toxicity due to improved aqueous solubility of the polar compounds, which may also have been more toxic (Thomas et al., 1995).

Toxicity of aromatic hydrocarbons

Studies with aromatic hydrocarbons found narcotic effects for many components (Donkin et al., 1989; Donkin et al., 1991). Lee et al. (1972) found no toxic effect after exposure to and accumulation of mineral oil by blue mussels in contrast to exposure to the aromatic compound tetralin. Wraige (1997) and Smith (2002) investigated the toxicity of several derivatives of *cyclo*hexyltetralin. Table 2-2 compiles their data, showing water and tissue concentrations which caused a toxic effect of 50% (WEC_{50} and TEC_{50}) in combination with aqueous solubilities. The concentration of aromatic hydrocarbons in the tissues of blue mussels reduced SFG over three orders of magnitude and without an apparent threshold effect (Widdows et al., 1987).

Dibenzothiophene is the only sulphur-containing aromatic compound tested for sublethal toxic effects in blue mussels. This three-ring compound is more toxic than the three-ring hydrocarbon phenanthrene (Donkin et al., 1989). Like oxygen- or nitrogen-containing heterocyclic aromatic compounds, organic sulphur compounds do induce narcosis (Donkin and Widdows, 1990). However, additional specific mechanisms of toxicity have been proposed (Rhodes, 2002; Colavecchia et al., 2004).

Table 2-2 Aqueous solubility [µg/L] (20-25°C in high purity water), WEC_{50} and TEC_{50} values in µg/L and µmol/L ([1]Donkin et al., 1991, [2]Donkin et al., 1989, [3]Smith, 2002, [4]Wraige, 1997, [5]Hassett et al., 1980).

Compound	Aqueous solubility [µg/L]	WEC_{50}		TEC_{50}	
		[µg/L]	[µmol/L]	[mg/kg wet wt]	[mmol/kg wet wt]
Toluene[1]	578600	2347	25.51	16	0.17
n-Propylbenzene[1]	52170	862	7.18	27	0.23
n-Pentylbenzene[2]	3850	123	0.83	94	0.63
n-Heptylbenzene[2]	269	93	0.53	35	0.20
n-Octylbenzene[2]	71	79	0.41	82	0.43
6-*cyclo*hexyltetralin[3,4]	109	24	0.11	44	0.21
7-*cyclo*hexyl-1-methyltetralin[3,4]	45	42	0.18	58	0.25
7-*cyclo*hexyl-1-propyltetralin[3,4]	23	62	0.24	138	0.54
Biphenyl[1]	7000	295	1.92	16	0.10
Naphthalene[1]	30640	922	7.20	31	0.24
Phenanthrene[1]	1180	148	0.83	31	0.17
Dibenzothiophene[1,5]	1470[5]	94	0.51	14	0.08

2.3 Procedures for assessing the toxicity of mixtures

Most toxicological data available are derived from experiments with single substances with single species. In the environment, mixtures of contaminants are the reality. However, a simple transfer from single compound toxicological data to the behaviour of a compound as a part of a mixture is not possible. In toxicological theory, there are two concepts which try to predict and explain how chemicals will behave in and contribute to a mixture.

2.3.1 The theoretical concept of Concentration Addition

The concept of Concentration Addition goes back to the pharmacologist Loewe (Loewe and Muischnek, 1926, in Grimme et al., 2000). Concentration Addition applies when all compounds in question are exerting their toxicity via the same biochemical mechanism of action. Thus, the overall toxic effect of a mixture remains constant as long as the sum of all toxic units remains constant, which can be expressed in the formula:

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1$$

The terms c_i are the concentrations of the individual compounds in the mixture, which causes a total effect of x%. ECx_i are the concentrations of these individual compounds at which they cause the effect x if applied as a single compound (Loewe, 1926, after Grimme et al., 2000).

This concept also implies that an individual substance in a mixture can be replaced by another chemical with the same mechanism of action (as long as the corresponding toxic unit remains constant) without changing the overall toxicity of the mixture. If all individual c_i/ECx_i

fractions (toxic units) add up to 1, Concentration Addition occurs. A value below or above 1 implies a different behaviour of the toxicity of the mixture in question and is often taken as indicative of an antagonistic or synergistic effect, respectively.

2.3.2 The theoretical concept of Independent Action

In contrast to Concentration Addition, the alternative concept for mixture toxicity of Independent Action applies to those mixtures in which the compounds cause a joint toxic effect via different modes of action. This concept goes back to Bliss (1939) (cited in Grimme et al., 2000). Basically, the concept says that the toxic effect of a chemical remains the same when a second substance is present. The second substance does not interfere with the toxic effect caused by the first compound. Only those components which are present in a concentration at which they do cause a toxic effect as a single toxicant contribute to the overall toxicity (Independent Action is also called Response Addition). Thus, threshold values of chemicals can be defined in contrast to Concentration Addition (Grimme et al., 2000).

2.3.3 Toxic effects of mixtures of hydrocarbons

It is scientific consensus that the concept of concentration addition can be used for compounds with a similar mode of toxic action (Grimme et al., 2000). This can either be the case for substances which exert a toxic effect at the identical molecular mechanism of action within the target organism or for compounds which cause narcosis (e.g. Hermens et al., 1984; e.g. Backhaus et al., 2003). Estimates say that 60-70% of all industrial compounds can cause narcosis (Verbruggen et al., 2000). Moreover, studies have shown that many substances with a specific mode of action cause unspecific narcosis if applied below the concentration necessary for the specific toxicity (van Loon et al., 1997). This is obviously very important when dealing with complex mixtures of organic compounds which may contain specifically acting chemicals like PAHs in minor concentrations.

The fact that mixtures are also toxic in low concentrations of the individual constituents has been shown before. Hermens et al. (1984) found joint toxic effects of mixtures of hydrocarbons on a sublethal level. For mixtures in which each component was present in very small amounts (EC_{01}), a significant overall toxic effect could be determined for both similarly and dissimilarly acting chemicals in an algae bioassay (Grimme et al., 2000). This was confirmed with bioassays using *Daphnia magna* and the luminescent bacterium *Vibrio fisheri* as test organisms (Grimme et al., 2000). In a study of a mixture of 16 dissimilarly acting chemicals at low concentrations, a joint toxic effect was caused which was higher than each single effect and which was predictable with the Independant Action concept (Faust et al., 2003). Thus, it is crucial to include all components of a mixture in the toxicity considerations even if they are present in very small amounts. In the often unresolved complex mixtures of contaminants in environmental samples, it is obviously very hard to

determine whether the mixture consists of similarly or dissimilarly acting substances. Even if the compounds are identified, there may be no information regarding their modes of toxic action (Walter et al., 2002).

Experiments with mixtures of known composition, for which predictions of toxicity were calculated, showed that in general Concentration Addition and Independent Action provide good results for mixtures of similarly and dissimilarly acting chemicals. In some cases it turned out that the differences between the two concepts are quite small, with Concentration Addition usually resulting in slightly higher predicted toxicity. Concentration Addition should be used for risk assessment procedures like e.g. for regulatory purposes because especially the predictions for mixtures with low concentrations are not as reliable with Independent Action (Grimme et al., 2000).

Theoretically, Independent Action and Concentration Addition are counterparts and can give quite different predictions especially when compounds in a mixture are present in low concentrations. Thus, the two mixture toxicity concepts provide two distinct models for evaluation purposes which can be used for comparison and as a reference since environmental mixtures probably exert an intermediate toxicity (also called partially additive) (Walter et al., 2002; Backhaus et al., 2003). One has to bear in mind that experimental evidence for both concepts is derived from single-species laboratory biotests and refers to one biological endpoint tested (Backhaus et al., 2003). Thus, an environmental mixture of toxicants which acts not only via different mechanisms but also causes effects on different biological endpoints will be even harder to assess. That is why some scientists argue for the use of Concentration Addition as the “general solution” to prediction problems (Berenbaum, 1985).

2.4 Hydrocarbon composition of crude oils and unresolved complex mixtures

In general, different crude oils consist of different mixtures of hydrocarbons. Major compound classes are alkanes and mono-, di- and triaromatic hydrocarbons. In addition, there are compound classes containing nitrogen, sulphur and/or oxygen atoms (NSO compounds).

Several factors influence the exact composition of a crude oil, including the composition of the sedimentary organic precursor material in the source rock, geological heating and pressure conditions in the sedimentary basin during oil generation, which also determine the maturity of an oil, and subsequent alteration such as in-reservoir biodegradation (Welte et al., 1997).

Oil is generated from the macromolecular sedimentary organic material called kerogen, which presumably evolves by polycondensation and defunctionalisation reactions of

biological precursors during early diagenesis at mild temperature and pressure conditions or by selective preservation of biopolymers (e.g. Tissot and Welte, 1984; Tegelaar et al., 1989; Rullkötter and Michaelis, 1990). New geopolymers may evolve so that kerogen is a combination of resistant biomacromolecules, geomacromolecules, sulphur-rich material and incorporated low-molecular-weight biomolecules. From this kerogen, hydrocarbons are thought to be released with increasing burial and heating in the sedimentary basin. The temperature increases with burial depth until, during catagenesis, thermal energy is sufficient to cause certain hydrocarbon fragments to break off. The size of hydrocarbons generally decreases with increasing maturity due to these cracking reactions. Oil is usually produced at temperatures between 100 and 150°C at 2.5 to 4.5 km burial depth (Killops and Killops, 2005).

Since cracking reactions are essentially responsible for the generation of oil hydrocarbons, structure diversity is enhanced compared to biosynthesis of organic components. Often, positional isomers are present in their respective thermodynamic stability (Warton et al., 1997). Exceptions from this are possible, in particular if a less stable isomer was present in high concentration as a precursor in the sedimentary organic matter.

For the identification of hydrocarbons, gas chromatographically resolved peaks usually are required. However, many compounds coelute and the structures of the coeluting compounds are still largely unknown. The more complex a mixture of hydrocarbon isomers is, the more coelutions are likely to occur. For example, there are three possible structural isomers for *n*-octyltoluene (C_9-benzenes). These can usually be separated by conventional gas chromatographic methods. In contrast, there are six possible structural isomers for *n*-heptylxylenes and another six isomers for *n*-hexyltrimethylbenzene (C_9-benzenes). These 15 isomers all elute very closely to each other due to their similar physico-chemical properties. Thus, they overlap in the chromatogram and only the components with the highest concentrations are visible as separate peaks on top of the unresolved mixture. For less complex mixtures, the individual compounds are perceivable in the mass chromatograms of important structural fragments in the GC-MS analysis. However, the more complex the mixture gets and the lower-concentrated each constituent is present, not even mass chromatograms can be of much help to disentangle the various coeluting compounds. Thus, highly concentrated low-molecular-weight homologues are often identifed whereas the higher homologues with their greater number of possible isomers are masked by the vast amount of overlapping hydrocarbons present at low concentrations, resulting in the chromatographically unresolved “hump“.

2.4.1 Composition of aromatic hydrocarbons in crude oils

In order to give a structured overview of the chemical constituents of the aromatic fractions of crude oils, relevant compound classes are described below, starting with monoaromatic hydrocarbons and moving on to polyaromatic molecules. Cited studies refer to different oils than those used in the present study but they provide a good generalised overview over the composition of several compound classes.

Monoaromatic hydrocarbons in crude oils

Benzene and alkylated derivatives are widely studied as well as alkylated toluenes and xylenes. They are reported in crude oils with carbon numbers up to 40, which corresponds to the carbon number range of alkanes. The benzene ring has one to four sidechains. In general, the examined sidechains are straight or isoprenoid in structure. Sometimes there are additional ring-methyl groups (Petrov, 1987). Low-molecular-weight alkylbenzenes are usually the most abundant aromatic compounds in a crude oil (Killops and Killops, 2005). Indane and tetralin (Figure 2-2) were also found in many crude oils. Alkylated species are rarely reported, e.g. an isoprenoid C_{15} sidechain attached to the alicyclic ring of indane (Petrov, 1987). Tricyclic monoaromatic compounds are usually homologues of octahydrophenanthrene (Figure 2-2). Monoaromatic steroids (Figure 2-2) are products of the diagenesis of steroids (Peters and Moldowan, 1993).

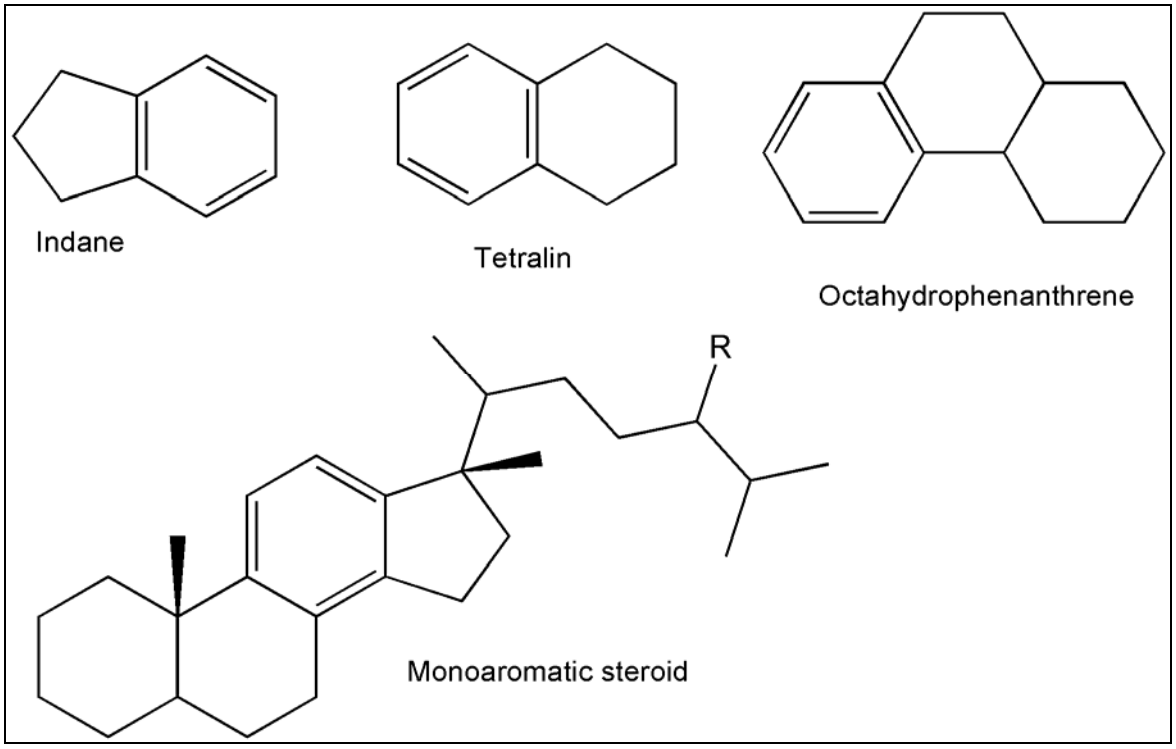


Figure 2-2 Structures of monoaromatic hydrocarbons reported in crude oils (without additional side chains).

As intermediates between mono- and diaromatic hydrocarbons, biphenyl (two benzene rings connected by a carbon-carbon bond) and methylbiphenyls are reported to be less concentrated than the diaromatic naphthalenes (Petrov, 1987).

Polyaromatic hydrocarbons in crude oils

Naphthalene (see Figure 2-1) itself is usually not present in crude oils in high amounts. Methylnaphthalenes and especially di- and trimethylnaphthalenes are present in higher concentrations while ethylnaphthalenes are not as abundant. Tricyclic diaromatic compounds were found in oil but the methylated homologues appear to be more important in coals than in crude oils (Petrov, 1987).

Triaromatic compounds are dominated by phenanthrene (see Figure 2-1) and its homologues. As for alkylnaphthalenes, substitution of phenanthrene and higher PAHs takes the form of polymethyl and ethyl sidechains in contrast to long chains for monoaromatic compounds (Killops and Killops, 2005). Numerous C_1- to C_5-alkylphenanthrenes were identified in various oils and isopropyl and butyl sidechains were also found (Petrov, 1987).

With increasing maturity, monoaromatic steroids can aromatise to triaromatic compounds. Aromatic hopanes have also been reported. Higher polyaromatic hydrocarbons and their methyl- and ethylhomologues are present in crude oils, too (Petrov, 1987). However, they are usually less abundant than the mono- to tri-aromatic compounds (Killops and Killops, 2005).

2.4.2 Composition of aromatic sulphur compounds in crude oils

Beside hydrocarbons, crude oils especially oils with a high sulphur content, also contain organic sulphur compounds. In general, the sulphur-containing compounds are less abundant than the pure hydrocarbons. They are not homogeneously distributed in the oil fractions as most elute in the NSO fraction (Strausz et al., 1990). In liquid chromatographic separations, the aromatic sulphur compounds elute in the fraction of aromatic hydrocarbons due to their similar polarity. In many separation schemes, aliphatic sulphur compounds like thiolanes and thianes (Figure 2-3) elute in the aromatic hydrocarbon fraction, too.

Analogous to the hydrocarbons, there are mono-, di- and triaromatic components, thiophene, benzothiophene and dibenzothiophene, respectively (Figure 2-3). Similar to the aromatic hydrocarbons, polymethylated and alkylated homologues occur.

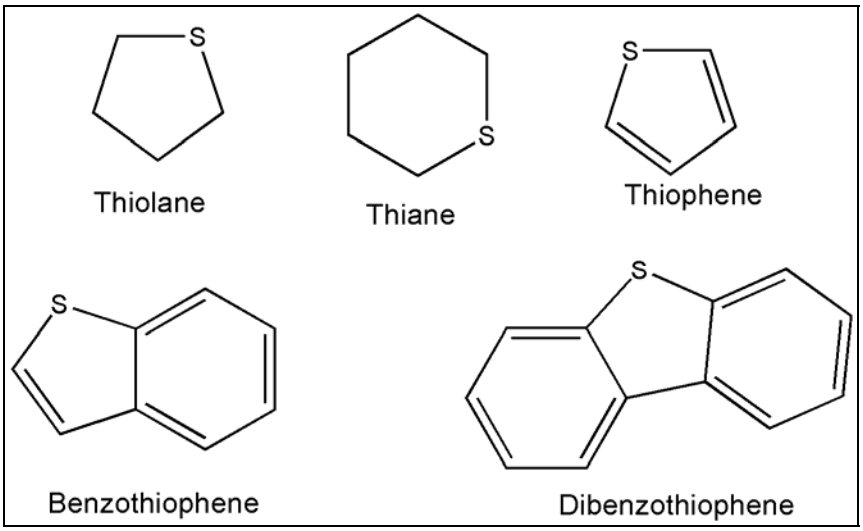


Figure 2-3 Structures of sulphur compounds reported in crude oils (without side chains).

2.4.3 Compositional studies of unresolved complex mixtures of hydrocarbons in crude oils

Although aromatic compounds are the main topic of this study, results of compositional studies of aliphatic unresolved complex mixtures from crude oils are briefly discussed as well. They highlight the diversity of structures present in hydrocarbon UCMs and can also partly be applied to aromatic UCMs. Since aliphatic and aromatic hydrocarbons are presumably both released from kerogen, they have a common source. Thus, structures found in the aliphatic UCM may be analogous to compounds within the aromatic UCM or attached to an aromatic moiety.

Characterisation of aliphatic unresolved complex mixtures

Studying the UCM in the total hydrocarbon fraction of a heavily degraded oil from Alaska, Killops and Al-Juboori (1990) showed that it consisted of about 40% acyclic compounds, 42% mono- and 15% dicyclic compounds. Chemical oxidation studies (with CrO_3) with this UCM produced primarily aliphatic structures (*n*-acids, α,ω-di-acids, isoprenoid acids). To explain the predominance of aliphatic structures after chemical oxidation, it was suggested that the UCM consisted of cyclic structures which were partly connected by alkyl chains (Killops and Al-Juboori, 1990).

Gough and Rowland (1990) carried out a similar study with several oils and obtained similar results. The major substances after chromium trioxide (CrO_3) oxidation of the aliphatic UCMs were found to be *n*-acids. From this, previous assumptions that the UCM consists primarily of highly branched alkanes were proven to be wrong. The mechanism of CrO_3 oxidation leads to a bond cleavage at a tertiary carbon atom. From the resulting structures, T-branched alkanes, for example 7-*n*-hexylnonadecane, were considered as possible precursors. They might be products of chain scission and recombination during oil generation from kerogen (Gough and Rowland, 1990).

Considering the UCMs´ resistance to biodegradation, slow degradation of selected compounds in degradation experiments was taken as a prerequisite whether these compounds are possible ingredients of UCMs or not. It was tested how well synthesised T-branched alkanes were degraded by *Pseudomonas fluorescens* within 25 days. The experiment showed that T-branched alkanes are more resistant to biodegradation than *n*-alkanes and monomethylalkanes and comparable to the degradation rates of an UCM and highly branched acyclic isoprenoids. Thus, further evidence was established that T-branched alkanes comprise part of the UCM (Gough and Rowland, 1990; Gough et al., 1992). This was proven by Warton et al. (1997), who examined the aliphatic fractions of five crude oils and found T-branched alkanes in all of them. Carbon numbers ranged from C_{10} to C_{25} and many isomers were found, especially for the C_{18} compounds. Quantifying the T-branched alkanes eluting between the *n*-C_{19} and *n*-C_{20} alkanes in the GC showed that they comprised approximately 3% of the crude oil alkanes. The abundance of the isomers of the T-branched alkanes in the oils reflected the stability of the compounds. This indicates that equilibration processes are involved, suggesting an origin from geosynthetic activity (Warton et al., 1997). However, possible precursors for some branched alkanes have been found in biological systems, as well (Warton et al., 1997 and references therein). On the whole, there is not enough evidence to be sure about a single formation mechanism of T-branched alkanes.

The results for constituents of aliphatic UCMs are briefly summarised in Table 2-3. Cyclic structures with alkyl bridges have been reconstructed from structure fragments. T-branched alkanes have been found in crude oils. Consequently, it is probable that they form part of the aliphatic UCM, as well, but there are still numerous unidentified compounds. T-branched alkanes may be aliphatic substituents attached to aromatic compounds but no such compound has been identified so far.

Table 2-3 Evidence for the occurrence of compound groups in aliphatic UCMs of crude oils and respective references.

Compound group	Evidence by	Reference
Cyclic structures, partly connected by alkyl chains	Chemical oxidation	Killops and Al-Juboori (1990)
T-branched alkanes	Chemical oxidation	Gough and Rowland (1990)
	Biodegradation	Gough and Rowland (1990) and Gough et al. (1992)
	Identification in oils	Warton et al. (1997)

Characterisation of aromatic unresolved complex mixtures

The aromatic fraction of the UCM of a heavily degraded oil from Alaska was dominated by (substituted) single ring benzenoid systems (Killops and Al-Juboori, 1990). In order to characterise the side chains of aromatic compounds of the aromatic UCM of a biodegraded

crude oil from the Gippsland Basin, Australia (Leatherjacket-1, biodegradation level 4 after Peters and Moldowan, 1993), oxidation experiments were carried out with ruthenium tetroxide (RuO_4) (Warton et al., 1999). The UCM was separated into a monoaromatic, a diaromatic and a triaromatic fraction. RuO_4 oxidises alkylaromatic compounds at the substituted aromatic C-atom. Thus, side chain lengths and structures can be determined. Resolved components in the monoaromatic fraction were identified as *n*-alkyltoluenes and *n*-alkylxylenes. Oxidation of the monoaromatic fraction yielded mainly *n*-acids but also methyl derivatives, isoprenoid and cyclic structures. Thus, as previous studies have shown (e.g. Killops and Al-Juboori, 1990), alkylaromatic compounds are important in an aromatic UCM.

Warton et al. (1999) identified resolved compounds in the diaromatic and in the triaromatic hydrocarbon fraction as diverse polymethylnaphthalenes and -phenanthrenes, respectively. Products of RuO_4 oxidation suggest *cyclo*hexylalkylbenzenes, phenylalkyl-benzenes, alkylindanes and alkyltetralins as possible precursors for the diaromatic hydrocarbon fractions as well as *cyclo*hexylphenylalkylbenzenes and alkylbiphenyls for the triaromatic hydrocarbon fraction. Alkyl chains between ring systems probably have varying numbers of carbon atoms (Warton et al., 1999).

Subsequently, the oxidation products were reduced and afterwards deuterated. The emerging hydrocarbons were analogous to the common hydrocarbons of the aliphatic fractions of crude oil. Thus, Warton et al. (1999) suggested that these common hydrocarbons and the alkyl chains attached to the aromatic rings have the same source. They proposed that fatty acids react via an alkylation mechanism with aromatics to form alkylaromatic compounds during oil generation. These alkylaromatics become defunctionalised to yield petroleum hydrocarbons.

Recently, Sutton et al. (2005) identified a novel C_{26} 17-desmethyl triaromatic steroid in a UCM from a Tia Juana Pesado crude oil from Venezuela.

Possible model compounds for an aromatic UCM are alkyltetralins (Smith et al., 2001). Several alkyl*cyclo*hexyltetralins were synthesised and tested for their resistance to degradation by *Pseudomonas fluorescens*. The tetralins were hardly degraded, compared to a reference *n*-C_{25} alkane, which was degraded almost completely. Thus, these alkyl*cyclo*hexyl-tetralins were considered suitable model compounds for aromatic UCMs.

All in all, chemical oxidation of an aromatic UCM revealed a vast number of alkylsubstituted aromatic compounds and the variation of side chain lengths and structures. It appears that alkyl bridges between ring structures are common in the components of the UCM. Thus, derivatives of phenylbenzene, alkyl*cyclo*hexylbenzene, indanes and tetralins comprise part of an aromatic UCM. Results are briefly summarised in Table 2-4. They were used as a starting point for the investigation of the aromatic compounds of the oils used in this study (Chapter 5).

Table 2-4 Evidence for the occurrence of compound groups in aromatic UCMs and respective references.

Compound group	Evidence by	Reference
Alkyltoluenes and -xylenes Polymethylnaphthalenes Polymethylphenanthrenes	Resolved peaks in mass chromatograms of oil samples	Warton et al. (1999)
*Cyclo*hexylalkylbenzenes Phenylalkylbenzenes Alkylindanes and -tetralins *Cyclo*hexylphenylalkylbenzenes Alkylbiphenyls	Chemical oxidation experiments	Warton et al. (1999)
C_{26} 17-desmethyl triaromatic steroid	Isolated from oil	Sutton et al. (2005)
*Cyclo*hexylalkyltetralins	Model compounds synthesised for biodegradation test	Smith et al. (2001)

2.5 The solubility of apolar hydrocarbons in aqueous systems

Hydrocarbons are hydrophobic substances with low aqueous solubilities. Components will dissolve in water to a small extent depending on their physico-chemical properties until an equilibrium is reached either with an overlying oil film or oil droplets. Since the bioavailability of a given xenobiotic to marine organisms is always connected with its aqueous solubility (Fent, 1998), a few considerations concerning solubility will be introduced here. For the exposure of blue mussels in the toxicity tests in this study, aqueous solutions of aromatic hydrocarbon mixtures were prepared.

Shaw (1977) distinguished three possible forms of hydrocarbons in water. Firstly, hydrocarbons can be truely dissolved (water soluble fraction, WSF). Aqueous solutions of crude oils differ in composition from the original crude oil. Usually, crude oil mixed with seawater results in solutions enriched in one- and two-ring aromatic hydrocarbons. Secondly, colloids with molecular aggregates smaller than 1 µm can be formed (water accommodated fraction, WAF). Colloids must not necessarily float to the surface but can stay in suspension. They are formed for example by wave activity after an oil spill. Thirdly, oil particles may remain larger than 1 µm. The larger oil particles float to the surface. Adjacent water is not necessarily saturated with dissolved hydrocarbons (Shaw, 1977).

2.5.1 Factors affecting the solubilisation of hydrocarbons in mixtures

Several factors influence the aqueous solubility of hydrocarbons, including size/surface area, polarity and degree of substitution. Molecular weight, molecular size and surface area are very closely linked to each other. The structure of hydrocarbons can have a significant impact on their water solubility. In general, as the molecular weight of a hydrocarbon increases, the water solubility decreases. With increasing polarity, aqueous solubility

increases, too. The degree of alkyl substitution on an aromatic ring system usually diminishes aqueous solubility (Smith et al., 2001).

There are several studies concerning the water solubility of whole crude oils as complex mixtures of hydrocarbons (Betton, 1994). These studies indicate that the interaction of components in such mixtures can cause significant changes in the solubilities of their individual constituents. For example, some more soluble compounds can affect partition coefficients and help to dissolve other, less soluble hydrocarbons (co-solution). Because of these interactions, the behaviour of a compound in a mixture may not correspond to its behaviour predicted from pure component data.

When dealing with the aqueous solubility of complex mixtures of hydrocarbons, Raoult´s law has to be taken into account. Raoult´s law states that the solubility of a compound present in an ideal mixture is equal to the solubility of the pure compound multiplied by its mole fraction in the mixture. Thus, as the mole fraction of a component in a mixture decreases, so does the water solubility of that component (Luthy et al., 1997 in Booth, 2004; Booth, 2004). In the case of a crude oil, in which a compound is just one of thousands, the mole fraction is very small. Thus, some compounds with a relatively high aqueous solubility like naphthalene may be less concentrated than expected in the aqueous solution of a crude oil. In contrast, this phenomenon can result in the relative enrichment of less soluble hydrocarbons such as PAHs or highly alkylated aromatic compounds (Booth, 2004 and references therein). For aqueous solubilities of selected hydrocarbons, the reader is referred back to Table 2-2.

A complex mixture of hydrocarbons such as a crude oil can probably not be regarded as a mixture with ideal behaviour, implying that Raoult´s law may not be fully applicable (Banerjee, 1984). However, mixtures of structurally related liquids were observed to be close to ideal behaviour (Booth, 2004 and references therein). Therefore, the fractions of aromatic hydrocarbons used in this study can be regarded as more ideal than the whole oil.

2.5.2 Aqueous solubility of aromatic unresolved complex mixtures

In a preliminary study of the aqueous solubility of a monoaromatic UCM from a Gullfaks North Sea crude oil, Smith (2002) determined a value of 560 µg/L from a single analysis. In a recent study by Booth (2004), the aqueous solubilities of a monoaromatic and a total aromatic hydrocarbon fraction of a weathered Tia Juana Pesado crude oil (both clearly dominated by a UCM) were investigated. Extracts of the aqueous solutions prepared were analysed by GC-MS. The water solubility and composition of the two aromatic hydrocarbon fractions deviated extremely by up to two orders of magnitude. Despite the resulting high standard deviations of the 50 mL aliquots of the aqueous solutions analysed, it turned out that the total aromatic hydrocarbon fraction had a considerably higher solubility than the less

complex monoaromatic mixture. Values were 293 ± 342 µg/L (n = 10) for the total aromatic UCM and 57 ± 21 µg/L (n = 9) for the monoaromatic UCM.

The lower solubility of the monoaromatic UCM was explained by the fact that the compounds in this mixture contain only one aromatic ring and several aliphatic and/or alicyclic moieties. Since saturated hydrocarbons are known to have much lower water solubilities than aromatic compounds of a similar size, substitution of the benzene ring with large saturated groups would be expected to reduce the water solubility significantly. In contrast, many constituents of the total aromatic hydrocarbon fraction contain more than one aromatic ring and relatively few aliphatic and/or alicyclic moieties. Thus, compounds with one aromatic ring are less soluble than the corresponding polyaromatic compounds with the same number of carbon atoms. This results in the overall higher aqueous solubility of the aromatic UCM compared to the monoaromatic one (Booth, 2004).

The gas chromatograms of the aqueous solutions indicate a higher molecular weight range in the total aromatic hydrocarbon mixture than in the monoaromatic one. Next to the explanations given above, linking these results with the considerations of Raoult´s law can provide a second explanation. In the case of the total aromatic UCM, the presence of polyaromatic compounds will reduce the mole fraction of the monoaromatic compounds. Therefore, compared to the monoaromatic UCM, their water solubility will be reduced. It should be noted that the hydrocarbons present in the original monoaromatic UCM are also present in the original total aromatic UCM as the former is an isolate of the latter. Some polyaromatic components may have a higher concentration in the aromatic UCM than some monoaromatic compounds. Thus, and with regard to Raoult´s law, it is possible that a hydrocarbon with a lower water solubility than another hydrocarbon (each as a pure compound) is more soluble when they are both present in the complex mixture (Booth, 2004). In addition, Booth (2004) found more resolved compounds in the aqueous solution of the monoaromatic UCM than in that of the total aromatic UCM. In the aqueous solution of the total aromatic UCM, no four- and five-ring PAHs were detected.

3 Materials, experimental methods and analytical techniques

In this chapter, materials and methods used in the present study are presented in detail. The materials section (3.1) starts with the oils used and their properties, followed by the description of the blue mussels from two North Sea sites. In Section 3.2, the preparation of oil fractions, the toxicity test, the method of sample extraction and the quantification strategy are described.

3.1 Materials

3.1.1 General characteristics of the used crude oils

In this section, the four used crude oils from two different oil provinces are described with general geochemical parameters. Their level of biodegradation was determined from several parameters of compounds in the C_{15+} saturated hydrocarbon fraction.

3.1.1.1 The Monterey Formation

The Monterey Formation in California is a well-studied deposit of marine sediments rich in organic matter. It was deposited during the Miocene (about 24 to 5 million years before today) on a marine continental margin with a palaeowater depth of 500 to 1500 m (Isaacs and Rullkötter, 2001). Today, it is buried up to 3300 m depth. Oil generation occurred mainly 6-3 million years before today, towards the end of the Miocene. All the oils are quite heavy and have a high sulphur and nitrogen content (Tennyson and Isaacs, 2001). The Monterey Formation is the principal source rock for the oils in the Santa Maria Basin, where the two oils used in this study come from (Orcutt field). The undegraded oil comes from the API Well Number 04-083-02368 in a depth of 1174 m (Tennyson and Isaacs, 2001). The degraded oil from the API Well Number 04-083-02322 in 957 m depth (Tennyson and Isaacs, 2001). It was shown that these two oils were generated from the same source rock and have the same maturity level (Rullkötter et al., 2001a). Thus, the main difference in the composition of the crude oils should be derived from the in-reservoir biodegradation of one of the oils. For example, the gas chromatograms of the C_{11+} fractions of the saturated hydrocarbons show that the *n*-alkanes are removed from the degraded oil compared to the undegraded oil (Figure 3-1).

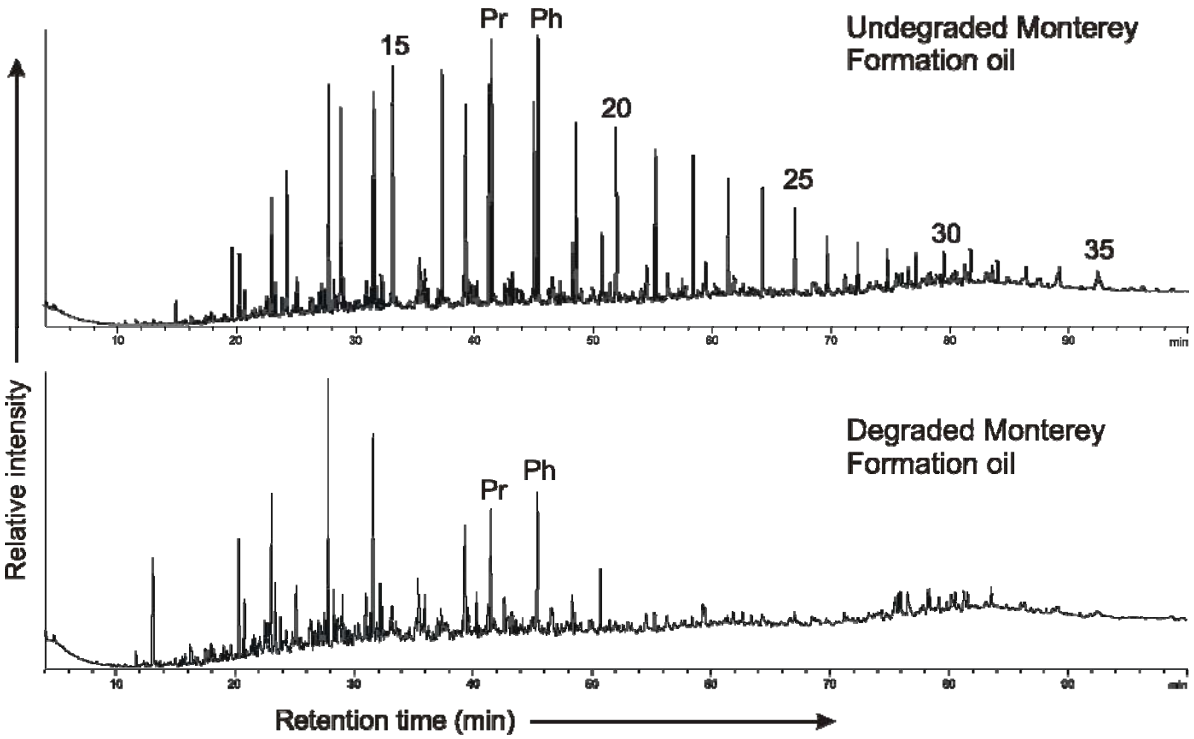


Figure 3-1 Gas chromatograms of the C_{11+} fractions of saturated hydrocarbons of the two Monterey Formation oils from the Orcutt field.

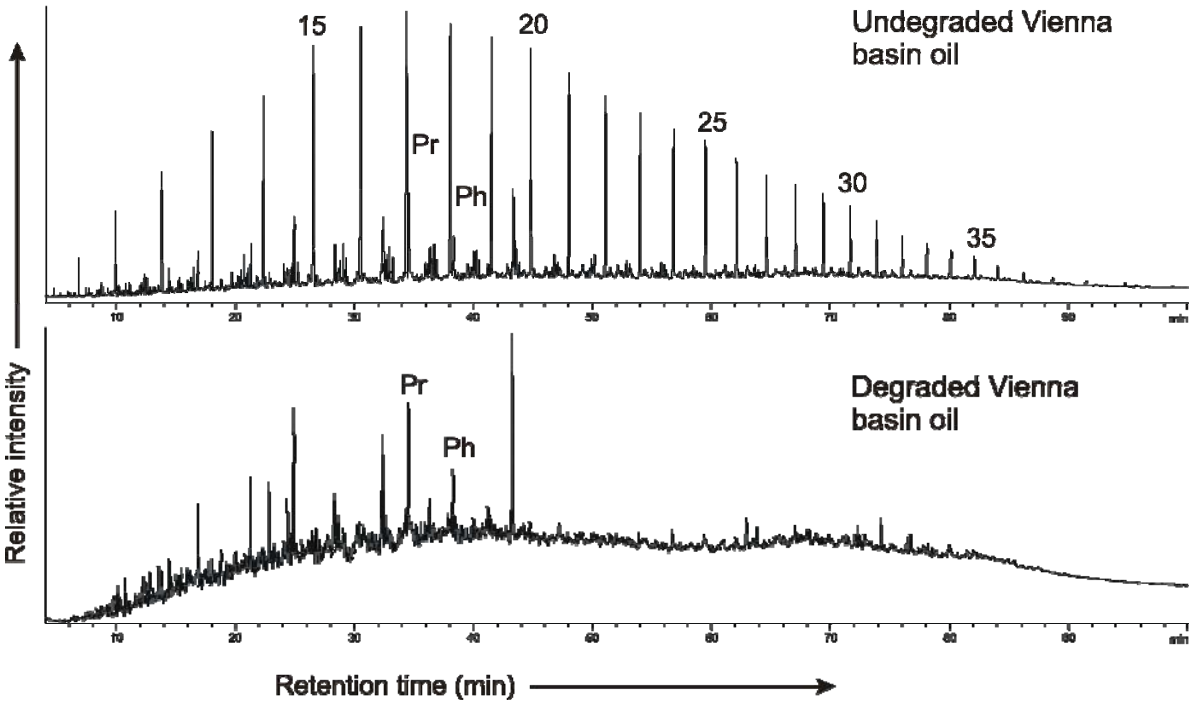


Figure 3-2 Gas chromatograms of the C_{11+} fractions of saturated hydrocarbons of the two Vienna basin oils.

3.1.1.2 The Vienna basin

The Vienna basin is the most important oil province in Austria and probably also of Central Europe (Welte et al., 1982; Plein, 1994). It lies at the boundary of the Alps and the Carpathians. Below the intramontane basin are Neogene sediments up to a depth of 5000 m (Miocene-Pliocene, about 24 to 2 million years before today). The oil apparently is generated from pre-Tertiary sediments (more than 65 million years old) below the Neogene sediments (Welte et al., 1982).

In general, the oils have a high maturity level. In the Vienna basin, the geothermal gradient is high with up to 50°C/km depth (Tissot and Welte, 1984). The oil reservoirs are situated at 800 to 5500 m depth (Welte et al., 1982). The well locations of the oils used have not been disclosed. Many oils are in-reservoir biodegraded, at least down to approximately 1700 m reservoir depth where it grows too warm for degrading bacteria (65-70°C). The gas chromatograms of the C_{15+} fractions of the saturated hydrocarbons show that the biodegraded oil lacks the *n*-alkanes compared to the undegraded oil (Figure 3-2). The Vienna basin oils are very similar in composition apart from the biodegradation effects. For example, all oils contain only small amounts of steranes and triterpanes (Welte et al., 1982). The sulphur content is low (less than 1%).

3.1.1.3 Comparison of the oils and their level of biodegradation

Some information about the four crude oils used in this study is listed in Table 3-1. In general, the Vienna basin oils contain more aliphatic/alicyclic hydrocarbons and less polar compounds than the Monterey Formation oils. This difference mirrors the lower sulphur and nitrogen content of the Vienna basin oils.

The pristane/*n*-heptadecane ratio (pri/*n*-C_{17}) is a maturity parameter which decreases with increasing maturity since *n*-alkanes are more abundantly formed than isoprenoids during catagenesis. However, if an oil is biodegraded, the *n*-alkanes are removed before the isoprenoids, resulting in high pri/*n*-C_{17} ratios until the *n*-alkane is virtually gone which renders the ratio inapplicable. The pri/*n*-C_{17} ratios of the two undegraded oils indicate that the Vienna basin oil is more mature but since the oils are derived from different organic materials, the parameter is not necessarily suitable for comparison. The two sterane isomer ratios in Table 3-1 are common maturity parameters (Peters and Moldowan, 1993). The two Monterey Formation oils have medium values, whereas high values were determined for the Vienna basin oils, demonstrating their higher maturity. The MPI-1 methylphenanthrene index (Radke, 1987), however, is similar for all four oils. The discrepancies in the different maturity parameters are due to the difficulties of applying sterane maturity parameters to oils from silica-rich source rocks such as the Monterey Formation, usually leading to an underestimation of the maturity (Rullkötter et al., 2001b). In addition, it has been reported that samples of different maturity can show identical MPI-1 ratios (Peters and Moldowan,

1993). However, several other maturity parameters point towards a moderate maturity of the Monterey Formation oils (Rullkötter et al., 2001b) and a high maturity of the Vienna basin oils (Welte et al., 1982).

Table 3-1 Basic data and geochemical parameters for the four crude oils used in this study. Data for the Monterey Formation oils are for the oils used in this study. Data for the Vienna basin oils are average data for Vienna basin oils unless idicated otherwise ([1]Rullkötter et al., 2001a; [2]Tennyson and Isaacs, 2001; [3]this study; [4]OMV, [5]Rullkötter et al., 2001b), sat = fraction of saturated hydrocarbons, aro = fraction of aromatic hydrocarbons.

Parameter	Undegraded Monterey Formation oil	Degraded Monterey Formation oil	Undegraded Vienna basin oil	Degraded Vienna basin oil
Depth [m]	1174[2]	957[2]	2000-3000[4]	max. 1500[4]
API gravity	22.7[1]	22.3[1]	29[4]	22.6[4]
Sulphur content [%]	2.91[1] / 3.09[3]	2.99[1] / 3.11[3]	0.20[3]	0.24[3]
% sat/aro/polar	30/29/42[1]	29/32/40[1]		
	29/24/47[3]	27/26/47[3]	67/23/10[3]	51/26/23[3]
Pri/*n*-C_{17}	1.64[1]	4.21[1]		
	1.62[3]	5.33[3]	0.42[3]	7.39[3]
Pri/Phy	0.94[1]	0.80[1]		
	0.94[3]	0.87[3]	1.97[3]	1.71[3]
C_{29} steranes	0.40[5]	0.44[5]		
20S/(S + R)	0.33[3]	0.32[3]	0.46[3]	0.38[3]
C_{29} steranes	0.59[5]	0.62[5]		
ββ/(αα + ββ)	0.51[3]	0.56[3]	0.80[3]	0.68[3]
MPI-1	0.92[3]	0.91[3]	0.90[3]	1.01[3]

In-reservoir biodegradation means that bacteria were (or are) living in the reservoir and degraded a variety of oil components. Until recently, the major processes of in-reservoir biodegradation were thought to be aerobic (Palmer, 1993). Now, many anaerobic bacteria are known to degrade oil hydrocarbons and these processes are suggested to be more important than aerobic ones (Wilkes et al., 2000).

Biodegraded oils usually have a lower API gravity and an increased viscosity (Connan, 1984). They are depleted in hydrocarbons and enriched in NSO compounds (Palmer, 1993). These changes are particularly obvious for the degraded Vienna basin oil (Table 3-1**)**.

Biodegradation is a “quasi-stepwise” process, meaning that the different compounds classes are not equally susceptible to biodegradation. Rather, some compounds of a more easily biodegradable compound class may still be present when compounds of the next more resistant class are already removed. Peters and Moldowan (1993) established a scale of biodegradation levels, which is commonly used to describe the degree of biodegradation of oils (PM levels 1 to 10). The first compounds which are biodegraded are the *n*-alkanes (light biodegradation, PM level 1-3), followed by the acyclic isoprenoids such as pristane (C_{19}) and phytane (C_{20}) (moderate biodegradation, PM level 4-5). When steranes and hopanes are affected, the oil is classified as heavily biodegraded (PM level 6 and higher). Volkman et al.

(1984) found that during light and moderate biodegradation, alkylbenzenes are depleted and the distribution of dimethylnaphthalene isomers is altered.

In the biodegraded Monterey Formation oil, the *n*-alkanes are largely removed but the isoprenoids pristane and phytane are still present (see Figure 3-1 and Table 3-1). In addition, there were no visible differences concerning steranes or hopanes in the *m/z* 217 and *m/z* 191 mass chromatograms of the two oils. Thus, the oil is moderately biodegraded (PM level 4-5) according to the classification of Peters and Moldowan.

Concerning the level of biodegradation of the Vienna basin oil, this oil also does not contain *n*-alkanes and the isoprenoids pristane and phytane are only present in small amounts (see Figure 3-2 and Table 3-1). No differences in sterane and hopane content were previously found between Vienna basin oils of different stages of biodegradation (Welte et al., 1982). This shows that the oil is also moderately biodegraded after Peters and Moldowan (1993).

However, many compound classes are not included in this classification scheme (and others), in particular aromatic compounds. One reason for this probably is the fact that the degradation of individual compounds depends on the array of microorganisms present, which is different from one environment to another (Alexander, 1999).

In general, two-and three-ring aromatic compounds are more easily degraded than four- and five-ring PAHs. The more alkyl chains are attached to the aromatic nucleus, the more resistant the compound is against biodegradation. Certain positions of substitution may influence susceptibiliy to biodegradation but no general rule can be applied for the various substitution patterns of the aromatic compound classes. Long-chain alkylated aromatic hydrocarbons are more susceptible to biodegradation than aromatic compounds with short or branched alkyl chains due to the similarity of the aliphatic moiety to *n*- or branched alkanes, respectively (Alexander, 1999). Thus, although susceptibility of oil compound classes to biodegradation can be partly generalised as shown above, degraded oils from different reservoirs vary in composition of compound classes and individual isomers.

Overall, the major differences between the two oil pairs are that the Monterey Formation oils are heavier oils with a higher sulphur (and nitrogen) content. The Monterey Formation oils contain steranes in higher amounts than the Vienna basin oils. With regard to biodegradation, the saturated hydrocarbon fractions indicate that the two degraded oils are both moderately biodegraded and largely lack *n*-alkanes. More detailed information about the distribution of aromatic hydrocarbons in each oil can be found in Chapter 5.

3.1.2 Blue mussels (*Mytilus edulis*) collected from two North Sea sites

A blue mussel biotest was applied in this study in order to determine the toxicity of several aromatic hydrocarbon mixtures isolated from crude oils. Blue mussels were collected from two sites close to the respective laboratory, Port Quin in Cornwall for the experiments in

Plymouth University and the Jade Bay near Wilhelmshaven for the experiments in the Terramare Research Centre in Wilhelmshaven. The biological endpoint monitored was the feeding rate, which is a sensitive indicator for mussel health and growth potential (Widdows et al., 1995, see chapter 2).

Blue mussels are wide-spread in the North Sea and other temperate regions of the northern hemisphere (Gosling, 1992). Widdows et al. (1995) sampled mussels from all over the British coast for analysis of growth and contaminant level. Prior to that, they compared the physiological response and scope for growth (SFG) of blue mussels from clean sites in North Scotland and South England (approximately 1000 km distance) to ensure differences were not due to genetic variation. No differences between clean sites were found, illustrating that mussels from different parts of the North Sea can indeed be compared. In the whole set of samples, differences in SFG could be attributed to environmental contamination. On the whole, SFG declined from north to south, mirroring the major inflow of clean water from the North Atlantic via the north of Scotland as well as the overall increase in environmental contamination with increasing urbanisation and industrialisation towards the south. Mussels from estuarine areas and sites near industrial activity had reduced SFG values, mainly caused by aromatic hydrocarbons and tributyltin in the mussel tissue.

3.1.2.1 Port Quin, Northwest Cornwall, GB

Port Quin is a small rocky cove on the Northwest coast of Cornwall, about 60 km from Plymouth. It has been used as a clean reference site before (Widdows et al., 2002; Donkin et al., 2003; Crowe et al., 2004). High SFG values, including high feeding rates, of the blue mussels reflect the high levels of health. Tissue concentrations of several contaminants in mussels from Port Quin (sampled in July 1996) were very low, e.g. 2-3 ring PAH concentrations as determined with HPLC were 0.32 µg/g dry weight (Widdows et al., 2002). For more data on xenobiotics in blue mussel tissue from Port Quin, see Table 3-2.

Table 3-2 Levels of selected compounds in mussel tissue from Port Quin and Jade Bay; data from: [1]Widdows et al., 2002; [2]Bakker et al., 2005; n.d. = not detected.

Compound	Port Quin[1] [µg/g dry weight]	Jade Bay[2] [µg/g dry weight]
Copper	7.40	≈ 7
Zinc	202	40 -100 (steadily declining from 1996-2002)
Mercury	0.10	usually < 0.3 but high value in 2000 with 0.5
Cadmium	1.05	< 1
Lead	1.70	< 1
Lindane	n.d. (< 0.001)	0.02 (average 1996-2002)
Chlorinated benzenes	Sum of 25 compounds: n.d. (< 0.002)	Sum of 6 compounds: 0.02-0.17
Hexachlorobenzene	n.d. (< 0.001)	0.0005 (in 1998)
Tributyltin	0.05	0.2 (in 1999)
Dibutyltin	0.04	~ 0.02

Donkin et al. (2003) measured mussel feeding rates from Port Quin directly and after a 24 hours depuration period. The feeding rates were not significantly different, indicating low stress, i.e. good conditions in their natural habitat. The absence of contaminants was also obvious in the GC-MS total ion current chromatograms of tissue extracts.

In November 2003, approximately 400 mussels (shell length 12-15 mm) were sampled from the intertidal zone of their rocky habitat for the toxicity tests in this study. Tissue extracts of control mussels exposed to acetone in the course of the experiments did not show a UCM or major contamination.

3.1.2.2 Jade Bay, Wilhelmshaven, German North Sea Coast

For the experiments performed at the Terramare Research Centre in Wilhelmshaven, mussels were sampled from the intertidal zone of the Jade Bay about 1 km from the institute. Since the Northern German coast is not rocky, mussels were collected from stones and small rocks on a sandy shore between two small moles. Literature data on the concentrations of xenobiotics in blue mussels from the Jade Bay are scarce. The Wadden Sea Quality Status Report 2004 (Bakker et al., 2005) provides some data from several years´ sampling in the whole area of the Jade basin (Table 3-2). Values in Bakker et al. (2005), however, do not refer directly to the site where mussels were collected for this study.

Background values for heavy metals (Cd, Cu, Pb, Zn) in blue mussels are similar to values found for Port Quin mussels. Mercury values are slightly higher for the Jade Bay in 2000. Regarding chlorinated organic xenobiotics, hexachlorobenzene is similarly concentrated in mussels from both sites whereas the summed concentration of all chlorinated organics is about one order of magnitude higher in the Jade Bay (Table 3-2). The concentration of tributyltin is about four times higher in mussels from the Jade Bay whereas the metabolic product dibutyltin is present at a similar level at the two sites (Widdows et al., 2002; Bakker et al., 2005).

Very close to the site of mussel sampling at Wilhelmshaven and further north towards the open sea, PAH concentrations in surface sediments sampled in 1997 were analysed (Rinne et al., 2005). Overall, the 16 EPA-PAHs were below natural background values and were chiefly attributed to combustion sources. Thus, industrial activities and shipping in the area do not introduce major amounts of petroleum hydrocarbons into the marine ecosystem. Concentration of summed PAHs ranged between 6 and 800 ng/g dry sediment. For comparison, in the Dutch Wadden Sea sediments, levels for the 16 EPA-PAHs were around 500 ng/g (Bakker et al., 2005) and in the German Bight up to a maximum of 1000 ng/g (Biselli et al., 2005). No sediment data are available for Port Quin. Literature data show that at the site near Wilhelmshaven, which was chosen for the collection of blue mussels, sediments contain low to average amounts of xenobiotics compared to average values for

the German North Sea. It was thus considered a suitable sampling site for blue mussels as test organisms in this study.

In March 2004, approximately 400 mussels (shell length 12-15 mm) were sampled from the intertidal zone for the toxicity tests in this study and approximately 200 mussels (shell length 12-15 mm) were sampled from the same site for more toxicity tests in November 2004. Tissue extracts of control mussels exposed to acetone in the course of the experiments did not show a UCM or major contamination.

3.2 Experimental methods and analytical techniques

The aromatic hydrocarbon fractions of four crude oils were chosen to be tested for their toxicity. The whole oils were separated in order to obtain total aromatic hydrocarbon fractions, which were further separated to gain monoaromatic hydrocarbon fractions. The different aromatic hydrocarbon fractions were analysed by GC-FID and GC-MS in order to identify resolved compounds as well as to characterise different distributions of compounds and compounds classes in the unresolved complex mixtures. For toxicity tests, the aromatic hydrocarbon mixtures were dissolved in acetone and mixed with seawater. Then, blue mussels (*Mytilus edulis*) were exposed to these toxicant solutions. The feeding rates of the mussels were determined and subsequently, the tissue was extracted to determine body burdens of hydrocarbons. Samples were analysed by GC-FID and GC-MS in order to quantify and identify accumulated hydrocarbons.

3.2.1 Preparation of oil fractions

3.2.1.1 Precipitation of *n*-hexane insoluble compounds

Approximatately 500 mg whole oil were exactly weighed into a round bottom flask and dissolved in 500 µL dichloromethane. A 20-fold excess of *n*-hexane was added to ensure precipitation of *n*-hexane insoluble components (asphaltenes) in the refrigerator overnight. The sample was filtered over extracted cotton wool and sodium sulphate to obtain the *n*-hexane soluble compounds (maltenes). Each flask and the corresponding funnel with cotton wool were rinsed a few times with *n*-hexane until the eluting solvent was colourless. The residue of asphaltenes was eluted with dichloromethane. The solvents were evaporated, samples blown to dryness with nitrogen and weighed. Maltenes were used for further separation. Figure 3-3 shows a simplified scheme of whole oil separation into fractions of different polarity.

3.2.1.2 Medium pressure liquid chromatography (MPLC)

Column chromatography was carried out with an automated system, which was developed for semipreparative separations of sediment extracts and oil samples into the three groups of aliphatic and alicyclic hydrocarbons, aromatic hydrocarbons and polar

heterocompounds or NSO compounds (Radke et al., 1980). For each sample, there is a precolumn (100 mm x 10 mm) with thermally deactivated silica gel 100 (particle size 63-200 µm) which retains the polar compounds. The separation between aliphatic and aromatic compounds takes place on the main column (silica gel as in precolumns). Separation is monitored by a refractive index detector and a UV-vis detector (wavelength: 259 nm).

The fraction of aliphatic and alicyclic hydrocarbons is eluted with 32 mL *n*-hexane (flow rate 8 mL/min). Afterwards, the flow direction is reversed and the aromatic hydrocarbons are eluted from the main column with 70 mL *n*-hexane (flow rate 12 L/min). The polar compounds can be eluted from the precolumn with dichloromethane/methanol (10% v/v) by an external device. The solvent is evaporated and the samples blown to dryness with nitrogen before the fractions are weighed.

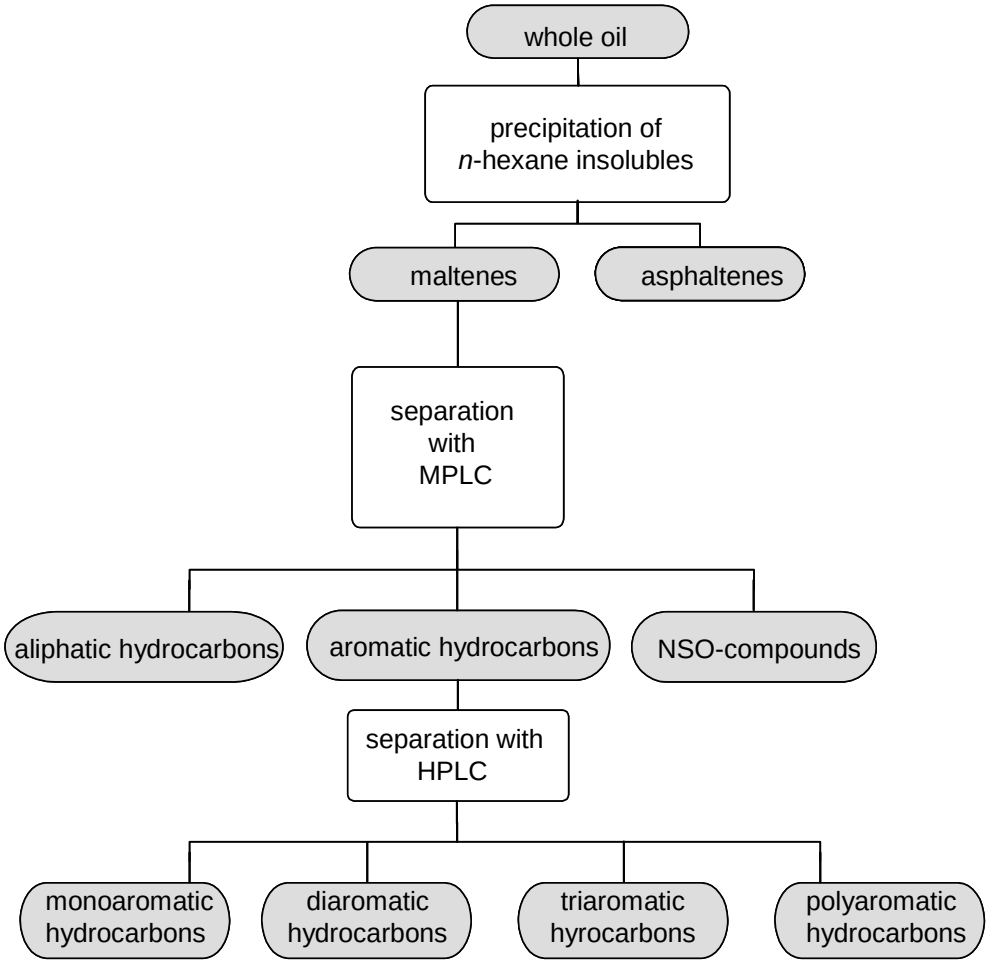


Figure 3-3 Simplified scheme for the isolation of the aromatic hydrocarbon fractions.

3.2.1.3 Normal-phase HPLC fractionation

Normal-phase HPLC separation was performed using a Beckman gold HPLC system with a pump module 126, a diode array detector 168 (DAD) and a computerised control and data evaluation system (Karat 32). UV-vis detection was performed mainly at 254 nm for

polyaromatics and 206 nm for monoaromatics. The DAD provided absorption spectra over the range of 190-550 nm.

For separation of the aromatic hydrocarbon fraction into mono-, di-, tri- and polyaromatic subfractions, a semipreparative Hypersil APS-2 column (250 mm x 10 mm x 10 μm) in series with a Hypersil APS-2 guard column (50 mm x 10 mm x 5 μm) was utilised (both ThermoHypersil-Keystone). With this column material (silica gel with aminopropyl groups) it is possible to separate aromatic hydrocarbons according to double bond equivalents (Killops, 1986). A column oven for the main column was set to 25°C in order to improve reproducibility. For all separations, *n*-hexane was used as eluting agent. Between the runs, the HPLC system was flushed with dichloromethane.

At first, a method for obtaining less complex mixtures of hydrocarbons for GC-MS identification was developed. A standard mixture of aromatic compounds was prepared and typical retention times were determined. *n*-Hexane (100%) with a flow rate of 1 mL/min for 60 minutes was used as eluent. The fraction collector was set to one-minute cuts. Standard compounds were identified by their relative retention time on the HPLC system. In addition, each one-minute-fraction was analysed by GC to identify the standard compounds in each fraction by comparing GC retention times.

Afterwards, total aromatic hydrocarbon fractions were separated and a selected number of samples was analysed by GC-MS in order to define the HPLC retention time at which diaromatic compounds appeared first. Since alkylbenzothiophenes eluted before alkylnaphthalenes, the retention time of these diaromatic sulphur compounds defined the cut between the monoaromatic and diaromatic fraction. For the cut between diaromatic and triaromatic fractions, the retention time of alkyldibenzothiophenes was used. From this, a preparative method for a clean separation of the monoaromatic hydrocarbon fraction from higher aromatic components was designed. 15 mg of the total aromatic fraction were injected per run. Fractions were collected according to retention times and UV absorption minima (if visible) as described above. The solvent was 100% *n*-hexane with different flow rates (Table 3-3) and as before, fractions were collected each minute.

Table 3-3 HPLC method for separating monoaromatic hydrocarbons from polyaromatic compounds using *n*-hexane as solvent.

Time programme	Flow rate	duration
0 – 15 min	1.0 mL	15 min
15 – 55 min	0.5 mL	40 min
55 – 70 min	1.0 mL	15 min
70 – 80 min	5.0 mL	10 min

One-minute-fractions were combined into fractions of mono-, di-, tri- and polyaromatic hydrocarbons, respectively (Table 3-4). The process was repeated to allow isolation of a

sufficient quantity of monoaromatic fraction of each oil. All aromatic hydrocarbon subfractions were analysed by GC and GC-MS. It is possible, however, that some compounds in the samples were not GC-amenable such as high-molecular-weight compounds and thus eluded detection.

Table 3-4 One-minute-cuts were combined to give mono-, di-, tri- and polyaromatic hydrocarbon fractions obtained by combining one-minute subfractions according to HPLC separation in Table 3-3.

Hydrocarbon fraction	From minute fraction	until minute fraction
Monoaromatic	20-21	30-31
Diaromatic	31-32	46-47
Triaromatic	47-48	59-60
Polyaromatic	60-61	79-80

3.2.1.4 Determination of sulphur in oil samples and hydrocarbon fractions

Total sulphur content of the whole oil, the fractions of aliphatic/alicyclic hydrocarbons and aromatic hydrocarbons were measured, using a LECO SC 444 elemental analyser. Sulphur is oxidised to sulphur dioxide at a temperature of 1400°C. Sulphur dioxide is measured via infrared detection. An integrated computer calculates the sulphur content from the intensity-time signal and the amount of sample with a calibration curve. Two calibrations were carried out, one for a sulphur content around 0.2% and one for amounts around 3%, using standardised reference materials containing 0.5% and 1% sulphur, respectively.

In order to confirm instrument performance, whole oil measurements were repeated once for samples with a high sulphur content (Monterey Formation oils) and twice for those with a low sulphur content (Vienna basin oils). Standard deviations were within ± 3% for a high sulphur content and within ± 5% for low values for oils and standards. Due to small amounts of sample, aliphatic and aromatic hydrocarbon fractions were only measured once. Sample amounts varied between 40 mg and 100 mg.

3.2.2 Ecotoxicological methods

Toxicity experiments including the preparation of test solutions and exposure and maintenance of mussels were conducted in a constant temperature room at 15°C. In general, experimental details were the same for the setups used in Plymouth and in Wilhelmshaven. A slightly modified method from Smith (2002) was used. Table 3-5 gives an overview over all toxicity tests performed in the course of this study.

3.2.2.1 Collection and maintenance of mussels (*Mytilus edulis*)

Blue mussels of 12-15 mm size were collected from the intertidal zone at Port Quin, Cornwall, UK, for the experiments performed at Plymouth laboratories (set Ply, experiments 1 and 2, see Table 3-5). Mussels were cleaned of epibionts and sediment and held in glass tanks in natural seawater from the Plymouth Sound (pipeline to the laboratories). All

glassware used was rinsed with deionised water and seawater before use for seawater solutions and mussels. Tanks were provided with air supplies. For the experiments performed at Wilhelmshaven (sets Whv-I and Whv-II, experiments 3 to 6), mussels were collected from the intertidal zone 1 km away from the Terramare Research Centre. Mussels were cleaned and held in glass tanks in natural seawater from the North Sea (pumped into a settling pond outside the laboratories and then stored at 15°C) and supplied with air. Mussels were acclimated to laboratory conditions for at least 7 days before use in any experiment. Conditions were kept constant since there are indications that blue mussels may react to environmental changes by slightly modifying their biochemical or physiological performance (Hawkins and Bayne, 1992).

In both sets, mussels were fed with an algal culture of *Isochrysis galbana*, which is a small brown flagellate (5-6 µm). The Instant Algal® concentrate was produced by Reed Mariculture, California, and bought from Cellpharm and Varicon Aqua Solutions (both Malvern, GB). It is commonly used for feeding in shellfish hatcheries.

Table 3-5 Overview over the nominal aqueous concentrations of the oil fractions applied in the six experiments of the three toxicity test sets, Ply = experiments carried out in Plymouth, Whv I and Whv-II = experiments carried out in Wilhelmshaven.

Set label	Experiment number	Oil fraction applied to mussels	Nominal aqueous concentrations of oil fractions [µg/L]
Ply	1	Total aromatic hydrocarbon fractions of the Monterey Formation oils	10, 50, 100, 200 and 500
	2	Monoaromatic hydrocarbon fractions of the Monterey Formation oils	50, 100 and 200
Whv-I	3	Total aromatic hydrocarbon fractions of the Vienna basin oils	50, 100, 200 and 500
	4	Monoaromatic hydrocarbon fractions of the Vienna basin oils	50, 100 and 200
Whv-II	5	Total aromatic hydrocarbon fraction of the degraded Monterey Formation oil	100 and 200
	6	Total aromatic hydrocarbon fraction of the degraded Vienna basin oil	100 and 200

3.2.2.2 Preparation of toxicant solutions

Toxicant solutions were prepared using 45-50 µm filtered seawater. Acetone was used as a solubility mediator to avoid inhomogeneity of aqueous solutions of hydrocarbon mixtures (see Chapter 2.5). Glass aspirators were filled with 10 L seawater and magnetically stirred to create a vortex. The test compounds were dissolved in acetone and 100 µL were discharged directly into the seawater, ensuring that any emulsion was held in the vortex until it was dissolved. Stock solutions of monoaromatic hydrocarbons were prepared with concentrations of 5 to 20 mg/mL acetone, of which 100 µg/L were added to 10 L seawater to give concentrations between 50 and 200 µg/L. The total aromatic hydrocarbon fractions with

concentrations of 10 to 500 µg/L seawater were prepared from hydrocarbon stock solutions in acetone, containing between 1 and 50 mg/mL.

For establishing control feeding rate data, pure acetone (100 µl) was also added in the same manner to 10 L of seawater. Previous studies by Donkin et al. (1989) and Wraige (1997) demonstrated that acetone has no effect upon mussel feeding rate in the concentration used (0.001% v/v). The aspirators were closed with seal-tight lids to prevent evaporation of volatile compounds. The solutions were stirred for 2 h before use and directly used for the exposure experiments in order to minimise loss of aromatic hydrocarbons from solution.

3.2.2.3 Exposure of mussels to toxicants

The experimental setup used (Donkin et al., 1991) was modified by Wraige (1997) and Smith (2002). A simplified scheme can be found in Figure 3-4. 7 mussels (12-15 mm) were placed in a 2 L tall glass beaker with 1.4 L of toxicant solution (see 3.2.2.2). Water movement was maintained by a small magnetic stirrer without the stirrer bar touching the mussels. The top of each beaker was loosely covered with a lid to prevent particles from falling into the beakers. Air was supplied via tubes and the mussels were continuously fed with an algal culture of *Isochrysis galbana*, controlled by means of a peristaltic pump. All exposure concentrations and controls were performed in duplicates, i.e. 14 mussels were exposed to each toxicant solution at a certain concentration.

The static system used is inferior to a flow-through system regarding several aspects but isolation of the aromatic hydrocarbon fractions and in particular the monoaromatic ones from the oils is too time-consuming to produce sufficient material for using a flow-through system with its required large volume of contaminated water.

3.2.2.4 Measurement of mussel feeding rate

After the 24 h exposure period, mussels were transferred to a smaller beaker each, containing 200 mL of toxicant solution. A 30 minute acclimatisation period was allowed for the mussels to re-open their valves and resume pumping. Then, a predetermined volume of algal culture was added to each beaker giving a concentration of 24000-30000 cells/mL. Each beaker was carefully stirred to ensure an even distribution of the dead algae. A 20 mL aliquot was removed using a syringe and placed in a 20 mL glass vial. After 30 minutes, a further 20 mL aliquot was removed and a third one after another 15 minutes (Smith, 2002). The cell numbers of these samples were determined in triplicate, using a Beckman Coulter Z 2 counter (Ply), an industrial model D Coulter Counter (Whv-I) or a Casy 1 cell analyser system (Whv-II). The model Z 2 Coulter Counter and the Casy system were set to measure particles between 3 and 11 µm, the model D Coulter Counter could only be set to measure particles greater than 3 µm. For the Casy 1 system, samples had to be diluted (1:10) with Isoton® to keep to the calibrated range of cell counts.

From the difference in cell counts, the feeding rate was calculated. The following equation was used,

$$\text{Feeding Rate [L/h]} = ((V * 60 \text{ min}) / \text{time}) * \ln(Nt_0) - \ln(Nt_1)$$

where V is the volume of 0.2 L toxicant solution per mussel, time denotes the time in minutes between sampling for cell counting (30 min) and Nt_0 and Nt_1 are the mean algal cell counts directly after adding the algae to the mussel (t_0) and after 30 minutes (t_1).

As a control, a beaker with the same volume of algae solution but without mussel was also set up with each feeding rate measurement.

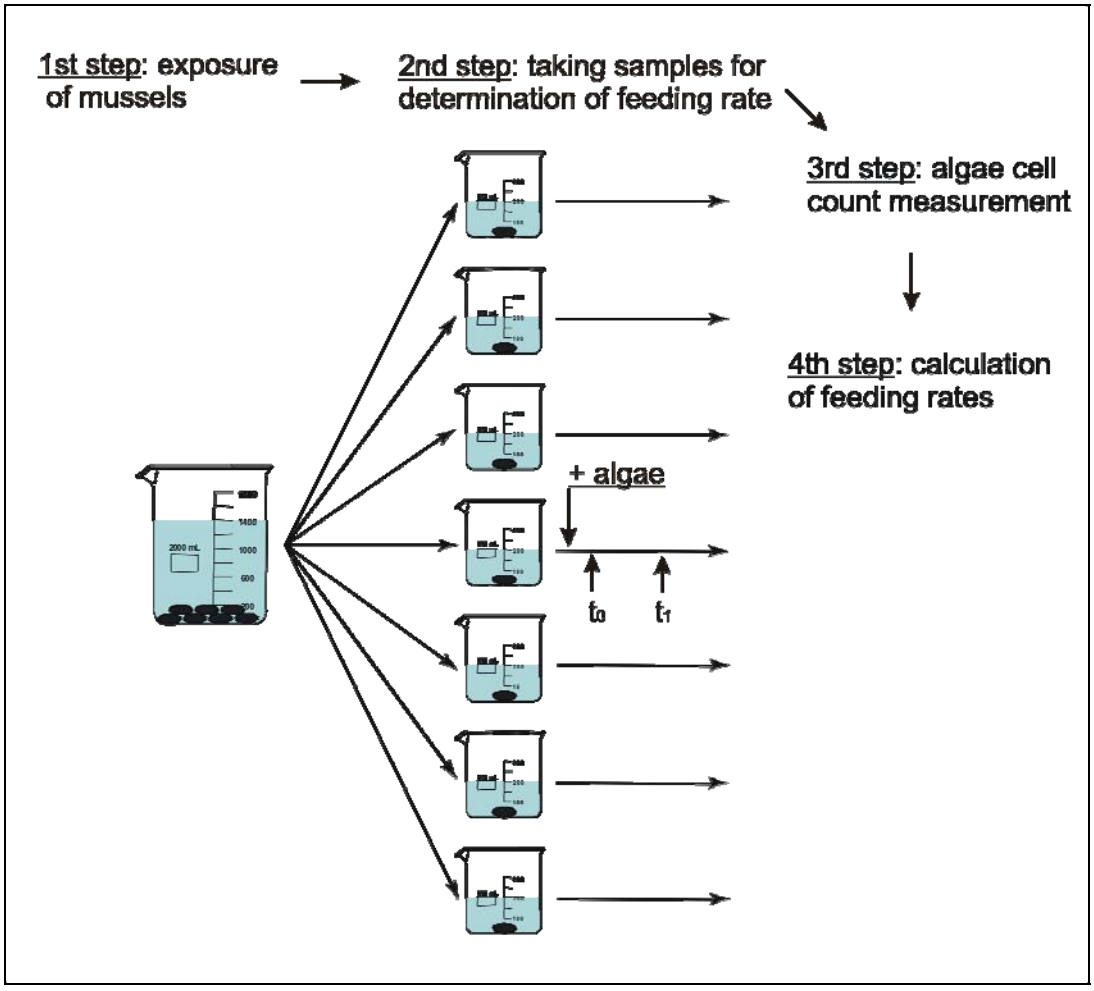


Figure 3-4 Scheme for the exposure to toxicant solutions and subsequent determination of feeding rates, t_0 and t_1 denoting the sampling of aliquots for the algae cell count.

3.2.2.5 Standardisation of toxicity test

As a toxicity test standard, a toxicant solution of *n*-nonane (100 µg/L seawater) was prepared in the same manner as the other toxicant solutions. This exposure was performed at the beginning of the experiments and was conducted identically to the other exposure experiments. Previous studies showed that *n*-nonane is toxic to blue mussels and the toxicant solution was expected to reduce the feeding rate by 50% (Donkin et al., 1989).

3.2.2.6 Statistical examination of feeding rate data

Firstly, the feeding rates of the 7 mussels from one exposure were tested for normal distribution with standardised skewness and kurtosis. In a few cases, outliers were defined due to e.g. the death or inactivity of individual mussels, thus the resulting number of feeding rates is 6 for some groups. Mean feeding rate values and corresponding standard errors were calculated. The standard error is the standard deviation divided by the square root of the number of values, in this case 6 or 7 mussels, which thus gives a measure of the biological variability of the individual animals.

Subsequently, statistical difference between each set of two duplicates was tested with analysis of variance (ANOVA). Groups of control mussels exposed to acetone were analysed for statistical difference (with ANOVA). Groups without statistical difference were pooled to give a mean feeding rate value. Groups of mussels exposed to toxicants were analysed for statistical difference compared to the pooled controls (with ANOVA). ANOVA was calculated after Fisher´s least significant procedure at the 95% confidence level ($p = 0.05$). For statistical data analysis, StatGraphics Plus Version 5.1 (Manugistics Inc.) was used.

3.2.3 Extraction of samples from the toxicity tests

3.2.3.1 Determination of dry weight conversion factors for the mussels

Approximately 0.5 g of mussel tissue was weighed into a small crucible made from aluminium foil and heated for 16 h at 105°C. The difference in mussel tissue was determined by back-weighing the crucibles and the ratio wet-to-dry weight was calculated. Thus, conversion factors were gained. The procedure was performed several times to aquire mean conversion factors. For the mussels collected from Cornwall in November 2003, the mean conversion factor was 6.3. Mussels collected from Wilhelmshaven in March 2004 had a conversion factor of 9.2 and mussels from November 2004 a factor of 8.4. In general, differences in wet-to-dry weight ratios may derive from variations of the lipid, protein and glycogen content of blue mussels during a yearly cycle but these are not necessarily different in March compared to November (Zandee et al., 1980). The conversion factors were used to translate accumulation of hydrocarbons in µg/g wet weight to dry weight since most of the literature data refer to dry weights.

3.2.3.2 Extraction of mussel tissue

Mussels from Ply experiments were frozen at -20°C, dissected and weighed before analysis. Mussel tissue from Whv-I and Whv-II experiments was dissected before storage in vials at -20ºC until required for analysis. After thawing, the whole mussel tissue of the seven mussels, which had been in the same exposure vessel, (usually about 0.5 g) was hydrolysed. An internal standard compound (ISTD) was added (see 3.2.4.1).

Once cooled, 10 mL of *n*-hexane were added and tubes vigorously shaken for 5 minutes. The samples were then centrifuged at 2000 rpm for 10 minutes and the upper *n*-hexane layer transferred to a conical flask. This process was repeated. A few samples formed gelatine-like matter and were extracted a third time. The hexane extract (20 mL) was dried over anhydrous sodium sulphate overnight and filtered over Na_2SO_4 on cotton wool. The solvent was removed with a rotary evaporator and with a gentle stream of nitrogen to 100 µL sample volume. These total organic extracts were then analysed by gas chromatography.

3.2.3.3 Separation of polar compounds from tissue extracts

The total organic extracts of mussel tissue contained a lot of compounds from the mussels themselves. Cholesterol was the dominant component. Thus, extracts were separated by column chromatography into fractions of polar and apolar substances for more precise quantification of accumulated hydrocarbons.

For this, a Pasteur pipette was filled 4 cm high with silica gel 60 (particle size 40-63 µm, Merck), which had been activated at 190°C for 2 h and then deactivated with 5% pure water. The silica gel was rinsed with 2 mL of *n*-hexane/dichloromethane (10:1), then the 100 µL total organic extract were applied. The fraction of non-polar compounds was eluted with 4 mL of this solvent mixture. The fraction of polar compounds was eluted with 4 mL of a dichloromethane/methanol (1:1) mixture, followed by 2 mL of methanol. The solvent was evaporated and the extract transferred to a microvial in a final sample volume of 100 µL. The fractions of apolar hydrocarbons were analysed by GC and GC-MS.

3.2.3.4 Re-extraction of toxicant solutions

500 mL of selected toxicant solutions (Table 3-6) were extracted three times with 50 mL dichloromethane. An internal standard (tetradecyl-octahydroanthracene) was added to the toxicant solutions from Whv-II. The solvent was evaporated and extracts transferred to a microvial and further evaporated to a final sample volume of 100 µL. The samples were then analysed by GC and some by GC-MS.

Table 3-6 Re-extracted toxicant solutions of aromatic hydrocarbon fractions, MA: monoaromatic hydrocarbon fraction, ARO: total aromatic hydrocarbon fraction.

Toxicant solution	Nominal aqueous concentrations [µg/L]	Experiment
Control	0	Experiment 5
MA of undegraded Vienna basin oil	100	Experiment 4
ARO of degraded Vienna basin oil	500	Experiment 3
ARO of degraded Vienna basin oil	50, 100, 200	Experiment 6
ARO of degraded Monterey Formation oil	50, 100, 200, 500	Experiment 5

3.2.4 Analytical techniques and quantification scheme

3.2.4.1 Standardisation

Internal standards (ISTD) were added after the toxicity test to the tissue samples because the standard compounds could have affected the toxicity tests in an unknown way. To the tissue samples of Ply and Whv-I experiments, deuterated methylnaphthalene and squalane were added as test ISTD at the beginning of the tissue extration. However, methylnaphthalene was too volatile and there was a high and irregular loss of this compound so that it was not further used. Recovery of squalane was 75% and 88% for procedural blanks. In some tissue samples though, only low amounts of the added squalane were recovered. Squalane may have a lower extraction efficiency than the aromatic hydrocarbons from the mussel tissue (Emma L. Smith, personal communication, 2004).

Due to the difficulties with methylnaphthalene and squalane, tetradecyl-octahydroanthracene was used as a standard compound for the tissue samples in setup Whv-II. Recovery of tetradecyl-octahydroanthracene was 69% and 93% for procedural blanks.

3.2.4.2 Technical details of GC analysis

GC analysis was performed with an HP 5890 gas chromatograph applying the following conditions:

GC	**HP 5890 Series II**
Injector	Gerstel® KAS 3 cold injection system
	T-Programme: 60°C (0.08 min) $\rightarrow$ 8°C*s^{-1} $\rightarrow$ 300°C (60 s)
Injection volume	1 µL (autosampler)
Carrier gas	Helium, constant pressure (12 psi = 0.83 bar)
Column	DB-5 fused silica column (J&W) 30 m length x 0.25 mm inner diameter; film thickness 0.25 µm temperature programme: 60°C (1 min hold time) to 305°C (50 min hold time) at 3°C*min^{-1}
Detector	FID; synthetic air: 300 mL*min^{-1}, H_2: 40 mL*min^{-1}, N_2: 30 mL*min^{-1}

Data were recorded and analysed with HPChemStation version A 09.01 software (Hewlett Packard).

3.2.4.3 Technical details of GC-MS analysis

GC-MS analysis was performed with a Finnigan SSQ 710B mass spectrometer applying the following conditions.

GC	**HP 5890 Series II**
Injector	Gerstel® KAS 3 cold injection system
	T-Programme: 68°C (0.08 min) → 10°C*s^{-1} → 350°C (2 min) splitless
Injection volume	1 µL or 2 µL (autosampler)
Carrier gas	Helium, constant pressure (12 psi = 0.83 bar)
Column	DB-5HT fused silica column (J&W), 30 m length x 0.25 mm ID; film thickness 0.1 µm
	temperature programme: 60°C (2 min) to 305°C (50 min) at 3°C*min^{-1}

MS	**Finnigan SSQ 710 B**
Electron energy	70 eV
Scanrate	1 scan*s^{-1}
Scanrange	50-650 u

Data were recorded and analysed with the *ICIS 7.1* software (Finnigan) and a computerised system (Digital).

3.2.4.4 External quantification of total body burdens from GC-FID analysis

The amounts of the aromatic UCM accumulated by the mussels were quantified from the FID gas chromatograms using an external calibration graph due to the difficulties encountered using the internal standard compounds mentioned above. The retention time range of 6-70 min of the apolar fraction of the mussel tissue was integrated for quantification. This interval was chosen because the visible “hump“ occurred in this retention time interval. In addition, some natural mussel tissue components like squalene eluted later than 70 min and thus were kept from affecting the quantification.

An external calibration graph with dodecylbenzene was used for quantification of body burdens of hydrocarbons. Dodecylbenzene combines both aliphatic and aromatic moieties and represents a typical component of the aromatic hydrocarbon mixtures. In addition, it elutes in the middle of the “hump“ and the integrated time interval and thus represents a large range of compounds in the “hump“. The regression ($y = 6241.3 * x + 243.1$, $r^2 = 0.9999$) was used for the whole range of body burdens.

Ideally, an external calibration should be performed with compounds as similar as possible to the analyte. In the case of an unresolved complex mixture, this is an almost impossible condition since the composition is largely unknown. It was attempted to use the

respective aromatic hydrocarbon fraction for quantification. For example, a calibration with several concentrations over a range from 0.1 to 5.0 µg/mL of a total aromatic fraction of one of the used oils was accomplished. However, the calibration range did not include the very low concentrations which had been found in some tissue samples. Thus, the concentration range of the total aromatic fraction did not represent the range of accumulated compounds. A possible explanation for this is that due to solubility partitioning processes during preparation of the toxiciant solutions, the original aromatic hydrocarbon fraction was not identical anymore to the hydrocarbon mixture accumulated within the mussel tissue. Another reason is that several high-molecular-weight compounds probably were not GC-amenable.

Quantification was performed using Chemstation™ (Hewlett Packard) software. Before integration of peak areas, a GC blank was automatically substracted from the samples to correct for the column bleed. The tissue extract concentration was determined from the total peak area, using the calibration curve. Body burden concentrations were calculated via the wet weights of the tissue samples. The background signal of compounds in the apolar fractions of the control mussel tissue samples were pooled for each set of experiments and are shown in Table 3-7.

Table 3-7 Mean background body burdens of control mussels with standard deviation and number of mussel group samples in the three sets of experiments, Ply = experiments carried out in Plymouth, Whv-I and Whv-II = experiments carried out in Wilhelmshaven.

Set of experiments	Pooled body burden of control mussels	sd	Number of control mussel groups
Ply	23.6 µg/g wet weight	1.5	2
Whv-I	18.6 µg/g wet weight	3.8	7
Whv-II	20.2 µg/g wet weight	3.5	4

The mean value was subtracted from the total amount of compounds in the exposed mussels of the respective set of experiments. In this way, the control mussels have a body burden of 0 µg/g. Some groups of mussels, which had accumulated very small amounts of compounds, have body burden values close to or below 0 µg/g. Since a few control mussel samples from set Ply were contaminated in the lab only two samples were used for the determination of the background signal.

3.2.4.5 Decontamination of contaminated apolar fractions of mussel tissue

Unfortunately, several mussel tissue samples from the toxicity tests (Ply) with the degraded Monterey Formation oil fractions and from some control experiments were contaminated with hydrocarbons during the separation of polar and apolar compounds. The contamination would have been quantified along with the accumulated aromatic compounds by the quantification method used. Thus, the samples had to be decontaminated.

It turned out that the contamination were purely saturated hydrocarbons. The samples were decontaminated by MPLC, set to the same conditions as before. For this step, anthracene-d_{10} was added to quantify the losses during MPLC separation and subsequent evaporation of solvent. One set of samples (mostly from experiments with the total aromatic hydrocarbon fraction) had recoveries around 60%. Another set of decontaminated samples (mainly from experiments with the monoaromatic hydrocarbon fraction) had basically no recoveries (around 10%) due to a problem with the evaporation unit. Thus, only the samples with recoveries around 60% are included in the results section.

3.2.4.6 Quantification of toxicant solution extracts

Tetradecyl-octahydroanthracene was added as ISTD to most toxicant solution samples. An external calibration curve with several concentrations of the aromatic hydrocarbon fraction of the degraded Vienna basin oil was determined on the gas chromatograph, as well. The results obtained by the external calibration were compared to the results from internal calibration. With a few exceptions, results were very similar (Table 3-8).

Table 3-8 Aqueous solutions of total aromatic hydrocarbon fractions and the ratio of the results from the external and the internal quantification method. Explanation of sample label: Whv-II = second set of experiments carried out in Wilhelmshaven, d = degraded oil, V = Vienna basin oil, M = Monterey Formation oil, followed by the nominal aqueous concentration (µg/L).

Toxicant solution sample	Ratio
Seawater control	0.96
Whv-II-dV-50	1.52
Whv-II-dV-100	0.78
Whv-II-dV-200	1.18
Whv-II-dM-50	1.12
Whv-II-dM-100	0.90
Whv-II-dM-200	0.76
Whv-II-dM-500	1.00

Values from the external calibration are used in Chapter 6 because they were quantified by the same method as the other samples without ISTD and are therefore more comparable.

3.2.4.7 Quantification of individual compounds in mass chromatograms

Total body burdens were externally quantified in the GC-FID chromatograms. Quantification of individual compounds in the GC-MS traces was also carried out with an external calibration. It has to be pointed out at this time that quantification of individual compounds was not the main focus of this work and thus, quantification strategies were not ideal. Therefore, the calculated amounts of individual compounds were used mainly for comparison between samples and are only semiquantitative. They were very tentatively related to literature data and to the amounts of total body burden derived from GC-FID analysis.

Individual components were quantified via the peak areas of diagnostic fragments (Table 3-9). This allowed the quantification of compounds which coelute with other constituents of the "hump" in the GC-FID chromatogram, especially in the very complex original oil fractions. Peak areas were integrated using the MS system software ICIS 7.1 (Finnigan).

Table 3-9 Diagnostic mass fragments of aromatic compounds used for quantification.

Compound	**Diagnostic fragment, *m/z***
Anthracene-d_{10}	188.2
Phenanthrene	178.2
C_1-Phenanthrenes (4 isomers)	192.2
C_2-Phenanthrenes (12 isomers, partly coelutions)	206.2
Dibenzothiophene	184.2
C_1-Dibenzothiophenes (3 isomers)	198.2
C_2-Dibenzothiophenes (7 isomers)	212.2

For external calibration, several concentrations of anthracene-d_{10} between 0.1 µg/ml and 20 µg/mL were analysed. This calibration was used for the phenanthrenes. A second calibration curve was aquired with dibenzothiophene, which was used for the sulphur-containing compounds. For analysis of parent and alkylated PAHs, the calibration curves of parent compounds are commonly applied to their alkylated homologues (e.g. Carls et al., 1999). Figure 3-5 presents the two calibration curves with regression lines. The respective regression coefficients indicate a high quality of the calibration.

With each suite of samples analysed by GC-MS, a standard mixture containing 10 µg/mL of anthracene-d_{10} and dibenzothiophene was measured, too. The ratio of the peak area derived from the calibration curve and the peak area of the standard compound in the standard mixture was calculated to correct for the daily deviations of the MS detector. With this factor, the amount of selected aromatic hydrocarbons in each sample was calculated. For the sulphur-containing compounds, the calibration curve of dibenzothiophene was used. Calculations with peak areas were done analogously to the quantification of pure hydrocarbons.

This calculation provided concentrations of the extracts of the tissue samples. In a last step, the wet weight of mussel tissues was included in the calculation. Usually, the amounts of C_0- to C_2-phenanthrenes or dibenzothiophenes, respectively, were added for comparison. For the calculation of ratios of phenanthrene to dibenzothiophene and methylphenanthrenes to methyldibenzothiophenes, peak areas were used directly.

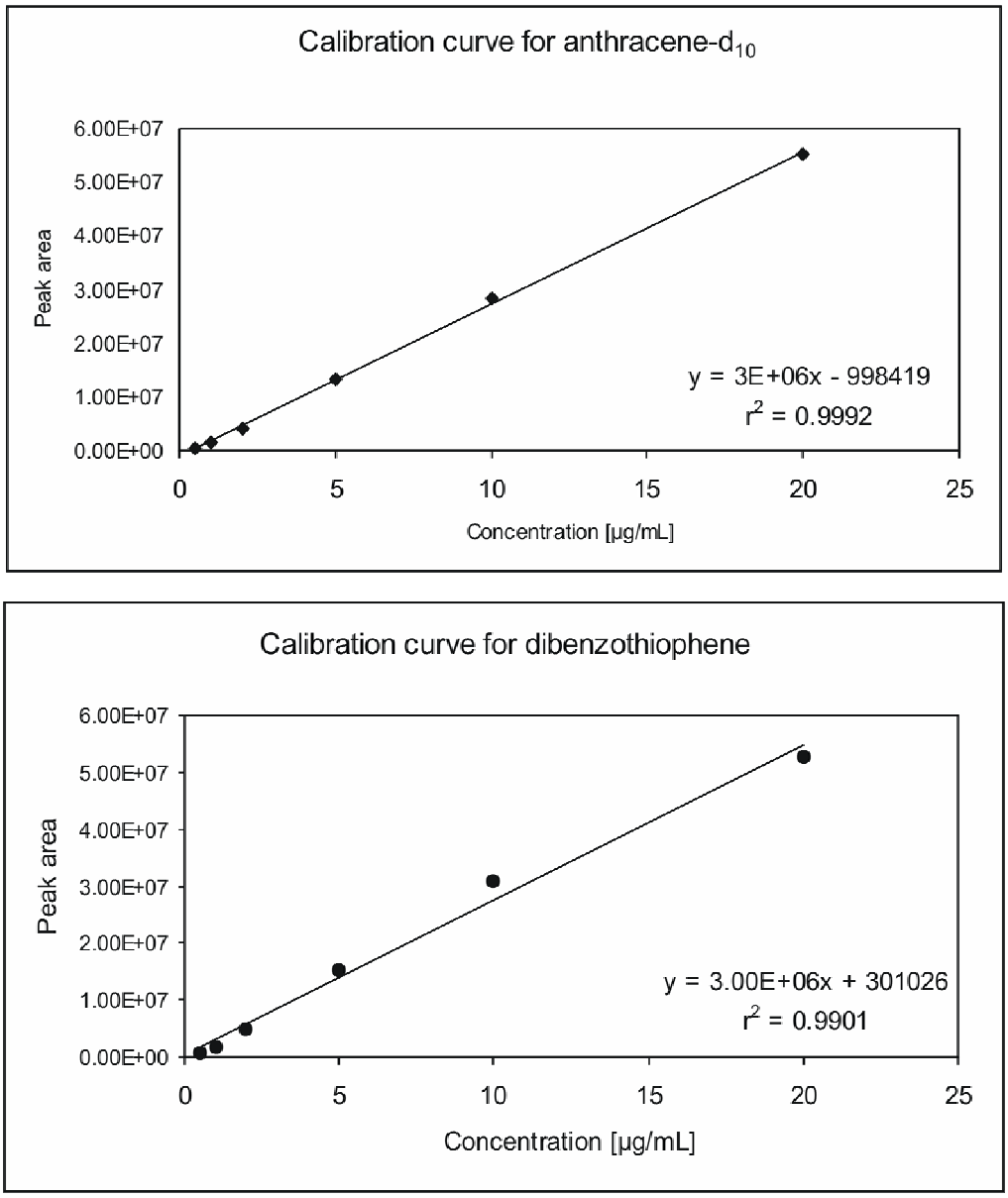


Figure 3-5 Calibration curves for anthracene-d_{10} (top) and dibenzothiophene (bottom) for GC-MS analysis, using *m/z* 188.2 and *m/z* 184.2, respectively.

3.2.4.8 Background compounds in procedural blanks

The determination of background values due to laboratory contaminants and sample workup is an important aspect of organic trace analysis. It helps to ascertain whether detected substances were indeed constituents of the original sample or entered during laboratory processes. Typical steps for the generation of such artefacts are solvent evaporation, time-consuming steps such as the manual clean-up column and auto-extraction of the septum in the sample vial caps by dichloromethane, especially at elevated temperatures during the summer months. In a few samples, a particular source of aliphatic contamination was impure *n*-hexane, which was tested for purity only randomly before but routinely after this incident.

Some typical contaminants of the laboratory background components such as phthalates and a homologous series of low concentrations of C_{18} to C_{31} *n*-alkanes can be seen in the total reconstructed ion current chromatogram (RIC) of the apolar fraction of a procedural blank (Figure 3-6). The procedural blank also suggests C_{20} and C_{22} fatty acids as potential contaminants during the extraction (Figure 3-6). In addition, there are minor amounts of siloxanes present, which may have come from the GC column (Figure 3-6).

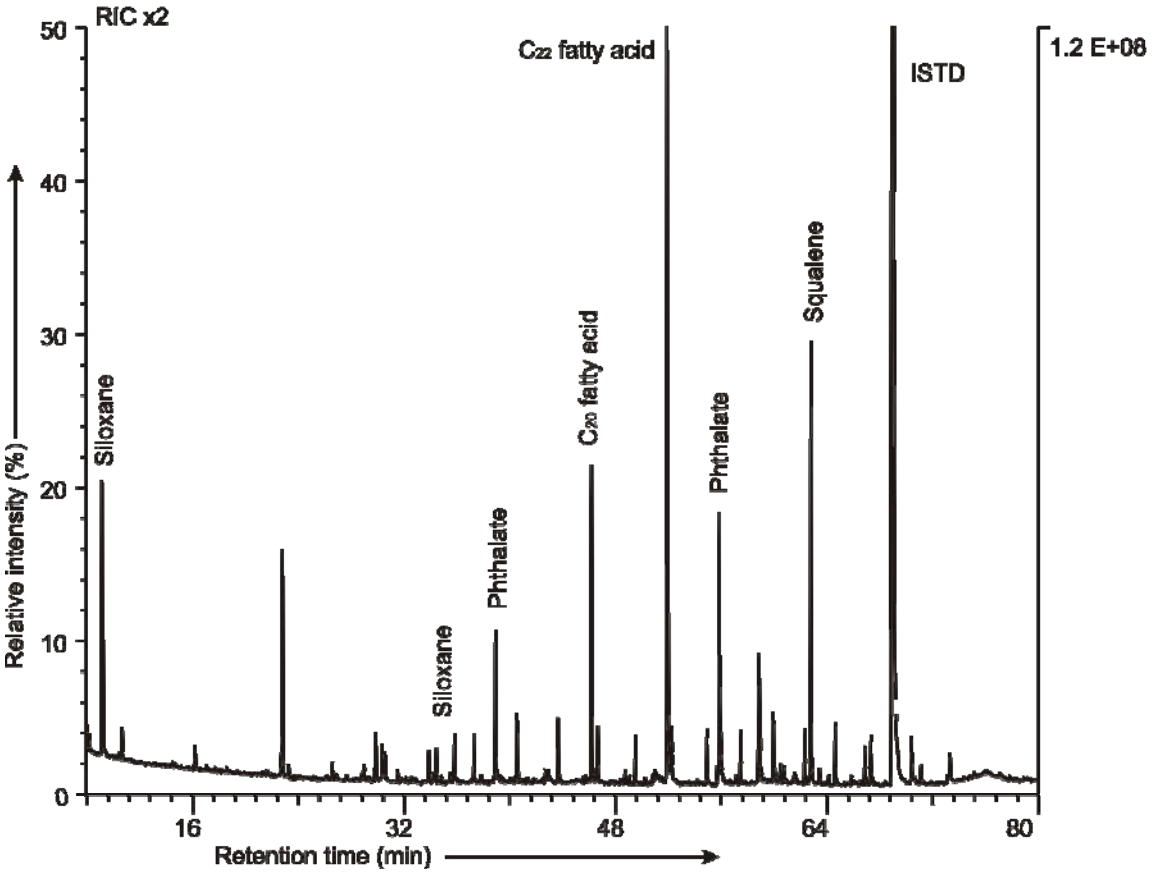


Figure 3-6 Background compounds in partial reconstructed ion current chromatogram of the apolar fraction of a procedural blank, ISTD: internal standard tetradecyloctahydroanthracene

4 Results and discussion – toxicity of aromatic hydrocarbon fractions from crude oils

Aromatic hydrocarbons are known to cause toxic effects on marine organisms. Various studies have shown that aromatic hydrocarbons are compounds of most concern in crude oils from an ecotoxicological point of view (Grant and Briggs, 2002). On the one hand, many ecotoxicological studies of selected PAHs in the laboratory as well as in field investigations report severe toxic effects (e.g. Rice et al., 1977 and references therein). On the other hand, many laboratory studies with aqueous solutions of whole oils showed toxic effects of low-molecular-weight aliphatic and aromatic (i.e. mono- and diaromatic) hydrocarbons (e.g. Neff et al., 2000). Different approaches in the studies impede the determination of an overall picture of the relative toxicity exerted by the different aromatic compound classes and in particular of the effect caused by a complex mixture of low- and high-molecular-weight compounds.

In this study, toxicity tests with the blue mussel *Mytilus edulis* were conducted with several mixtures of aromatic hydrocarbons isolated from four crude oils. The monoaromatic hydrocarbon mixtures and the total aromatic hydrocarbon mixtures from each oil were tested to determine the relative toxicity of monoaromatic and polyaromatic hydrocarbon mixtures from crude oils. Acetone was used as a carrier to dissolve not only low- but also higher-molecular-weight compounds of the various aromatic compound classes, as also performed by Smith (2002), who showed that a monoaromatic UCM was toxic to blue mussels by reducing the feeding activity.

In this chapter, the results of the toxicity tests performed in this study are presented and discussed. Two of the four oils come from the Monterey Formation in California, the other pair of oils is from the Vienna basin. In each oil pair, one oil is moderately biodegraded (for a general description of the oils see Section 3.1). Three sets of experiments were performed in the course of this study, one in Plymouth and two in Wilhelmshaven (Table 4-1).

Table 4-1 Toxicity experiments performed in this study, Ply = experiments carried out in Plymouth, Whv-I and Whv-II = experiments carried out in Wilhelmshaven.

Set label	Experiment number	Oil fraction applied to mussels
Ply	1	Both Monterey Formation oils; total aromatic hydrocarbon fractions
	2	Both Monterey Formation oils; mono-aromatic hydrocarbons
Whv-I	3	Both Vienna basin oils; total aromatic hydrocarbon fractions
	4	Both Vienna basin oils; mono-aromatic hydrocarbons
Whv-II	5	Degraded Monterey Formation oil; total aromatic hydrocarbon fraction
	6	Degraded Vienna basin oil; total aromatic hydrocarbon fraction

This chapter starts with results from the toxicity tests with the total aromatic hydrocarbon fractions of the four oils on the one hand (4.1) and the toxicity tests with the monoaromatic

hydrocarbon fractions of the four oils on the other hand (4.2). In Section 4.1, the results from the repeated exposures with the total aromatic hydrocarbon fractions of the biodegraded Monterey Formation and Vienna basin oils are included (Whv-II). Dose-response relationships of mussel feeding rates with the nominal aqueous concentrations of aromatic hydrocarbons and the accumulated amounts of aromatic hydrocarbons in the mussel tissue are presented and discussed (Section 4.3). A short summary follows in Section 4.4.

4.1 Toxicity of total aromatic hydrocarbon fractions to blue mussels

The crude oils were separated via automated column chromatography into fractions of aliphatic/alicyclic hydrocarbons, aromatic hydrocarbons and polar compounds. The fractions of total aromatic hydrocarbons were dissolved in acetone and seawater solutions prepared at different nominal concentrations between 10 and 500 µg/L.

Blue mussels were exposed to these toxicant solutions for 24 hours and their feeding rates measured. Subsequently, mussel tissue was extracted and the accumulated hydrocarbons were quantified. Control mussels were exposed to an non-toxic concentration of acetone (Donkin et al., 1989; Wraige, 1997; Smith, 2002). The feeding rates of control mussels from the three sets of experiments are listed with their respective standard errors in Table 4-2. The quantity of control mussels (n) varied for the three sets (Table 4-2). Statistical differences of feeding rates of exposed mussels were calculated at the 95% limit (see Section 3.2.2.6). Statistically significant differences depend on the mean value and on the standard deviation of compared groups.

Table 4-2 Mean feeding rates of control mussels and the reduction of feeding rates by duplicate exposures to *n*-nonane [nominal aqueous concentration 100 µg/L]; asterisks indicate statistically significant differences from mean feeding rate of control mussels.

Set	Mean feeding rates of control mussels	Feeding rate reduction by *n*-nonane
Ply	0.26 L/h ± 0.02	46% * (n = 7)
	n = 55	46% * (n = 7)
Whv-I	0.22 L/h ± 0.02	68% * (n = 7)
	n=68	14% (n = 7)
Whv-II	0.19 L/h ± 0.03	32% (n = 5)
	n = 25	11% (n = 7)

For all sets of experiments, the standard compound *n*-nonane (at the concentration of 100 µg/L) with known toxicity was applied to the blue mussels (n = 7) in order to note differences in sensitivity and behaviour of the mussels in general and to assess comparablility to previous data by Donkin et al. (1989, 1991), Smith (2002) and Wraige (1997). Expected reduction of feeding rate by 100 µg/L *n*-nonane was approximately 50% (Emma Smith, personal communication and unpublished data, 2003).

4.1.1 Toxicity tests with two Monterey Formation oil fractions (experiment 1)

Blue mussels exposed to aqueous solutions of total aromatic hydrocarbon fractions accumulated unresolved complex mixtures of compounds in contrast to control mussels (Figure 4-1). The most abundant compound in both chromatograms is squalene, which is a biogenic component in mussel tissue (de Zwaan and Mathieu, 1992) and was also present in the blanks (see Figure 3-6). The bottom chromatogram in Figure 4-1 shows that the mussels took up not only individual resolved compounds but also compounds from the unresolved complex mixture. This signifies the environmental relevance of these unresolved compounds. They can be accumulated by the mussels and consequently can cause damage since it is known that tissue concentrations are more important than nominal aqueous concentrations (Baussant et al., 2001b).

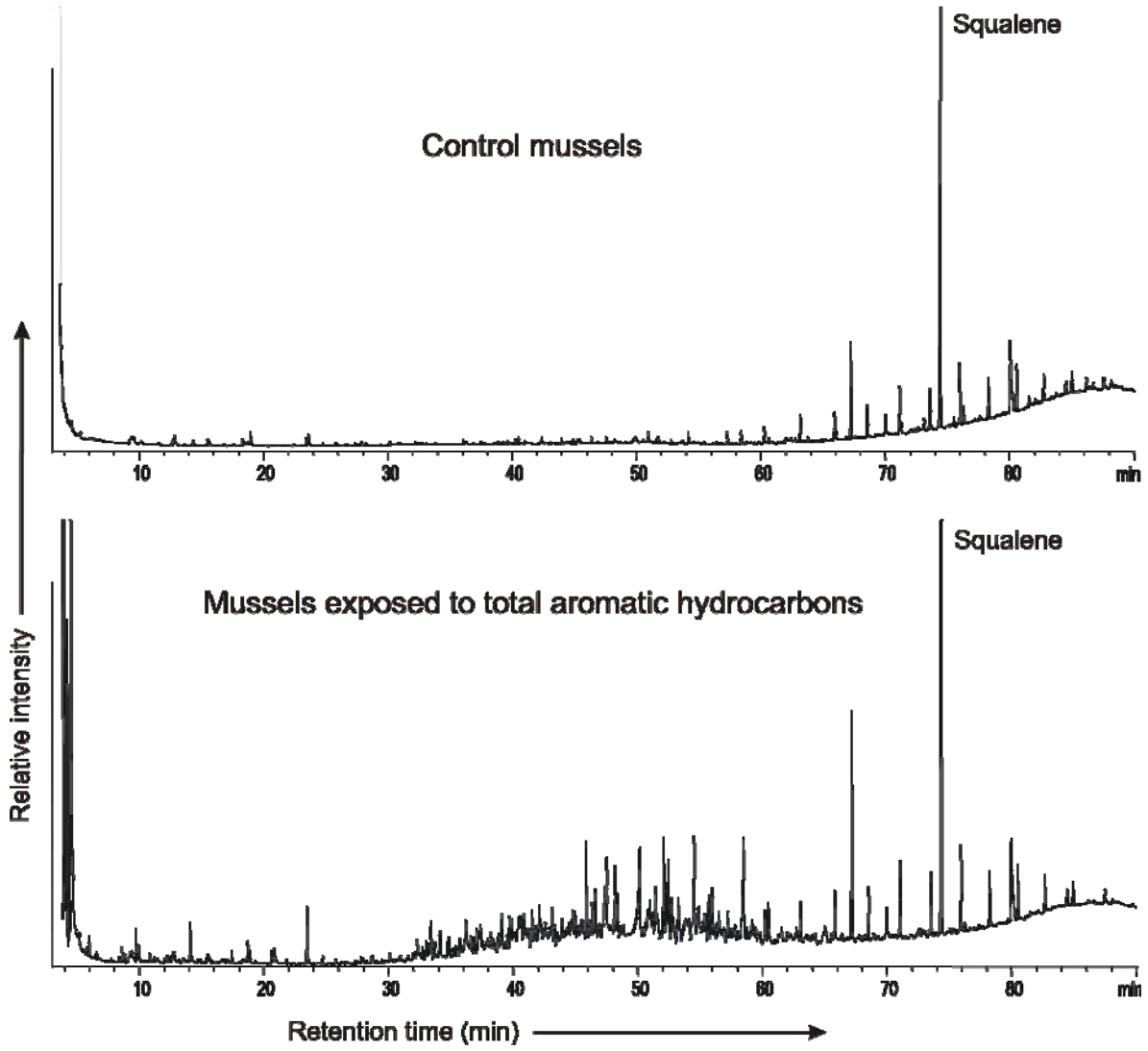


Figure 4-1 Gas chromatograms of the apolar fraction of tissue extracts from control mussels (top) and mussels exposed to total aromatic hydrocarbons of the undegraded Monterey Formation oil (bottom).

For quantification of the amounts of accumulated compounds, the mean body burden value of control mussels, which is derived from biogenic compounds, was subtracted from

the body burden values of the exposed mussels. Thus, mussels which had not accumulated any hydrocarbons during the exposure have body burden values (= tissue concentration) close to zero, with slight differences in the content of biogenic compounds and the method of quantification leading to values below zero in a few cases. All body burden data are shown after subtraction of the control background value (see Table 3-7). Since all seven mussels of one duplicate were pooled together for extraction, there was only one sample per exposure experiment for quantification. In this chapter, solely the quantitative aspect of the body burdens will be investigated. Their composition will be discussed in Chapter 6.

The results of the exposure experiments to the two total aromatic hydrocarbon fractions of the Monterey Formation oils are listed in Tables 4-3 and 4-4. For each exposure concentration, two sets of seven mussels were used. The duplicates are shown in the tables.

Table 4-3 Nominal aqueous concentrations [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of control mean [%], reduction of feeding rate [%] and body burdens [µg/g ww] of mussels exposed to the total aromatic hydrocarbon fraction of the undegraded Monterey Formation oil, no red.: no reduction since FR > 100%.

Nominal aqueous concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	55	0.26 ± 0.02	100	0	0
10	7	0.23 ± 0.03	88	12	-3.6
10	7	0.27 ± 0.04	102	no red.	n.d.
100	7	0.20 ± 0.03	77	23	22.5
100	7	0.17 ± 0.02	66	34	14.1
200	7	0.16 ± 0.02	61	39	42.9
200	6	0.11 ± 0.02	44	56	31.8
500	7	0.17 ± 0.03	63	37	19.7
500	7	0.27 ± 0.04	102	no red.	177.5

Table 4-4 Nominal aqueous concentrations [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of control mean [%], reduction of feeding rate [%] and body burdens [µg/g ww] of mussels exposed to the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil, no red.: no reduction since FR > 100%.

Nominal aqueous concentration [µg/L]	n	Feeding rate[L/h]	FR [%]	Reduction of FR [%]	Body burden[1] [µg/g ww]
0 (Controls)	55	0.26 ± 0.02	100	0	0
50	7	0.32 ± 0.03	123	no red.	n.d.
50	7	0.19 ± 0.03	73	27	14.0
100	7	0.30 ± 0.02	114	no red.	82.3
100	7	0.17 ± 0.02	63	37	97.5
200	7	0.18 ± 0.04	67	33	43.6
200	7	0.16 ± 0.02	61	39	32.8
500	7	0.21 ± 0.04	78	22	7.1
500	7	0.22 ± 0.05	82	18	47.6

[1] The tissue samples of the mussels used in the experiments with the degraded Monterey Formation oil were contaminated with aliphatic compounds during the clean-up step. Body burdens were calculated after decontamination (see Section 3.2.4.5).

The data from Tables 4-3 and 4-4 are plotted in Figures 4-2 and 4-3.

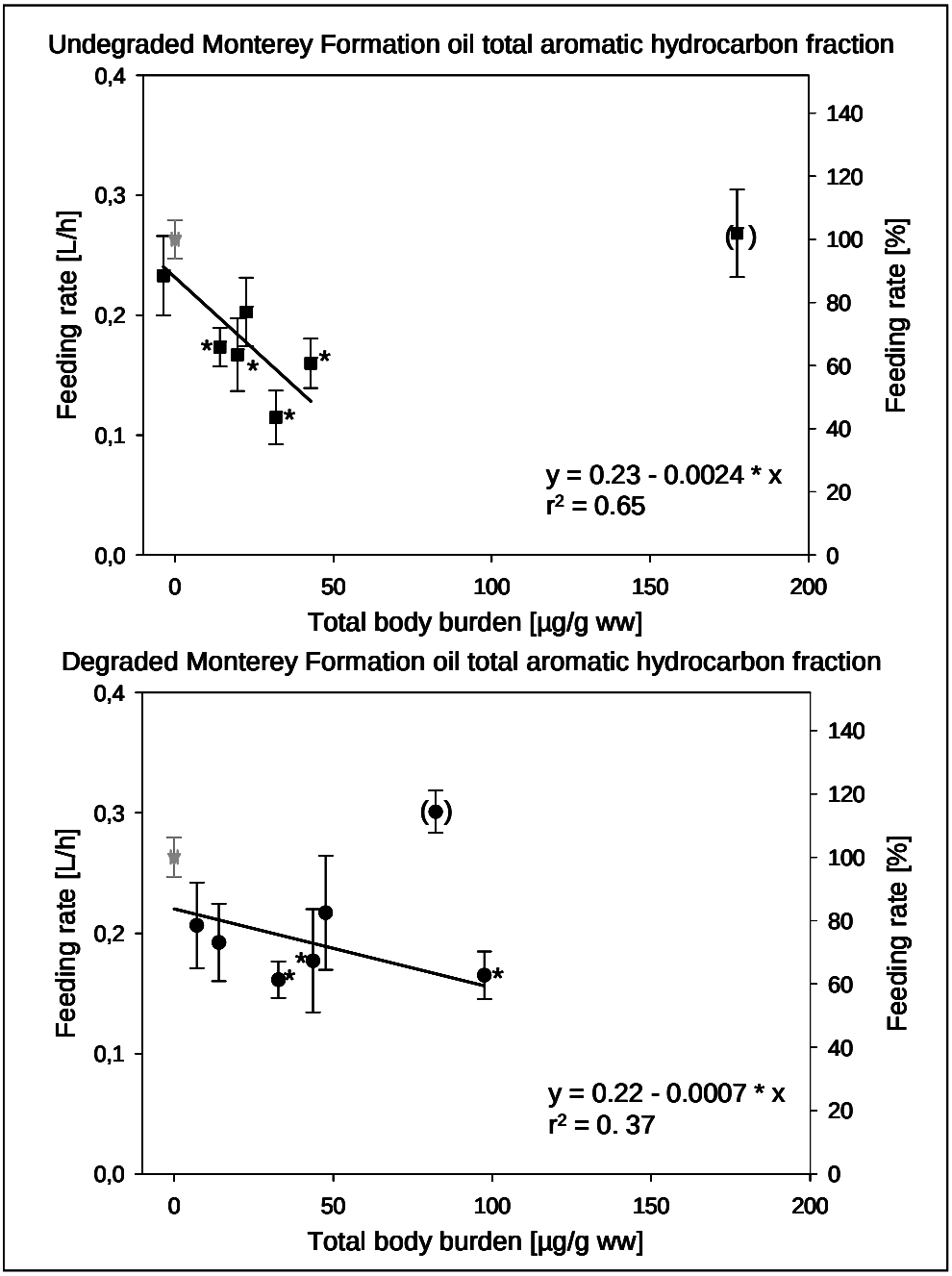


Figure 4-2 Total body burden-feeding rate curves from the experiments with the two total aromatic hydrocarbon fractions of the Monterey Formation oils. The control mean (grey triangle) and data points are shown with error bars (se) for feeding rate mean values. Asterisks denote statistically significant differences from the control mean feeding rate. The tissue concentration of the control is shown ± standard deviation (n = 2). Regression lines, regression equations and coefficients are also shown. Data points in parenthesis are considered outliers and were not included in the calculation of the regression.

Most groups of mussels exposed to the aqueous solutions of total aromatic hydrocarbons of the undegraded Monterey Formation oil took up body burdens up to approximately 50 µg/g wet weight (Figure 4-2, top). The feeding rate decreases rapidly until the tissue concentration of 50 µg/g wet weight is reached. One group of mussels retains a

high feeding rate in spite of an extraordinarily high body burden of 177 µg/g wet weight. It was considered an outlier (in parenthesis).

The bottom plot in Figure 4-2 shows the tissue concentrations in the mussels exposed to the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil. The feeding rate declines whereas tissue concentrations increase to 100 µg/g wet weight. The decline is less pronounced compared to the experiments with the undegraded oil fraction. Similar to the top diagram, one data point shows a high feeding rate in spite of a high body burden. It does not fit into the general trend and was also considered an outlier (in parenthesis). Outliers were not included in data analysis. For both plots, regression lines with regression equations and coefficients are given. The higher regression coefficient for the experiment with the undegraded oil fraction indicates a stronger correlation of increasing tissue concentration and feeding rate reduction. This and the statistically significant differences in feeding rates indicate a possible higher toxicity compared to the degraded oil fraction.

In the diagrams displaying nominal aqueous concentrations and mussel feeding rates, the mussels exposed to the total aromatic hydrocarbon fraction of the undegraded Monterey Formation oil (Figure 4-3, top) at the nominal aqueous concentration of 10 µg/L did not show a reduction of feeding rate compared to the control value. This concentration appeared to be too low and was not used again. At nominal aqueous concentrations of 100 µg/L and 500 µg/L, the feeding rate of one duplicate group was reduced compared to the control, exhibiting typical variations for duplicate vessels in this experimental setup. Mussel feeding rates after exposure to the degraded oil fraction (Figure 4-3, bottom) also show some differences between duplicates. For example, at the concentration of 50 µg/L, one set of seven mussels shows a decline in feeding rate and the other mean value increases.

Asterisks in Figures 4-2 and 4-3 denote statistically significant differences compared to the mean feeding rate of the control mussels[2]. No statistically significant differences in feeding rates may well be due to biological variance. Fluctuations in mean feeding rates of 2-5% were observed before (Wraige, 1997) and can be attributed to the natural variations in the feeding behaviour of the animals.

[2] Note: All figures show standard errors for feeding rate values but calculation of statistically significant differences is based on standard deviations.

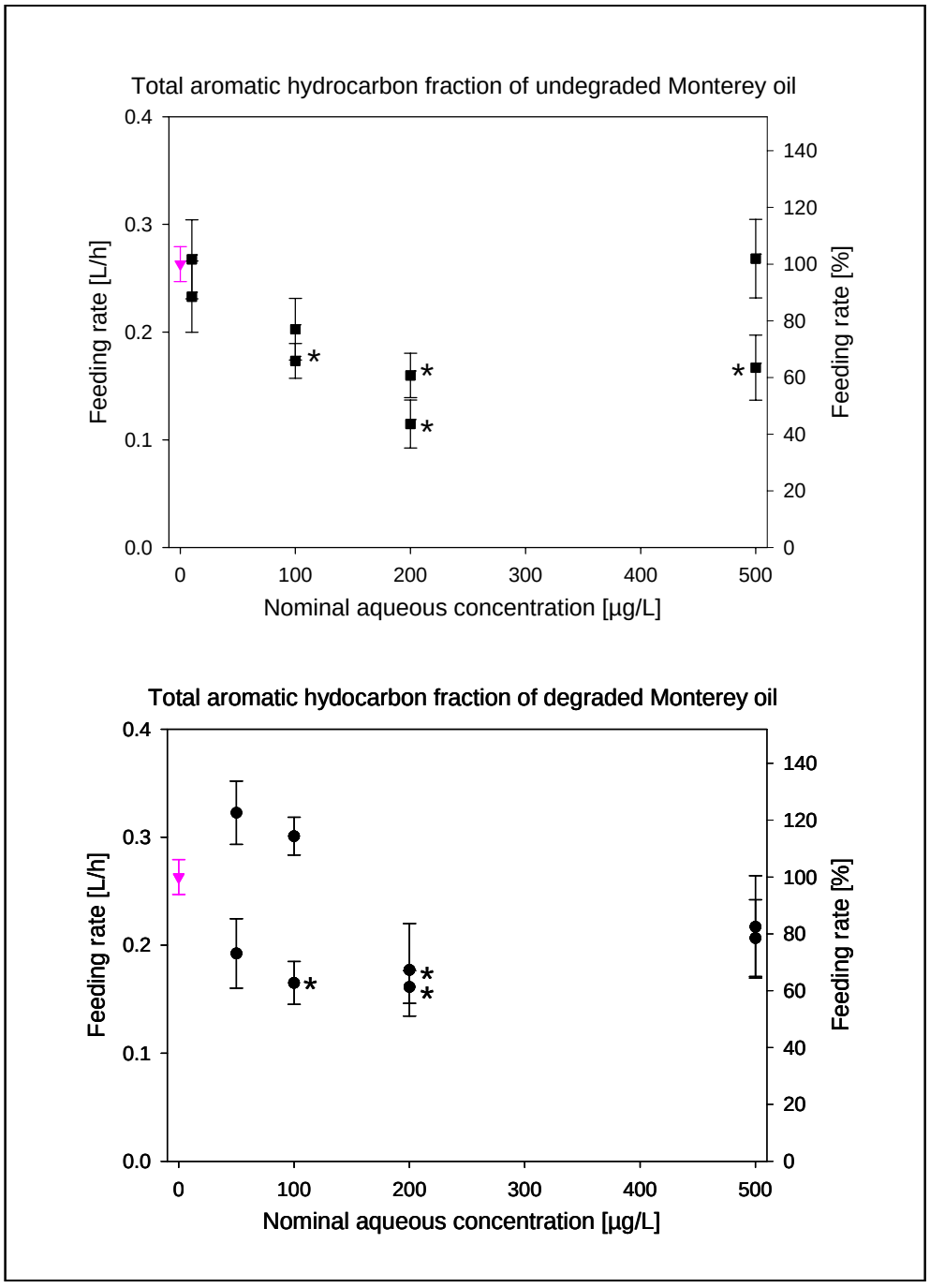


Figure 4-3 Nominal aqueous concentration-feeding rate diagrams for the toxicity tests with the two Monterey Formation oil total aromatic hydrocarbon fractions. The control mean (grey triangle) and data points are shown with error bars (se). Asterisks denote statistically significant differences from the control mean.

The dose-response relationships in Figure 4-2 and Figure 4-3 show a decline in feeding rate with increasing aqueous and tissue concentrations but slight differences are apparent. For the undegraded Monterey Formation oil fraction (top), the lowest feeding rate appears at an exposure to 200 µg/L of the undegraded oil fraction (Figure 4-3). In one group of mussels exposed to the 500 µg/L nominal concentration of the total aromatic hydrocarbon fraction of the undegraded Monterey Formation oil, the feeding rate is reduced in a similar range compared to the reduction at 200 µg/L. The other group, however, does not show an

inhibition. The reason for this is the extent of accumulation of aromatic compounds in the tissue. Uptake by mussels from the aqueous phase can be influenced by many factors (*cf.* Chapter 2.1). For example, if the mussels stopped feeding during the exposure to hydrocarbons, they would have aquired less hydrocarbons. Figure 4-4 shows the uptake of total aromatic hydrocarbons into the mussel tissue.

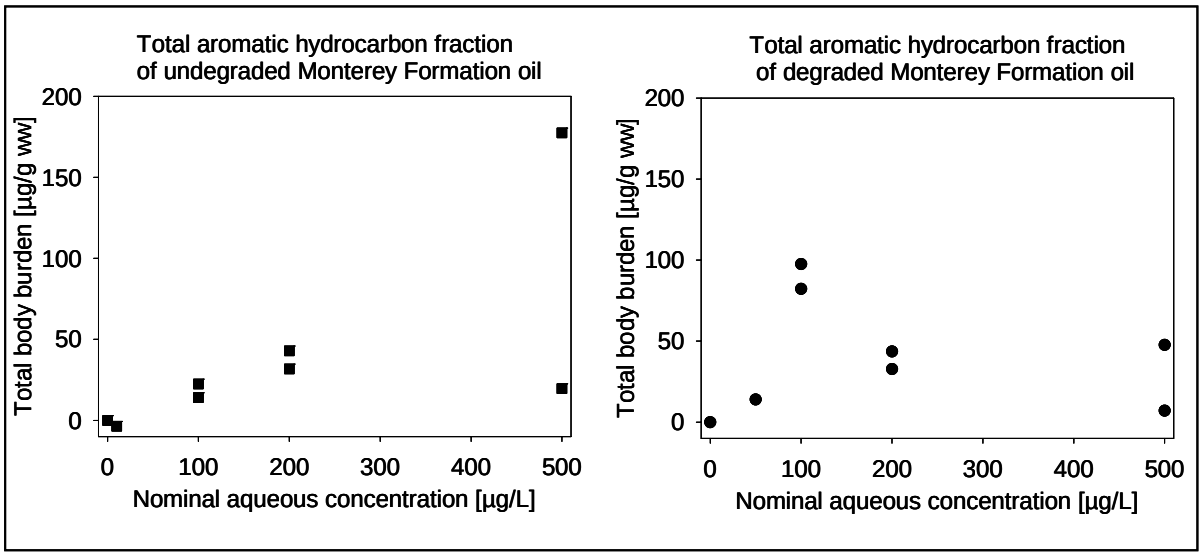


Figure 4-4 Uptake patterns of total aromatic hydrocarbon fractions of undegraded (left) and degraded (right) Monterey Formation oils.

The left diagram shows the data from the tests with the hydrocarbon mixture from the undegraded oil. With the exception of the highest nominal concentration (500 µg/L), there is a higher accumulation with higher exposure concentration. The group of mussels with the very high body burden from the solution of the undegraded oil is the above mentioned outlier with a high mean feeding rate value in spite of an elevated body burden.

On the right hand side, the uptake pattern for the degraded oil fraction shows no proportionality with the highest uptake from a nominal aqueous concentration of 100 µg/L. The mussels exposed to a nominal aqueous concentration of 500 µg/L have not taken up more than those exposed to 200 µg/L, indicating that the actual concentration of the two solutions was similar. The extent of accumulation from the solution at a nominal aqueous concentration of 200 µg/L was very similar for the two oil fractions (around 40 µg/g ww).

Excursus: solubility considerations

The mussels exposed to the highest aqueous concentrations at 500 µg/L do not show further reduced feeding rates compared to those exposed to 200 µg/L. This can be explained by their tissue concentrations, which also do not increase further. This indicates the occurrence of a different phenomenon, which overlaps with the toxic effect: the solubility cutoff for high-molecular-weight hydrocarbons (*cf.* Section 2.5).

In the case of the total aromatic hydrocarbon mixtures of the two Monterey Formation oils, the solubility limit appears to be exceeded. An oily layer of aromatic compounds on top of the seawater was observed during the preparation of the 500 µg/L solutions. Differences in the aqueous solubilities of aromatic compounds on the one hand and a maximum limit of dissolved hydrocarbons as a whole on the other hand can result in discrepancies between nominal and actual concentrations. It is possible that compounds, which dissolved in the water at lower concentrations and which acted as co-solvents, did not dissolve at the higher concentrations. They remained in the hydrophobic layer on top of the water. This possible difference in composition of the hydrocarbon solution probably influences its toxicity.

Shaw (1977) mentioned that water adjacent to an oil film is not necessarily saturated with hydrocarbons. Thus, the actual concentration of aromatic hydrocarbons was probably lower than the nominal concentration of 500 µg/L. Taking into account Raoult´s law (that the mole fraction of a compound in a mixture must be taken into account, see Section 2.5), it is likely that in the solution with a nominal aqueous concentration of 500 µg/L certain compound classes were less homogeneously dissolved than in the 200 µg/L solution.

Hokstad et al. (2000) found in a biotest with several aqueous solutions of crude oils that not necessarily the solution with the highest concentration is the most toxic one since toxicity strongly depends on the relative chemical composition of the aqueous solution. From this follows that the lower toxic effect observed for the exposure to the 500 µg/L solution in this study can either be due to a lower overall concentration or due to the absence of specific compounds.

Excess material of several toxicant solutions of the two Wilhelmshaven sets and some additionally prepared ones were extracted with dichloromethane and their actual concentrations were determined. In general, the actual concentrations of the aromatic hydrocarbon fraction in water were lower than the nominal concentrations (Table 4-5).

The seawater sample contains some background compounds. Most of them have the same GC retention time as those in the blank, indicating that they represent the laboratory background (*cf.* Section 3.2.4.8). This background value was not subtracted from the actual aqueous concentrations since the single sample might not be representative.

The actual concentrations in the solutions of the total aromatic fraction of the degraded Vienna basin oil indicate that the solution with a nominal aqueous concentration of 200 µg/L contained only slightly more hydrocarbons than the solution of nominally 50 µg/L. The solution with a nominal concentration of 500 µg/L contained about 10 times as much hydrocarbons as the solution with nominally 50 µg/L but still only half the nominal amount.

Table 4-5 Nominal and measured concentrations of selected aqueous solutions from various exposure experiments; “No experiment” indicates that the solutions were prepared especially for re-extraction and quantification of total hydrocarbons.

Nominal concentration	Oil	Fraction	Experiment	Measured concentration
0 µg/L	-	seawater blank	Experiment 5	15 µg/L
100 µg/L	undegraded Vienna basin oil	monoaromatic hydrocarbons	Experiment 4	86 µg/L
50 µg/L	degraded Vienna basin oil	total aromatic hydrocarbons	No experiment	21 µg/L
200 µg/L	degraded Vienna basin oil	total aromatic hydrocarbons	No experiment	34 µg/L
500 µg/L	degraded Vienna basin oil	total aromatic hydrocarbons	Experiment 3	244 µg/L
50 µg/L	degraded Monterey Formation oil	total aromatic hydrocarbons	No experiment	23 µg/L
100 µg/L	degraded Monterey Formation oil	total aromatic hydrocarbons	Experiment 5	37 µg/L
200 µg/L	degraded Monterey Formation oil	total aromatic hydrocarbons	Experiment 5	79 µg/L
500 µg/L	degraded Monterey Formation oil	total aromatic hydrocarbons	No experiment	93 µg/L

The measured concentrations in the aqueous solutions of the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil increase with the nominal concentrations although most of them are quite low. Although these solutions do not include the ones used in experiment 1, this analysis shows that in general the actual aqueous concentration was lower than the nominal one. Some solutions with different nominal aqueous concentrations have almost the same actual concentrations. In addition, some actual concentrations were not proportional to their respective nominal concentrations. One reason for this discrepancy between nominal and measured aqueous concentrations is that not all compounds in the oil fractions were GC-amenable so that the measured concentrations do not include all the compounds which were weighed at first to give the nominal concentrations.

The 500 µg/L concentration of total aromatic hydrocarbons was chosen because is lies within the range of maximum solubility of a monoaromatic UCM isolated from a biodegraded Gullfaks crude oil, which was determined to be 560 µg/L (Smith, 2002). However, this refers to 25°C and distilled water, whereas both decreasing temperature and increasing salinity are known to reduce aqueous solubility. In this experiment, seawater at 15°C was used.

Booth (2004) recently showed that concentration and composition of aqueous solutions of aromatic unresolved complex mixtures deviate extremely. Mean aqueous solubility of an aromatic UCM isolated from a crude oil was 293 ± 342 µg/L. In the present study, acetone was used as a carrier to enhance solubilisation of the apolar compounds in the aqueous phase and thus to minimise the high variability of the hydrocarbon solubilities in the mixtures. Since 500 µg/L is at or even above the upper limit of solubility of the unresolved complex

mixture and indeed oily layers were observed on the solutions during their preparation, the nominal aqueous concentration-response curves were replotted without the data for the highest concentrations (Figure 4-5).

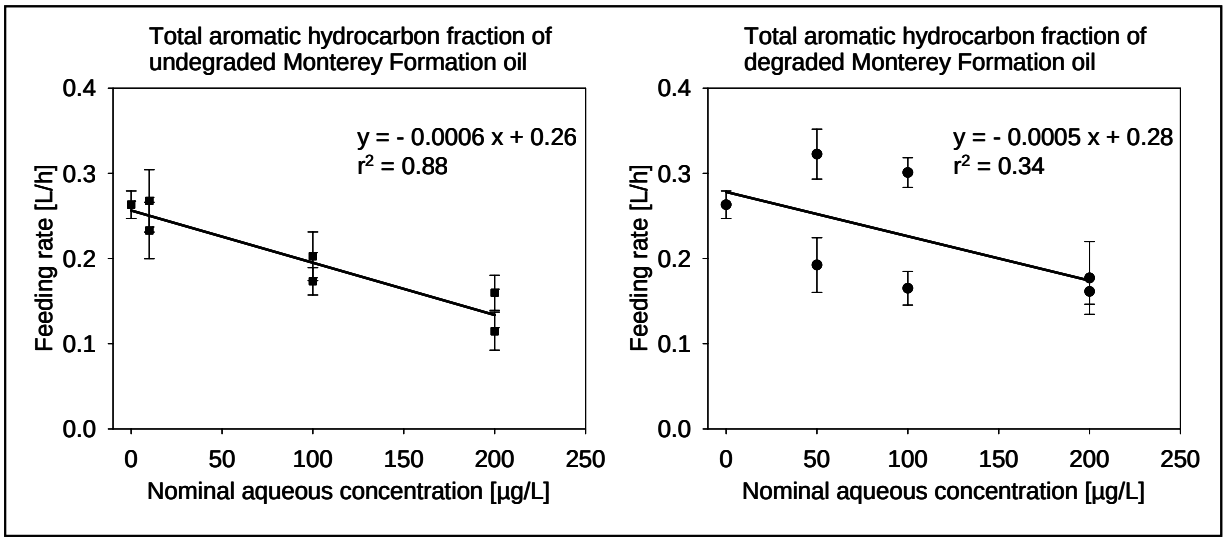


Figure 4-5 Regression of nominal aqueous concentration-feeding rate curves without the data for the nominal 500 µg/L solution.

The equations of linear regressions of the nominal aqueous concentration-feeding rate curves of the total aromatic hydrocarbon mixtures of the two Monterey Formation oils are very similar to each other with a negative slope of 0.0006 and 0.0005. The regression line for the undegraded Monterey Formation oil fraction (Figure 4-5, left) shows a much better regression coefficient than that for the degraded oil fraction (Figure 4-5, right). Thus, the correlation between increasing aqueous concentration and declining feeding rate is better for the undegraded oil, which may suggest that the undegraded oil fraction is slightly more toxic than the degraded one. Overall, the fractions appear to be similarly toxic.

This discussion highlights the difficulty of comparing aqueous solutions of crude oil fractions. Since narcotic effects are known to depend on tissue concentrations of accumulated compounds, the focus of this study was rather laid on the composition of tissue samples.

Comparing the experiments with the two oil fractions, similarities and differences can be seen. Body burdens of up to approximately 50 µg/g wet weight were accumulated by the majority of mussels within the 24-hour exposure period. Higher amounts were taken up by one mussel group exposed to 500 µg/L undegraded oil fraction and both duplicates exposed to 100 µg/L degraded oil. The higher amounts did not lead to a further significant reduction of feeding rate.

The results are in accordance with data from Wraige (1997), who also found a levelling off of the reduction of feeding rates at higher tissue concentrations. Additional analyses

showed that gill tissue concentrations correlated better with the reduction of feeding rates than total body burdens, especially at high concentrations. This is probably due to the fact that the gills, which are the presumable site of toxic action, have a certain capacity for compounds which cannot be exceeded. Thus, further accumulated hydrocarbons stay in the other parts of the mussel tissue where they do not have an effect on the feeding rate. Until saturation is reached, body burden analysis does correlate well with gill tissue concentration (Wraige, 1997). This saturation was reached after approximately 40 hours exposure time in Wraige´s study. In order to avoid saturation of gills and resulting levelling-off of the toxic effects, exposure was performed for ony 24 hours in the experiments of this study.

4.1.2 Toxicity test with the degraded Monterey Formation oil fraction (experiment 5)

The first set of toxicity tests was performed in Plymouth and the second in Wilhelmshaven with blue mussels from North Sea sites close to the respective laboratories. In order to test the comparability of the toxicity tests, several exposures of blue mussels to aqueous solutions of the total hydrocarbon fractions of the two degraded oils were repeated with mussels from the German North Sea site. Nominal concentrations of 100 and 200 µg/L were applied in order to avoid difficulties with the solubility at higher concentrations. Results from the repeated exposures to the degraded Vienna basin oil fraction are described after the next section, which presents the results of the toxicity tests with the Vienna basin oil fractions (4.1.3).

The results of the repeated exposure to the total aromatic hydrocarbon fractions of the degraded Monterey Formation oil are shown in Table 4-6.

Table 4-6 Nominal aqueous concentrations [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of control mean [%], reduction of feeding rate [%] and body burden [µg/g wet weight] of the repeat experiments with the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil, no red.: no reduction since FR > 100%.

Nominal aqueous concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww)
0 (Controls)	25	0.19 ± 0.03	100	0	0
100	6	0.22 ± 0.06	114	no red.	34.3
100	6	0.22 ± 0.06	115	no red.	51.5
200	6	0.14 ± 0.06	72	28	52.4
200	7	0.13 ± 0.04	67	33	22.2

The tissue concentration after 24 hours exposure time lies in the range of 50 µg/g wet weight but in general, the datapoints are scattered and the high feeding rates do not necessarily correlate with low body burdens (Figure 4-6). For example, of the two groups of mussels which took up around 50 µg/g one exhibits a high and the other one a low feeding rate. These feeding rates are not statistically significantly different from each other and not

different from the control, suggesting that the accumulated hydrocarbons did not have a significant effect on the feeding rates.

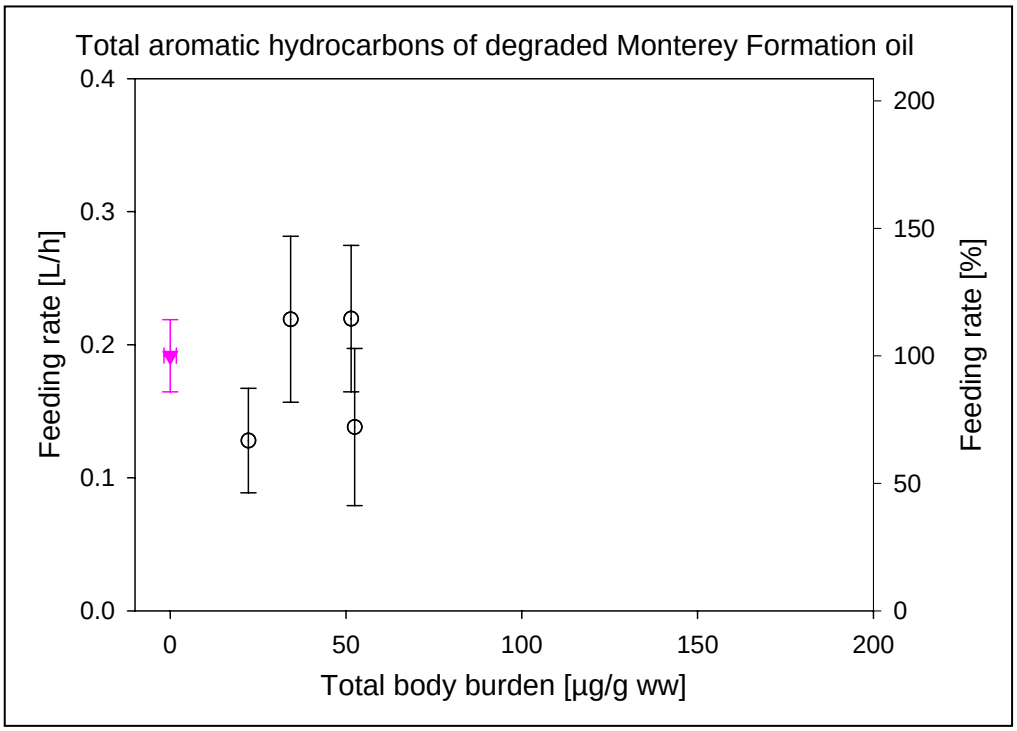


Figure 4-6 Tissue concentration-response diagram of the repeated exposure to total aromatic hydrocarbon fractions of the degraded Monterey Formation oil. The control mean (grey triangle) and data points are shown with error bars (se) for feeding rate means. The tissue concentration of the control is shown ± standard deviation (n = 4).

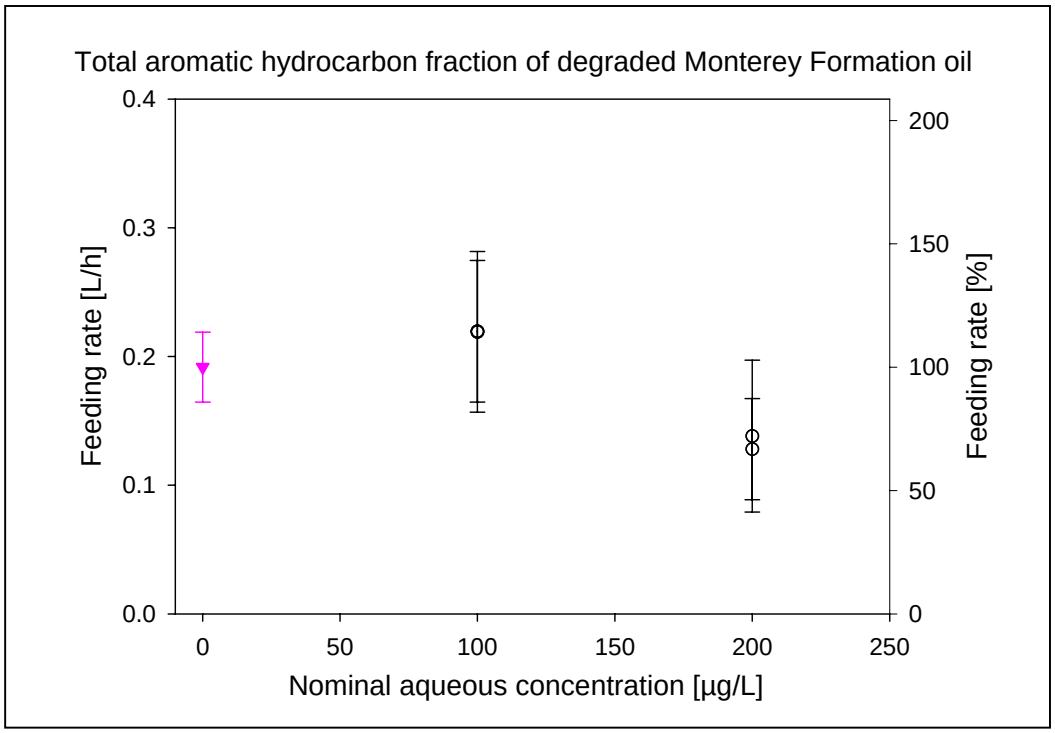


Figure 4-7 Nominal aqueous concentration-feeding rate diagram of the exposure experiment with the degraded Monterey Formation oil fraction (experiment 5). The control mean (grey triangle) and data points are shown with error bars (se).

The nominal aqueous concentration-feeding rate plot (Figure 4-7) shows a slight increase in feeding rate at the nominal concentration of 100 µg/L. At the nominal concentration of 200 µg/L, feeding rates of both duplicates decline by around 30% compared to the control value. However, this feeding rate reduction cannot be perceived in the tissue concentration-response curve, which does not show a consistent trend. The feeding rate values of the four groups of mussels exposed to the hydrocarbons were not statistically different from the untreated controls. This can be explained by a higher biological variance of the mussels from Wilhelmshaven compared to the Cornish ones, as indicated by the larger error bars.

The uptake pattern (Figure 4-8) of the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil shows a similar extent of accumulation from the two toxicant solutions. The body burdens range between 20 and 60 µg/g wet weight. The measured concentrations of the nominally 100 and 200 µg/L solutions were 37 and 79 µg/L, respectively (see Table 4-5). Uptake by the mussels does not reflect these different concentrations, which highlights the existence of many other factors that influence uptake of chemicals from water.

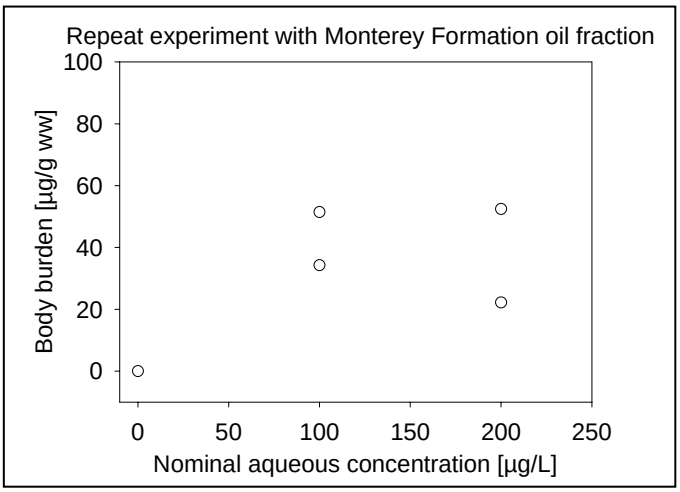


Figure 4-8 Uptake pattern of the exposure experiment with total aromatic hydrocarbon fraction of the degraded Monterey Formation oil.

Comparison of experiment 1 and experiment 5

Comparing the exposure experiment of Cornish mussels to the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil to the experiment with German mussels, it can be seen that the reduction of feeding rate with increasing aqueous concentration is similar. Due to a lower control mean value, the percentage of reduction is not as high as in experiment 1.

Comparing the uptake of hydrocarbons, one can see that many mussels accumulated similar amounts of hydrocarbons in the experiments. Apparently, saturation of the gill tissue of the mussels has been reached at around 60 µg/g.

Figure 4-9 shows the regression lines of the nominal aqueous concentration-feeding rate data of the two experiments with the degraded oil fractions. The equations are quite similar to each other and the regression coefficient is higher for experiment 5 (right). This indicates that there is a relationship between higher concentration of contaminants and a declining feeding rate. However, there is no statistically significant difference of feeding rates in experiment 5. Thus, the toxic effect was only reproduced to a certain extent with mussels from different coastlines since the intensity of the feeding rate reduction was variable. It can be concluded that the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil caused a reduction of feeding rate with increasing concentration although the German mussels appear to be less sensitive.

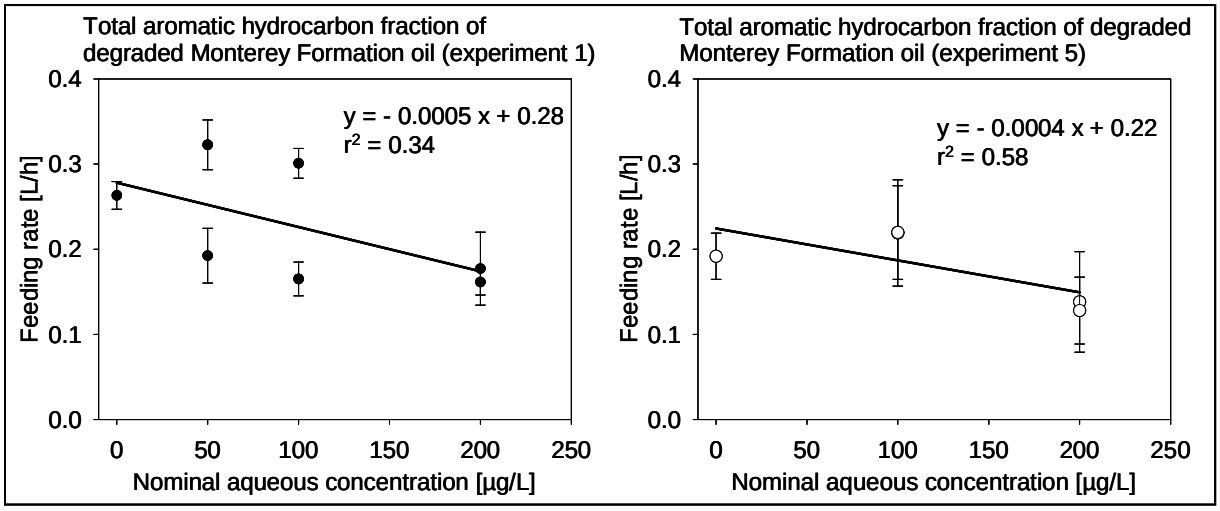


Figure 4-9 Nominal aqueous concentration-feeding rate curves from the tests with the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil with linear regression of experiments 1 and 5.

4.1.3 Toxicity test with the Vienna basin oil fractions (experiment 3)

The results of the exposure experiments with the two total aromatic hydrocarbon fractions of the Vienna basin oils are shown in Tables 4-7 and 4-8.

Table 4-7 Nominal aqueous concentration [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of controls [%], reduction of feeding rate [%] and body burdens of mussels exposed to the total aromatic hydrocarbon fraction of the undegraded Vienna basin oil, no red.: no reduction since FR > 100%.

Nominal aqueous Concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	68	0.22 ± 0.02	100	0	0
50	7	0.21 ± 0.06	93	7	12.6
50	7	0.24 ± 0.05	111	no red.	15.9
100	7	0.16 ± 0.03	74	26	10.9
100	7	0.14 ± 0.02	62	38	5.1
200	7	0.17 ± 0.03	78	22	33.3
200	7	0.18 ± 0.05	80	20	31.9
500	7	0.19 ± 0.05	85	15	106.5
500	7	0.20 ± 0.05	91	9	82.5

Table 4-8 Nominal aqueous concentration [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of controls [%], reduction of feeding rate [%] and body burdens of mussels exposed to the total aromatic hydrocarbon fraction of the degraded Vienna basin oil, no red.: no reduction since FR > 100%.

Nominal aqueous Concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	68	0.22 ± 0.02	100	0	0
50	7	0.22 ± 0.07	102	no red.	8.2
50	7	0.20 ± 0.03	91	9	11.8
100	6	0.12 ± 0.03	56	44	2.1
100	7	0.18 ± 0.04	81	19	17.7
200	6	0.14 ± 0.04	64	36	30.6
200	7	0.23 ± 0.04	102	no red.	137.6
500	7	0.16 ± 0.03	75	25	178.6
500	7	0.19 ± 0.03	88	12	83.5

The tissue concentration-feeding rate and the nominal aqueous concentration-feeding rate curves of the exposure experiments with the Vienna basin oil fractions are displayed in Figures 4-10 and 4-11.

Four groups of mussels exposed to the aromatic hydrocarbon fraction of the undegraded Vienna basin oil did not accumulate many hydrocarbons (Figure 4-10, top). Their highly variable feeding rates of 62% to 111% scatter around the control value. Two groups of mussels accumulated hydrocarbons of around 40 µg/g tissue and both have slightly reduced feeding rates compared to the control mussels. Two other groups of mussels contain more than 100 µg/g wet weight aromatic hydrocarbons but do not show reduced feeding rates.

After exposure to the degraded oil fraction (Figure 4-10, bottom), tissue concentrations of five groups of mussels are below 50 µg/g. The feeding rates of the three groups of mussels with the lowest body burdens range from 56% to 102%. One group exhibits a very low feeding rate without any accumulation of hydrocarbons. Three groups of mussels have accumulated high amounts of hydrocarbons but do not show reduced feeding rates. Regarding the range up to 50 µg/g wet weight, a trend of decreasing feeding rate is perceivable for the degraded oil fraction. However, the facts that the feeding rates are not statistically different from the control and that there are three groups of mussels with relatively high feeding rates in spite of high body burdens, do not support this perception.

In both experiments, the mussels with the lowest feeding rates (62% and 56% of the control value) did not accumulate many hydrocarbons (body burdens between 2 and 10 µg/g wet weight, respectively). Thus, these mussels must have had a different reason why they filtered water to such a small extent. There are no statistically significant differences in control and exposed mussels. In addition, the regression lines for both plots have no slope and the regression coefficients are near zero, indicating that there is no relationship between amount of hydrocarbons and the feeding rate.

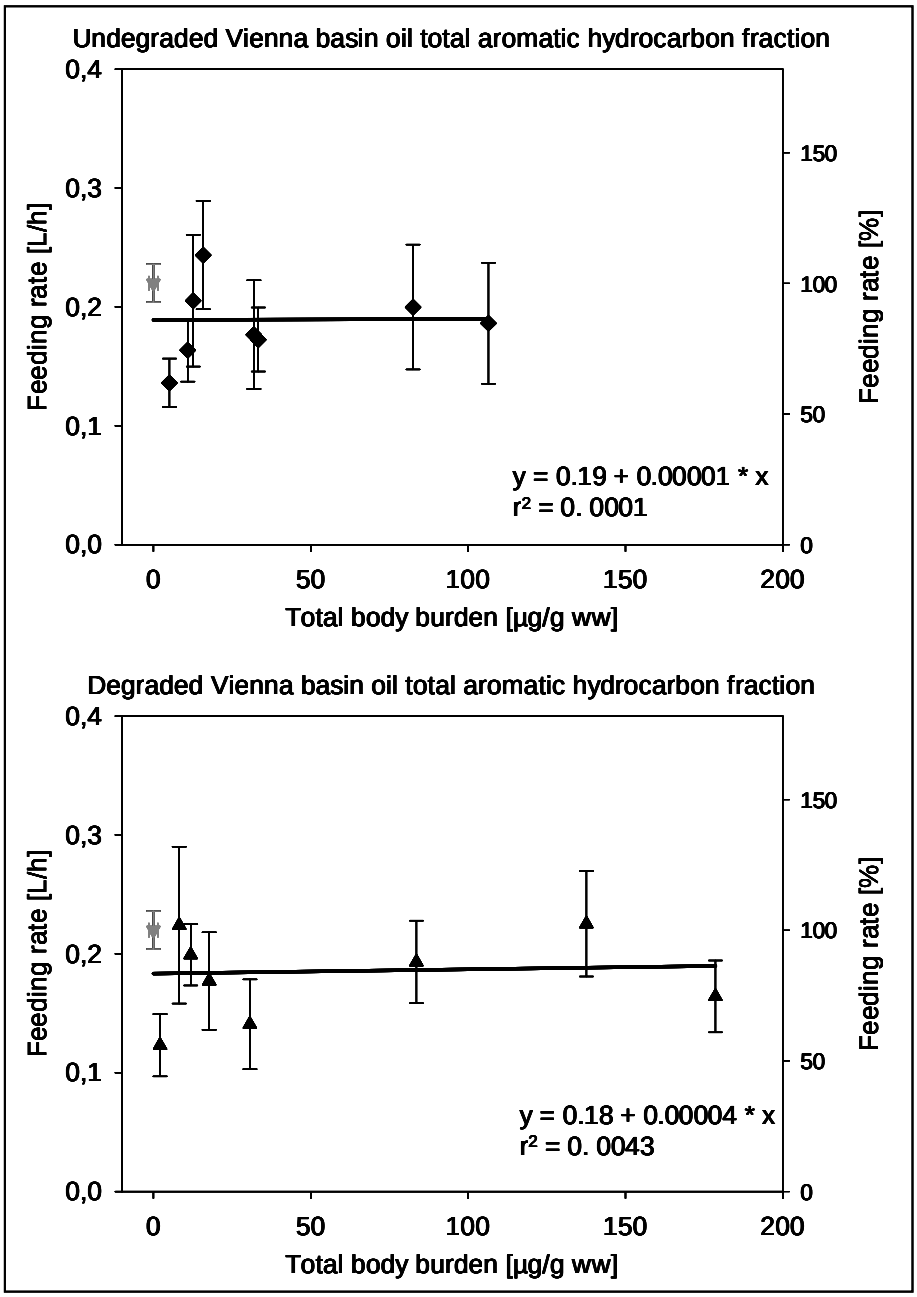


Figure 4-10 Total body burden-feeding rate curves of the two total aromatic hydrocarbon fractions of the Vienna basin oils. The control mean (triangle) and data points are shown with error bars (se) for feeding rate mean values. The tissue concentration of the control is shown ± standard deviation (n = 7). Regression lines, regression equations and coefficients are also shown.

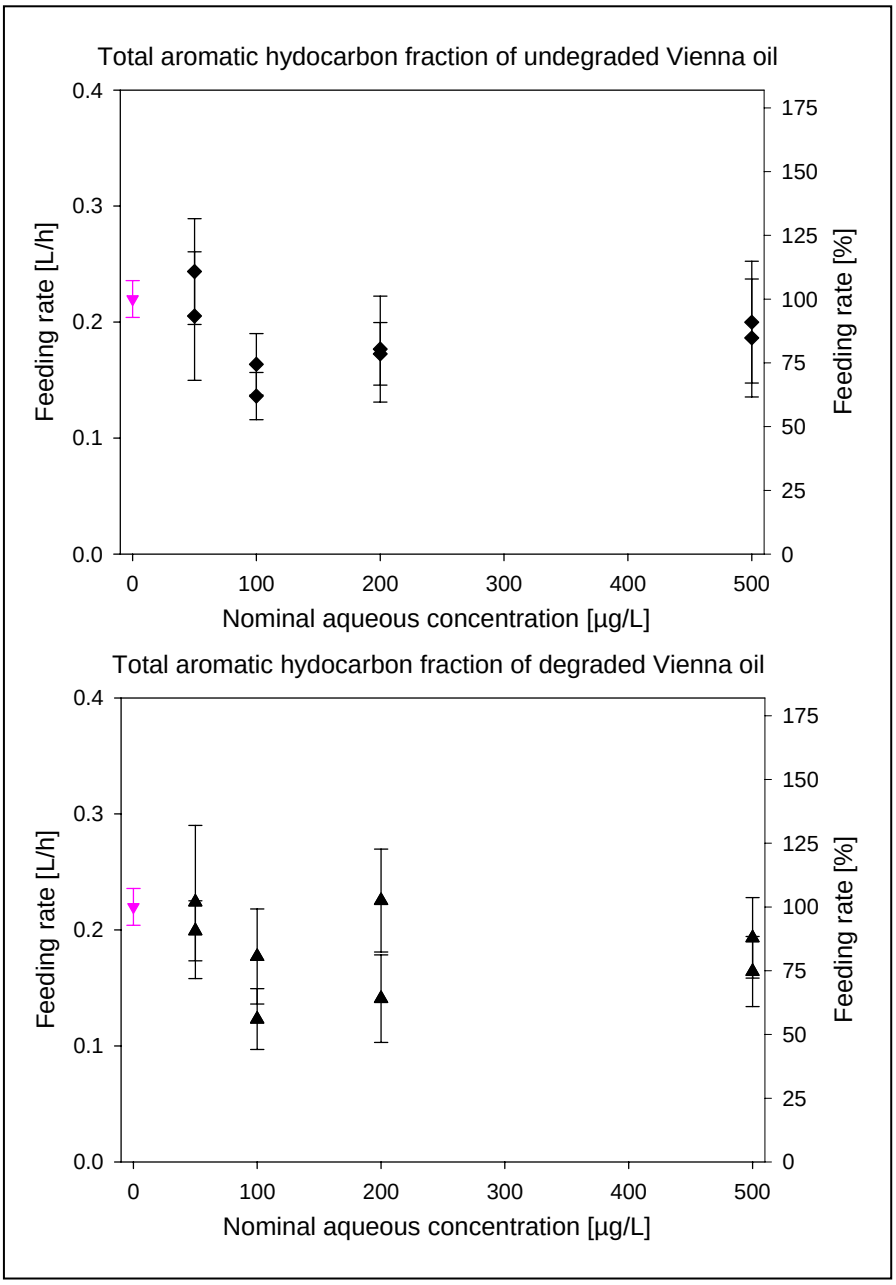


Figure 4-11 Nominal aqueous concentration-feeding rate diagrams of the two Vienna basin oil total aromatic hydrocarbon fractions. The control mean (triangle) and data points are shown with error bars (se).

The aqueous concentration-feeding rate plots (Figure 4-11) look very similar to each other. At a nominal concentration of 50 µg/L, the feeding rates scatter around the control feeding rate. At the nominal concentration of 100 µg/L, the feeding rates decline but at 200 and 500 µg/L, no further reduction is observed. The mussels exposed to the aromatic hydrocarbon mixture from the degraded oil basically exhibit the same pattern. The lowest feeding rate values can be found at the concentration of 100 µg/L for both oil fractions.

The majority of mussels exposed to the total aromatic fractions from the Vienna basin oils did not respond with reduced feeding rates to high concentrations of compounds. Basically all mussels exposed to the aromatic hydrocarbons exhibited similar feeding behaviour. Taking everything into account, it is not possible to discern a consistent reduction

of feeding rate with higher tissue concentration of the aromatic compounds from the undegraded Vienna basin oil. This means that there is no toxic effect observable.

Table 4-9 Nominal aqueous concentrations [µg/L], number of mussels (n) combined as duplicates, mean feeding rate (FR) values with standard errors (se) and as percentage of control [%] of mussels exposed to the total aromatic hydrocarbon fractions of the Vienna basin oils.

Undegraded Vienna basin oil				Degraded Vienna basin oil			
Aqueous concentration [µg/L]	**n**	**Feeding rate [L/h]**	**FR [%]**	**Aqueous concentration [µg/L]**	**n**	**Feeding rate[L/h]**	**FR [%]**
0 (Controls)	68	0.22 ± 0.02	100	0 (Controls)	68	0.22 ± 0.02	100
50	14	0.22 ± 0.03	100	50	14	0.21 ± 0.03	93
100	14	0.15 ± 0.02	68	100	13	0.15 ± 0.03	68
200	14	0.17 ± 0.03	78	200	13	0.19 ± 0.03	88
500	14	0.19 ± 0.04	88	500	14	0.18 ± 0.02	82

Statistical analysis showed that for this experiment, the duplicates could be combined to groups of 14 mussels (Table 4-9). The corresponding dose-response diagram (Figure 4-12) displays the similarity of the curves. The overall trend of a lacking feeding rate reduction is clearly visible for both oils. There is virtually no relationship between increasing nominal aqueous exposure and mussel feeding rate in the experiments with the Vienna basin oils.

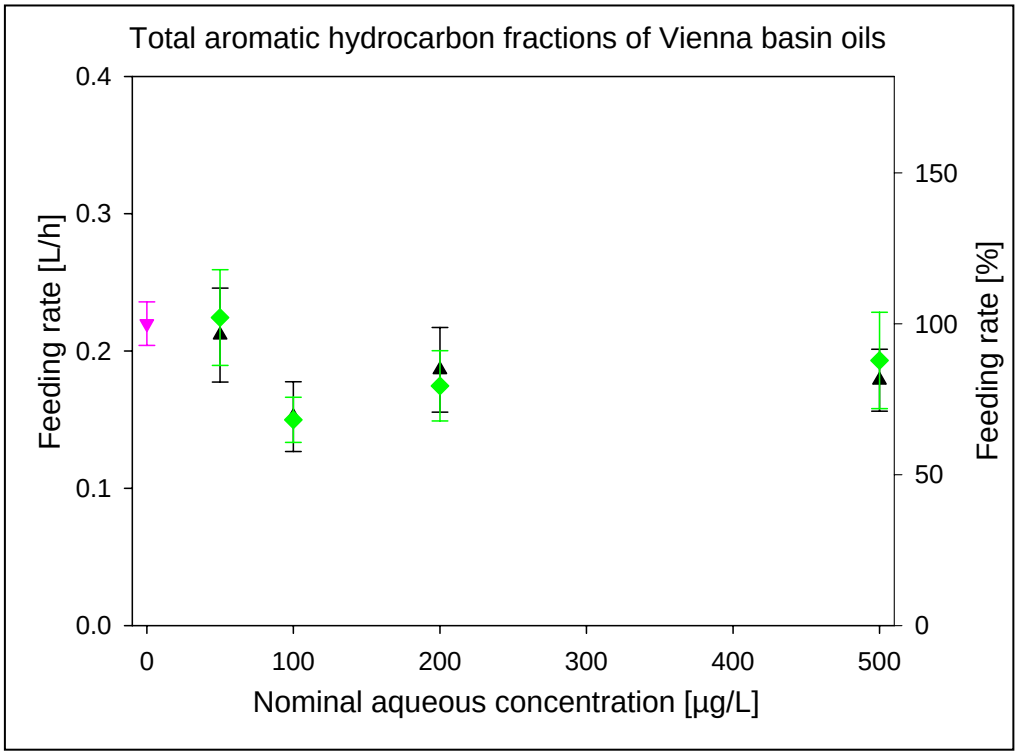


Figure 4-12 Nominal aqueous concentration plotted against feeding rates of the combined duplicates of mussels exposed to the total aromatic hydrocarbon fractions of the two Vienna basin oils (green: undegraded). The control mean (triangle) and data points are shown with error bars (se).

For the highest nominal aqueous concentrations at 500 µg/L, an oily layer of the undegraded oil aromatic hydrocarbon mixture was visible on top of the seawater during the preparation of these toxicant solutions. Thus, the same phenomenon appears to have

occurred as for the Monterey Formation oil total aromatic hydrocarbon fractions (see *Excursus: Solubility considerations* in Section 4.1.1). This strongly signifies that the actual concentration was lower than the nominal concentration. An oily layer was not observed on the toxicant solution of the hydrocarbons from the degraded oil. A few samples of toxicant solution were re-extracted in order to determine their actual concentration. One of the solutions randomly sampled was the one with a nominal concentration of 500 µg/L of the undegraded oil. Analysis revealed a concentration of 244 µg/L. This shows that in this case the concentration was indeed significantly lower than the nominal one.

The accumulation patterns for the two Vienna basin oils show higher uptake into mussel tissue with increasing nominal aqueous concentrations (Figure 4-13). Only small amounts of aromatic compounds were accumulated from the 50 and 100 µg/L solutions. The highest uptake of the undegraded oil fraction added up to around 100 µg/g ww. For the degraded oil fraction, two body burdens are much higher than the body burdens after exposure to the undegraded Vienna basin oil.

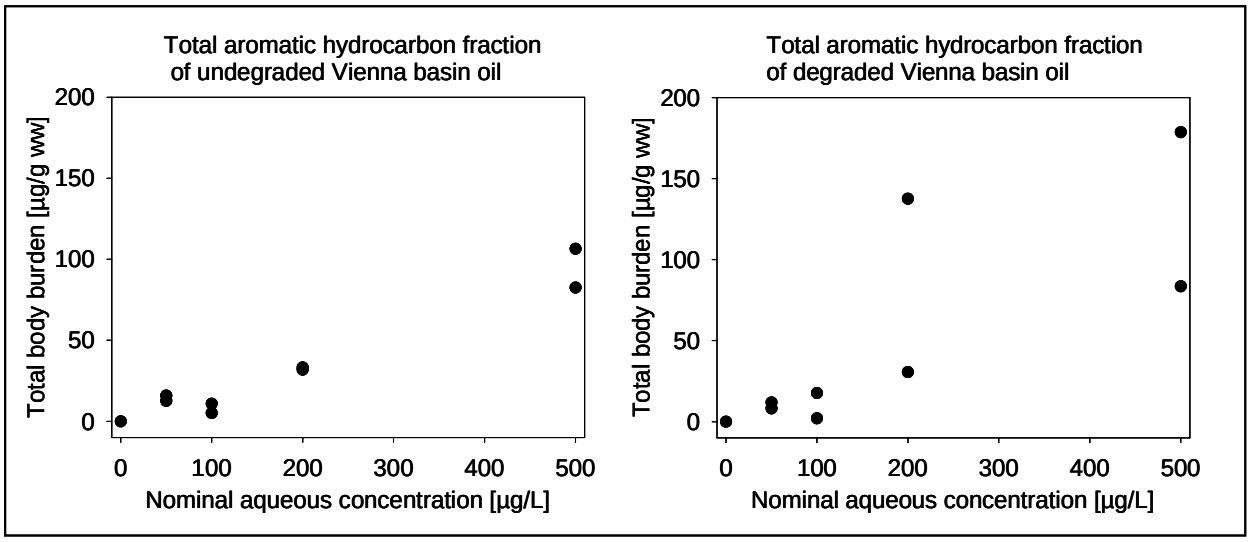


Figure 4-13 Uptake patterns for total aromatic hydrocarbon fractions of undegraded (left) and degraded (right) Vienna basin oils.

4.1.4 Toxicity test with the total aromatic hydrocarbon fraction from the degraded Vienna basin oil (experiment 6)

Table 4-10 shows the results of the exposure of German blue mussels exposed to the total aromatic hydrocarbon fractions of the degraded Vienna basin oil (experiment 6).

Table 4-10 Nominal aqueous concentrations [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of control mean [%], reduction of feeding rate [%] and body burden [µg/g wet weight] of the repeat experiments with total aromatic hydrocarbon fraction of the degraded Vienna basin oil, no red.: no reduction since FR > 100%.

Nominal aqueous concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	25	0.19 ± 0.03	100	0	0
100	6	0.32 ± 0.08	201	no red.	4.6
100	6	0.24 ± 0.07	124	no red.	5.3
200	6	0.28 ± 0.07	147	no red.	45.4
200	7	0.34 ± 0.07	176	no red.	28.8

All groups of mussels have higher feeding rates than the control mussels although some accumulated hydrocarbons up to 45 µg/g wet weight (Table 4-10). The dose-response relationships are plotted in Figures 4-14 and 4-15.

The tissue concentration-response relationship for the exposure of German blue mussels to the Vienna basin oil fraction shows a scatter of data points at a high feeding rate level (Figure 4-14). A statistically significant difference from controls was observed for one group of mussels. The error bars of the feeding rate are very large. This suggests that the high feeding rates are not related to the amount of hydrocarbons present in the tissue.

The aqueous concentration-feeding rate plot (Figure 4-15) shows an increasing feeding rate with higher concentration of hydrocarbons. This is surprising and to the author´s knowledge has not been reported before. Possible explanations are discussed in Section 4.3.

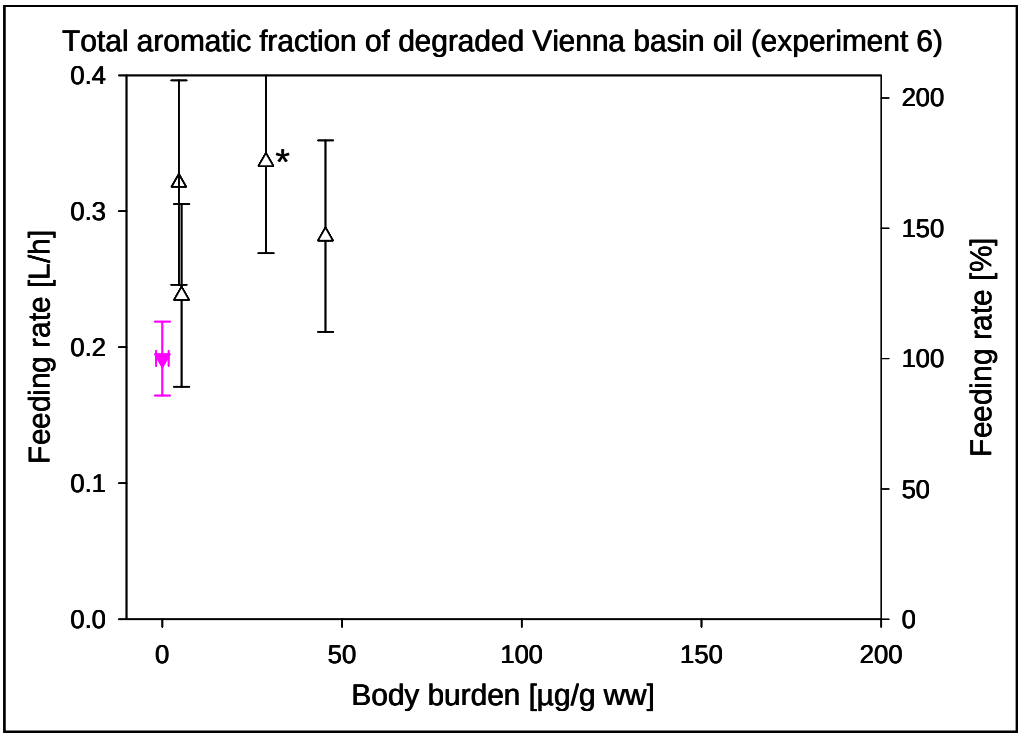


Figure 4-14 Tissue concentration-response diagram of the repeated exposure of mussels to the total aromatic hydrocarbon fraction of the degraded Vienna basin oil. The control mean (triangle) and data points are shown with error bars (se) for feeding rate means. The tissue concentration of the control is shown ± standard deviation (n = 4).

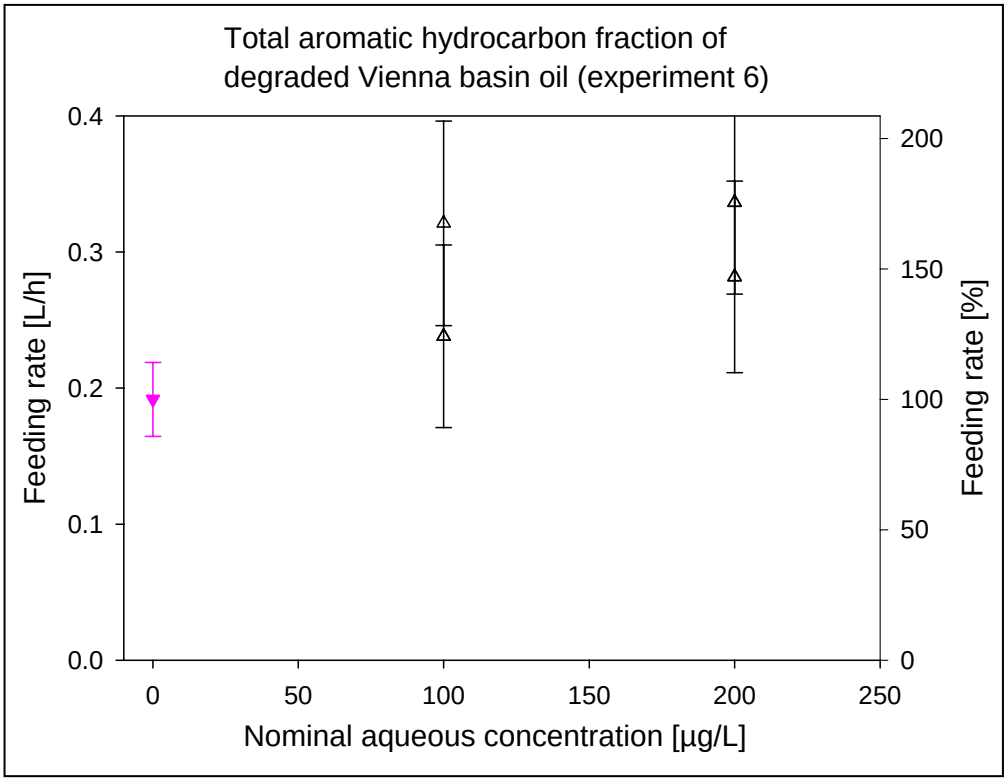


Figure 4-15 Nominal aqueous concentration-feeding rate diagram of the exposure experiment with the degraded Vienna basin oil fraction (experiment 6). The control mean (triangle) and data points are shown with error bars (se). Asterisks indicate statistical significant differences from the control mean value.

The uptake pattern for the biodegraded Vienna basin oil aromatic hydrocarbons (Figure 4-16) shows little accumulation at a nominal concentration of 100 µg/L and a higher uptake at 200 µg/L.

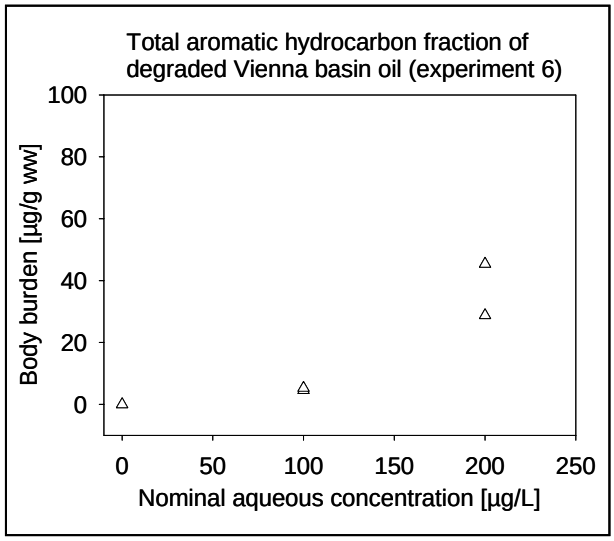


Figure 4-16 Uptake pattern of the exposure experiment with total aromatic hydrocarbon fraction of the degraded Vienna bain oil.

Comparison of experiments 3 and 6

Comparing the first exposure of German mussels to the total aromatic hydrocarbons of the degraded Vienna basin oil to the second one, some differences are obvious. Whereas the first exposure to the degraded Vienna basin oil fraction does not show consistently declining feeding rates with higher exposure concentrations, the repeat experiment suggests a stimulation of feeding rate compared to the controls. However, the error bars are very large for the mussel feeding rates in experiment 6 (see Figure 4-15). Thus, the feeding rates may be high due to different reasons such as a very high biological variance. In addition, if the control value was not that low the feeding rates would rather appear wide-spread than increasing.

Comparing the uptake pattern from the experiments with the Vienna basin oil aromatic hydrocarbon fractions with the repeat experiment, it is obvious that uptake was similarly low from the 100 µg/L solution. With one exception at 200 µg/L, accumulation was similar for the two experiments.

Thus, one can conclude that the Vienna basin oils did not cause a reduction of feeding rates of mussels from Wilhelmshaven. This strengthens the hypothesis that the German mussels used in the toxicity tests were relatively insensitive towards hydrocarbon contamination.

4.1.5 Differences in mussel responses to exposure to the four total aromatic hydrocarbon fractions

Table 4-11 summarises the responses of the mussels exposed to the total aromatic hydrocarbon fractions of the four crude oils.

Table 4-11 Summary of mussel uptake of and feeding rate response to the total aromatic hydrocarbon fractions of the different oils, M: Monterey Formation oil, V: Vienna basin oil.

Experiment number	Oil	Uptake range [µg/g ww]	Feeding rate	Toxic?
1	Undegraded M	0-43	reduction	Toxic to Cornish mussels
	Degraded M	0-98	reduction	Toxic to Cornish mussels
5	Degraded M	0-52	possible reduction	Maybe toxic to German mussels
3	Undegraded V	0-107	no reduction	Not toxic to German mussels
	Degraded V	0-179	no reduction	Not toxic to German mussels
6	Degraded V	0-45	possible stimulation	Not toxic to German mussels

The tissue concentration-response curve for the undegraded Monterey Formation oil aromatic fraction shows the clearest reduction of feeding rates with higher tissue concentrations (Figure 4-2, top). There is a steep linear decline, which illustrates the toxic effect. The tissue concentration-response relationship for the degraded Monterey Formation oil fraction also demonstrates that increasing body burden causes a decline of mussel feeding rate (Figure 4-2, bottom). The regression line shows a less steep decline than for the undegraded oil but data are not tight enough to ascertain a higher toxicity for the undegraded oil. The regression line of the tissue concentration-response curve for the exposure to the undegraded Monterey Formation oil fraction shows the steepest decline and the highest correlation coefficient of all four exposure experiments with total aromatic hydrocarbon fractions (*cf.* Figures 4-2 and 4-10).

The Vienna basin oil fraction experiments yield correlation coefficients near zero, thus no mathematical correlation between tissue concentration and feeding rate was determined. In each diagram for the Vienna basin oil mixtures, there are several groups of mussels which do not show reduced feeding rates in spite of high amounts of aromatic hydrocarbons in their tissue and some with reduced feeding rates without having accumulated high body burdens. This means that a toxic effect was not observed.

Comparing experiments 5 and 6, it was observed that some mussels exposed to the Monterey Formation oil fraction displayed reduced feeding rates. This was not observed in any mussel exposed to the Vienna basin oil fraction. In both experiments, similar amounts of tissue concentrations were accumulated. In experiment 5 with the total aromatic fraction of

the Monterey Formation oil, two groups of mussels show reduced feeding rates with tissue concentrations of 20 and 50 µg/g accumulated hydrocarbons. In the experiment with the total aromatic fraction on the degraded Vienna basin oil, though, all feeding rates were high regardless of the amount of hydrocarbons taken up. On the one hand, this may indicate a disturbance of feeding activity caused by the Vienna basin oil fraction. On the other hand, this may indicate that some individual German mussels responded to the hydrocarbons from the Monterey Formation oil with reduced feeding rates in contrast to the German mussels exposed to the Vienna basin oil fraction. This may imply a higher toxicity of the Monterey Formation oil fraction compared to the Vienna basin oil.

Another indication that the hydrocarbons from the Monterey Formation oil were indeed toxic to the German blue mussels is provided when one takes into account the reduction of feeding rate caused by the test compound *n*-nonane. The feeding rate of mussels in set Ply was reduced by around 46% whereas the feeding rate of mussels in set Whv-II was reduced by around 30% (Table 4-2). Thus, the hydrocarbons of known toxicity caused a stronger toxic effect to the Cornish mussels than to the German mussels. This implies that the German mussels are less sensitive than the Cornish mussels and that a given toxicant causes less reduction of feeding rates to the German mussels. Analogous to *n*-nonane, the reduction of feeding rates due to the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil was lower for German mussels than for Cornish ones. Thus, the less intense decline of feeding rates of German mussels caused by the Monterey Formation oil fraction may indicate a toxic effect.

In Cornish mussels, both Monterey Formation oil fractions caused a decline in feeding rate. It can be assumed that the total aromatic hydrocarbon fractions of both Monterey Formation oils would cause a toxic effect with increasing concentration although the German mussels appear to be less sensitive.

From Table 4-11 follows that the total aromatic hydrocarbon fractions of two Vienna basin oil fractions did not cause a reduction of feeding rate of the blue mussels used although some mussels accumulated high body burdens. Several tissue concentrations are even higher than after exposure to the total aromatic hydrocarbon fraction of the Monterey Formation oils. Thus, the difference in toxicity does not derive from a different extent of accumulation of compounds. One possible reason is that the Monterey Formation oils are more toxic than the Vienna basin oils.

Differences in the toxicity of oils have been reported before, owing to differences in their composition (e.g. Rice et al., 1977). Increases in toxicity are usually attributed to higher amounts of aromatic hydrocarbons in the oils studied (e.g. Rice et al., 1977; Neff et al., 2000). Besides the relative amount of aromatic hydrocarbons in crude oils, other factors can

influence whole oil toxicity such as the method of preparing the aqueous solutions needed for exposure experiments of marine organisms. These different methods can yield very differently composed aqueous solutions (e.g. Hokstad et al., 1999). Thus, depending on the composition of the aqueous solutions of aromatic hydrocarbons, different aromatic hydrocarbon fractions can have different potentials of toxicity. In this study, pure aromatic hydrocarbon mixtures were used in order to compare similarly composed mixtures of this priority class of contaminants. Many aromatic compounds are proven toxicants (e.g. Donkin et al., 1989; Donkin et al., 1991). There may be a certain compound group responsible for the toxic effect which is missing in the Vienna basin oils but present in the Monterey Formation oils. Discussion of the composition of tissue samples of mussels exposed to the total aromatic hydrocarbon fractions will follow in Chapter 6. However, it is highly unlikely that one single compound group in such a complex mixture as an aromatic hydrocarbon fraction of a crude oil is chiefly responsible for the toxic effect. This is particularly true since aromatic hydrocarbons are known to act via a similar toxic mechanism. Consequently, they exert a joint toxic effect as described by the concept of concentration addition (*cf.* Section 2.3). Thus, the question remains why the German mussels, which accumulated aromatic compounds, did not respond to the toxicants with reduced feeding rates.

Comparing the three mean feeding rate values of control mussels from the three sets (Table 4-2), the German mussels have lower feeding rate values. The mean value of the mussels from Cornwall (0.26 L/h) is statistically different from the feeding rate value of German mussels from set Whv-II (0.19 L/h). However, due to the intermediate value of mussel feeding rates in set Whv-I (0.22 L/h), the three mean values taken together are not statistically different from each other.

There may be several reasons for the low mean feeding rates of the German mussels. In general, low feeding rates of blue mussels are an indication that the mussels are not healthy (e.g. Widdows et al., 1995). This is why this biological endpoint was used as an indicator for mussel health after exposure to toxicants. Analogously, low feeding rates of mussels in the field indicate a reduced health. Thus, the health of the mussels from Jade Bay near Wilhelmshaven appears to have been already slightly affected. Another indication that the health of the German mussels was impaired is the number of inactive mussels. The percentage of inactive or dead mussels in experiments 5 and 6 is very high (9%) compared to the other two sets of experiments with 1% for the Cornish mussels and 2% for the mussels in setup Whv-I.

This and the lower feeding rates of control mussels from Jade Bay compared to Cornish mussels may indicate that the German mussels were not healthy at the beginning of the experiments. For example, they may have been exposed to narcotically acting xenobiotics from the North Sea near Wilhelmshaven and consequently had reduced feeding rates.

However, background levels of several contaminants were not very high in the North Sea near Wilhelmshaven (*cf.* Section 3.2). In addition, mussels from the Jade Bay were checked for background contamination prior to use, revealing no contamination (see e.g. extract of control mussels in Figure 4-1). However, there may have been toxicants present in the mussel tissue which were volatile or low concentrated and thus escaped notice.

Another possible reason for the low feeding rates of the German mussles is that the 10 day acclimatisation period may have been too short. All control groups within one set varied slightly over time but there is no chronological de- or increasing trend perceivable, which would imply a systematical error in the experiments.

Seasonal differences may also play a role. Mussels of set Whv-I were collected in March and the mussels for the other sets, Ply and Whv-II, in November 2003 and November 2004. The weather may have been quite different in Cornwall and North Germany in two subsequent autumns, though, placing the mussels at different stages of their yearly cycle. This can affect various biological responses. For example, the amount of lipids in mussel tissue is known to vary over the course of a year (Zandee et al., 1980). In addition, bivalves have been observed to display a yearly cycle in the uptake of contaminants due to changes in their reproductive cycle and lipid content (Hellou et al., 2002). This can influence uptake or distribution of the lipophilic contaminant (Hellou et al., 2002).

Wraige (1997) reported variations between a first exposure and a repeat experiment with blue mussels from the same site which were collected in April 1994 and November 1994. The first experiment with 4-propyl-octane showed significantly reduced feeding rates compared to untreated controls after 96 hours exposure (30% reduction). The repeat experiment did not show significantly reduced feeding rates (15% reduction). Tissue analysis later revealed a lower extent of accumulation in the repeat experiment. This is not the case here, but another explanation for the different biological responses may be that the composition of the accumulated hydrocarbons differs between experiments 3 and 6 due to the difficulty of preparing aqueous solutions of hydrocarbons reproduceably.

It has been shown that an increase in toxic effect does only take place as long as gill tissue concentrations are rising (Wraige, 1997). Afterwards, the presumed site of toxic action seems to be saturated and toxicants seem to be distributed within other parts of the body. A possible explanation for the lack of feeding rate reduction may be that an unknown previous exposure in the Jade Bay saturated the site of action in the gills before the experiments were conducted with the mussels. If indeed there was previous contamination of the mussel from the Jade Bay this possibly even caused a certain adaptation and tolerance of the mussels to contaminated water and elevated tissue concentrations.

Wraige (1997) mentioned that mussels can become desensitised after exposure to contaminants. In addition, other studies have shown that pre-exposure of mussels to e.g. naphthalene enhanced the elimination of this compound, suggesting amplified excretion after exposure (in Widdows et al., 1983; in Widdows and Donkin, 1992). This may be a strategy of the mussels to reduce their sensitivity to toxic hydrocarbons. This process has been observed before for e.g. fish becoming adapted to PAHs (Fent, 1998). It is also possible that adaptation to a certain pollutant is not specific for these compounds alone but to a wider range of chemicals (cotolerance) (Fent, 1998).

The lower feeding rates of the control mussels from Jade Bay compared to the mussels from Cornwall may well be an indication for a less clean habitat. Widdows et al. (1995) found declining Scope for Growth in North Sea mussels the further south they worked, which mirrors the increasing extent of contamination in the North Sea. The low feeding rates of control mussels from Wilhelmshaven are a strong indication that the mussels were either not healthy or had become desensitised. Thus, they were not as sensitive as the Cornish mussels. If the Vienna basin oil fractions were applied to mussels as sensitive as the ones from Cornwall they probably would induce a toxic effect.

4.2 Toxicity of monoaromatic hydrocarbon fractions to blue mussels

In order to gain more information about the question of the relative toxicity of the aromatic subfractions, the monoaromatic fractions were isolated from the total aromatic fractions of the four crude oils by HPLC. These four monoaromatic hydrocarbon fractions were applied to blue mussels in the same way as the total aromatic hydrocarbon fractions, employing aqueous solutions of nominal concentrations between 0 and 200 µg/L. Results from these toxicity test are presented and compared here, followed by discussion of the results for all eight mixtures of aromatic hydrocarbons (Section 4.3). For mean feeding rates of control mussels, the reader is referred back to Table 4-2.

4.2.1 Toxicity test with the Monterey Formation oil fractions (experiment 2)

The results of the toxicity tests with the two monoaromatic hydrocarbon fractions of the Monterey Formation oils are listed in Tables 4-12 and 4-13. Several tissue samples of mussels exposed to the degraded oil fraction were contaminated and body burdens could not be determined.

Table 4-12 Nominal aqueous concentration [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of controls [%], reduction of feeding rate [%] and body burdens of mussels exposed to the monoaromatic hydrocarbon fraction of the undegraded Monterey Formation oil, no red.: no reduction since FR > 100%.

Nominal aqueous concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	55	0.26 ± 0.02	100	0	0
50	6	0.12 ± 0.03	46	54	50
50	7	0.31 ± 0.04	117	no red.	2
100	7	0.28 ± 0.04	105	no red.	1
100	7	0.18 ± 0.01	67	33	86
200	7	0.27 ± 0.04	101	no red.	4
200	7	0.20 ± 0.03	74	26	11

Table 4-13 Nominal aqueous concentration [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of controls [%], reduction of feeding rate [%] and body burdens of mussels exposed to the monoaromatic hydrocarbon fraction of the degraded Monterey formation oil, no red.: no reduction since FR > 100%, n.d.: not determined.

Nominal aqueous concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	55	0.26 ± 0.02	100	0	0
50	7	0.27 ± 0.04	104	no red.	n.d.
50	7	0.32 ± 0.04	121	no red.	n.d.
100	7	0.29 ± 0.03	110	no red.	n.d.
100	7	0.21 ± 0.04	81	19	39
200	7	0.24 ± 0.05	93	7	n.d.
200	7	0.21 ± 0.03	81	19	32

The dose-response relationships are shown in Figure 4-17 and Figure 4-18.

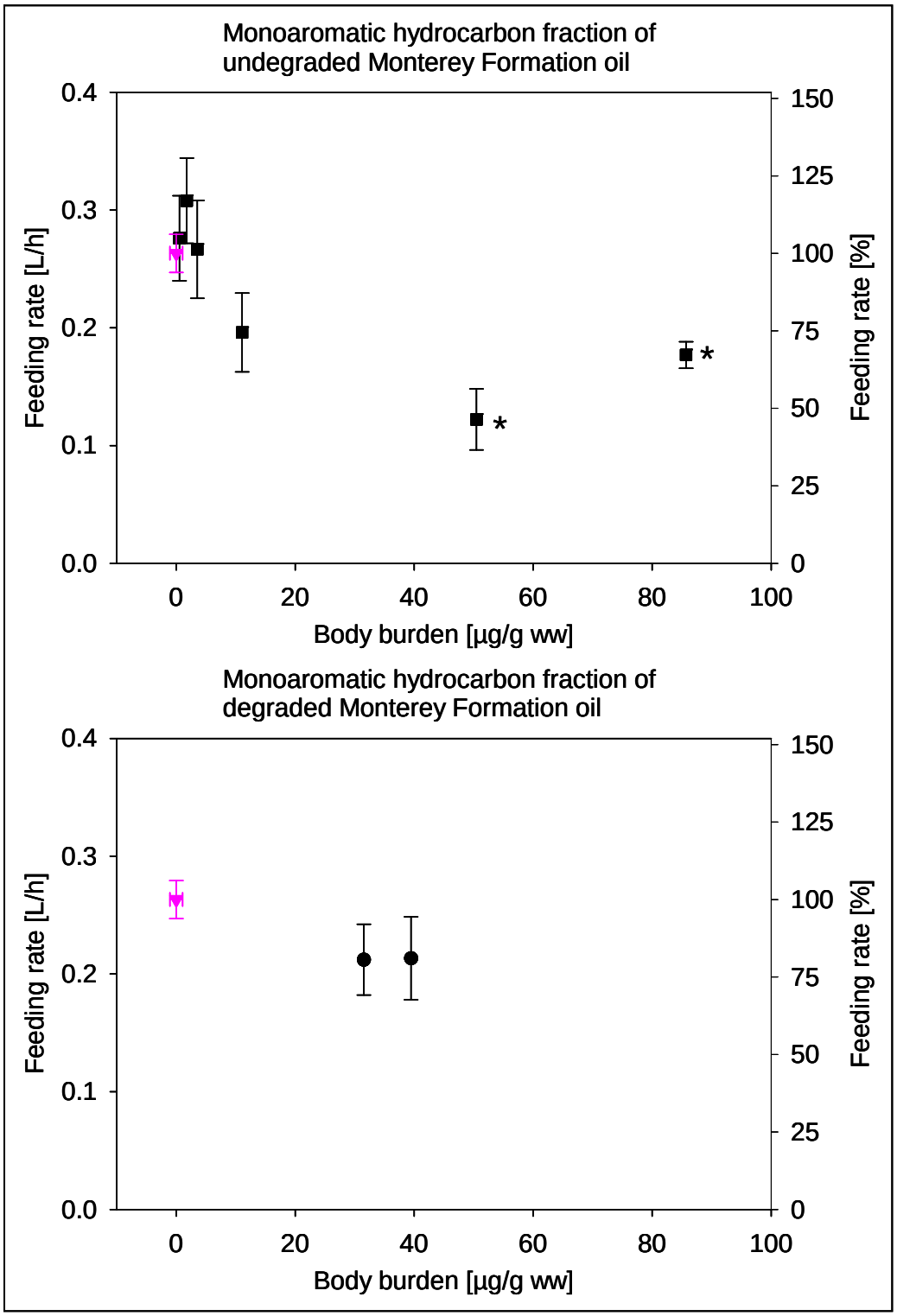


Figure 4-17 Body burden-feeding rate plots of the two monoaromatic hydrocarbon fractions of the Monterey Formation oils. The control mean (grey triangle) and data points are shown with error bars (se) for feeding rate mean values. Asterisks denote statistically significant differences from the control mean feeding rate. The tissue concentration of the control is shown with standard deviation (n = 2).

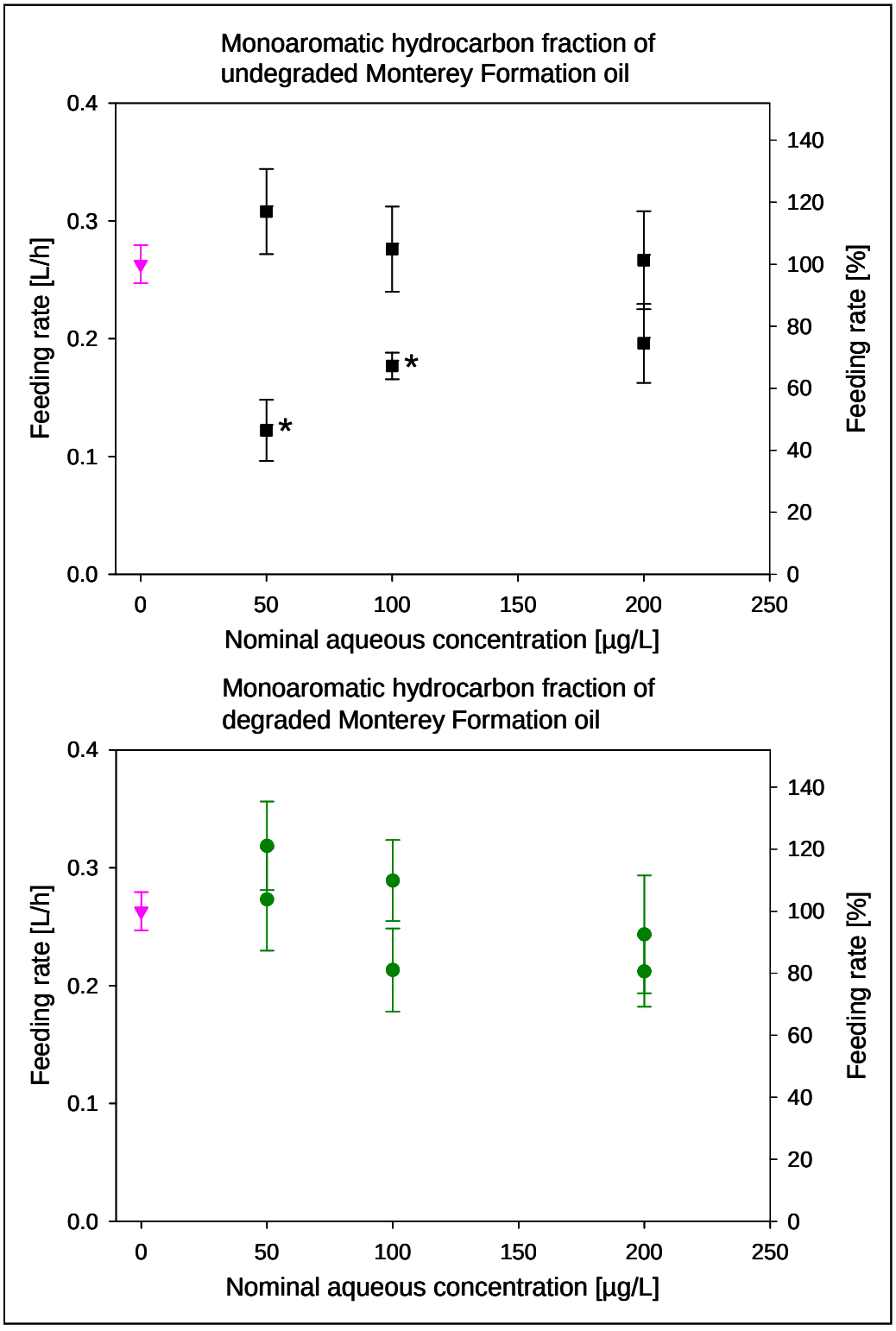


Figure 4-18 Nominal aqueous concentration-feeding rate curves of the two monoaromatic hydrocarbon fractions of the Monterey Formation oils. The control mean (grey triangle) and data points are shown with error bars (se) for feeding rate mean values. Asterisks denote statistically significant differences from the control mean feeding rate.

The accumulation of hydrocarbons from the undegraded Monterey Formation oil monoaromatic hydrocarbon fraction caused a reduction of feeding rate (Figure 4-17, top). The lowest feeding rate is reduced by over 50% compared to controls at a body burden of 50 µg/g wet weight. The higher body burden of 86 µg/g wet weight caused a reduction of feeding rate by 33%.

Most of the tissue samples from the experiments with the degraded oil fraction became contaminated during the clean-up step. However, the gas chromatograms of the total extracts do not show accumulated compounds in the tissue. Three of the lost four samples did not show a reduced feeding rate and there was no statistical difference to the control. The remaining two values in the diagram (Figure 4-17, bottom) show a reduction of feeding rate but unfortunately, there are not enough values to perform a valid regression of the data.

The plot of the monoaromatic hydrocarbon fraction of the undegraded Monterey Formation oil shows a slight decline in feeding rate with higher aqueous concentrations (Figure 4-18, top) but the lowest feeding rate value was determined at the lowest nominal aqueous concentration of 50 µg/L. The monoaromatic hydrocarbon fraction of the degraded Monterey Formation oil shows a continuously decreasing feeding rate with increasing nominal aqueous concentration of hydrocarbons (Figure 4-18, bottom). However, no feeding rate value is statistically different from the control mean value.

Both nominal aqueous concentration-feeding rate curves (Figure 4-17) show a slight increase of feeding rates in one group of mussels at a nominal aqueous concentration of 50 µg/L and 100 µg/L which is not statistically different from the control mean. Similar increases in mean feeding rates were observed before (2-5%; Wraige, 1997) and can be attributed to the natural fluctuations in feeding behaviour of the animals. All in all, the feeding rates decrease with increasing concentrations of monoaromatic hydrocarbons, thus showing a toxic effect.

The uptake patterns of monoaromatic hydrocarbons from the Monterey Formation oils are displayed in Figure 4-19. Only two groups of mussels have taken up considerable amounts of hydrocarbons from the undegraded Monterey Formation oil (left), the other four groups do not show elevated tissue concentrations. At 200 µg/L nominal toxicant solution, both mussel groups have not accumulated much. One possible reason for this is that the solubility limit was reached for the monoaromatic UCM, similar to the total aromatic hydrocarbon fractions discussed above (Section 4.1.1). However, no oily layer had been observed during preparation of the solution of monoaromatic compounds. Smith (2002) found an aqueous solubility of 560 µg/L for a monoaromatic UCM, whereas Booth (2004) reported the highly variable aqueous solubility to be much lower at 57 ± 21 µg/L. Even with the help of acetone as a solubility mediator, the loading of the water with 200 µg/L

monoaromatic hydrocarbons may have exceeded the solubility, resulting in a much lower actual concentration (*cf.* Sections 2.5 and 4.1.1).

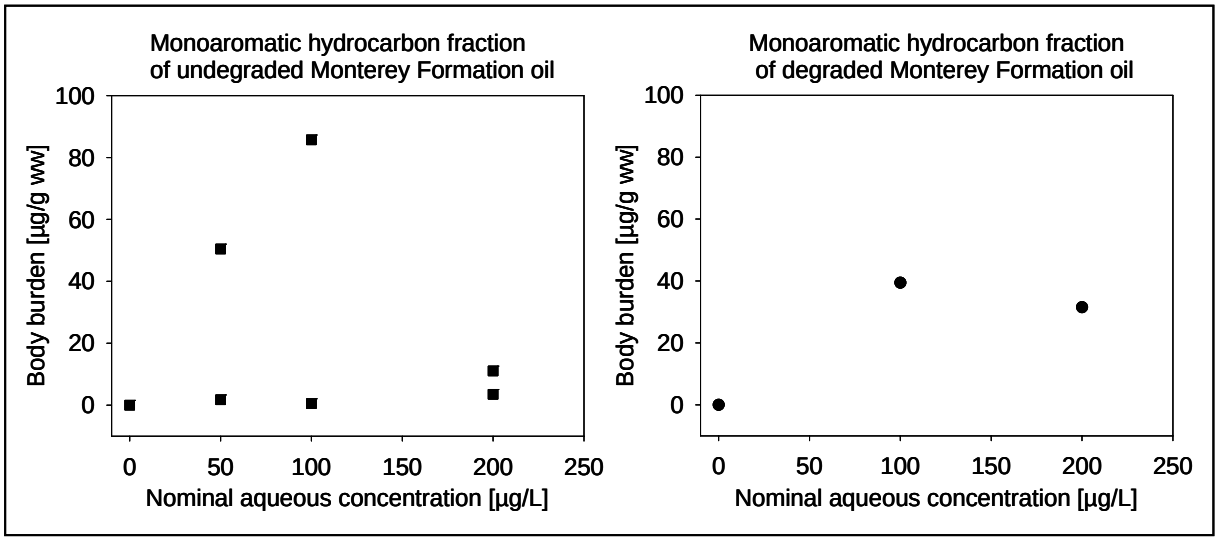


Figure 4-19 Uptake patterns of monoaromatic hydrocarbon fractions of undegraded (left) and degraded (right) Monterey Formation oils.

On the right hand side in Figure 4-19, the uptake of monoaromatic hydrocarbons of the degraded oil by two groups of mussels can be seen. The tissue concentrations of 32 and 39 µg/g wet weight are similar to the second highest body burden aquired from the monoaromatic fraction of the undegraded Monterey Formation oil (50 µg/g wet weight).

4.2.2 Toxicity test with Vienna basin oil fractions (experiment 4)

The results of the toxicity tests with the two monoaromatic hydrocarbon fractions of the Vienna basin oils are given in Tables 4-14 and 4-15.

Table 4-14 Nominal aqueous concentration [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of controls [%], reduction of feeding rate [%] and body burdens of mussels exposed to the monoaromatic hydrocarbon fraction of the undegraded Vienna basin oil, no red.: no reduction since FR > 100%.

Nominal aqueous concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	68	0.22 ± 0.02	100	0	0
50	7	0.22 ± 0.03	100	0	-2
50	7	0.19 ± 0.05	86	14	-3
100	6	0.24 ± 0.04	110	no red.	-7
100	7	0.27 ± 0.05	122	no red.	-6
200	6	0.17 ± 0.04	76	24	11
200	7	0.16 ± 0.03	74	26	4

Table 4-15 Nominal aqueous concentration [µg/L], number of mussels (n), mean feeding rate (FR) values with standard errors (se), as percentage of controls [%], reduction of feeding rate [%] and body burdens of mussels exposed to the monoaromatic hydrocarbon fraction of the degraded Vienna basin oil, no red.: no reduction since FR > 100%.

Nominal aqueous concentration [µg/L]	n	Feeding rate [L/h]	FR [%]	Reduction of FR [%]	Body burden [µg/g ww]
0 (Controls)	68	0.22 ± 0.02	100	0	0
50	7	0.17 ± 0.04	79	21	3
50	7	0.14 ± 0.04	65	35	1
100	7	0.21 ± 0.03	97	3	21
100	7	0.14 ± 0.03	65	35	10
200	7	0.18 ± 0.04	84	16	14
200	7	0.26 ± 0.04	117	no red.	32

The monoaromatic hydrocarbon fraction of the undegraded Vienna basin oil was not accumulated to high amounts with only two samples containing compounds in higher concentrations than the control mean (Table 4-14). The compounds from the degraded oil fraction were taken up to a higher extent (Table 4-15). For both exposure experiments, feeding rate values are not statistically significantly different from the control mean value. The dose-response relationships are shown in Figure 4-20 and Figure 4-21. Due to the low accumulation from the undegraded oil fraction, the tissue concentration-feeding rate plot cannot be interpreted properly. Biological responses may have been caused by toxic volatile or low concentrated compounds, which may have escaped detection after extraction of the tissue samples.

The reason for the high variability of feeding rates is probably biological variance, especially since one group of mussels has a body burden below zero but also shows a lower feeding rate than the control mean (Figure 4-20, top). The nominal aqueous concentration-feeding rate curve in Figure 4-21 show slightly reduced feeding rates at the highest aqueous concentration.

The hydrocarbon fraction of the degraded Vienna basin oil (Figure 4-20, bottom) shows a totally different pattern. The feeding rates increase although there are body burdens of monoaromatic compounds around 40 µg/g wet weight. This increase in feeding rates is also visible in the aqueous concentration-response curve of the degraded oil (Figure 4-21, bottom).

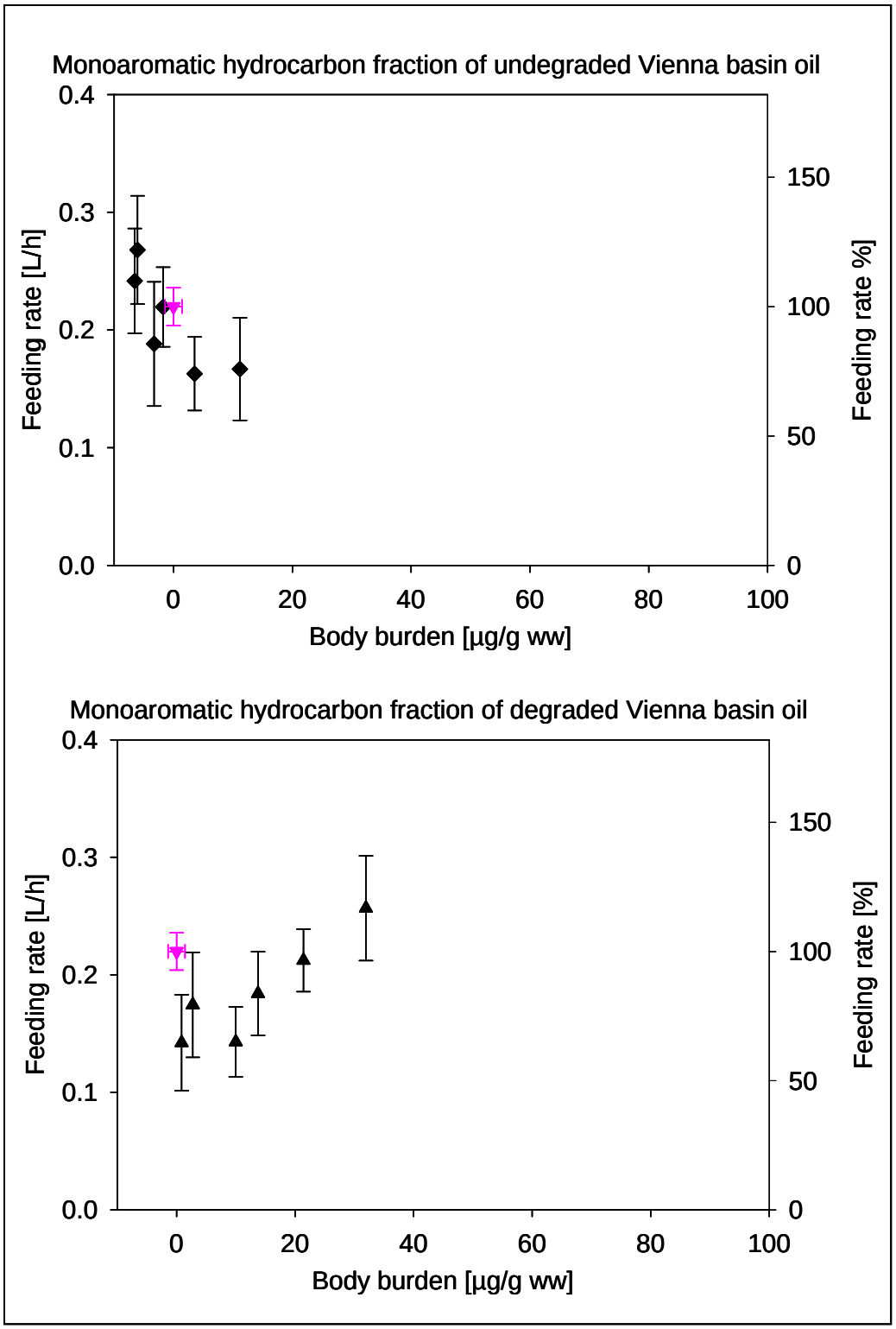


Figure 4-20 Body burden-feeding rate curves of the two monoaromatic hydrocarbon fractions of the Vienna basin oils. The control mean (triangle) and data points are shown with error bars (se) for feeding rate mean values. The tissue concentration of the control is shown with standard deviation (n = 7).

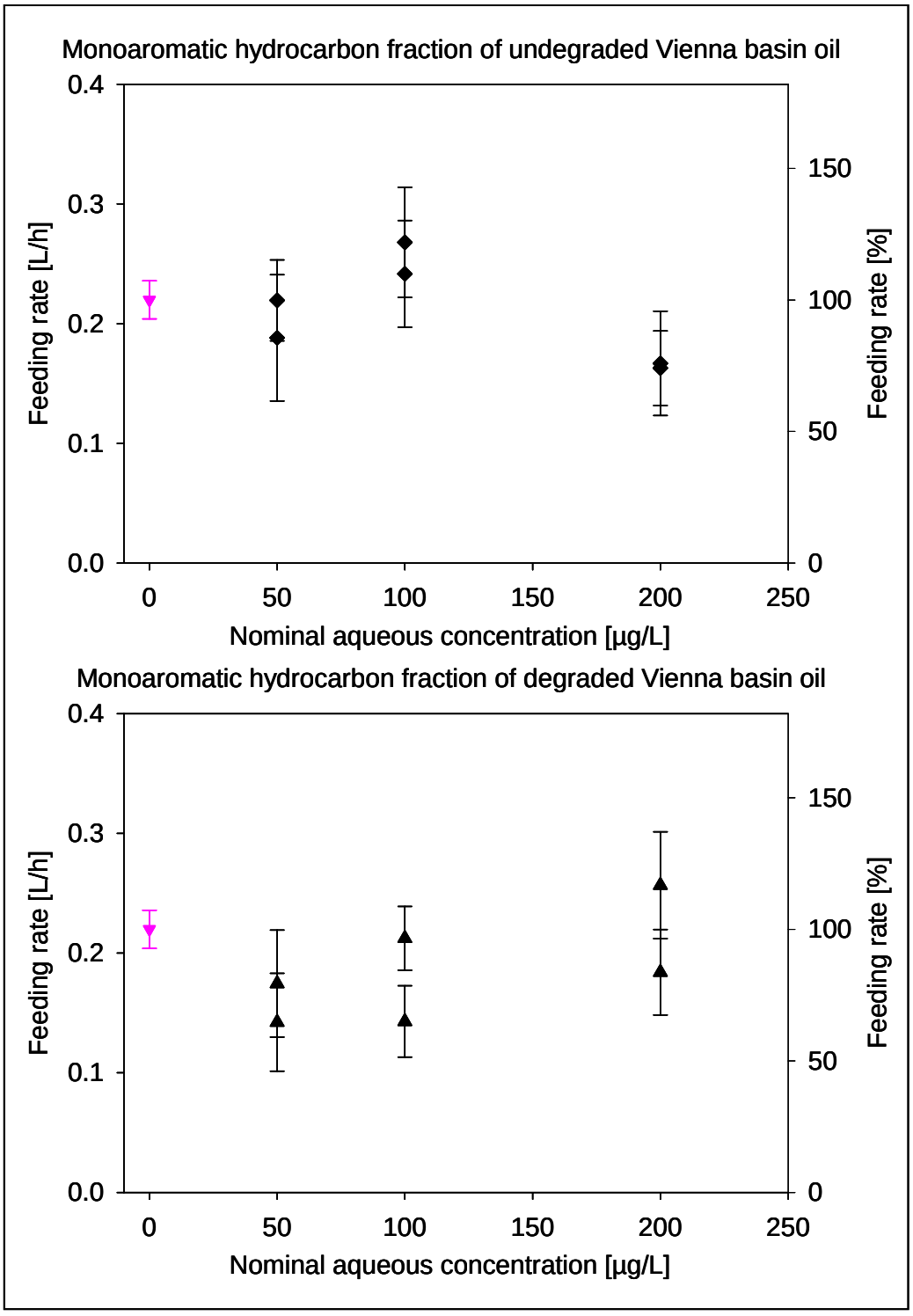


Figure 4-21 Nominal aqueous concentration-feeding rate curves of the two monoaromatic hydrocarbon fractions of the Vienna basin oils. The control mean (triangle) and data points are shown with error bars (se) for feeding rate mean values.

The uptake patterns in Figure 4-22 illustrate the low accumulation of monoaromatic compounds from the Vienna basin oil fractions. This is in particular true for the undegraded oil fraction although the solution with a nominal concentration of 100 µg/L contained 85 µg/L monoaromatic compounds, as determined by re-extraction of the solution (see Table 4-5). Thus, mussels seem to have stopped feeding during exposure or did not accumulate

hydrocarbons due to other reasons. Compounds from the degraded oil (Figure 4-22, right) were taken up in linear relation to the concentration in the aqueous solution (100 and 200 µg/L).

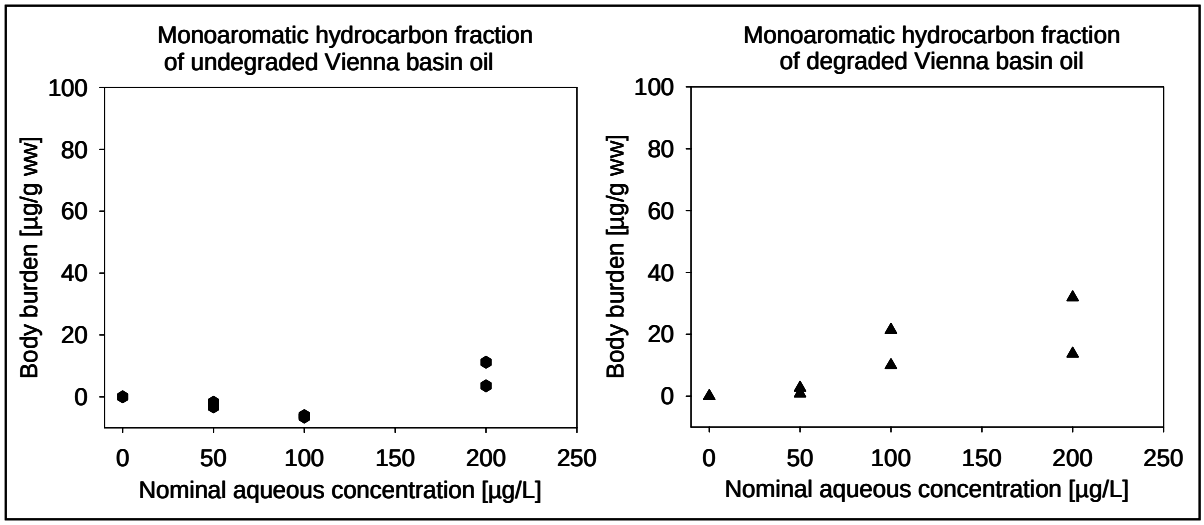


Figure 4-22 Uptake patterns of monoaromatic hydrocarbon fractions of undegraded (left) and degraded (right) Vienna basin oils.

4.2.3 Differences in mussel response to the four monoaromatic hydrocarbon fractions

Table 4-16 summarises the results of the toxicity tests with the monoaromatic hydrocarbon fractions from the four crude oils.

Table 4-16 Summary of hydrocarbon uptake by exposed mussels and feeding rate response to the monoaromatic hydrocarbon fractions of the different oils.

Experiment number	Oil	Uptake range [µg/g ww]	Feeding rate	Toxic?
2	Undegraded M	0-86	reduction	Toxic to Cornish mussels
	Degraded M	0-39	reduction	Toxic to Cornish mussels
4	Undegraded V	0-11	no reduction	Not toxic to German mussels
	Degraded V	0-32	stimulation	Not toxic to German mussels

Aqueous and tissue concentration-response curves for the exposure of mussels from Cornwall to monoaromatic hydrocarbon fractions revealed a reduction of feeding rates with increasing accumulation of monoaromatic hydrocarbons from the undegraded Monterey Formation oil. The steepest decline in feeding rate reduction was caused by the undegraded Monterey Formation oil fraction.

The mussels exposed to the monoaromatic compounds from the Vienna basin oils did not take up many hydrocarbons from the undegraded oil fraction. The monoaromatic

hydrocarbons from the degraded Vienna basin oil were accumulated to the same extend as those from the degraded Monterey Formation oil. Surprisingly, accumulation of monoaromatic compounds from the degraded Vienna basin oil correlates with increased feeding rates. Looking back to experiment 6 with the total aromatic hydrocarbon fraction of this Vienna basin oil (see Section 4.1.4), the same effect was observed in the nominal aqueous concentration-response relationship. Thus, it may be the case that some monoaromatic compounds in the degraded Vienna basin oil can induce a stimulation of mussel feeding rates.

It is known from other species that low level contamination of oil can induce an increased immune response, leading to a stimulation of the observed parameter. For example, Gordon and Prouse (1973) found stimulation of photosynthesis in phytoplankton at low level oil exposure (10-39 µg/L) and subsequent supression at higher levels (60-200 µg/L). Anderson (1977) cited several studies in his review which mention a stimulation of respiratory rate in fish at low concentrations, following a decline in respiration at higher concentrations of some oil mixtures. In general, toxicants sometimes stimulate reproduction at low concentrations, rather than reduce it. The reason is largely unknown, maybe it is caused by suppression of a secondary stress by the toxicant at low concentrations (Kooijman, 1998).

Hawkins and Bayne (1992) summarized results which indicate that increased feeding rates of blue mussels after environmental changes such as the composition of organic compounds in the natural seawater do not necessarily lead to higher food acquisition and an elevated state of mussel health. Rather, generation of pseudo-facies increased, indicating an incomplete use of the aquired food. This shows that there are instances in which high feeding rates do not necessarily indicate healthy mussels but rather signify that the mussels are affected in some way by the change in their environment (Hawkins and Bayne, 1992). Maybe the presence of contaminants affects the mussels in a similar way, so that the high feeding rates observed in experiments 4 and 6 do not point towards a high state of mussel health but to a disturbance in feeding behaviour.

If indeed there are compounds in the aromatic hydrocarbon fractions which stimulate mussel feeding rates, this may mask any adverse effect which is caused by toxic compounds, thus resulting in a pattern of feeding rates which does not indicate a clear trend towards the reduction of feeding rates. These presumably monoaromatic hydrocarbons should be more highly concentrated in the degraded Vienna basin oil since the undegraded oil fraction did not show a similar stimulation of feeding rates. However, since all Vienna basin oil samples did not induce large toxic effects, some of these stimulating compounds may be present in both oils and some may be polyaromatic, as well. Unfortunately, accumulation from the aqueous solutions of monoaromatic hydrocarbons yielded too low tissue concentrations to perform compound-targeted GC-MS analysis.

Previous experiments with blue mussels have not shown a consistently increasing feeding rate with higher contaminant concentration (to the author´s knowledge). A single case of higher feeding rate compared to the control mean value was reported before (2-5%; Wraige, 1997). The similar shape of the aqueous concentration-response curves of a second experiment with the total aromatic hydrocarbon fraction of the same oil (see Figure 4-15) indicates that the increasing feeding rate may not be an isolated occurrence but a measurable effect. Maybe the proportion of monoaromatic compounds was different in the toxicant solutions in experiment 4 from that of experiment 6. The supposedly stimulating compounds may have been present to a higher extent in the repeat experiment solution than in the first experiment with the Vienna basin oil, which could explain the similarity of the curves in Figures 4-15 and 4-20.

Mussels exposed to the monoaromatic hydrocarbon mixture from the degraded Vienna basin oil show an increasing feeding rate with higher body burdens. This does not mean that the mussels preferred the contaminated water to clean water but implies a disturbance in normal feeding activity. This disturbance may lead to a reduction of feeding rates at higher levels of hydrocarbon contamination. In addition, aromatic compounds may have influenced a biochemical mechanism inside the mussels to which the mussels react with increased filtering. For example, if the energy acquisition efficiency from the food is disturbed, mussels may react with increased feeding in order to compensate the deficit and maintain the net gain of energy.

Another possible explanation is that the mussels may have sensed contamination of the water and tried to avoid uptake of contamination with a slightly reduced filtering activity during the exposure period. Wraige (1997) mentioned that mussels may stop feeding in contaminated waters. In this study, this may be indicated by the low uptake of hydrocarbons from the solution at a measured concentration of 85 µg/L (nominal aqueous concentration of 100 µg/L). However, there is not enough evidence for any of these possible biochemical effects, which were not the focus of this study.

The question remains why the Vienna basin oil monoaromatic compounds do not seem to reduce mussel feeding rate when other monoaromatic single compounds and mixtures have been found to induce narcotic effects on blue mussels. Donkin et al. (1989, 1991), Wraige (1997), Smith (2002) and the present study show various examples for reduced feeding rates due to monoaromatic hydrocarbons (*cf.* Table 2-2). It is possible that a short-term stimulation of feeding rates veiles an underlying reduction of feeding rates but this has never been observed before for feeding rates of blue mussels. However, mixture toxicity is a relatively new realm of research and its complexity poses a lot of challenges, including overlapping effects and effects at low concentrations. Thus, establishing cause-effect relationships is very difficult. The increasing feeding rate may be due to a stimulating effect of

some accumulated compounds but there is not enough data to establish a cause-effect relationship.

As has been discussed in Section 4.1.5, a likely explanation for the absence of feeding rate reduction in hydrocarbon-exposed German mussels is a reduced sensitivity of the German mussels compared to Cornish mussels. This difference in biological response of Cornish and German mussels was observed again in the experiments with the monoaromatic hydrocarbon fractions, which strengthens this line of argumentation. If the German mussels were indeed less sensitive or otherwise affected they may have reacted to hydrocarbon contamination in hereto unknown ways such as an increasing feeding rate as was observed in experiment 4.

4.3 Discussion of toxic effects of different mixtures of aromatic hydrocarbons

4.3.1 Calculation of mixture toxicity of the undegraded Monterey Formation oil fractions

One possible way of comparing the strength of the toxic effects caused by the aromatic hydrocarbon mixtures of the four oils is the comparison of calculated regressions of the dose-response curves. Often, one finds sigmoidal or exponential fittings for dose-response curves, usually on half-logarithmic scales, covering several orders of magnitude of aqueous concentrations. This is also the case for mixtures although one could also assume that the diverse nature of a mixture with hundreds of components with individual toxicities could result in such an overlap of single sigmoidal curves that the emerging picture looks more like a broad linear graph. In particular, the steeply increasing part of a sigmoidal curve without the almost invisible increasing toxicity at very low concentrations and the levelling-off towards high concentrations could be approximated with a first-order regression line.

Due to the above mentioned solubility constraints, it was not possible to cover several orders of magnitude of aqueous concentrations in this study. Exponential and linear regressions did not show large differences in the correlation coefficients, thus not clearly favouring one model. The reason for this is that the concentration range applied in this study more or less represents the almost-linear part of the sigmoidal curve. In addition, Kooijman (1998) argues in favour of a linear function as a first approximation of tissue concentration-response curves.

In order to compare the toxicity of the total aromatic hydrcarbon fraction of the undegraded Monterey Formation oil to literature data, the WEC_{50} (the aqueous concentration which causes 50% of a toxic effect measured) was calculated for the total aromatic hydrocarbon fraction. For this calculation, the feeding rates were plotted against the

logarithmic nominal aqueous concentrations (Figure 4-23). Due to the observed immiscible layers on top of the water and the abovementioned solubility considerations, exposure to 500 µg/L was not included. The calculated WEC_{50} value for blue mussels is 343 µg/L. This is in the range of previously determined WEC_{50} values for monoaromatic compounds, e.g. phenanthrene has a WEC_{50} of 148 µg/L and naphthalene of 922 µg/L (see Table 2-2; Donkin et al., 1989).

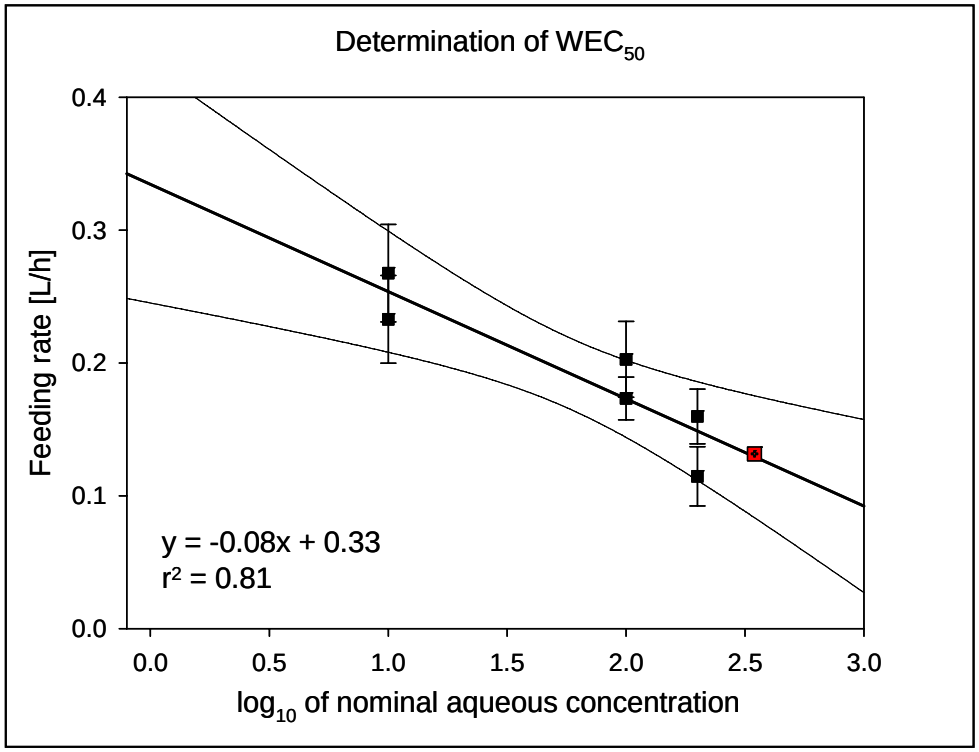


Figure 4-23 Nominal aqueous concentration (log_{10}) and feeding rate with error bars (se) of mussels exposed to the total aromatic fraction of the undegraded Monterey Formation oil. Regression line and confidence intervals are shown. The equation for the regression line was used to calculate the WEC_{50}, shown as red square.

The TEC_{50} (the tissue concentration which causes 50% of a toxic effect measured) was calculated for the total aromatic hydrocarbon fraction of the undegraded Monterey Formation oil (without the outlying values). In Figure 4-24, the feeding rates were plotted against the logarithmic tissue concentrations. The calculated TEC_{50} value for blue mussels is 119 µg/g wet weight. Confidence intervals are also shown. The value is in the range of previously determined TEC_{50} values for monoaromatic compounds and slightly higher than for polyaromatic compounds (Donkin et al., 1989, 1991; Wraige, 1997; Smith, 2002).

Concerning the monoaromatic hydrocarbon fraction of the undegraded Monterey Formation oil, the wide spread of the nominal aqueous concentration data prevented the determination of a WEC_{50} value. A TEC_{50} value of 116 µg/g wet weight was calculated from the regression line of the logarithmic tissue concentrations. Figure 4-25 shows the plot and the confidence intervals.

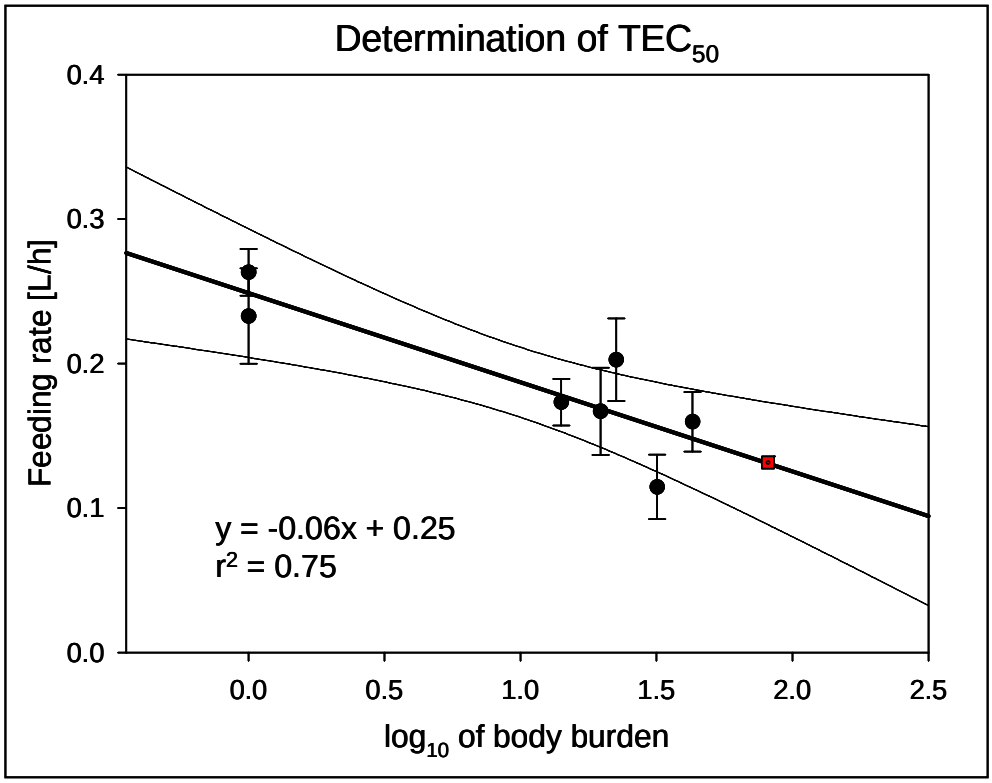


Figure 4-24 Body burden (log_{10}) and feeding rate with error bars (se) of mussels exposed to the total aromatic fraction of the undegraded Monterey Formation oil. Regression line and confidence intervals are shown. The equation for the regression line was used to calculate the TEC_{50}, shown as the red square.

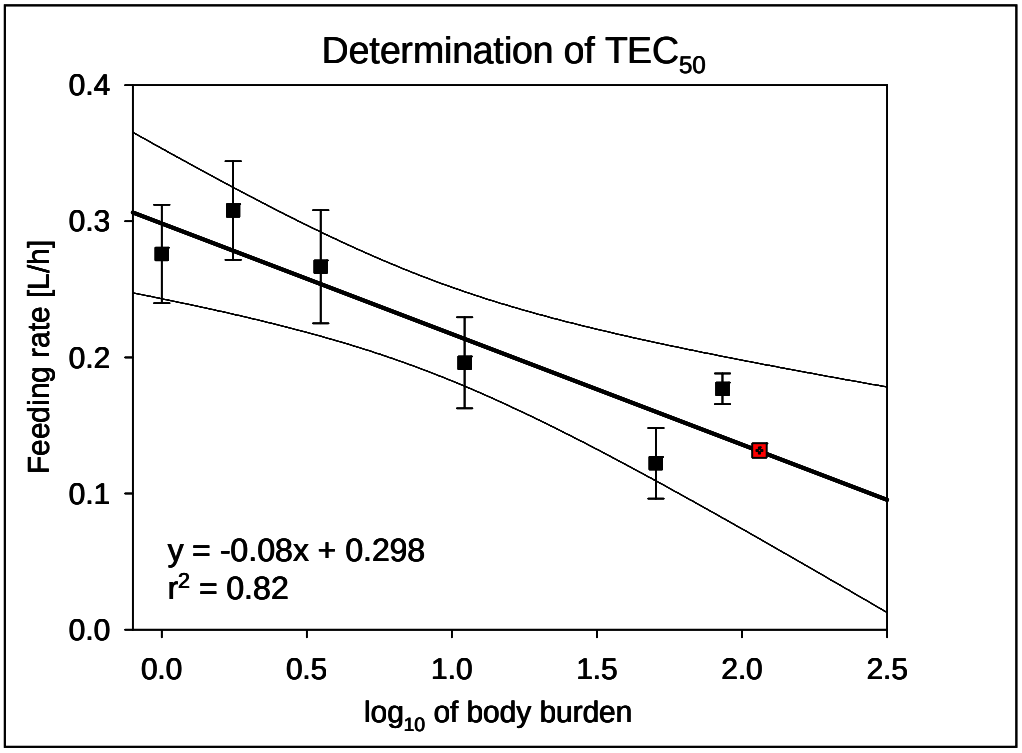


Figure 4-25 Body burden (log_{10}) and feeding rates with error bars (se) of mussels exposed to the monoaromatic fraction of the undegraded Monterey Formation oil. Regression line and confidence intervals are shown. The equation for the regression line was used to calculate the TEC_{50}, shown as red square.

Smith (2002) determined a TEC_{50} value of 500 µg/g wet weight for the monoaromatic hydrocarbon fraction of a crude oil but the confidence intervals were quite high due to extrapolation of data. Smith (2002) suggested that the actual value would be lower. The TEC_{50} value of the undegraded Monterey Formation oil fraction is much lower than the one determined by Smith (2002). In addition, the value is closer to TEC_{50} values of model monoaromatic UCM hydrocarbons. Alkylated benzenes have TEC_{50} values between 35 and 82 µg/g wet weight (Donkin et al., 1989; Donkin et al., 1991) and alkylated *cyclo*hexyl-tetralins have TEC_{50} values between 44 and 138 µg/g wet weight (Wraige, 1997; Smith, 2002). From these TEC_{50} values follows that the Monterey Formation oil monoaromatic fraction exerts a mixture toxicity of a strength similar to that of individual monoaromatic compounds.

4.3.2 Comparison of the toxicity of the total and monoaromatic hydrocarbon fractions from the undegraded Monterey Formation oil

In general, it is established knowledge that the BTEX compounds and naphthalenes are the major reason for toxicity of fresh crude oils. For example, in a study with several nonweathered and in-laboratory weathered oils, monoaromatic hydrocarbons were chiefly responsible for the lethal toxicity of fresh oils towards four marine animals whereas polyaromatic compounds were the major toxicants in weathered oils (Neff et al., 2000). In addition, Donkin et al. (2003) found that the monoaromatic subfraction of an aromatic UCM, which had been accumulated by blue mussels in the field, was more toxic than the polyaromatic subfraction.

However, some scientists emphasise the low aqueous solubility of high-molecular-weight compounds, the resulting low bioavailability and consequently also the low toxic potential of these high-molecular-weight polyaromatics (Bobra et al., 1983). This has been disputed by others (Baussant et al., 2001a) who found that the more hydrophobic PAHs are slowly released into water from oil droplets, so that with extended exposure times high-molecular-weight compounds gain the opportunity to exert toxicity. Other scientists conceded that immediately after an oilspill, one- and two-ring aromatic compounds are highly concentrated in the environment and cause toxic effects, but they point out that the long-term toxicity is predominantly due to three- to five-ring polyaromatic hydrocarbons, which are for example continuously washed out of oiled sediments (Peterson et al., 2003; Short et al., 2003). Rice et al. (1977) stated that the monoaromatic compounds are the least toxic ones and that acute toxicity increases with increasing molecular size towards the four- and five-ring aromatics. Some go so far as to call volatile monoaromatics environmentally irrelevant since they evaporate quickly whereas the polyaromatics accumulate in the marine ecosystems (Heintz et al., 2000). These polyaromatics do not only include parental PAHs but also alkylated ones, which can also cause long-term damage (e.g. mutagenic effects, in Thomas et al., 2002). In

addition, alkylated PAHs are present in oils in higher amounts than the unsubstituted aromatic compounds (e.g. Carls et al., 2001).

These references show that different accounts of oil toxicity can be found in the literature whenever there are not only a few compounds dominating the composition of an oil. This indicates that the answer to the question which group of aromatic hydrocarbons is the most problematic in an ecotoxicological context depends on many aspects. For example, the toxicity of certain compounds may vary for different species and also on the type of toxic mechanisms under consideration.

Looking at the relative narcotic toxicity of mono- and polyaromatic hydrocarbon fractions from the Monterey Formation oil towards blue mussels, the calculated TEC_{50} values of the total aromatic hydrocarbon fraction (119 µg/g wet weight) and the monoaromatic hydrocarbon fraction (116 µg/g wet weight) of the undegraded Monterey Formation oil reveal that the two hydrocarbon fractions are similarly toxic. The monoaromatic TEC_{50} value fits well with previously determined data of alkylsubstituted monoaromatic compounds; 2- and 3-ring PAHs such as naphthalene, phenanthrene and dibenzothiophene were found to be more toxic (Donkin et al., 1989). Thus, the TEC_{50} of the total aromatic hydrocarbon fraction represents a combined toxicity of toxic PAHs, intermediately toxic monoaromatic compounds and some less toxic aromatic compounds.

Monoaromatic compounds comprise about 55% of the total aromatic hydrocarbon fraction of the undegraded Monterey Formation oil. If this percentage is used in order to find out how toxic the remaining aromatic compounds are, it turns out that all other compounds combined give a TEC_{50} of 123 µg/g wet weight (calculated after Faust et al., 2003).

$$119\,\mu g = \left(\frac{55\%}{116\,\mu g/g} + \frac{45\%}{X} \right)^{-1}$$

$$123\,\mu g = X$$

This is of course only a broad calculation since the different aqueous solubilities of mono- and polyaromatic compounds lead to changes in the relative proportions of the aromatic subfractions as will be shown in Chapters 5 and 6. Since the whole mixture includes substances with lower TEC_{50} values (more toxic) (see Table 2-2), other substances must be present with a higher TEC_{50} (less toxic). This ties in with the theory that there may be a toxicity cutoff for high-molecular-weight compounds (e.g. Donkin et al., 1991).

Overall one can conclude that although the monoaromatic UCM compounds are slightly more toxic, the whole aromatic hydrocarbon fraction of a crude oil can exert toxicity on an ecosystem if spilled or discharged.

The extrapolated TEC_{50} values from the experimental data can be compared to TEC_{50} values, which can be calculated for prediction purposes based on literature data. For the prediction, the relative amounts of the mono,- di-, tri- and polyaromatic subfractions (57%, 29%, 9% and 7% as determined by HPLC fractionation of the oil, followed by gravimetry) in the total aromatic hydrocarbon fraction were multiplied with an average TEC_{50} for each subfraction (Table 4-17), which was based on data summarised in Table 2-2.

Table 4-17 TEC_{50} values of aromatic hydrocarbons on feeding rates of blue mussels, values in bold print were used for calculated prediction ([1]Donkin et al., 1991; [2]Donkin et al., 1989; [3]Smith, 2002; [4]Wraige, 1997).

Compound	TEC_{50} [µg/g]
Toluene[1]	16
n-Propylbenzene[1]	27
n-Pentylbenzene[2]	94
n-Heptylbenzene[2]	35
n-Octylbenzene[2]	82
6-*cyclo*hexyltetralin[3,4]	44
7-*cyclo*hexyl-1-methyltetralin[3,4]	58
7-*cyclo*hexyl-1-propyltetralin[3,4]	138
Biphenyl[1]	16
Mean monoaromatic compounds	**57**
Naphthalene[1]	**31**
Phenanthrene[1]	**31**
Pyrene[1]	> 189
Fluoranthene[1]	627
Mean polyaromatic	**408**

Using these surrogate mean values, a predicted TEC_{50mix} was calculated for the total aromatic hydrocarbon fraction (after Faust et al., 2003):

$$TEC_{50mix} = \left(\frac{}{} + \frac{}{} + \frac{}{} + \frac{}{} \right)^{-1}$$

$$TEC_{50mix} = 45.3 \text{ µg/g}$$

Of course, using TEC_{50} data from very few single components as representatives of a whole group of aromatic compounds is a very coarse approximation and apparently, they are not representative for the whole range of compounds present. Their use resulted in an effect concentration lower than the experimentally derived TEC_{50} value of 119 µg/g. This implies that there are hydrocarbons in the mixture which are less toxic than the ones studied in the laboratory by Donkin et al. (1991) but are probably quite similar to the alkyl*cyclo*hexyl-tetralins studied by Wraige (1997) and Smith (2002). This emphasises the necessity to improve the understanding of the chemical properties of constituents of UCMs.

The predicted value of 45 µg/g is overestimating the mixture toxicity of the total aromatic hydrocarbon fraction compared to the experimentally derived TEC_{50} value of 119 µg/g. This is in agreement with other predictions based on the Concentration Addition Concept (Grimme et al., 2000; Backhaus et al., 2003; Faust et al., 2003). A possible reason is that the relative amounts of the aromatic subfractions are derived from the total aromatic hydrocarbon fraction isolated from the orginal oil. Thus, fractionation steps due to solubility and biological uptake have not been taken into account.

In contrast, the prediction of the TEC_{50} for the monoaromatic hydrocarbon mixture of the undegraded Monterey Formation oil resulted in a mixture TEC_{50} value similar to the values of individual monoaromatic hydrocarbons. These were alkylated monoaromatics, which are probably more similar in their structure to compounds present in the UCM than the parent PAHs used for the calculation above. This also demonstrates the necessity to learn more about the constituents of UCMs.

If the experimentally derived TEC_{50} value for the whole monoaromatic fraction (116 µg/g) is used instead of the mean TEC_{50} value derived from single substance tests with monoaromatic hydrocarbons, the predicted mixture toxicity TEC_{50mix} value of the total aromatic hydrocarbon fraction rises to 58 µg/g wet weight. This is still lower than the experimentally derived value for the total aromatic hydrocarbon fraction but if all subfractions in the equation were substituted with proper mean values instead of single substance data, the predicted value would be more precise. This again shows the need for “compound class” or “less complex mixture” data in predictive toxicology, at least for hydrocarbon mixtures.

However, although the EC_{50} values are interesting for comparing toxicity, one has to bear in mind that they are statistical parameters without a high environmental relevance. For example, they are different for each species and each investigated biological endpoint. In addition, since they give the concentration at which 50% of an effect occurred (e.g. growth inhibition, mortality and population damages) they are much too high to be used as “desired environmental concentrations” (Kooijman, 1998). Preferably, legislation should aim at environmental concentrations which do not cause detrimental effects. This was also called for by the “Oslo-Paris-Commission” (OSPAR) in 1998: Until the year 2020, concentrations “close to zero for man-made substances” shall be attempted.

4.3.3 Long-term effects of aromatic compounds and oil residues

Some ecotoxicologists argue that studies with short exposure periods have limited value due to the impossibility to detect long-term damages. This is true for many biological endpoints. However, for the baseline toxicity of narcosis, an increase in toxicity is usually observed until equilibrium of the contaminants is reached. Depending on the solubility and bioavailability of the narcotic, this can take 24 hours or more. When a critical body burden is reached or the site of toxic action is saturated, no further toxic effect is observed (Wraige,

1997), rendering longer exposure times rather ineffective. In addition, there seems to be a range of body burden in which narcosis typically occurs, no matter in which period of time the toxicants were accumulated (van Wezel and Opperhuizen, 1995).

In the experiments with the total aromatic hydrocarbon mixtures in the present study, most groups of mussels took up around 50 µg/g wet weight, which equals approximately 300-450 µg/g dry weight, depending on the set of mussels, which had slightly different conversion factors. This is in the range of environmental concentrations of hydrocarbons in blue mussels. For example, Smith (2002) found predominantly unresolved aromatic hydrocarbons at 392 µg/g dry weight in mussels from Whitby (UK), which was similar to the accumulated body burden of a monoaromatic UCM during her experiments (90 µg/g wet weight; 400 µg/g dry weight). In mussels from the French coast, total PAHs ranged between 0.1 and 300 µg/g dry weight (Widdows and Donkin, 1992). For other reference data, the reader is referred to Table 2-1.

In addition to the effects of short-term exposure, chronic effects of aromatic hydrocarbons can be severe indeed and must not be disregarded. Peterson et al. (2003) report an unexpected persistence of toxic subsurface oil and chronic exposures at a sublethal level which still affects wildlife in the Alaskan coastal ecosystem near the Prince William Sound where the *Exxon Valdez* grounded in March 1989. Carls et al. (2001) showed that oil, which was trapped in sediments, was released after a storm into overlying mussel beds and into the water in Prince William Sound and the Gulf of Alaska. The mussel bed “protected” the oil from weathering so that hydrocarbons have been released continuously into the adjacent mussels, the seawater and the deeper (> 2 cm) sediments. Thus, the oil has not lost its toxicity as suggested by Bence et al. (1996) but continues to affect the ecosystem (Short et al., 2003). It was estimated that it will take 30 years after the spill to reach background levels again (Carls et al., 2001).

Especially for long-term, often indirect effects it is important to understand the ecological significance of chronic contamination, for which Carls et al. (2001) state three ways of impact:

1. chronic exposure to oil compounds of species closely associated with the contaminated (often shallow) sediments;
2. delayed populational impacts of sublethal doses on health, growth and reproduction;
3. indirect effects of interaction and trophic cascades.

These indirect effects may initiate the decimation and even extinction of a population due to reduced health and reproduction, leading to community changes in an ecosystem. Mussels for example metabolise hydrocarbons only slowly, thus their high body burdens are taken up when eaten e.g. by sea otters and accumulate through the food-chain. In addition, cascades in the food-chain may lead to predators reducing the abundance of their prey and consequently the food of the prey is not controlled in number any more, which again may

lead to community changes (Peterson et al., 2003). Crowe et al. (2004) studied the community structure at six sites on the UK coast. At two sites, blue mussels contained UCMs of aromatic hydrocarbons (163 and 250 µg/g dry weight) and had low Scope for Growth values, indicating a reduced state of health. At these sites, the diversity of macrofaunal communities associated with mussels was reduced. This shows that contamination with aromatic hydrocarbons at much lower concentrations than the ones accumulated in this study after 24 hours can indeed lead to effects on the community level of an ecosystem.

Another aspect that is important when aromatic hydrocarbons are accumlated by organisms is the exposure time, which significantly influences the toxic mechanisms. In short-term experiments such as the ones performed in the present study, the dominating toxic effect will be narcosis due to membrane dysfunction whereas in long-term experiments, specific mechanisms of toxic action will become more important (Hermens et al., 1984). This indicates that even if the whole toxic effect in this study was caused due to narcosis by the sum of aromatic hydrocarbons, the overall toxic effect after long-term exposition (e.g. after an oilspill) can be much more severe. Short et al. (2003) studied long-term damage after the *Exxon Valdez* oil spill and found that low levels of long-term crude oil pollution affects the sensitive live stages of marine organisms, especially fish embryos. Each symptom may be hard to detect by itself but the cumulative effect can decrease productivity on the population level. Although immediately after a spill, one- and two-ring aromatic compounds are highly concentrated in the environment and cause toxic effects, the long-term toxicity is predominantly due to three-, four- and five-ring polyaromatic hydrocarbons, which are washed out of oiled sediments continuously (Peterson et al., 2003; Short et al., 2003). In addition, one has to bear in mind that not only parental PAHs but alkylated aromatic compounds can cause long-term damage, too (e.g. mutagenic effects, in Thomas et al., 2002) and that they are present in higher amounts than the unsubstituted aromatic compounds (e.g. Carls et al., 2001). In addition, it is known that not only benzene causes toxicity but alkylbenzenes, too. Analogously, the same is valid for substituted higher aromatic compounds as shown for alkylnaphthalenes (e.g. Anderson, 1977).

In addition to short-term narcosis and long-term specific toxicity at low concentrations, for many substances, it seems unlikely that their toxicity is limited to one specific mechanism in the pharmocological sense, i.e. interaction with a single specific receptor. Most individual components of a mixture have a specific primary target but may have a secondary toxic mechanism on top of the first. These “side-effects” will contribute to the overall toxicity and may interact with other compounds and their toxic mechanism. This becomes more probable the higher the level of organisation (e.g. whole organism is very high), which is used as a toxic parameter (Deneer et al., 1988). For the bioassay used in the present study, this means that the observed endpoint of a reduction of feeding rate may not only be influenced by

narcosis but the specific toxic mechanisms of certain components, too. This is also corroborated by the work of Walter et al. (2002), who calculated EC_{50} values of narcotic toxicities of naphthalene, biphenyl and fluoranthene in comparison to observed EC_{50} values for the specific toxicities of the same compounds on the algae *Scenedesmus vacuolatus*. The EC_{50} values for the baseline toxicity were higher than the ones for the specific toxic effect, indicating that narcosis may still be occurring while the specific mode of toxic action is active. This is reasonable since narcosis is probably due to passive diffusion into the membrane, which will only stop when equilibrium is reached and not because some molecules reached a different site of action in the organism. It has been shown that mixtures of dissimilarly acting toxicants in very low concentrations do indeed cause a joint toxic effect (Grimme et al., 2000; Walter et al., 2002; Backhaus et al., 2003). Thus, the toxic effect caused by a complex mixture of very low concentrated individual aromatic hydrocarbons will be mainly narcosis but some compounds may contribute with their special toxic mechanism.

4.4 Summary – toxicity tests with aromatic hydrocarbon fractions

Dose-response relationships were obtained for the exposure experiments with blue mussels and various complex mixtures of aromatic hydrocarbons isolated from crude oils, which were accumulated by the mussels in environmentally relevant amounts. The overall result of the toxicity experiments is that the aromatic hydrocarbon fractions of the Monterey Formation oils did cause acutely narcotic effects on blue mussels from the Cornish coast, as evidenced by decreasing feeding rates. In addition, there are indications that the Monterey Formation oil fractions disturbed mussel feeding rates from the German coast. The Vienna basin oil fractions apparently did not cause acutely narcotic affects on blue mussels from the German coast.

The reasons for the lack of clearly reduced feeding rates in the German mussels are uncertain, possible explanations are either differences in the sensitivity in the mussels which may be derived from an already affected state of health and consequently saturation of the site of toxic action for the mussels from the Jade Bay near Wilhelmshaven or a different composition of the oil fractions. This means that there may be a certain compound group responsible for the toxic effect which is missing in the Vienna basin oils but present in the Monterey Formation oils. In one case, activation of feeding was observed with increasing tissue concentration of aromatic compounds, which may indicate a phenomenon of masking of toxic effects so that no changes in feeding rates were determined.

The feeding rates of mussels exposed to the solutions at a nominal aqueous concentrations of 500 µg/L were not further reduced compared to the nominal aqueous concentrations of 200 µg/L. It was shown that this was due to excess loading of the aqueous solution and quantification of body burdens proved that the determination of tissue

concentrations is indispensable when attempting to establish cause-effect-relationships of biological responses.

The TEC_{50} values of the monoaromatic and total aromatic hydrocarbon fractions of the undegraded Monterey Formation oil showed that the two fractions have a similar toxic potential. That means that whenever oil is spilled into an ecosystem, all aromatic compounds combined and not only a few will exert toxicity.

5 Results and discussion – aromatic hydrocarbons in the four crude oils

Within the last decades, structure identification of crude oil components has been of major interest. This is particularly true for compounds which are easily accessible by conventional analytical techniques such as GC-MS. Compounds within the unresolved complex mixture (UCM) of a crude oil are hard to identify due to coelutions and resulting mixed mass spectra. Thus, for many aromatic compound classes, the less structurally diverse isomers of the lower-molecular-weight homologues have been identified but with increasing number of isomers, identification becomes more difficult. In addition, mass spectra of positional isomers often are almost identical to each other so that definite identification can only be achieved with coinjection of authentic standards, which usually have to be specifically synthesised.

In the present study, identification of compounds is based on their respective mass spectra and retention times in comparison with standard compounds and literature data. The complexity of the aromatic hydrocarbon fractions often prevents an unambiguous identification of compounds. Therefore, ion chromatograms of specific structural fragments within compound classes (Table 5-1) were used to gain information regarding their distribution. There are many unknown compounds in the oil fractions but their mass spectra do not provide reliable structural information due to the high background of unresolved compounds. Therefore, mass spectra of only a few unknown compounds are shown and several relatively large peaks could not be identified.

Table 5-1 Target compound classes and their characteristic molecular ions or key fragments, n = number of carbon atoms in side chain.

Compounds/compound class	Characteristic fragment
Alkylbenzenes	*m/z* 91.1
Alkyltoluenes	*m/z* 105.1
Alkylxylenes	*m/z* 119.1
Alkyltrimethylbenzenes	*m/z* 133.1
Alkylindanes	*m/z* 117.1
Alkylmethylindanes	*m/z* 133.1
Alkyltetralins	*m/z* 133.1
Monoaromatic steroids	*m/z* 253.3
C_0- to C_3-Naphthalenes	*m/z* 128.1 + 14n
C_0- to C_3-Phenanthrenes	*m/z* 178.2 + 14n
Triaromatic steroids	*m/z* 231.2
Alkylthiolanes	*m/z* 87.1
Alkylthianes	*m/z* 101.1
Alkylthiophenes	*m/z* 97.1
Alkylmethylthiophenes	*m/z* 111.1
Isoprenoid thiophenes	*m/z* 125.1
Alkylbenzothiophenes	*m/z* 147.1
Alkylmethylbenzothiophenes	*m/z* 161.2
C_0- to C_3-Dibenzothiophenes	*m/z* 184 + 14n

Monoaromatic compounds were analysed in the monoaromatic hydrocarbon fractions which were isolated by HPLC from the total aromatic hydrocarbon fractions. Polyaromatic hydrocarbons were investigated in the total aromatic hydrocarbon fractions and additionally in less complex diaromatic subfractions from the HPLC separation. Due to the laboratory procedures, compounds with a high volatility were lost or depleted so that they are not included in the analysis.

In this chapter, the compositions of the monoaromatic hydrocarbon fractions and of the total aromatic hydrocarbon fractions of the two oil pairs are analysed and discussed (Sections 5.1 and 5.2). In Section 5.3, the sulphur-containing compounds in the different oil fractions are investigated. Section 5.4 offers a short summary.

5.1 Distribution of monoaromatic hydrocarbons in the four crude oils

All of the four oils contain approximately 25% aromatic hydrocarbons (see Table 3-1), of which monoaromatic hydrocarbons represent the largest subfraction with around 50%. The monoaromatic hydrocarbons make up 10-14% of the whole oils. Monoaromatic compounds have one benzene ring, which can be substituted with aliphatic and alicyclic moieties. The main focus of this section are monocyclic compounds but dicyclic and polycyclic monoaromatic compounds are also touched upon.

Reconstructed ion chromatograms (RIC) of the monoaromatic hydrocarbon fraction of the four oils are shown in Figure 5-1. All chromatograms show a pronounced unresolved complex mixture with several seemingly resolved peaks. Most peaks, however, are not identifiable as individual compounds but actually result from coeluting with UCM components.

5.1.1 Monocyclic monoaromatic hydrocarbons in the Monterey Formation oils

Monoalkylated benzenes (*m/z* 91, Figure 5-2 a) in the undegraded Monterey Formation oil show a homologous series with alkyl chain lengths from C_6 to C_{25}. There is only a relatively small UCM visible. The distribution pattern of alkylated toluenes (*m/z* 105, Figure 5-2 b) is more complex, showing a triplet of major isomers with *n*-alkyl chain lengths from C_5 to C_{17}. These are the *ortho*-, *meta*- and *para*-substituted isomers, with the *meta*-substituted one eluting first, followed by the *para*- and *ortho*-alkyltoluenes (Ellis et al., 1992). In the undegraded Monterey Formation oil, the least abundant isomers are the *para*- and the most abundant isomers are the *ortho*-alkyltoluenes. The mass chromatograms for xylenes (*m/z* 119, Figure 5-2 c) show a high number of isomers. Figure 5-2 d (*m/z* 133) displays the more complex mass chromatogram for the alkyltrimethylbenzenes but no homologous series is visible. This shows that with increasing number of isomers in a compound class, the probability for coelutions increases and the UCM beneath the resolved compounds becomes more prominent. More complex structures, which may even structurally not be related, can

also produce the same fragment and may contribute to this “hump“ (e.g. Frysinger et al., 2003).

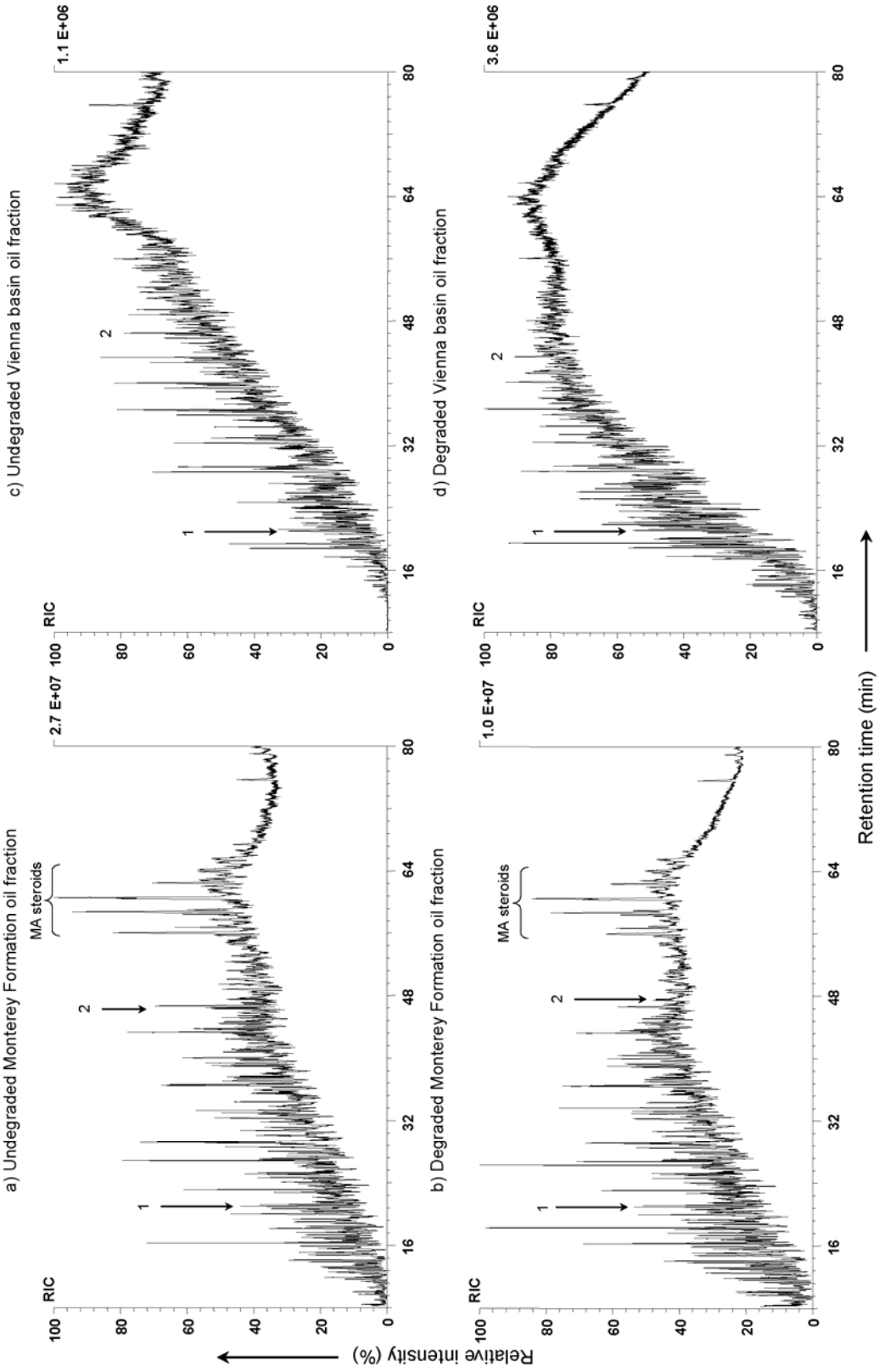


Figure 5-1 Partial reconstructed total ion current chromatograms (RIC) of monoaromatic hydrocarbon fractions of a) undegraded and b) degraded Monterey Formation oils, c) undegraded and d) degraded Vienna basin oils, 1: octylbenzene, 2: tetradecyltoluene, MA: monoaromatic.

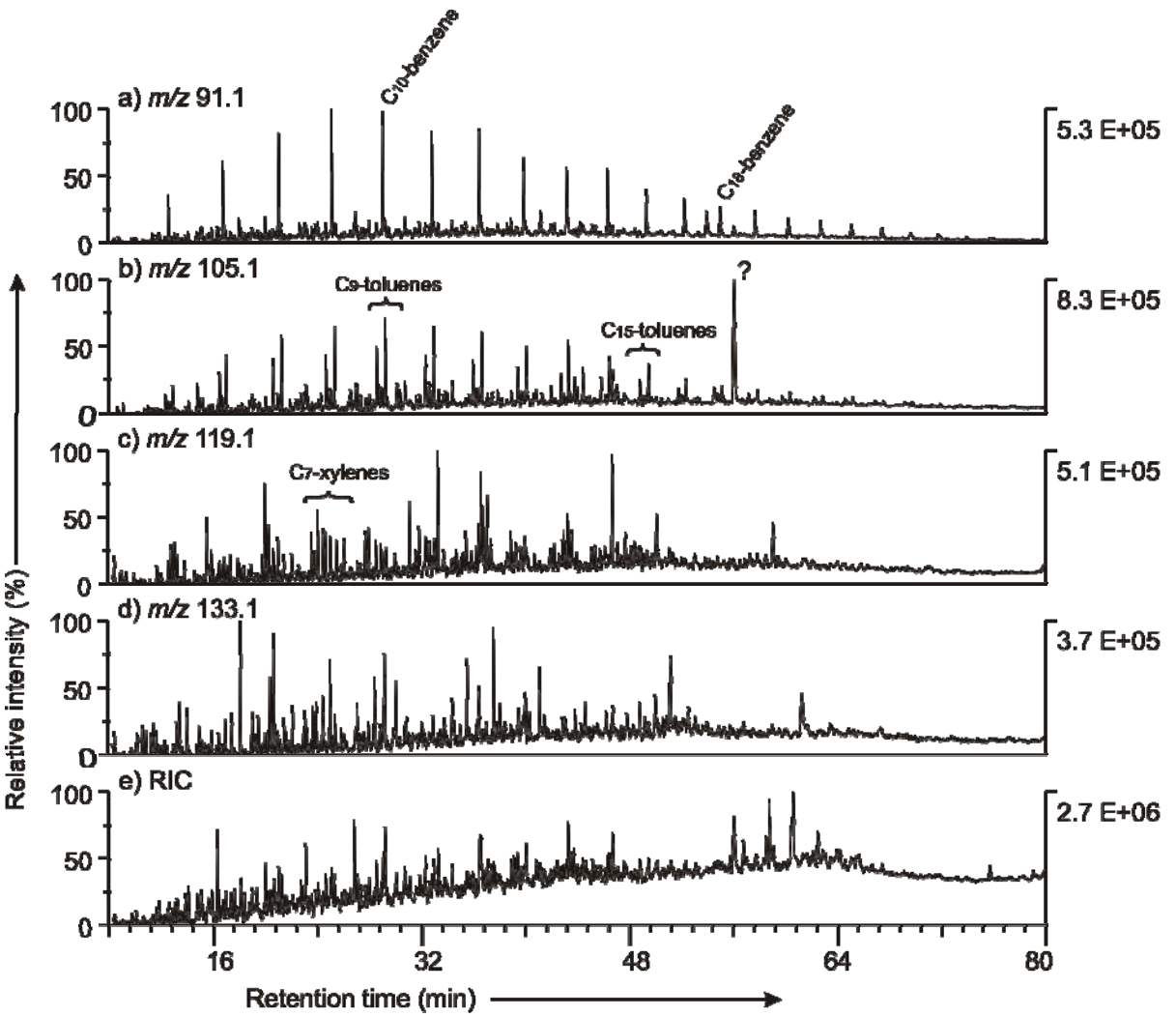


Figure 5-2 Partial reconstructed total ion current (e) and selected mass chromatograms of a) alkylbenzenes, b) alkyltoluenes (elution order: *m*-, *p*-, *o*-), c) alkylxylenes and d) alkyltrimethylbenzenes of the monoaromatic fractions of the undegraded Monterey Formation oil.

In the degraded Monterey Formation oil, monoalkylated benzenes (*m/z* 91, Figure 5-3 a) are present from 6 to 13 carbon atoms in the alkyl chain. Alkyltoluenes (*m/z* 105, Figure 5-3 b) comprise homologues with sidechains ranging from C_5 to C_{14}. For the shorter chain lengths, the same pattern of triplets can be observed as in the undegraded oil. With increasing length of the sidechain from C_{10} onwards, the *meta*- and *para*-isomers gradually disappear. However, this does not imply a generally higher susceptibility of these two positional isomers to biodegradation compared to the *ortho*-alkyltoluenes. Biodegradation studies have shown that different bacteria degrade different isomers at different rates. For example, Harms et al. (1999) isolated two strains of sulphate reducing bacteria from an oil-degrading enrichment culture, which had in common the ability to oxidise toluene and benzoates. Additionally, one strain utilised *o*-ethyltoluene and *o*-xylene in contrast to the other which preferred *m*-ethyltoluene and *m*-xylene. Another study showed that many bacteria degraded toluene and at slow rates also *p*-xylene but there were only some among them which could also degrade certain isomers of C_2- or C_3-benzenes (Spormann and

Widdel, 2000). In general, anaerobic bacteria degrade low-molecular-weight *o*- and *m*-alkylbenzenes better than *p*-substituted ones (Wilkes et al., 2000). This shows that various bacteria prefer different positional isomers of a substance. This can lead to a depletion of certain isomers in a biodegraded crude oil, depending on the microorganisms present in the reservoir. It is therefore not uncommon that the distribution of positional isomers differs from one degraded oil to another.

In-reservoir biodegradation clearly reduced the longer-chain alkylbenzenes and -toluenes in the degraded Monterey Formation oil compared to the undegraded oil. This is usually attributed to the similarity of compounds with long alkyl chains to aliphatic compounds, which are more susceptible to microbial degradation (Alexander, 1999). The compound labelled with a question mark in Figure 5-2 and Figure 5-3 is a branched (probably an isoprenoid) C_{20}-toluene.

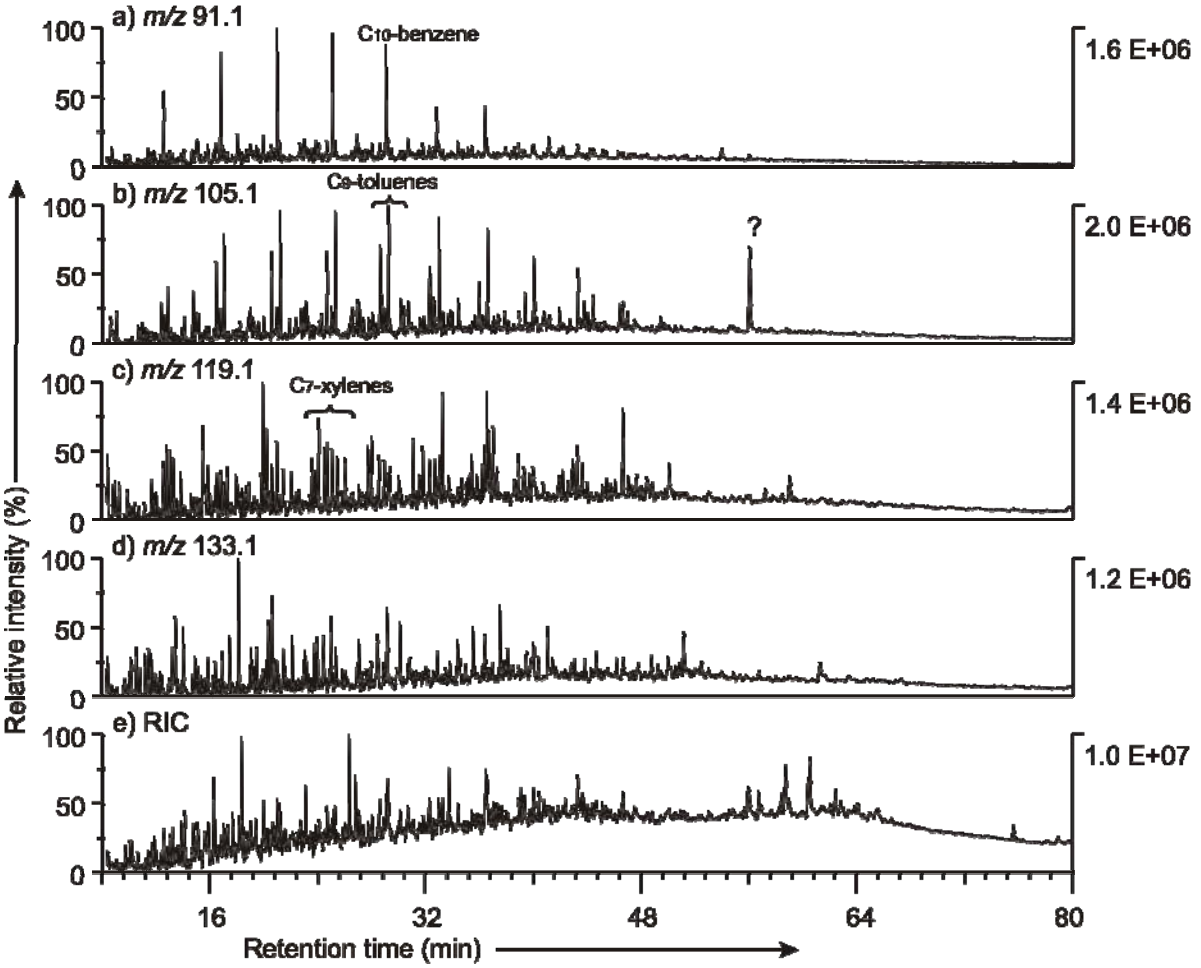


Figure 5-3 Partial reconstructed total ion current (e) and selected mass chromatograms of a) alkylbenzenes, b) alkyltoluenes (elution order: *m*-, *p*-, *o*-), c) alkylxylenes and d) alkyltrimethylbenzenes of the monoaromatic fractions of the degraded Monterey Formation oil.

Mass chromatograms for alkylated xylenes and trimethylbenzenes (Figure 5-3 c and d) of the degraded oil look similar to the ones from the undegraded oil, indicating a higher stability of these compounds towards biodegradation. This is consistent with the literature,

stating that the more sidechains are attached to the aromatic ring system, the more resistant the compound is to microbial attacks (Alexander, 1999). The mass chromatograms also demonstrate the difficulty of perceiving compositional changes in unresolved mixtures.

5.1.2 Distribution patterns of monocyclic monoaromatic hydrocarbons in the Vienna basin oils

Monoalkylated benzenes (*m/z* 91, Figure 5-4 a) in the undegraded Vienna basin oil show a homologous series with alkyl chain length from C_7 to C_{26}. The distribution pattern of alkylated toluenes (*m/z* 105, Figure 5-4 b) shows triplets of isomers with *n*-alkyl chain lengths from C_5 to C_{25}. For each carbon number, the *meta*-substituted isomers are present in higher concentrations than the *ortho*-isomers. The *para*-isomers are low in abundance.

The mass chromatograms for xylenes (*m/z* 119, Figure 5-4 c) show many isomers. Suites of isomers are present from C_6- to C_{15}-alkyl substituted xylenes. Figure 5-4 d (*m/z* 133) shows many isomers of alkyl-trimethylbenzenes.

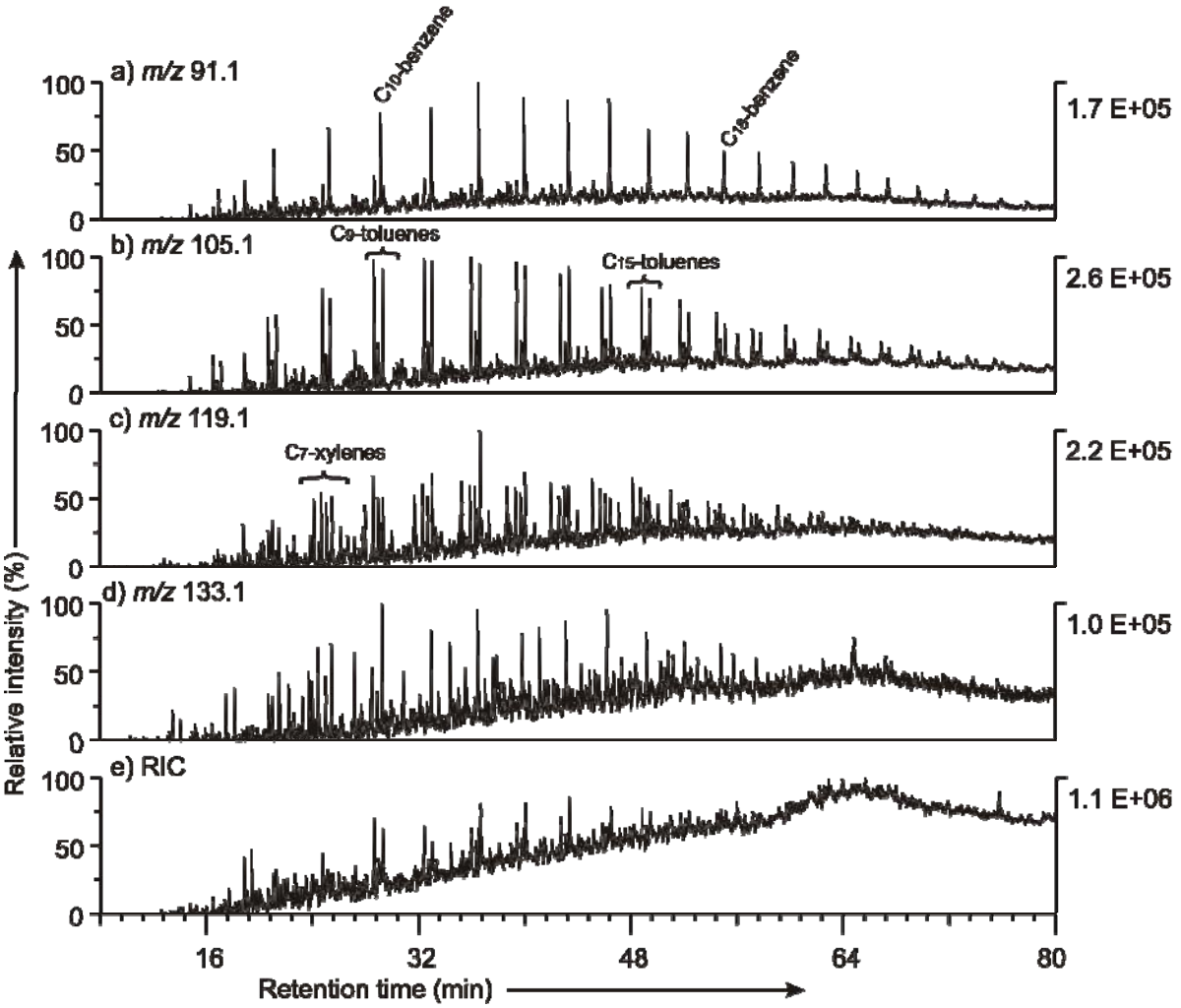


Figure 5-4 Partial reconstructed total ion current (e) and selected mass chromatograms of a) alkylbenzenes, b) alkyltoluenes (elution order: *m*-, *p*-, *o*-), c) alkylxylenes and d) alkyltrimethylbenzenes of the monoaromatic fractions of the undegraded Vienna basin oil.

In the degraded Vienna basin oil, resolved monoalkylated benzenes (*m/z* 91, Figure 5-5 a) are virtually absent. Instead, a prominent UCM is visible in the mass chromatogram due to the absence of highly concentrated resolved compounds usually used for normalisation. Alkyltoluenes (*m/z* 105, Figure 5-5 b) are present with sidechains ranging from C_5 to C_{14}. Concerning the three isomers, differences can be observed compared to the undegraded oil. The amount of the *meta*-substituted isomer decreases from the chain length of C_{11} onwards relative to the *ortho*-substituted isomer. In addition, from the chain length of C_{11} onwards, the *para*-isomer cannot be distinguished from the underlying UCM anymore. This indicates that in this case the *ortho*-substituted toluenes were more resistant to biodegradation by the bacteria present in the reservoir than the other two isomers. For both compound groups, the long-chain homologues are depleted with biodegradation.

The mass chromatogram for alkylated xylenes (*m/z* 119, Figure 5-5 c) shows suites of isomers from C_6- to C_{12}-alkyl chain lengths. The homologues with longer sidechains were apparently biodegraded. In the mass chromatogram for alkylated trimethylbenzenes (*m/z* 133, Figure 5-5 d) the higher homologues are depleted compared to the undegraded oil.

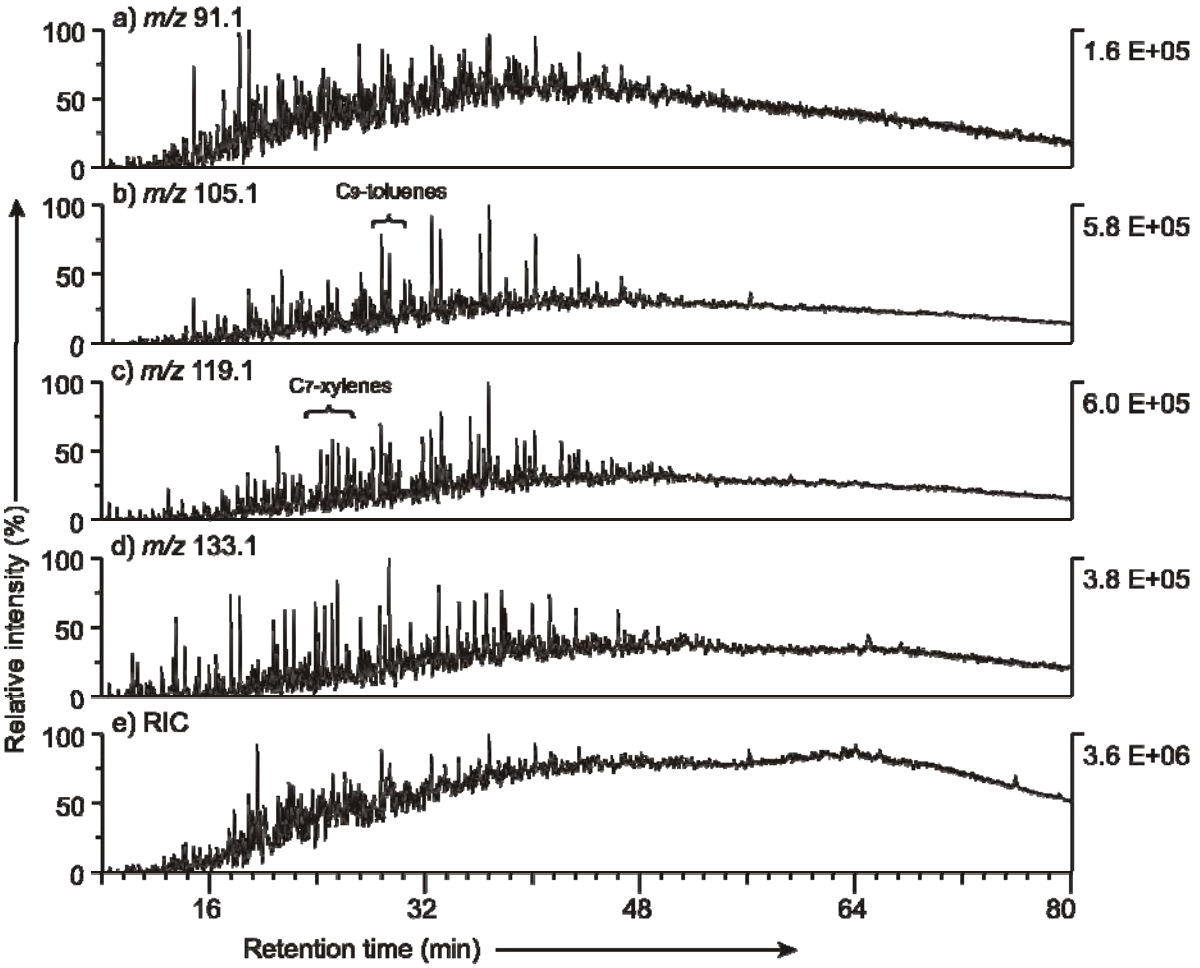


Figure 5-5 Partial reconstructed total ion current (e) and selected mass chromatograms of a) alkylbenzenes, b) alkyltoluenes (elution order: *m*-, *p*-, *o*-), c) alkylxylenes and d) alkyltrimethylbenzenes of the monoaromatic fractions of the degraded Vienna basin oil.

5.1.3 Distribution of other monoaromatic hydrocarbons in the four oils

Distribution of dicyclic monoaromatic compounds in the Monterey Formation oils

The mass chromatograms in Figure 5-6 a and b show alkylated indanes (*m/z* 117) and alkylated methylindanes and alkyltetralins (*m/z* 131) in the undegraded Monterey Formation oil. Identification of the methyl- and dimethylhomologues was achieved from their mass spectra. Higher homologues in particular in the *m/z* 131 chromatogram were tentatively identified by their later elution but the pattern is not completely regular, leaving the identification uncertain.

The substitution of indane with such long alkyl chains was unexpected since predominantly short alkyl chains are mentioned in the literature (Petrov, 1987). For instance, C_0- to C_2-indanes and -tetralins were detected in crude oils and the indanes were much more highly concentrated than the tetralins (Berthod et al., 1998). The C_0- to C_2- indanes and -tetralins did not correlate with age or reservoir depth of the oil samples studied (Berthod et al., 1998). In Green River shales, some long-chain tetralins were detected (Gallegos, 1973). In general, there are not many studies concerning the distribution of indanes and tetralins in crude oils and even less about their biodegradability.

There are minor differences between the two oil fractions. In particular, the long-chain alkylindanes appear slightly depleted due to biodegradation (Figure 5-7). This does not apply to the alkylated methylindanes and alkyltetralins. Differences between the oils are less pronounced than for monocyclic aromatic compounds, probably owing to the fact that higher substitution with the alicyclic moiety enhances resistance to microbial attack. Analogous to the alkylbenzenes, long-chain aromatic compounds are more susceptible to biodegradation than those with shorter alkyl chains (Alexander, 1999). One study on the aerobic biodegradation of alkylated tetralins showed that alkyl*cyclo*hexyltetralins were not easiliy biodegraded by the aerobic bacterium *Pseudomonas fluorescens* (Booth et al., 2002).

*Cyclo*hexylalkylbenzenes are present in both oils. Since their molecular weights are identical with alkylated indanes and tetralins and their retention behaviour is also very similar, identification of individual isomers is not possible without synthetic standards but typical fragments in a lot of mass spectra indicate the presence of *cyclo*hexylalkylbenzenes and possibly also *cyclo*pentylalkylbenzenes. Similar compounds, including several substances with changing number of carbon atoms in the bridge between the alicyclic and the aromatic moiety, were also found in Green River shales (Gallegos, 1973).

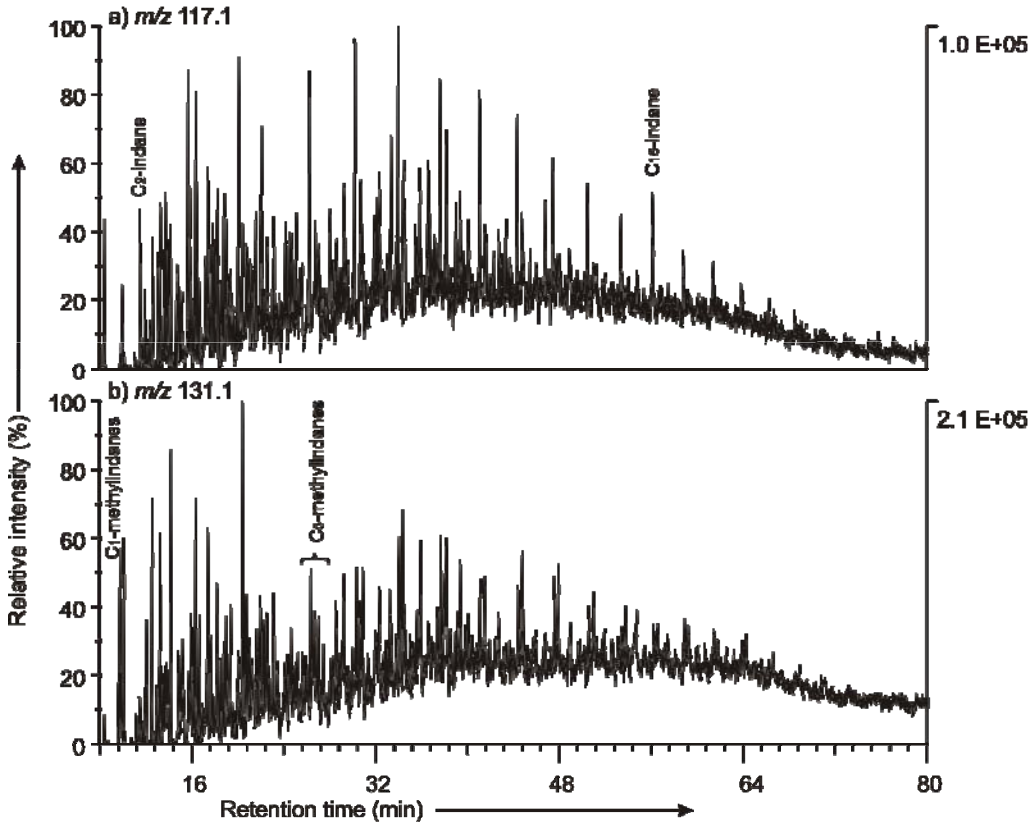


Figure 5-6 Partial mass chromatograms of a) alkylindanes and b) alkylmethylindanes and alkyltetralins of the monoaromatic fraction of the undegraded Monterey Formation oil.

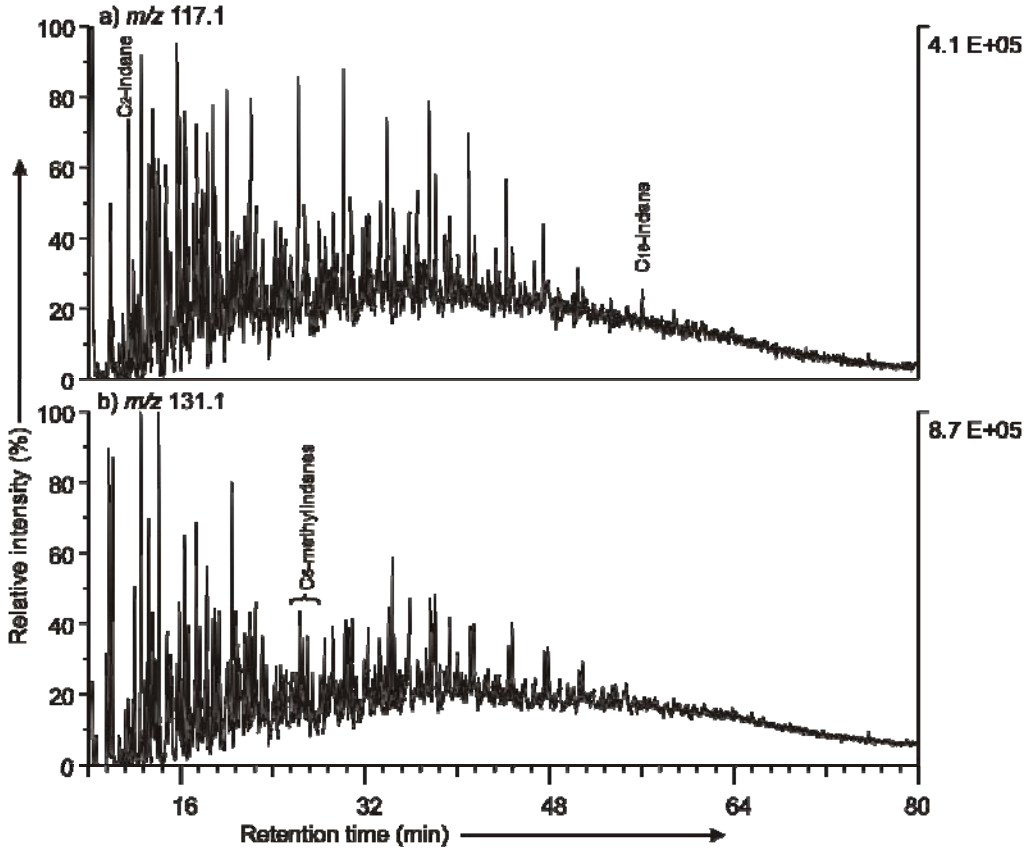


Figure 5-7 Partial mass chromatograms of a) alkylindanes and b) alkylmethylindanes and alkyltetralins of the monoaromatic fraction of the degraded Monterey Formation oil.

Figure 5-8 Partial mass chromatograms of a) alkylindanes and b) alkylmethylindanes and alkyltetralins of the monoaromatic fraction of the undegraded Vienna basin oil.

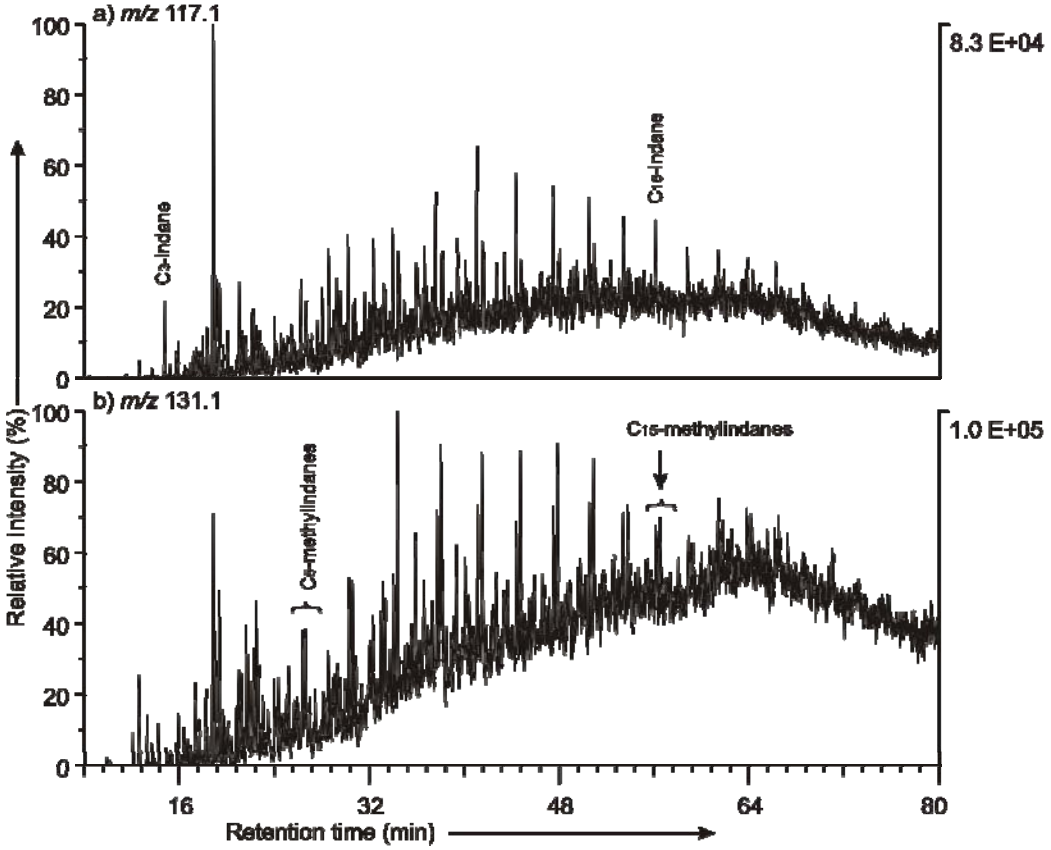
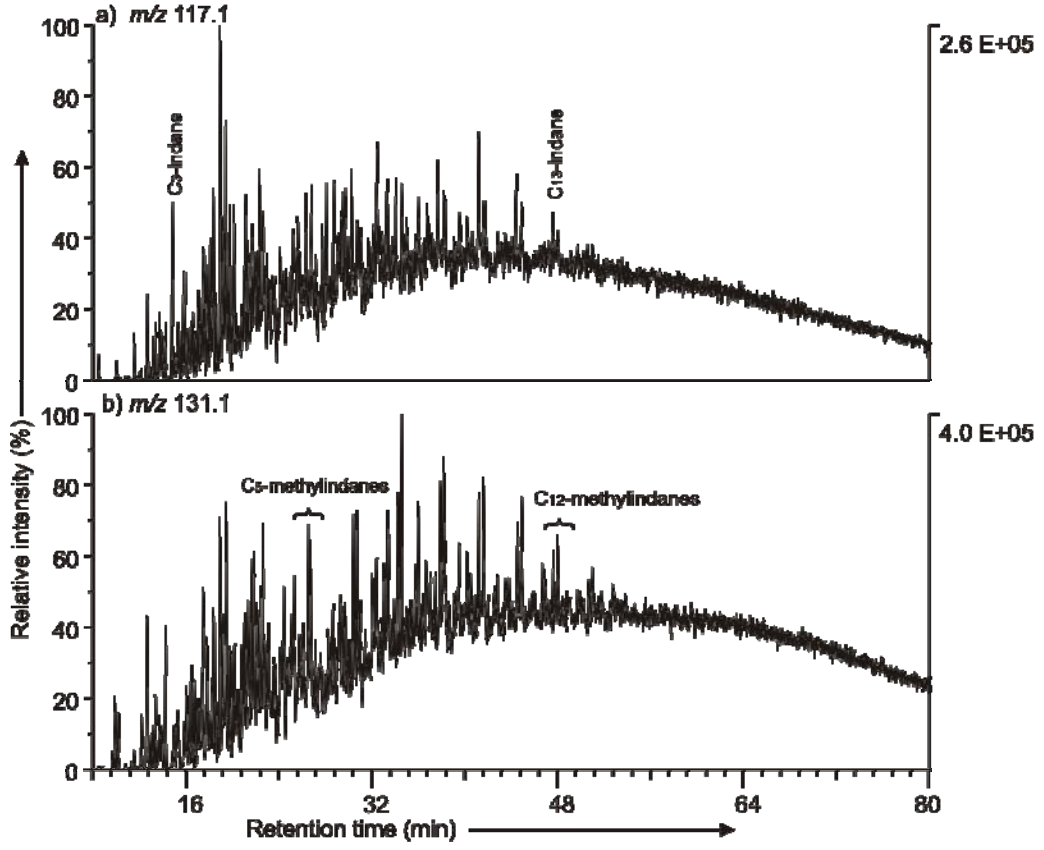


Figure 5-9 Partial mass chromatograms of a) alkylindanes and b) alkylmethylindanes and alkyltetralins of the monoaromatic fraction of the degraded Vienna basin oil.



Distribution of dicyclic monoaromatic hydrocarbons in the Vienna basin oils

Alkylated indanes and tetralins in the undegraded Vienna basin oil are shown with their typical mass chromatograms in Figure 5-8 a and b. Alkylated indanes up to C_{19} chains are present. In the retention time range from 30 to 50 minutes, a peak doublet is present after each tentatively identified *n*-alkylindane (*m/z* 117). For alkylated methylindanes (*m/z* 131), corresponding doublets of peaks can also be seen in Figure 5-8. These seem to be positional isomers but without the help of authentic standards, identification could not be achieved. *Cyclo*hexylalkylbenzenes are also present in both oils.

There are only minor differences between the dicyclic monoaromatic hydrocarbons of the undegraded and the degraded Vienna basin oil. Only the long-chain alkylindanes are slightly depleted but the alkylated methylindanes are scarcely affected (Figure 5-9). In the degraded oil, the UCM is more prominent than in the undegraded oil.

Distribution of monoaromatic steroids in Monterey Formation oils

C-ring monoaromatic steroids, which are derived from sterol precursors, are present in relatively high concentrations in the two Monterey Formation oils. Most of them show up already in the RIC trace, eluting between 54 and 65 minutes. The mass chromatogram of *m/z* 253 of the undegraded oil is shown in Figure 5-10 along with the RIC. The high abundance of steroid hydrocarbons in the Monterey Formation oils in general was reported before by Isaacs and Rullkötter (2001). Components were identified via their mass spectra and via the relative retention times reported by Peters and Moldowan (1993, pp. 76-79) and their numbering of compounds was adopted.

Table 5-2 gives the names of the corresponding steranes for identification of the monoaromatic steroids. Separation of the isomers was not as good in these samples with many coeluting components. Therefore, the presence of the isomers could not be verified in each case. With regard to the relative distribution of compounds, there was no visible difference between the two oils.

Since differences in steroid hydrocarbon distribution and aromatisation are used as maturity parameters for crude oils and since both Monterey Formation oils have a similar maturity, no big differences were observed for the aromatic steroids. In addition, differences in steroid distribution emerge at higher levels of biodegradation (Peters and Moldowan, 1993), although Connan (1984) reported monoaromatic steroids to be resistant to biodegradation.

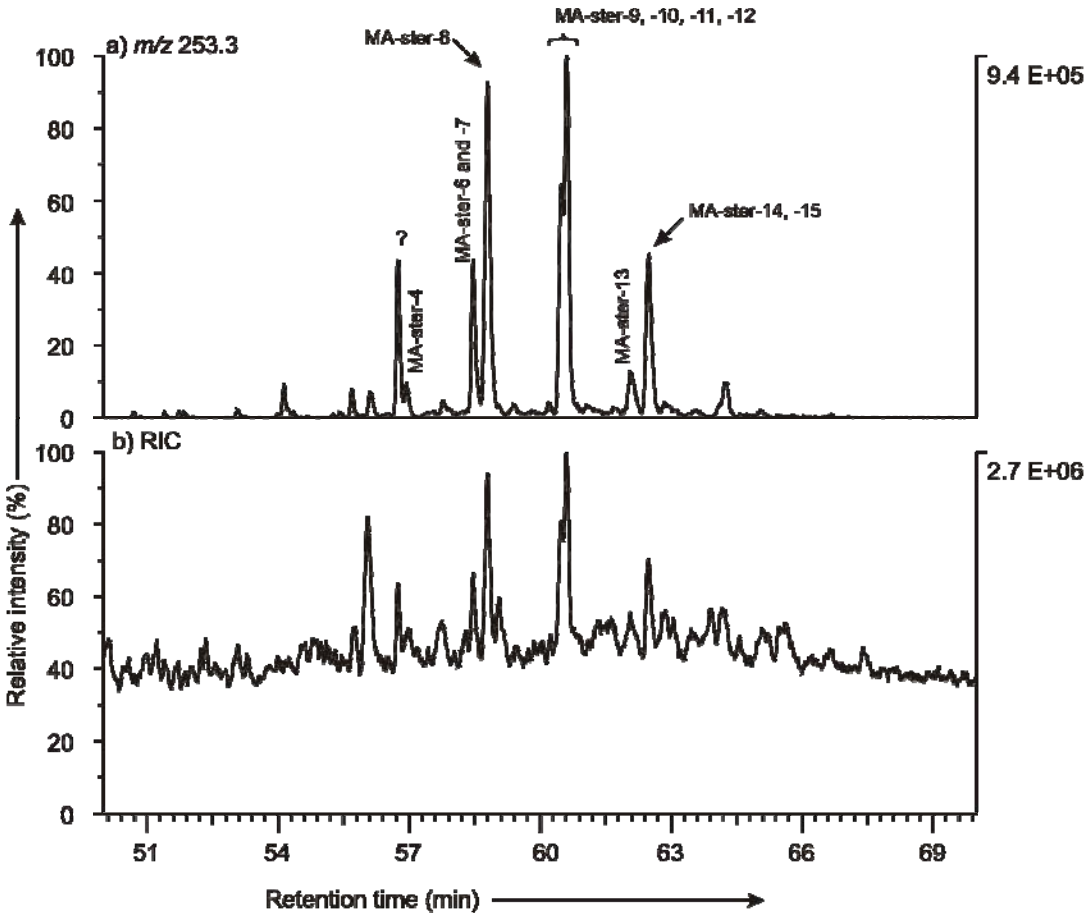


Figure 5-10 Partial reconstructed ion current (b) and mass chromatogram (a) of monoaromatic steroids (MA-ster) of the undegraded Monterey Formation oil, for peak labels see Table 5-2.

Table 5-2 Peak numbers and corresponding sterane for identification of monoaromatic steroids (MA-ster) after Peters and Moldowan (1993).

Peak number	Name of corresponding sterane	Carbon number	Peak number	Name of corresponding sterane	Carbon number
MA-ster-4	5β-cholestane 20S	27	MA-ster-11	24-methyl-5β-cholestane 20R	28
MA-ster-6	5β-cholestane 20R	27	MA-ster-12	24-ethyl-5β-cholestane 20S	29
MA-ster-7	5α-cholestane 20S	27	MA-ster-13	24-ethyl-5α-cholestane 20S	29
MA-ster-8	24-methyl-5β-cholestane 20S	28	MA-ster-14	24-methyl-5α-cholestane 20R	28
MA-ster-9	5α-cholestane 20R	27	MA-ster-15	24-ethyl-5β-cholestane 20R	29
MA-ster-10	24-methyl-5α-cholestane 20S	28	MA-ster-16	24-ethyl-5α-cholestane 20R	29

Distribution of monoaromatic steroids in the Vienna basin oils

C-ring monoaromatic steroids are present in very low concentrations in the two Vienna basin oils. The mass chromatogram of *m/z* 253 of the undegraded oil is shown in Figure 5-11. Identification of compounds was performed analogously to the Monterey Formation oil fractions (see above). The compounds are not present in sufficient amounts to give distinct peaks in the total ion current chromatogram. The low concentration of steranes in the Vienna basin oils was reported before by Welte et al. (1982).

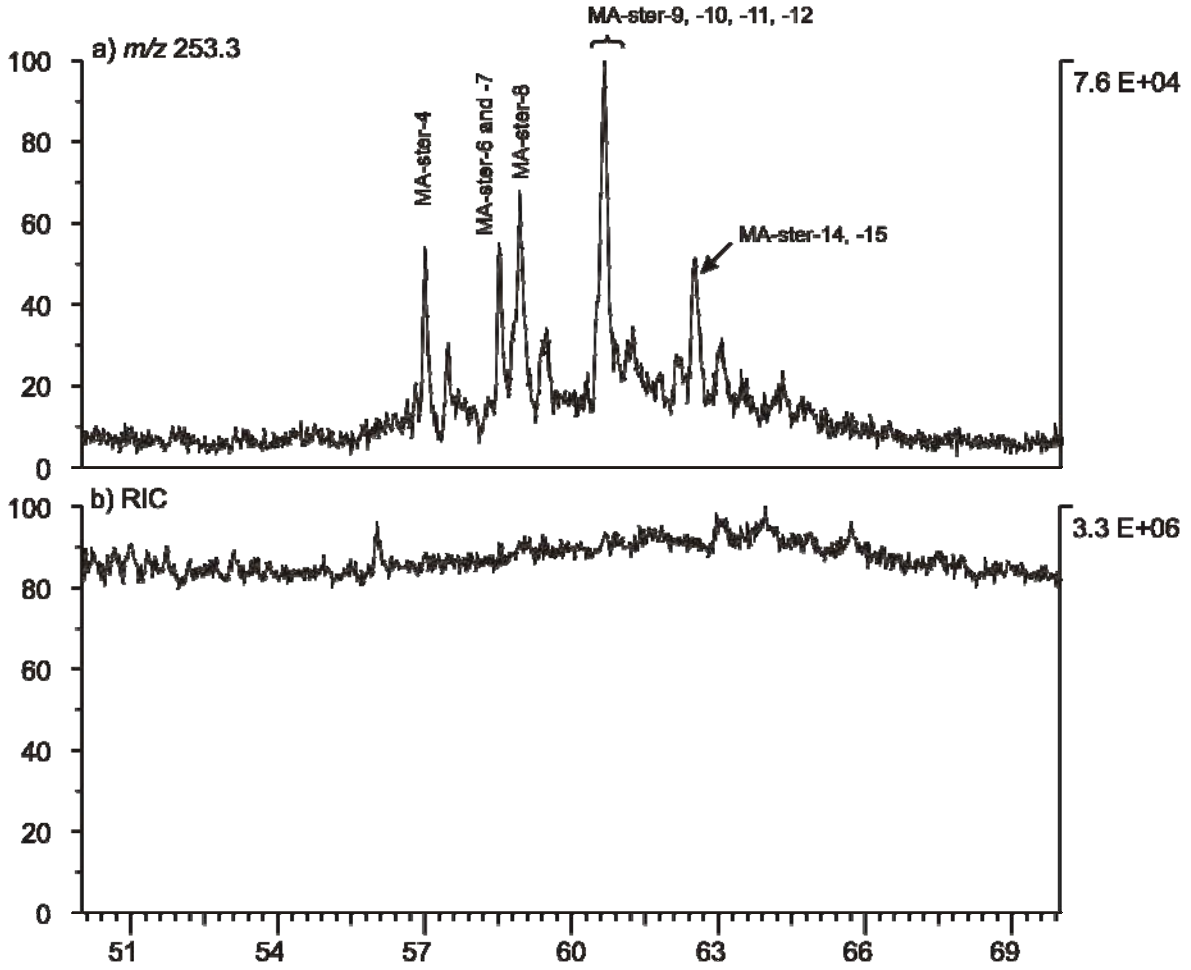


Figure 5-11 Partial reconstructed total ion current (b) and mass chromatogram (a) of mono-aromatic steroids (MA-ster) of the degraded Vienna basin oil, for peak labels see Table 5-2.

5.1.4 Comparison of Monterey Formation and Vienna basin oils

The undegraded oil from the Vienna basin contains benzenes, toluenes, xylenes and trimethylbenzenes with longer alkyl chains compared to the undegraded Monterey Formation oil. There are more resolved alkylxylenes and alkylated trimethylbenzenes present in the Vienna basin oils than in the Monterey Formation oils. These two compound groups are clearly affected by in-reservoir biodegradation with the longer-chain homologues removed or depleted in the degraded oils.

In the undegraded Vienna basin oil, the *meta*-positioned alkyltoluenes are most abundant. In contrast, *ortho*-alkyltoluenes are the chief toluenes in the undegraded Monterey Formation oil. With biodegradation of the homologues with longer chain lengths, the *ortho*-alkyltoluenes are less reduced compared to the other two positional isomers, which implies that susceptibility to biodegradation can be dependant on local factors such as the community of degrading microorganisms present in a reservoir.

Concerning alkylindanes, the undegraded oil from the Vienna basin contains more long-chain homologues than the Monterey Formation oil. In the undegraded Monterey oil, there are much more seemingly resolved homologues and isomers of indane than in the Vienna basin oils. Thus, the complex distribution patterns look different for the two oil pairs.

*Cyclo*hexylalkylbenzenes are present in all oils. This confirms the results of chemical oxidation studies by Warton et al. (1999) who concluded that *cyclo*hexylalkylbenzenes must have been present in the oils used. Beneath the resolved peaks, the UCM is relatively more pronounced in the Vienna basin oils. The most important differences and similarities concerning monoaromatic hydrocarbons in the four oils are summarised in Table 5-3.

Table 5-3 Summarised results of the general composition of the monoaromatic hydrocarbons in the four oils used.

Compound class	Similarities	Differences
Monocyclic monoaromatics	o In both degraded oils, the long-chain substituted components are more strongly degraded.	o Different isomers of e.g. alkyltoluenes are dominating, depending on the origin of the oil. o The undegraded Vienna basin oil contains benzenes, toluenes, xylenes and trimethylbenzenes with longer alkyl chains. o Alkylated benzenes are more strongly depleted in the degraded Vienna basin oil.
Dicyclic monoaromatics	o In both degraded oils, the long-chain substituted components are more strongly degraded.	o High diversity of alkylated indanes, tetralins and alkylcyclohexyl-benzenes in all oils.
Monoaromatic steroids		o The Monterey Formation oils contain high amounts whereas the Vienna basin oils contain low amounts.
UCM as a whole	o A “hump“ is present underneath the resolved peaks in the mass chromatograms of all oils.	

The fact that the alkylbenzenes are almost completely removed from the degraded Vienna basin oil suggests that the Vienna basin oil is more heavily biodegraded than the Monterey Formation oil. The other monoaromatic compound classes give no such indication.

To sum up, all four oils contain a high diversity of monoaromatic compounds. Each oil shows slightly different patterns for several compound groups. Benzenes, toluenes, xylenes and indanes substituted with long alkyl chains are depleted in the biodegraded oils.

5.2 Distribution of polyaromatic hydrocarbons in the four crude oils

Alkylated naphthalenes and phenanthrenes are major components in crude oils. In contrast to alkylated monoaromatic compounds, they do not have long sidechains. They are rather substituted by methyl and ethyl groups. Hydrocarbons with four and more aromatic rings are present in oils in very small amounts only (Killops and Killops, 2005). Parent PAHs were not found in the fractions of the four oils except phenanthrene and dibenzothiophene, as in the UCMs analysed by Booth (2004). Naphthalene and the methylnaphthalenes were partly lost during solvent evaporation, owing to their relatively high volatility. Figure 5-12 a-d shows the total aromatic fractions of the four oils with several identified compounds and suites of compounds (Table 5-4).

Table 5-4 Peak labels of identified compounds in Figure 5-12.

Peak label	Compound
3	Methylnaphthalenes
4	C_2-Naphthalenes
5	C_3-Naphthalenes
6	Dibenzothiophene
7	Phenanthrene
8	Methyldibenzothiophenes
9	Methylphenanthrenes
10	C_2-Dibenzothiophenes
11	C_2-Phenanthrenes
12	C_3-Phenanthrenes
13	Squalene

5.2.1 Distribution of alkylnaphthalenes in the two Monterey Formation oils

Alkylated napthalenes are the major diaromatic compounds present in the oils. C_0- to C_5-alkylnaphthalenes in the form of polymethylnaphthalenes are common constituents in crude oils (e.g. Huang et al., 2004).

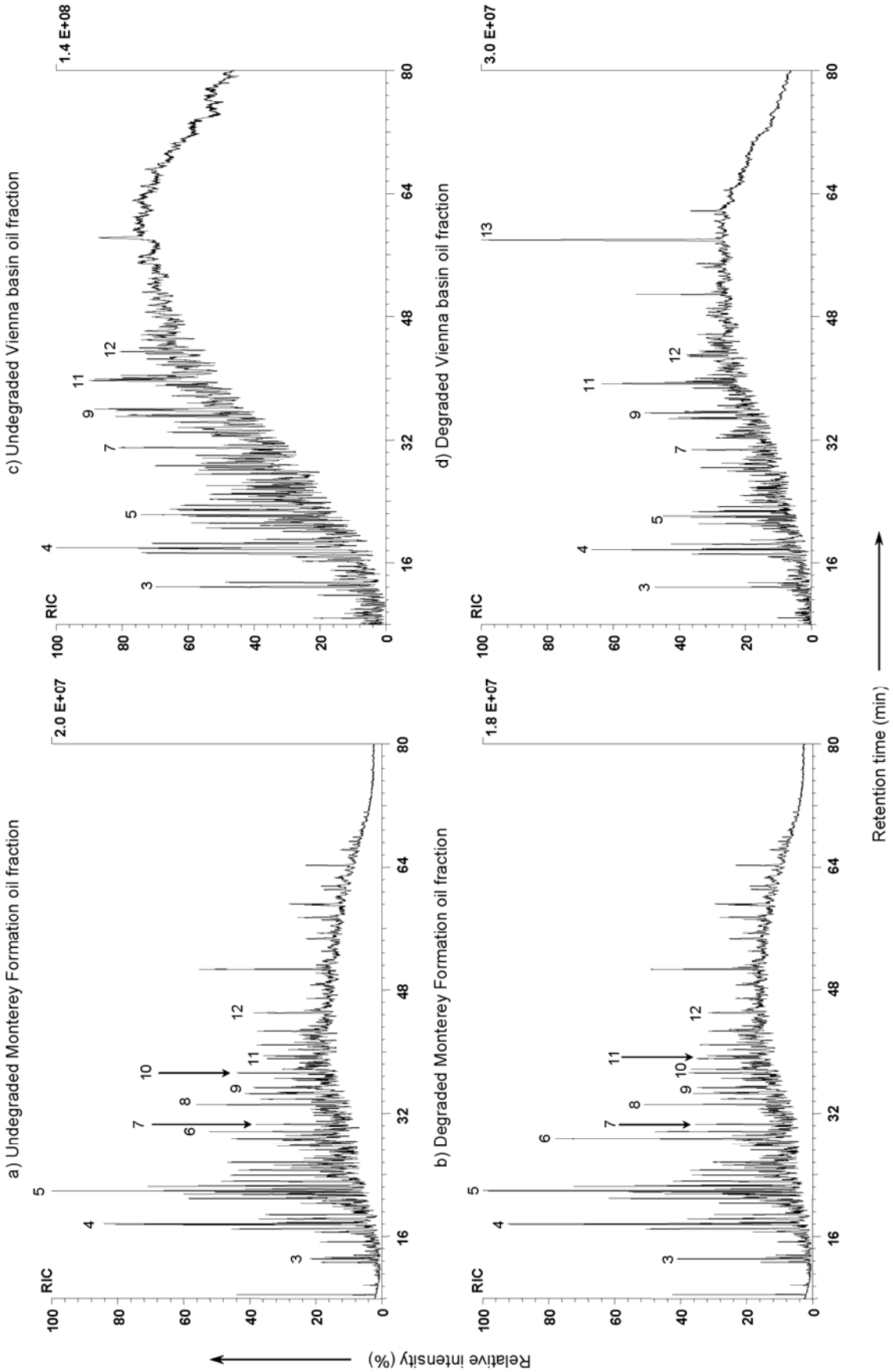


Figure 5-12 Partial reconstructed total ion current chromatograms (RIC) of total aromatic hydrocarbon fractions of a) undegraded and b) degraded Monterey Formation oils, c) undegraded and d) degraded Vienna basin oils, for peak labels see Table 5-4.

The mass chromatograms for C_2- (*m/z* 156) and C_3-naphthalenes (*m/z* 170) in the undegraded Monterey Formation oil is shown in Figure 5-13. Compounds were identified via their mass spectra and the relative retention times published by Rowland et al. (1986) and Ellis et al. (1994). C_3-naphthalenes also produce a *m/z* 156 fragment and consequently are visible in both mass chromatograms.

1,6-Dimethylnaphthalene (DMN) is the most abundant compound in the undegraded Monterey Formation oil and the two ethylnaphthalenes are the least abundant ones. This is also the case in the crude oil studied by Ellis et al. (1994). In the undegraded oils used by Rowland et al. (1986), the coeluting 1,7- and 1,3-DMN give the most intense signal. The distribution pattern for C_2-naphthalenes in the degraded Monterey Formation oil is very similar (not shown). Thus, the Monterey Formation oil has not yet reached the same state of biodegradation as an Australian oil, which was classified as PM level 5 by Fisher (2002).

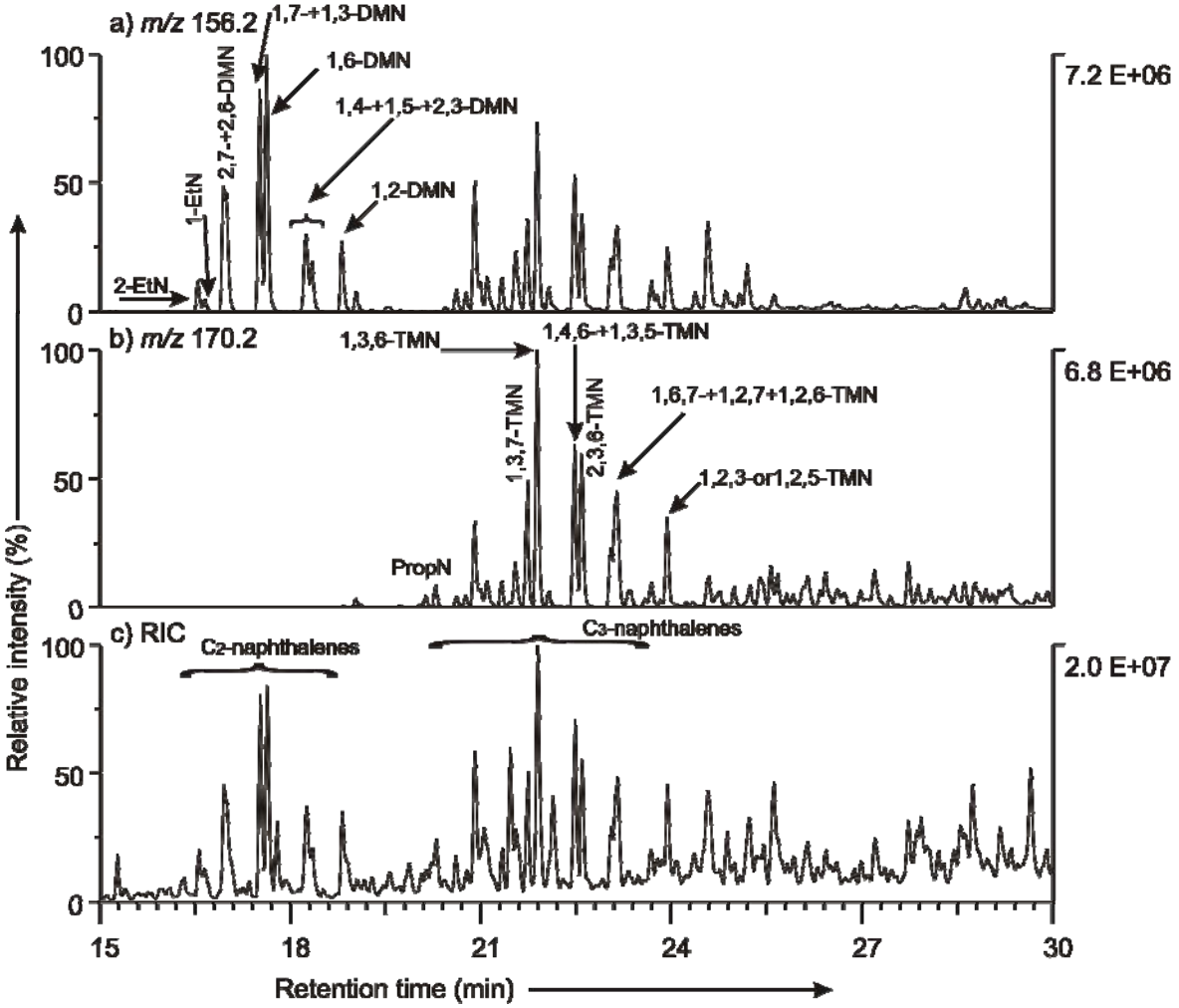


Figure 5-13 Partial mass chromatograms of a) C_2-naphthalenes, b) C_3-naphthalenes and c) reconstructed ion current of the undegraded Monterey Formation oil; EtN: ethylnaphthalene, DMN: dimethylnaphthalene, PropN: propylnaphthalene, TMN: trimethylnaphthalene.

Mass spectra and retention time comparisons with Ellis et al. (1994) and Huang et al. (2004) allowed identification of many C_3-methylnaphthalenes. After the coeluting 1,6,7-,

1,2,7- and 1,2,6-trimethylated isomers, there appears to be the 1,2,4-isomer missing in the Monterey Formation oils so that the subsequently eluting isomer may be 1,2,3- or 1,2,5-trimethylnaphthalene. The most abundant compound is 1,3,6-TMN as in the Australian oils studied (Rowland et al., 1986).

Differences between the two Monterey Formation oils were minor. Only the two isomers 1,2,6- and 1,2,5-trimethylnaphthalene are slightly reduced in the biodegraded oil. This is also the case in an oil studied by Rowland et al. (1986) but it is not in accordance with results of Huang et al. (2004), who found 2,3,6- and 1,3,6-trimethylnaphthalenes to be more susceptible to in-reservoir biodegradation in the Chinese oils studied. However, since different microorganisms are living in different environments, varying degradation rates are not unusual since they may depend on locally varying factors such as the nutrient supply (Alexander, 1999). The mass chromatograms for the higher homologues of naphthalene are dominated by unresolved complex mixtures.

5.2.2 Distribution of alkylnaphthalenes in the two Vienna basin oils

Naphthalene and the two methylnapthalenes are present in both oils. Regarding dimethylnaphthalenes, the coeluting 1,7- and 1,3-DMN and the coeluting 2,7- and 2,6-DMN give the most intense signals in the undegraded Vienna basin oil (not shown). The least concentrated compounds are the ethylnaphthalenes and the 1,2-DMN. Compounds were identified analogous to the Monterey Formation oils (see above).

In the degraded Vienna basin oil (Figure 5-14), the coeluting 1,7- and 1,3-DMN give the biggest signal in the mass chromatogram, too, but the relative amounts of the other isomers are lower than in the undegraded oil. 1,3- and 1,7-DMN were found to be more susceptible to biodegradation in in-laboratory degraded oils as well as for in-reservoir degraded oils (Rowland et al., 1986).

Regarding the C_3- substituted naphthalenes, the *m/z* 170 mass chromatogram gives the molecular ions of the isomers (Figure 5-14 b). The compound present in the highest concentration is 1,3,6-TMN in both oil fractions. The other isomers are present in lower amounts. This agrees with some Australian crude oils and with an in-laboratory degraded oil (Rowland et al., 1986; Fisher, 2002).

As for the Monterey Formation oils, the mass chromatograms for the polymethyl-naphthalenes are getting more and more complex with increasing alkylation.

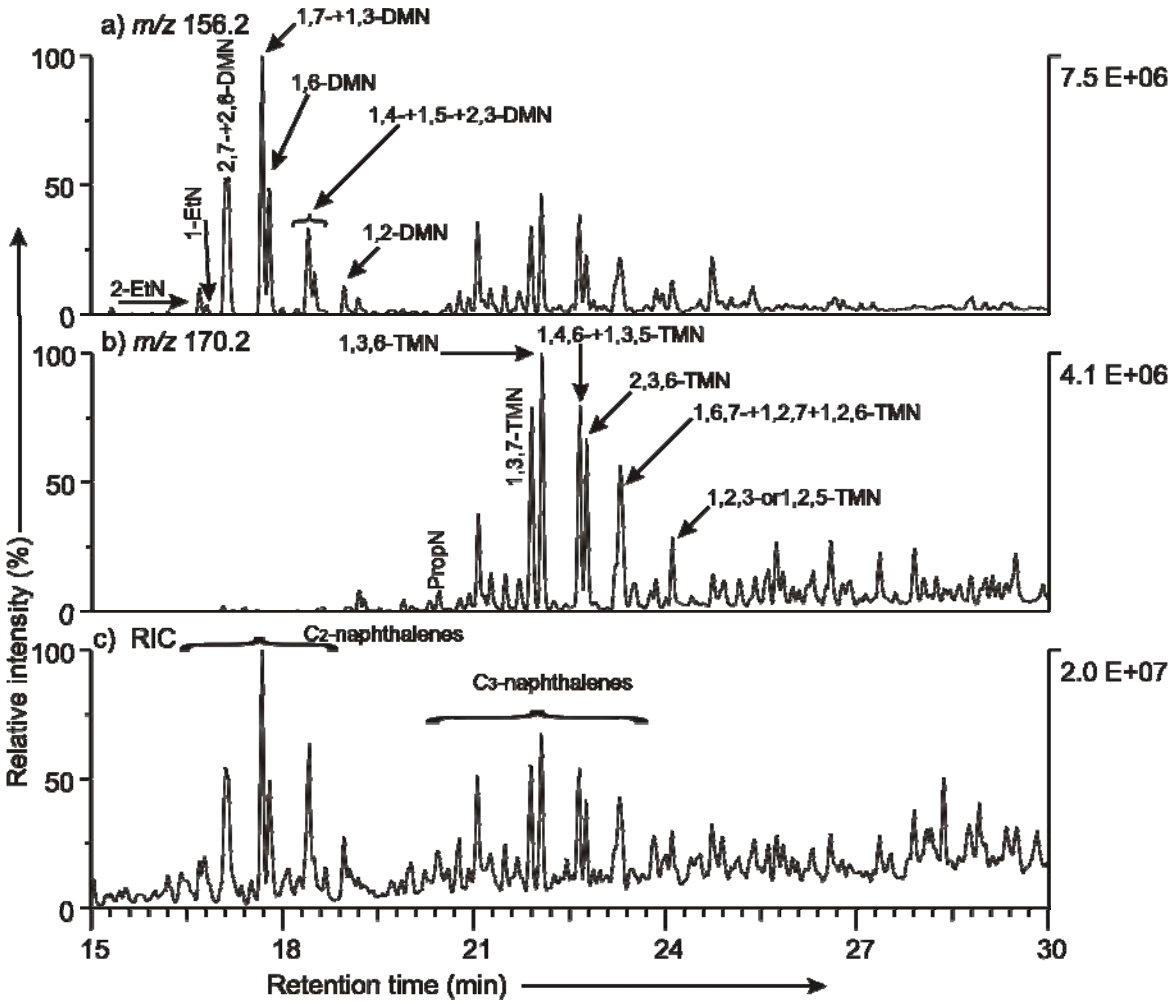


Figure 5-14 Partial mass chromatograms of a) C_2-naphthalenes, b) C_3-naphthalenes and c) reconstructed ion current of the degraded Vienna basin oil; EtN: ethylnaphthalene, DMN: dimethylnaphthalene, PropN: propylnaphthalene, TMN: trimethylnaphthalene.

5.2.3 Distribution of alkylphenanthrenes in the two Monterey Formation oils

Phenanthrene and the four methylphenanthrenes (MP) are present in both oils in very similar relations. The methylphenanthrene index is the same for the two oils, indicating no differences in maturity (see section 3.1.1). In both oils, 9-MP is the most and 1-MP the least abundant isomer. Huang et al. (2004) reported that the most pronounced changes in methylphenanthrene distribution occur between biodegradation levels 5 and 7. The degraded Monterey Formation oil had been classified as level 5 from several parameters mentioned in Section 3.1.1, so that no major changes were expected.

In contrast to the small number of isomers of methylphenanthrenes, the distribution of isomers of C_2-phenanthrenes is more complex. Ellis et al. (1994) attributed relative retention times to 21 structural isomers with several specifically synthesised methylphenanthrenes. Many coelutions occur. Figure 5-15 shows the mass chromatogram for C_2-phenanthrenes (*m/z* 206) and C_3-phenanthrenes (*m/z* 220) in the undegraded Monterey Formation oil. Compound identification follows the relative retention times published by Ellis et al. (1994).

Compared to the undegraded oil, the degraded Monterey Formation oil contains less 1,7-dimethyl-phenanthrene (DMP) and the combined signal of 9- and 2-ethylphenanthrene and the unknown TMP (peak number 16) is less intense, too. A similarly high susceptibility of 1,7-DMP to in-reservoir biodegradation was reported for Chinese oils. Subtle changes in ethylphenanthrene distribution, however, started only at higher biodegradation levels (Huang et al., 2004). As in the Monterey Formation oils, 1,9-DMP and the coeluting four isomers (3,9-, 2,10-, 3,10- and 1,3-DMP) have been observed to be most resistant to biodegradation (Fisher, 2002).

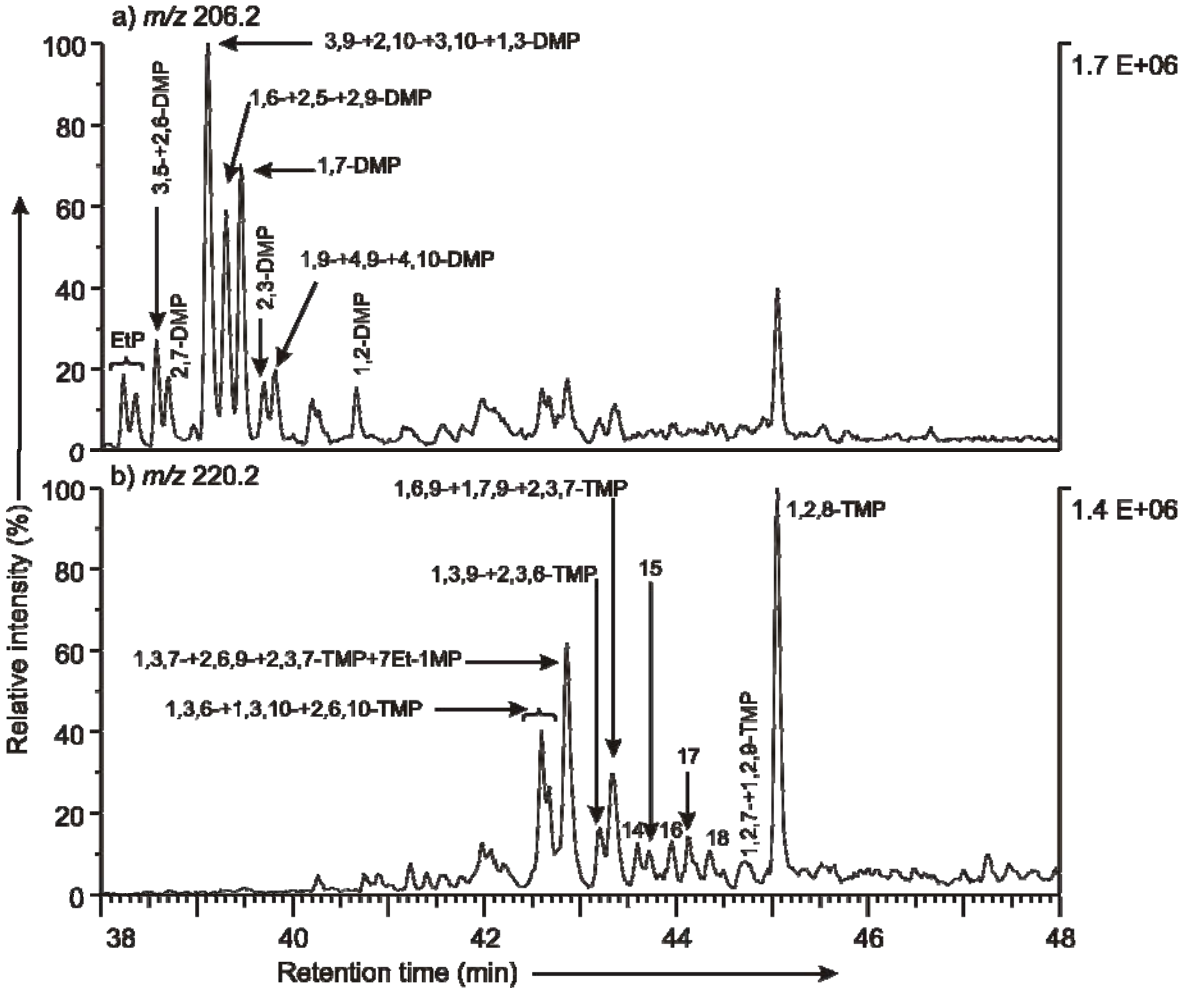


Figure 5-15 Partial mass chromatograms of a) C_2-phenanthrenes and b) C_3-phenanthrenes of the undegraded Monterey Formation oil; EtP: ethylphenanthrene, DMP: dimethylphenanthrene, TMP: trimethylphenanthrene, 14: 1,3,8-TMP, 15: 2,3,10-TMP, 16: unknown TMP, 17: 1,6,7-TMP, 18: 1,2,6-TMP.

In general, more highly alkylated aromatics are more resistant to biodegradation than less alkylated ones (Alexander, 1999), implying C_3-phenanthrenes should be less affected than the C_2-phenanthrenes. Huang et al. (2004) observed changes in the distribution pattern starting at biodegradation level 4 to 5. The isomer 1,2,8-trimethylphenanthrene (TMP) was the least resistant one (Huang et al., 2004). This isomer is also clearly depleted in the degraded Monterey Formation oil (not shown). In addition, the coeluting compounds 1,3,6-,

1,3,10- and 2,6,10-TMP are reduced in relative intensity. This again agrees with the observations made by Huang et al. (2004), who found these compounds to be depleted in degraded oils of level 5.

5.2.4 Distribution of alkylphenanthrenes in the Vienna basin oils

Phenanthrene and the four methylphenanthrenes are present in both oils. The methylphenanthrene index is similar for the two oils (see Section 3.1.1), indicating their similar maturity. In the undegraded Vienna basin oil, 2-MP is the most abundant isomer and the other three isomers are present in almost equal amounts. In the biodegraded oil, 9-MP is the most abundant isomer, 2-MP and 1-MP are reduced compared to the undegraded oil. This agrees with observations by Rowland et al. (1986), who found that 9-MP is more resistant to biodegradation than the other MPs. The fact that degradation of methylphenanthrenes has started in the degraded Vienna basin oil indicates that PM level 5 has been reached (Huang et al., 2004).

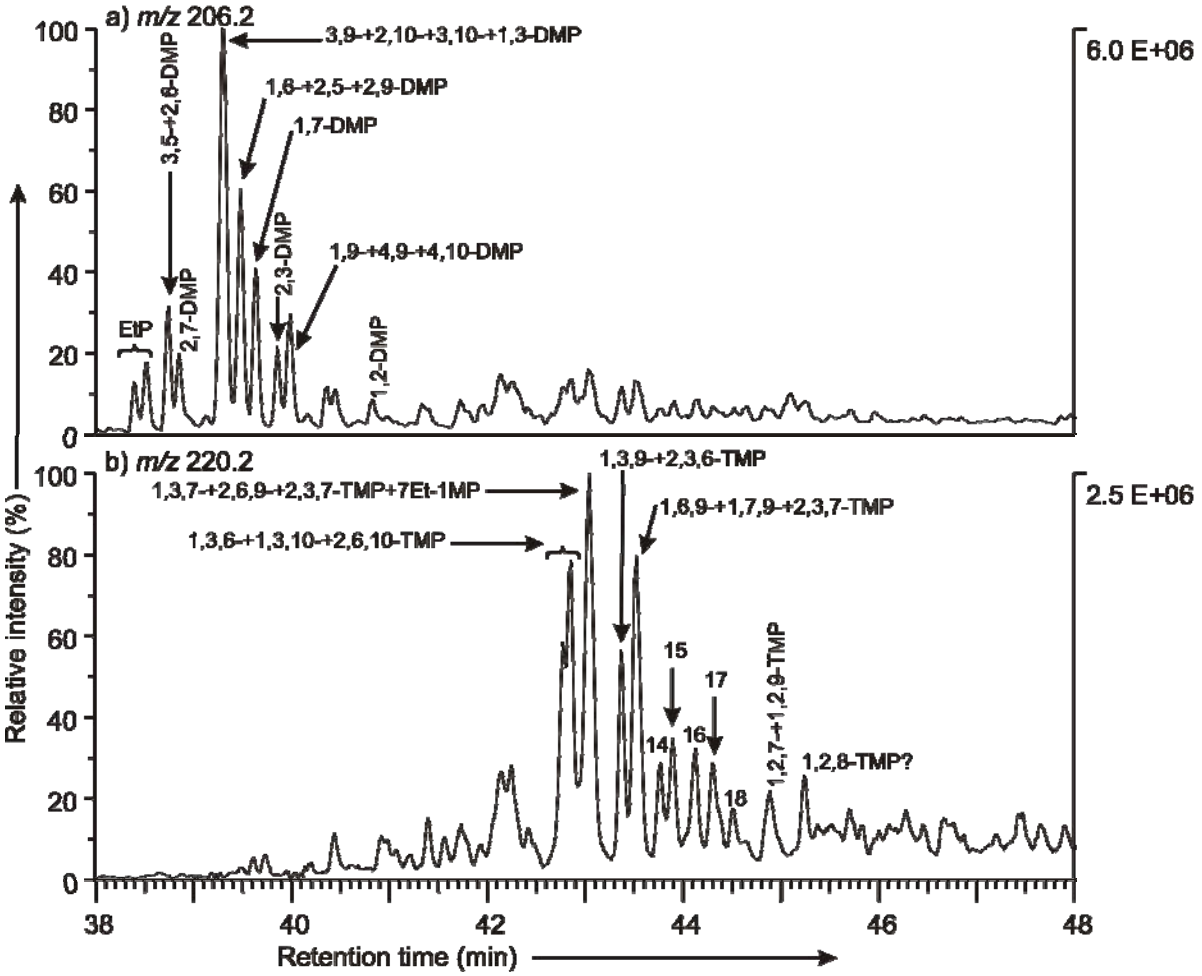


Figure 5-16 Partial mass chromatograms of a) C_2-phenanthrenes and b) C_3-phenanthrenes of the degraded Vienna basin oil; EtP: ethylphenanthrene, DMP: dimethylphenanthrene, TMP: trimethylphenanthrene, 14: 1,3,8-TMP, 15: 2,3,10-TMP, 16: unknown TMP, 17: 1,6,7-TMP, 18: 1,2,6-TMP.

The distribution of C_2- and C_3-phenanthrenes in the two Vienna basin oils is very similar to each other. The respective mass chromatograms of the degraded oil are shown in Figure 5-16. 1,7-DMP is less abundant than in the Monterey Formation oils. The other depleted isomers in the degraded Vienna basin oil comply with those in a suite of degraded Australian oils (Fisher, 2002), thus the extent of biodegradation can be ranked as PM level 5.

The mass chromatograms of C_3-alkylphenanthrenes of the undegraded (not shown) and the degraded oil (Figure 5-16) look similar except that the degraded oil contains relatively less of the coeluting isomers 1,2,7- and 1,2,9-TMP. The most easily biodegradable isomer (1,2,8-TMP) is not present in high amounts in any of the two oils and its presence in the degraded oil could not be ascertained due to a mass spectrum with too intense background signals. Since it was not detected in the undegraded oil, biodegradation cannot be the reason for its absence. Many compounds in crude oils evolved from biological precursor molecules, thus a specific precursor for 1,2,8-TMP may not have been present in the Vienna basin during oil formation. Studies have indeed suggested a specific precursor for 1,2,8-TMP, which is a likely product in the aromatisation of the tetracyclic triterpenoids *β*-amyrin and lupeol and of many diterpenoids (Carruthers and Douglas, 1957, in Budzinski et al., 1993). These compounds are derived from terrestrial higher plants. In general, the Vienna basin oils do not contain many components which evolved from terrestrial organic matter (Welte et al., 1982). A possible reason for this is the age of the source rock of the Vienna basin oils. Since the oil is thought to have been generated from pre-Cretaceous sediments (Welte et al., 1982), compounds deriving from angiosperms such as *β*-amyrin and lupeol did not exist or were probably not yet widely spread. This can explain the low abundance of 1,2,8-TMP in both Vienna basin oils.

Another possible explanation for the low amount of this isomer is a shift of methyl groups on the aromatic ring, resulting in the formation of 1,2,7-TMP and 1,2,6-TMP from 1,2,8-TMP with higher level of thermal maturity (Budzinski et al., 1993). This may explain the relatively low abundance of 1,2,8-TMP in the more mature Austrian oils in comparison to the less mature California oils. However, the signals of 1,2,7-TMP and 1,2,6-TMP in the Vienna basin oils are not elevated to such a great extent as to ascertain the occurrence of these methyl group shifts.

5.2.5 Triaromatic steroid hydrocarbons in the Monterey Formation oils

Several triaromatic steroids are present in the Monterey Formation oils. Compounds were attributed with the help of molecular ions and relative retention times by Peters and Moldowan (1993, p. 81). The representative mass chromatogram (*m/z* 231) of the undegraded oil is presented with the RIC in Figure 5-17. The mass spectra of the peaks on top of the UCM in the RIC were too unclean for identification. Similar to the monoaromatic steroids, triaromatic steroids are present in high concentrations as some are visible in the

total reconstructed ion chromatogram, eluting between 60 and 70 minutes. For peak labels see Table 5-5. As expected, there were no changes in the distribution of triaromatic steroids in the moderately biodegraded oil.

Table 5-5 Peak numbers and corresponding sterane for identification of triaromatic steroids (TA-ster) after Peters and Moldowan (1993).

Peak number	Name of corresponding sterane	Carbon number
TA-ster-4	Cholestane 20S	26
TA-ster-5	Cholestane 20R and 24-Methylcholestane 20S	26 and 27
TA-ster-6	24-Ethylcholestane 20S	28
TA-ster-7	24-Methylcholestane 20R	27
TA-ster-8	24-Ethylcholestane 20R	28

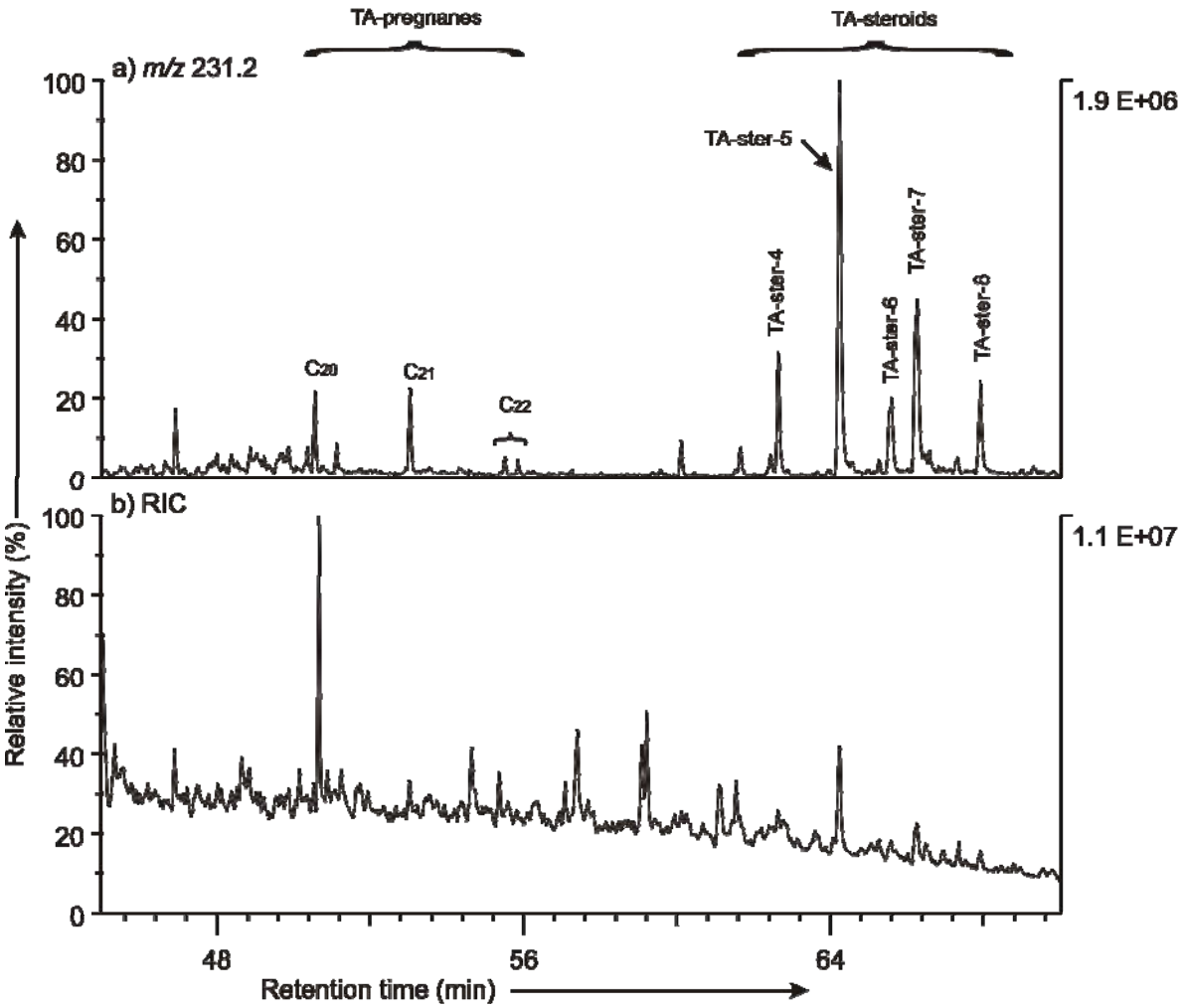


Figure 5-17 Partial reconstructed total ion current (b) and mass chromatogram (a) of triaromatic analogs of pregnanes and steroids (TA-ster) of the undegraded Monterey Formation oil, for peak labels see Table 5-5.

In both oils, the triaromatic analogues of C_0- to C_2-pregnanes (carbon skeletons of C_{20} to C_{22}, respectively) were identified in the retention time range between 50 and 60 minutes (Peters and Moldowan, 1993; Li and Jiang, 2001). Pregnanes have the tetracyclic carbon skeleton of steroids without the extended sidechain. The C_{20} and C_{21} homologues are common in crude oils whereas the C_{22} compounds is not as widespread. The triaromatic

alkylpregnanes are not easily degraded and may be even more resistant than the triaromatic steroids (Li and Jiang, 2001).

5.2.6 Triaromatic steroid hydrocarbons in the Vienna basin oils

Triaromatic steroids in the degraded Vienna basin oil are shown in the mass chromatogram (*m/z* 231) with the RIC in Figure 5-18. Steroids were attributed with the help of the assignments published by Peters and Moldowan (1993) but the dominant compound visible in the RIC was not identified. As for the monoaromatic steroids, concentration of the individual compounds was too low to be able to see the compounds directly in the RIC trace. With regard to the biodegradation level of the oils, there were no changes in the distribution of triaromatic steroids. Pregnane analogues were identified analogously to the Monterey Formation oil. The two C_{22} isomers were not detected in the Vienna basin oils. They usually become depleted or totally removed with higher maturity (Li and Jiang, 2001), which may be the reason why they are not present in the Vienna basin oils compared to the less mature Monterey Formation oils.

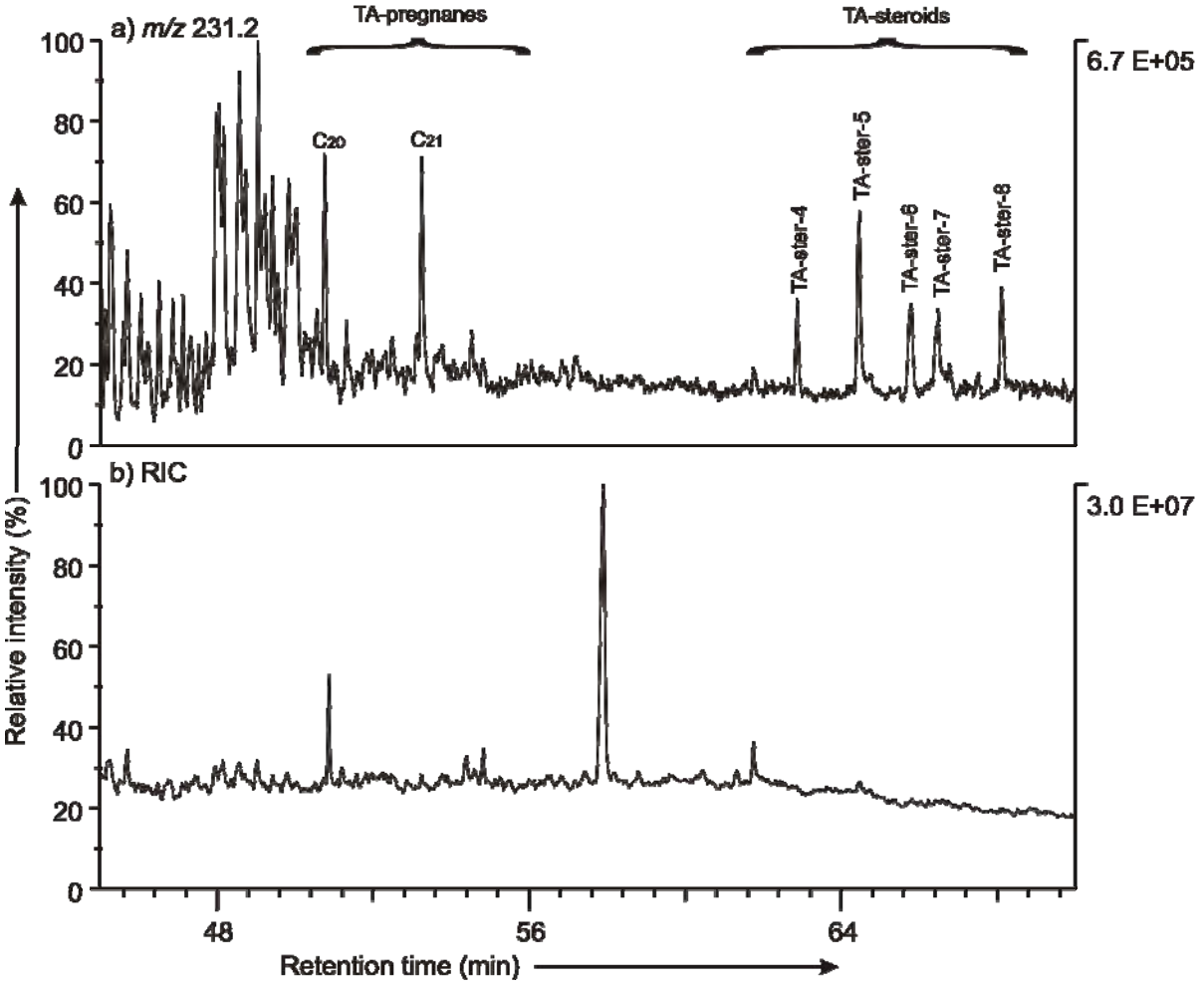


Figure 5-18 Partial reconstructed total ion current (b) and mass chromatogram (a) of triaromatic analogs of pregnanes and steroids (TA-ster) of the degraded Vienna basin oil, for peak labels see Table 5-5.

5.2.7 Comparison of polyaromatic hydrocarbons in the Monterey Formation and Vienna basin oils

Most alkylnaphthalenes are present in all oils in similar relative abundance but the most highly concentrated dimethylnaphthalene isomers differ. All four oils contain 1,3,6-trimethylnaphthalene as the most abundant C_3-naphthalene, indicating that it is not necessarily the one most susceptible to biodegradation as reported by Huang et al. (2004).

1- and 2-methylphenanthrene (MP) are slightly degraded in the degraded Vienna basin oil but not at all in the Monterey Formation oil. Therefore one can assume that biodegradation of MP has already begun in the Austrian oil in contrast to the Monterey Formation oil. Compared to the undegraded Monterey Formation oil, the degraded Vienna basin oil contains less 1,7- and 1,2-dimethylphenanthrene (DMP) and ethylphenanthrenes. However, the signals of 1,9-, 4,9- and 4,10-DMP and of 2,3-DMP have a slightly higher intensity. The high susceptibility to biodegradation of 1,7-DMP as well as the high resistance of isomers with a methyl group at position 9 or 10 were reported before (Huang et al., 2004). The absence of 1,2,8-trimethylphenanthrene from the two Vienna basin oils can be explained by the lack of biogenic precursors from terrestrial organic matter (see above).

The concentration of triaromatic steroids in the Monterey Formation oils is higher than in the Vienna basin oils. Both oil pairs do not show a change with increased biodegradation level. Relative distribution of steroids differed according to the origin of the oils (Welte et al., 1982; Isaacs and Rullkötter, 2001). In all oils, the coeluting (20R)-C_{26} and (20S)-C_{27} isomers give the highest signal. In the Vienna basin oils, the other four peaks are similarly intense whereas there is more variation in abundance in the Monterey Formation oils. Table 5-6 briefly summarises the results regarding the polyaromatic compound classes in the four oils.

Table 5-6 Summarised results of the general composition of the polyaromatic hydrocarbons in the four oils used.

Compound class	Similarities	Differences
Naphthalenes	o Most isomers are present in similar relative concentrations in the undegraded oils.	o The two degraded oils show different patterns of depletion of dimethyl- and trimethyl-naphthalenes.
Phenanthrenes	o The methyl- and dimethylphenanthrenes show that biodegradation of phenanthrenes has only just begun in both oils.	o Differences in isomeric composition can be derived from specific precursors depending on the origin of the oil (e.g. 1,2,8-TMP). o The same isomers are not equally susceptible to microbial degradation in the different environments.
Triaromatic steroids		o The Monterey Formation oils contain high amounts of triaromatic analogues of steranes and pregnanes whereas the Vienna basin oils contain low amounts.
UCM as a whole	o A “hump“ is present underneath the resolved peaks in the mass chromatograms of all oils.	

5.3 Distribution of sulphur-containing compounds in the four crude oils

Crude oils contain variable amounts of sulphur compounds. In general, total sulphur content of crude oils can be used to subdivide them into high sulphur oils (>1% S) and low sulphur oils (<1% S) (Orr and Sinninghe Damsté, 1990). The Monterey Formation oils have a relatively high total sulphur content around 3% (sd = 3%) and the Vienna basin oils have a total sulphur content below 0.5% (sd = 5%). In addition to the sulphur content of the whole oils, the sulphur content of the aliphatic/alicyclic and aromatic hydrocarbon fractions were determined (Table 5-7). The low sulphur content of the aliphatic hydrocarbon fractions was expected since sulphur compounds elute in the aromatic hydrocarbon fraction of MPLC separation due to their similar polarity. The total sulphur content of monoaromatic hydrocarbon fractions could not be determined due to small amounts of sample.

Table 5-7 Total sulphur content of whole oils, aliphatic/alicyclic and total aromatic hydrocarbon fractions (M = Monterey Formation oil, V = Vienna basin oil), 0.05% = quantification limit.

Total sulphur content [%] of	Undegraded M	Degraded M	Undegraded V	Degraded V
Whole oil	3.09	3.11	0.20	0.24
Aliphatic/alicyclic	< 0.05	< 0.05	< 0.05	< 0.05
Aromatic	3.94	3.72	0.46	0.12

5.3.1 Sulphur-containing compounds in the monoaromatic hydrocarbon fractions of the four crude oils

Due to the higher polarity of sulphur compounds compared to the pure hydrocarbons, sulphur-containing compounds elute in the aromatic hydrocarbon fraction during column chromatography. Therefore, some non-aromatic sulphur compounds were detected in the monoaromatic hydrocarbon fractions.

Sulphur compounds in monoaromatic hydrocarbon fractions of the Monterey Formation oils

In GC-MS analysis, alkylthiolanes and dialkylthiolanes give the structural fragment *m/z* 87, which is shown in Figure 5-19 a for the undegraded Monterey Formation oil. For each homologue with more than 14 carbon atoms in the sidechain, two peaks can be distinguished. For the lower homologues, an additional peak can be perceived eluting in between. Strausz et al. (1990) reported that the 2,5- dialkylthiolanes elute before monoalkylthiolanes, indicating that the last eluting isomer may be the monoalkylthiolane. The homologues with more than 20 carbon atoms in the side chain are lacking in the degraded oil.

The representative mass fragment for alkylmethylthiolanes and alkylthianes is *m/z* 101, which is shown in Figure 5-19 b. Alkylmethylthiolanes are reported to elute closely after the alkylthianes (Strausz et al., 1990). From this follows that the vaguely visible pattern of homologues represents the alkylmethylthiolanes as labelled in Figure 5-19 b. If this is the case the alkylthianes are virtually absent at least for the lower homologues but without

synthetic standards, attribution of structures is uncertain since there is a high unresolved background signal. The compounds with total carbon numbers of 20, 22 and 24, which were tentatively identified with the help of their molecular ions, are depleted in the degraded oil.

Sinninghe Damsté et al. (1987b) distinguished 19 isomers (including *cis*- and *trans*-isomers) of C_{16} dialkylthiolanes and dialkylthianes with the help of mass fragments derived from different sidechain length, which showed slight differences in retention behaviour of the isomers. Due to the complexity of the fraction in general and coelution with alkyltoluenes in particular, this it not possible here although it appears as if not all isomers found by Sinninghe Damsté and co-authors (1987b) in Rozel Point Oil (USA) are present in the Monterey Formation oils.

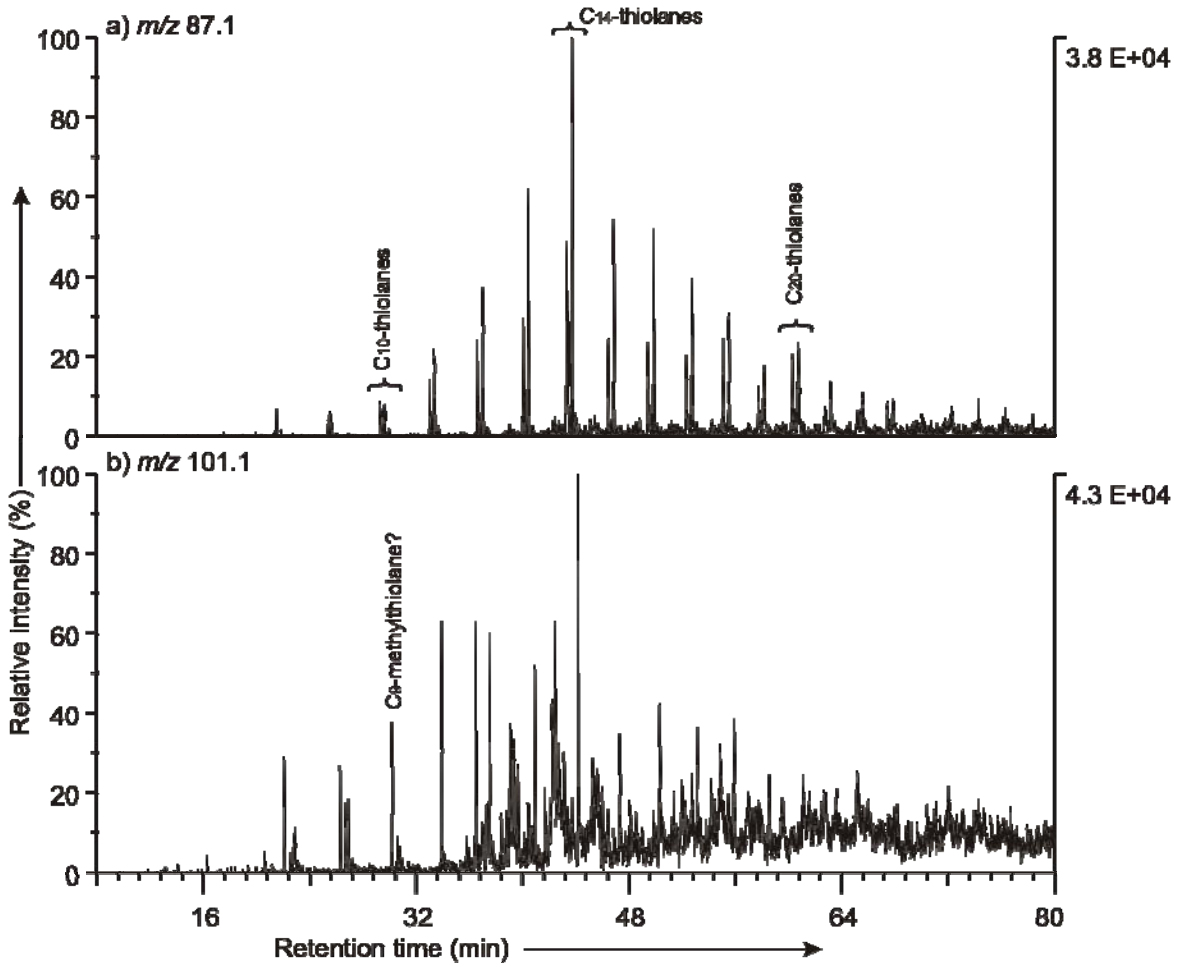


Figure 5-19 Partial mass chromatograms of a) alkylthiolanes and b) alkylmethylthiolanes and alkylthianes of the undegraded Monterey Formation oil.

Alkylthiolanes and alkylthianes were not present in an in-reservoir degraded crude oil, which was degraded to a higher extent than this Monterey Formation oil (Fedorak, 1990). In a laboratory study, alkane-degrading bacteria degraded several branched C_{11}-C_{18} alkyl-thiophenes, with degradation at the side chain as often observed for non-sulphur-containing hydrocarbons (Fedorak and Peakman, 1991).

In the two Monterey Formation oils, several individual alkylthiophenes, methylalkylthiophenes and dimethylalkylthiophenes were tentatively identified. There was no series of more than five homologues found in either oil. The compounds were identified as even-numbered alkylthiophenes but usually alkylthiophenes and methylalkylthiophenes are only visible in the mass chromatogram of their respective typical fragment ions and not in both (Figure 5-20).

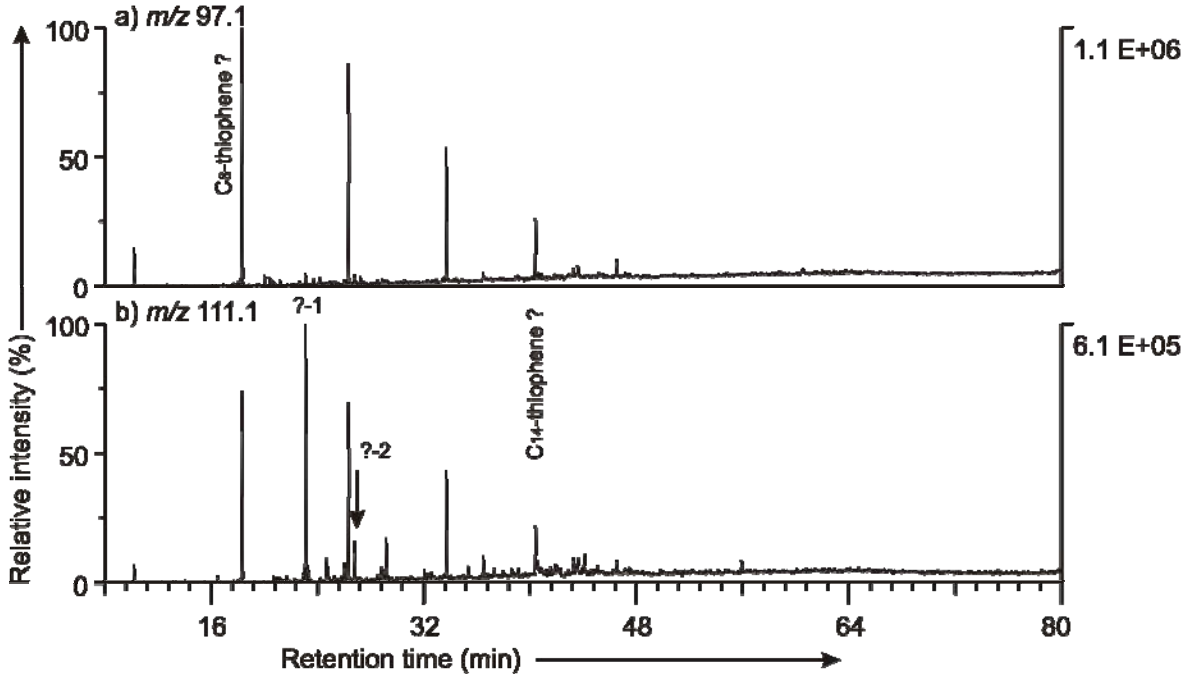


Figure 5-20 Partial mass chromatograms of a) alkylthiophenes and b) methylalkylthiophenes of the degraded Monterey Formation oil.

Two highly concentrated unknown components (?-1 and ?-2) were found in both oils, of which the mass spectra are shown in Figure 5-21. There are additional compounds coeluting but the molecular ions appear to be at *m/z* 222 for ?-1 (retention time 23:04 min) and *m/z* 236 for ?-2 (retention time 26:48 min), which gives a difference of one CH_2-unit, implying the two compounds may be structurally related with one additional methyl group in ?-2. Both spectra show the unusual loss of 40 u. A loss of a 40 u fragment was reported for chromans (Sinninghe Damsté et al., 1987a). The fragment may consist of three carbon atoms including one quaternary carbon atom and four hydrogen atoms. It is possible that the unknown compounds do not contain sulphur but are monoaromatic hydrocarbons with an additional alkyl ring (Simon C. Brassell, personal communication, 2005).

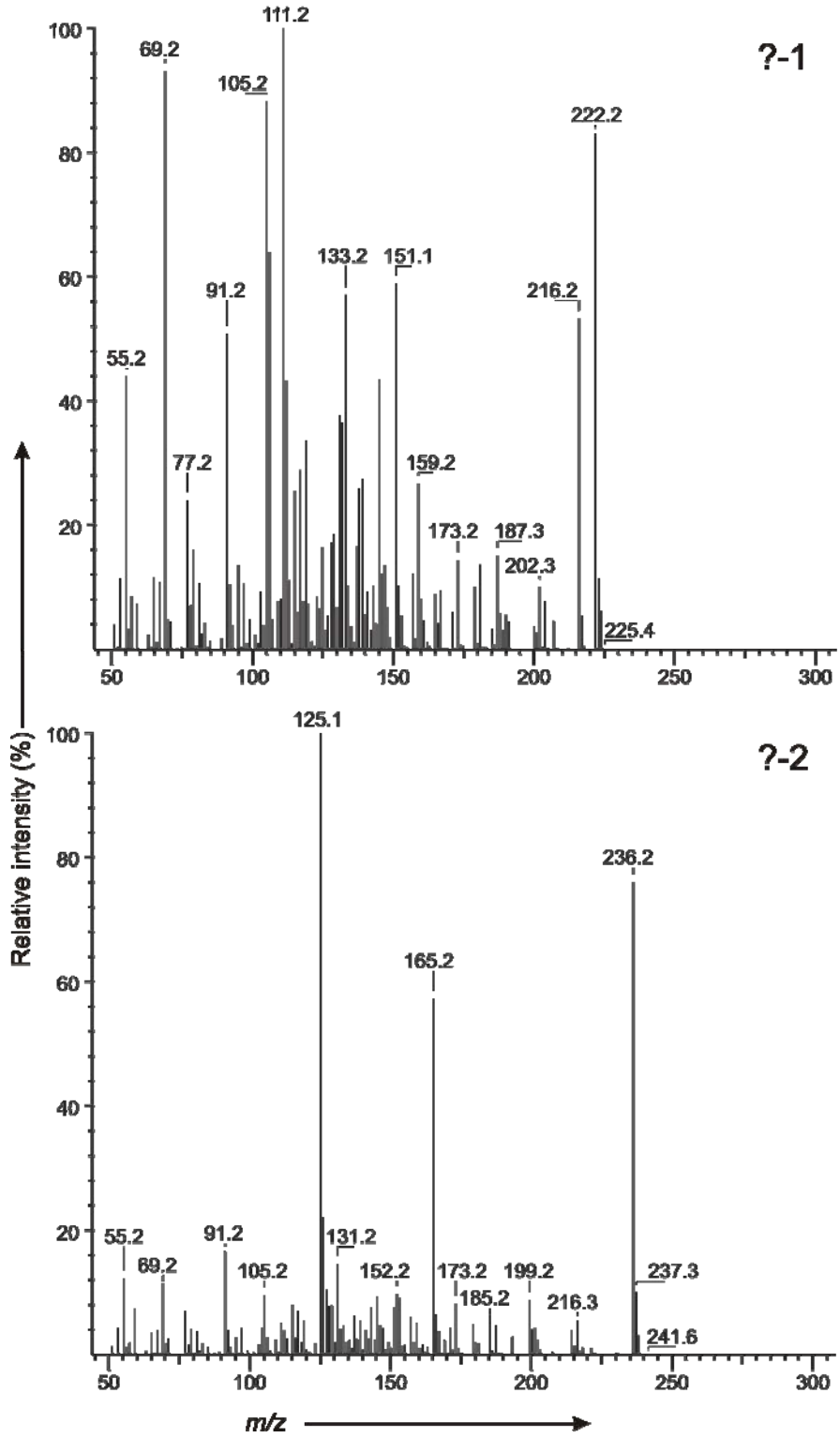


Figure 5-21 Mass spectra of two unknown compounds (?-1 and ?-2) eluting in the 111.1 *m/z* mass chromatogram of the degraded Monterey Formation oil (Figure 5-20).

Sulphur compounds in the monoaromatic hydrocarbon fractions of the two Vienna basin oils

In both Vienna basin oils, alkylated thiolanes, thianes and thiophenes were not detected.

5.3.2 Sulphur-containing polyaromatic compounds in the four crude oils

Sulphur-containing polyaromatic compounds in the Monterey Formation oils

With regard to the alkylbenzothiophenes, C_2 and C_3 homologues are most abundant in the two Monterey Formation oils (retention time around 16 min in Figure 5-22). Due to their high concentration, the mass chromatograms in Figure 5-22 are magnified in order to perceive the homologous series of alkyl- and alkylmethylbenzothiophenes in the undegraded oil. For all homologues, there are several isomers present. For the alkyl-methylbenzothiophenes with more than 6 carbon atoms in the side chains, peak doublets are visible. These may be formed by the two structural isomers of 4-alkyl-2-methylbenzothiophenes, which elute slightly before 2-alkyl-4-methylbenzothiophenes (Strausz et al., 1990).

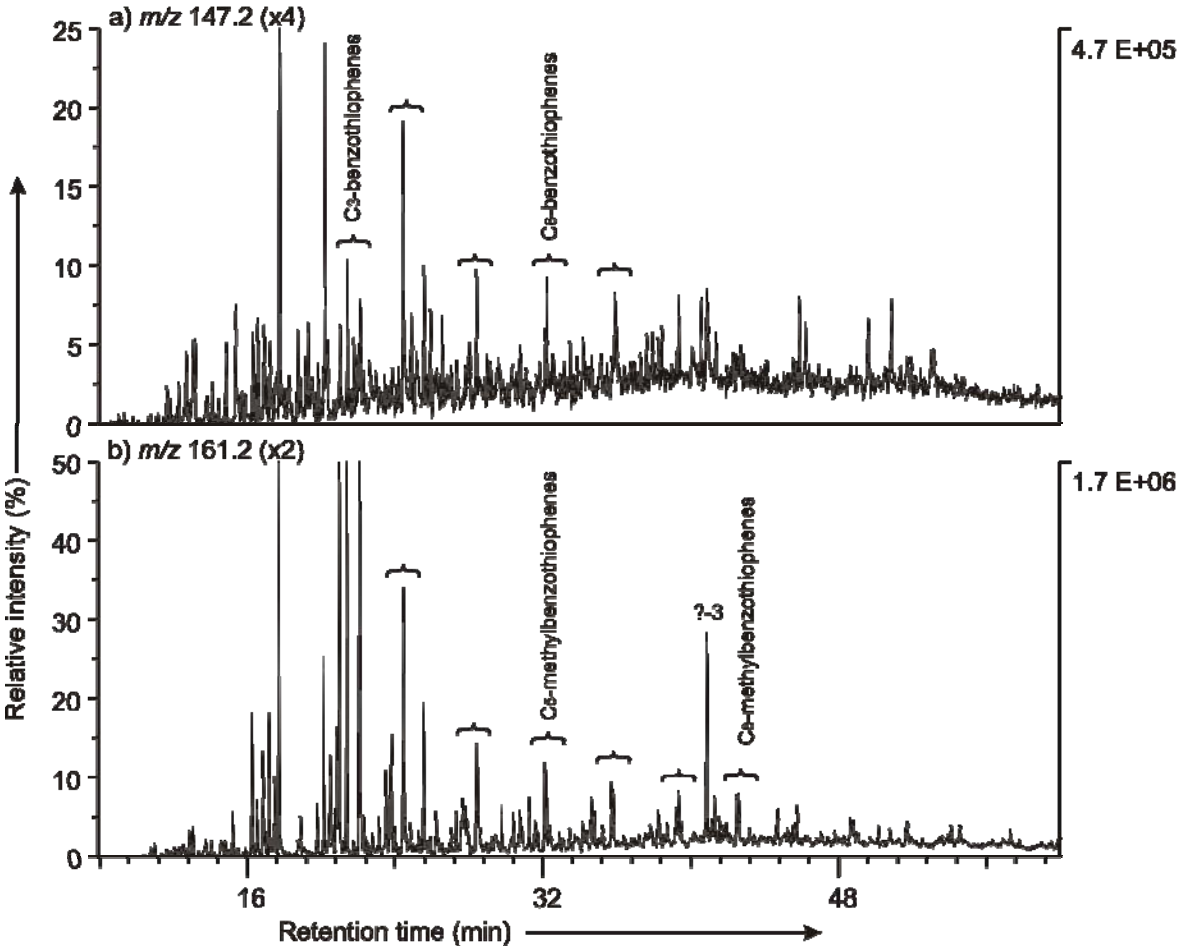


Figure 5-22 Partial mass chromatograms of a) alkylbenzothiophenes and b) methylalkyl-benzothiophenes of the undegraded Monterey Formation oil.

In both oils, an unknown compound (?-3) elutes between the C_7- and C_8-methylbenzothiophenes (retention time 43:16 min). The base peak in the mass spectrum (Figure 5-23) is *m/z* 105 but the compound is not visible in the *m/z* 105 mass chromatogram (see Figure 5-2). Either compound ?-3 is a polyaromatic compound which is not present in

the monoaromatic hydrocarbon fraction from which Figure 5-2 is derived or compound ?-3 has a very low concentration compared to the alkyltoluenes in Figure 5-2.

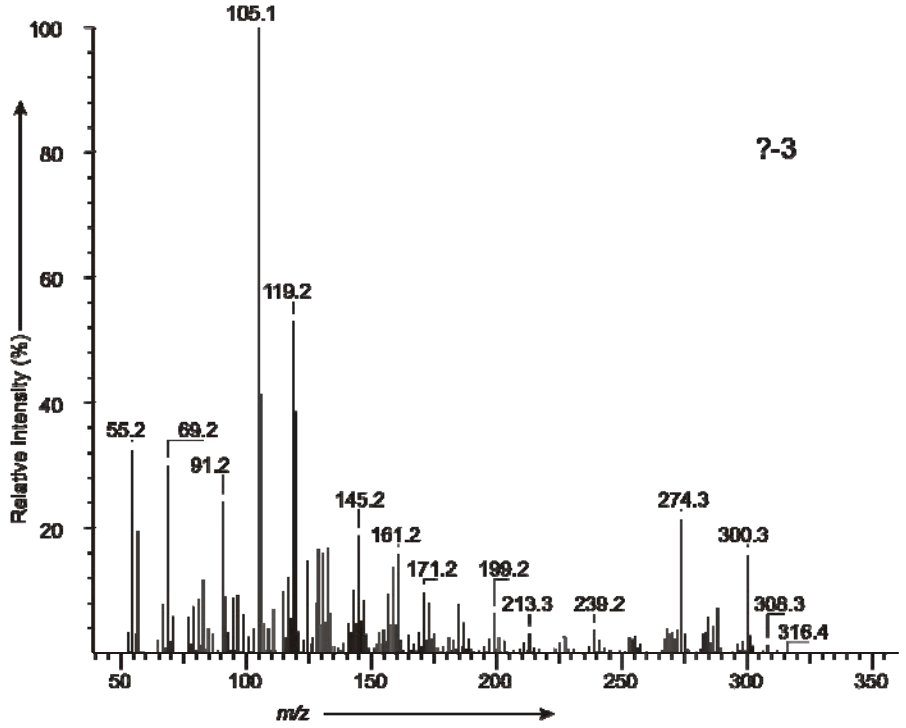


Figure 5-23 Mass spectrum of unknown compound (?-3) eluting in the 161.1 *m/z* mass chromatogram of the undegraded Monterey Formation oil.

Benzothiophene and methylbenzothiophenes are readily degraded by bacteria (Fedorak, 1990). The lower homologues of the methylbenzothiophenes are depleted in the degraded oil but one has to bear in mind that a loss due to the relatively high volatility may also have occurred.

Whereas thiophenes and benzothiophenes in crude oils may have long alkyl chains as substituents, the triaromatic compound dibenzothiophene (DBT) is mainly alkylated with methyl- and ethyl groups (Hughes, 1984). DBT and the four positional isomers of methyldibenzothiophenes are present in both oils. It can be degraded by bacteria (Fedorak, 1990), thus its presence denotes that biodegradation of sulphur-containing compounds has only started.

Figure 5-24 displays the C_2- and C_3-alkyldibenzothiophenes in the undegraded Monterey Formation oil. Four isomers of the C_2-dibenzothiophenes were identified using the relative retention times as estabilished with synthesised standards by Chakhmakhchev et al. (1997). Several high peaks in the mass chromatograms have corresponding signals in the RIC, indicating that at the respective retention times, the methyl- and dimethyldibenzothiophenes are dominant compounds in the aromatic hydrocarbon fraction in spite of the high unresolved background visible in the RIC.

Isomers substituted at position 1 are less stable than those substituted at C-3, which are less stable than 2-dimethyl-DBT, which again are less stable than 4- and 6-dimethyl-DBT. Differences in distribution patterns of methyl-DBTs and dimethyl-DBTs with biodegradation were not noted. Palmer (1993) stated that changes of alkyl-DBTs occurred at more advanced levels of biodegradation.

In addition to benzothiophenes and dibenzothiophenes, there is mass spectrometric evidence of higher aromatic sulphur compounds such as phenyldihydro-benzothiophenes and naphtho-thiophenes but due to the coelutions in the “hump“, no clean mass spectra could not be obtained for identification.

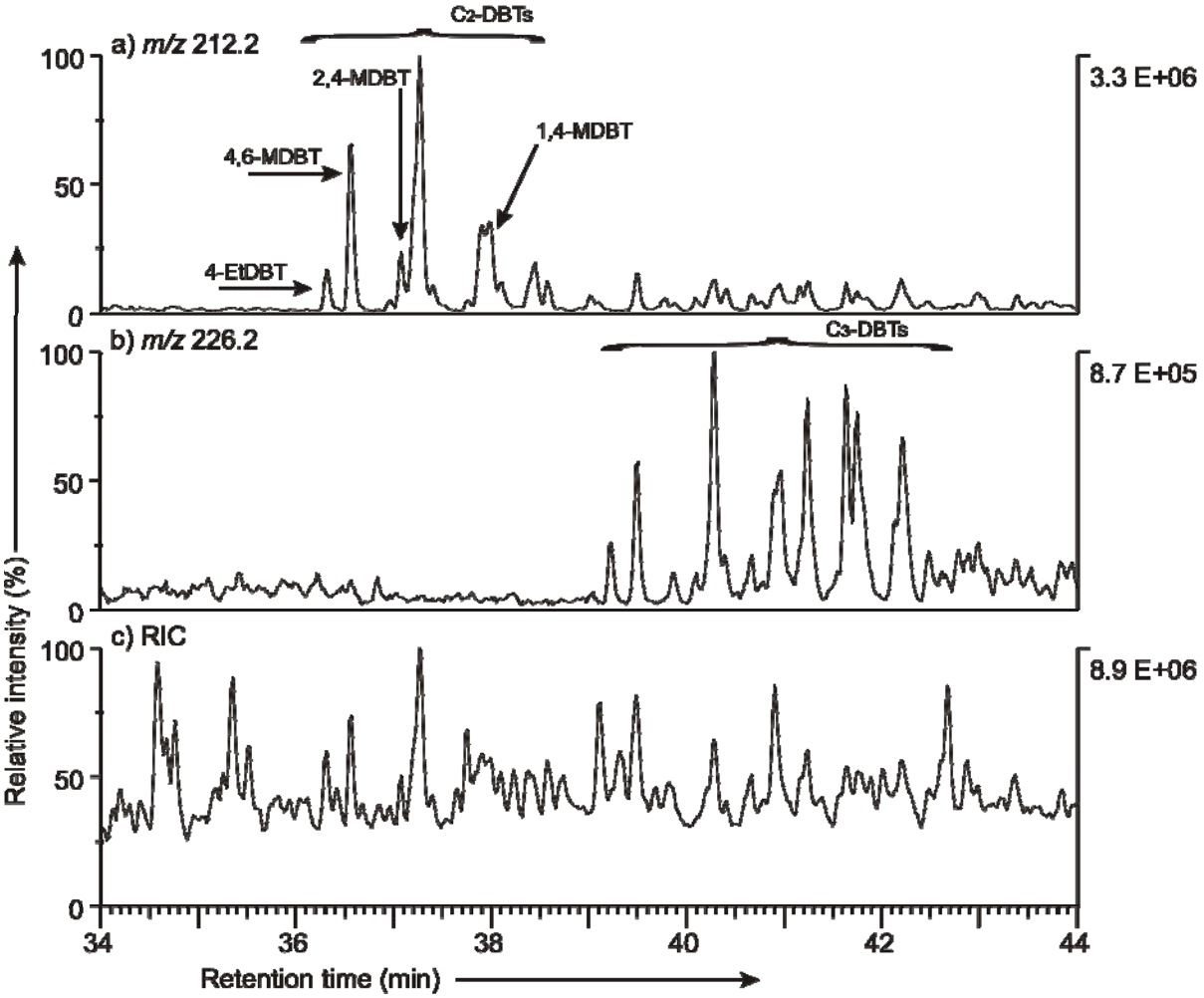


Figure 5-24 Partial mass chromatograms of a) C_2-dibenzothiophenes, b) C_3-dibenzothiophenes and c) reconstructed ion current of the undegraded Monterey Formation oil; M: methyl-, Et: ethyl-, DBT: dibenzothiophene.

Sulphur-containing polyaromatic compounds in the Vienna basin oils

Benzothiophenes in the Vienna basin oils were present in such small amounts that coeluting compounds masked the mass spectra and prevented identification. DBT and methyl-DBTs are present in the two Vienna basin oils but the mass spectra are not very clean either since coeluting compounds are present in higher abundance and mask the

spectra. With increasing maturity of oils, Radke (1987) observed a decrease in the amount of the less stable 1-methyl-DBT compared with the amount of 4-methyl-DBT. This seems to be the case here, too, in comparison to the less mature Monterey Formation oils but the mass chromatograms of the Vienna basin oils are not sufficiently neat to provide ample proof.

The pattern of alkylated dibenzothiophenes looks similar for both Vienna basin oils. Figure 5-25 presents the C_2- and C_3-homologues (*m/z* 212 and *m/z* 226) in the degraded Vienna basin oil. In contrast to the sulphur-rich Monterey Formation oil (*cf.* Figure 5-24), the dibenzothiophenes are not visible in the RIC, indicating their low relative concentrations.

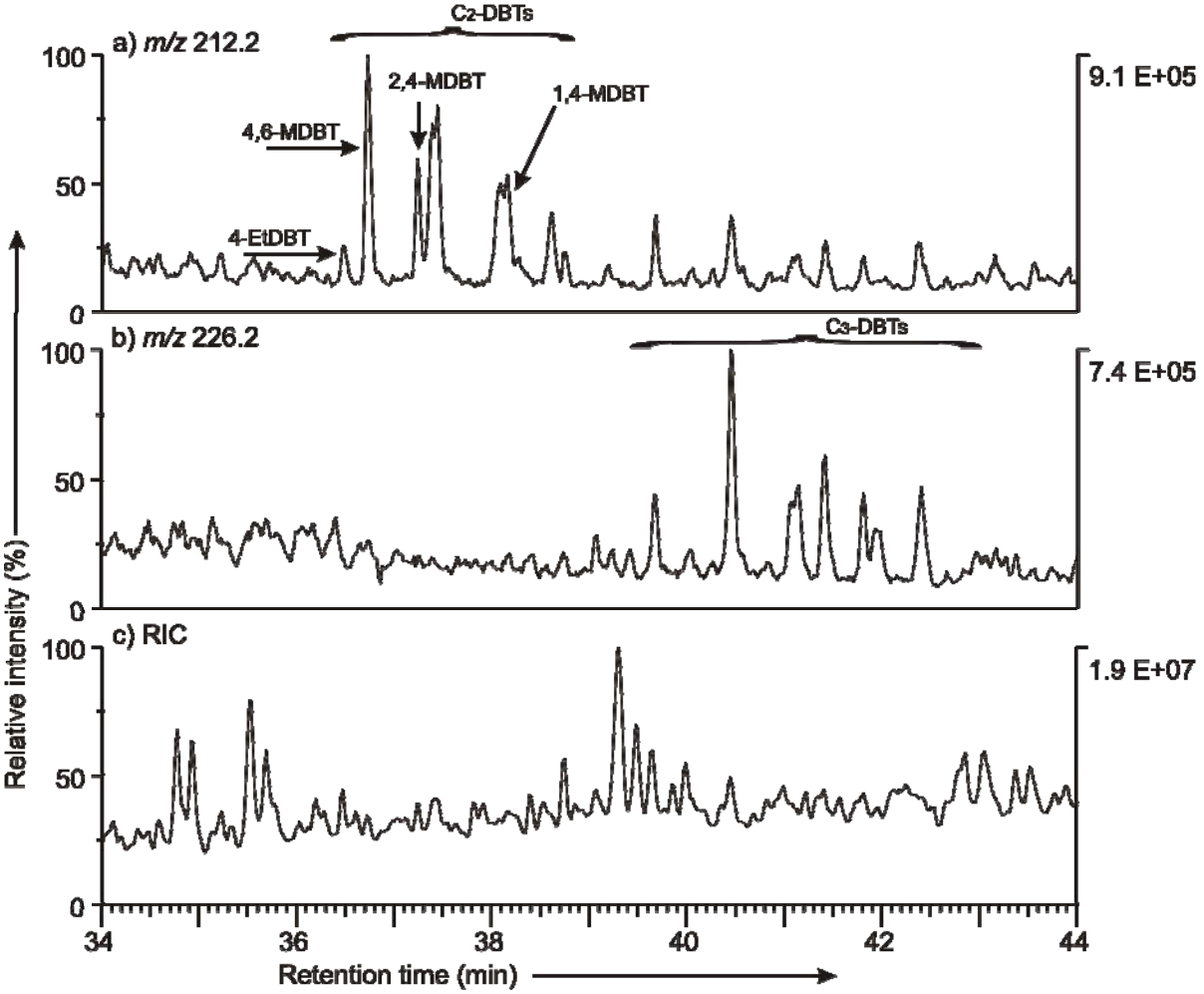


Figure 5-25 Partial mass chromatograms of a) C_2-dibenzothiophenes, b) C_3-dibenzothiophenes and c) reconstructed ion current of the degraded Vienna basin oil; M: methyl-, Et: ethyl-, DBT: dibenzothiophene.

5.3.3 Comparison of sulphur content and organic sulphur compounds in the Monterey Formation and the Vienna basin oils

The aromatic hydrocarbon fractions contain similar proportions of sulphur as the whole oils, including the non-aromatic sulphur compounds. The aromatic hydrocarbon fractions of the sulphur-rich Monterey Formation oils contain several thianes, thiolanes and thiophenes whereas the aromatic hydrocarbon fractions of the sulphur-lean Vienna basin oils contain no non-aromatic and monoaromatic sulphur compounds in readily detectable amounts. Benzothiophenes are present in all four oils but relative amounts in the sulphur-lean Vienna basin oils are very low. In the biodegraded Monterey Formation oil, methylbenzothiophenes are depleted. Hence, biodegradation of organic sulphur compounds has started.

Alkylated dibenzothiophenes are present in all four oils although in smaller diversity and abundance in the Vienna basin oils. 1-MDBT is depleted in the Vienna basin oils compared to the Monterey Formation oils due to the higher maturity of the Austrian oils.

Table 5-8 gives the ratios of phenanthrene to dibenzothiophene and methylphenanthrenes to methyldibenzothiophenes in the four oils. This comparison shows that the low sulphur content of the oils is reflected in the relation of triaromatic sulphur compounds to triaromatic hydrocarbons. The ratios for the methylated compounds are higher in all four oils, which can be attributed to the fact that there are four MP isomers but only three MDBT isomers, of which the peak areas were added. In addition, it is obvious that all ratios from the Vienna basin oils are much higher than the ones derived from the Monterey Formation oils. This mirrors the lower total sulphur content and the resulting lower concentration of aromatic sulphur compounds in the Austrian oils.

Table 5-8 Ratio of phenanthrene/dibenzothiophene and ratio of total methylphenanthrenes to total methyldibenzothiophenes in the total aromatic hydrocarbon fractions of the four oils.

Crude oil	Phen/DBT	MP/MDBT
Undegraded Monterey Formation oil	0.64	0.71
Degraded Monterey Formation oil	0.72	0.81
Undegraded Vienna basin oil	2.03	2.99
Degraded Vienna basin oil	4.69	7.31

There are also differences between the ratios of the undegraded oils and the degraded oils. The ratios of all four oils increase with biodegradation. This means that either more phenanthrenes or less dibenzothiophenes are present in the degraded oils compared to the undegraded ones. This is more obvious for the sulphur-lean Vienna basin oil pair. As for the Monterey Formation oil, biodegradation has only slightly altered the distribution of organic sulphur compounds.

Table 5-9 summarises the similarities and differences regaring the organic sulphur compounds in the sulphur-rich Monterey Formation oils and the sulphur-lean Vienna basin

oils. Since mass spectra were dominated by fragments of coeluting compounds of higher relative abundance, differences between the undegraded and the degraded Vienna basin oils were not perceived.

Table 5-9 Summarised results of the general composition of the organic sulphur compounds in the four oils studied.

Compound class	Similarities	Differences
Aliphatic and monoaromatic sulphur compounds		o The Vienna basin oils contain no aliphatic and monoaromatic sulphur compounds. o Some long-chain substituted components are depleted in the degraded Monterey Formation oil.
Diaromatic sulphur compounds	o Present in all four oils.	o Present in sulphur-lean oils in very low amounts. o Methylbenzothiophenes are degraded in sulphur-rich oil.
Triaromatic sulphur compounds	o The same isomers of MDBTs and DMDBTs are present in all oils. o The two degraded oils contain less DBTs in relation to the phenanthrenes.	o DBTs are more abundant in the Monterey Formation oils in relation to the phenanthrenes. o The higher maturity of the Vienna basin oils is indicated by the distribution of MDBTs.

5.4 Summary – composition of aromatic crude oil fractions

The monoaromatic and total aromatic hydrocarbon fractions of the four oils used in this study were analysed for their distribution patterns within several compound classes. A wide range of alkylated monoaromatic compounds was found in all four oils. This is particularly true for indanes and tetralins. In addition, the fragmentation pattern in the mass spectra of many compounds suggested their structures to be related to *cyclo*hexylalkylbenzenes and *cyclo*pentylalkylbenzenes, possibly with bridges of different numbers of carbon atoms. This confirms retrospective structure identification studies by Warton et al. (1999) who concluded from products of chemical oxidation experiments that alkylated indane, tetralins and *cyclo*hexylalkylbenzenes must be present in aromatic crude oil fractions.

Unresolved complex mixtures are dominant in all mass chromatograms of all oils. The less specific a typical fragment is for a compound class and the lower concentrated the resolved compounds are in the mass chromatogram, the more pronounced the UCM becomes.

Differences in composition between the oils studied were caused by

a) different origins and levels of maturity of the oils (moderately mature Monterey Formation oils versus mature Vienna basin oils),
b) the degree of biodegradation (nondegraded versus moderately biodegraded oils) and
c) the dissimilar susceptibility of individual isomers to various degrading bacteria (degraded Monterey Formation oil versus degraded Vienna basin oil)

and are summarised in Table 5-10.

The most obvious difference in oil composition with origin can be attributed to the sulphur content and resulting differences in the distribution of organic sulphur compounds. Sulphur compounds are present in a higher diversity and are more abundant in the aromatic fractions of the sulphur-rich Monterey Formation oils than in the sulphur-lean Vienna basin oils. Other differences can derive from e.g. differences in maturity and the composition of organic matter of the source rock.

In addition, the amount of steroids differs between the oils. They are more abundant in the Monterey Formation oils. Furthermore, individual isomers such as 1,2,8-TMP and the distribution patterns of the complex mixtures of dicyclic monoaromatic compounds depend on the origin of the oils. Maturity does not appear to have a major influence on the composition of the aromatic compounds investigated. Differences were detected for a few individual compounds only, for instance alkyldibenzothiophenes.

Table 5-10 Influence of differences in a) origin and maturity, b) biodegradation and c) susceptibility on distribution within selected compound classes, M: Monterey Formation oil, V: Vienna basin oil, n.d. = not determined due to coeluting compounds.

compound class	a) origin/maturity	b) biodegradation	c) susceptibility
Monocyclic monoaromatics	• Abundance of positional isomers	• Long-chain substituted homologues degraded, • no alkylbenzenes left in V	• Preference of different positional isomers
Dicyclic monoaromatics	• Abundance and presence of components	• Long-chain substituted homologues degraded	• n.d.
Naphthalenes	• Relative abundance of positional isomers	• M: Changes in C_3-naphthalenes • V: Changes in C_2- and C_3-naphthalenes	• No consistent trend
Phenanthrenes	• Abundance of individual isomers	• M: Ethyl- and some C_3-phenanthrenes depleted • V: Some C_1-and C_3-phenanthrenes depleted	• No consistent trend
Aromatic steroids	• Abundance of steroids in general	• No influence	• No change
Sulphur compounds without DBTs	• Aliphatic and monoaromatic compounds absent in the sulphur-lean oils	• M: Long-chain substituted homologues degraded	• n.d.
DBTs	• All isomers are less concentrated relative to phenanthrenes in the sulphur-lean oils. • Relative abundance of positional isomers due to maturity	• Lower P/DBT ratios in degraded oils indicate that biodegradation has started in both oils.	• n.d.

Biodegradation caused obvious alterations of the oils. In general, as often reported in the literature, most changes with biodegradation occurred in the monoaromatic compound groups. Overall, monoaromatic compounds are more susceptible to biodegradation than diaromatic ones, which are more susceptible than triaromatic compounds. The long-chain

monoaromatic compounds are attacked during initial biodegradation. The dicyclic monoaromatic compounds are less easily degraded, probably owing to the fact that the benzene ring has two additional substituents with the condensed saturated ring. This complies with the general principle that the more carbon atoms of the aromatic nucleus are substituted, the less susceptible it is to microbial attack. For the environmental relevance this implies that dicyclic monoaromatics such as indanes and tetralins can quickly accumulate in the enviroment.

As a general rule for di- and triaromatic compounds, methylated compounds are attacked prior to di- and trimethylated ones (e.g. Rowland et al., 1986; Palmer, 1993; Alexander, 1999). In all oils, methyl- and dimethylnaphthalenes as well as methyl- and dimethylphenanthrenes and higher homologues were still present, with individual isomers being depleted in the degraded oils. This indicates that as described before the overall level of biodegradation was moderate (PM level 4-5) for the two oils (Chapter 3.1 and in this chapter). Several compounds classes indicate that the two oils are equally biodegraded. One exception is the slight degradation of ethylphenanthrenes in the Monterey Formation oil, which was reported to start only at level 6 (Huang et al., 2004) and suggests an advanced level of biodegradation. However, this is the only indication that the Monterey Formation oil is more degraded than the Vienna basin oil. In contrast, the more severe depletion of alkylbenzenes and the slight depletion of methylphenanthrenes in the degraded Vienna basin oil indicate that this oil is possibly a bit more heavily degraded than the Californian oil. Overall, the majority of compounds suggests an equal or a slightly higher degree of biodegradation for the Vienna basin oil. For moderately degraded oils, this means that the Monterey Formation oil is level 4 and the Vienna basin oil is level 5.

Regarding the succession of the depletion of individual isomers, differences and similarities between the two oil pairs and literature data were found. For example, several trimethylphenanthrenes were depleted in the Vienna basin oil whereas several dimethylnaphthalenes were not visibly affected.

Biodegradation of sulphur aromatics usually starts with the parent compounds, followed by the methylated ones. In both degraded oils studied, the parent compounds were still present but the ratios to the corresponding phenanthrenes indicate that biodegradation of aromatic sulphur compounds has started.

All things considered, the oils studied concur with the general principles reported in the literature. Minor deviations can be explained by the fact that biodegradation is a quasi-stepwise process, which denotes that overlapping of typically succeeding steps is not unusual. In addition, one has to bear in mind that in disparate oil reservoirs different consortia of degrading bacteria are present, which may favour different components for microbial attack.

6 Results and discussion – aromatic compounds in aqueous solutions and accumulated in mussel tissue

In Chapter 4, the feeding rates of the mussels exposed to aromatic hydrocarbon fractions from crude oils were compared to the nominal aqueous hydrocarbon concentrations and to the concentration of total aromatic compounds accumulated in the mussel tissue. For the fractions isolated from the Monterey Formation oils, the overall reduction of mussel feeding rates correlated with an increasing amount of accumulated aromatic compounds (only a few mussel feeding rates did not concur with this general trend). The aromatic hydrocarbon fractions isolated from the Vienna basin oils did not appear to affect mussel feeding rates. In this section, the composition of aromatic hydrocarbons accumulated in the mussel tissue is investigated in terms of compound groups present in the original oils (see Table 5-1). In addition, the composition of aromatic hydrocarbons extracted from a few aqueous solutions of the oil fractions is briefly described.

The composition of the accumulated aromatic compounds in mussel tissue was studied in order to look for differences between the distribution of compound groups in the original oil fractions and in the mussel tissue samples. In addition, the accumulated compounds were investigated to find out whether there was any obvious compositional difference possibly explaining the low toxic response of the mussels from Jade Bay.

In general, a mixture of similarly acting toxicants causes a joint toxic effect. This can be measured and cause-effect relationships between the total amount of xenobiotics and the toxic effect are obtained. However, the attribution of the toxic effect to a certain compound group within the mixture is only rarely achieved (e.g. Carr et al., 2001; e.g. Reineke, 2003). This is usually only possible if a certain compound group constitutes a high proportion of the mixture such as BTEX compounds in fresh oils (e.g. Fuller et al., 2004) and not in a mixture of thousands of hydrocarbons each concentrated at a low level. Furthermore, nonspecific narcosis is caused by the majority of chemicals (van Wezel and Opperhuizen, 1995) and does not require a specific toxic mechanism (e.g. via a certain receptor). The low-molecular-weight aromatic hydrocarbons are absent from the oil fractions used in this study and the chromatograms of the samples indicate that many compounds are present at low concentrations in each fraction (see Chapter 5). Thus, it was anticipated that an attribution of the toxic effects to a certain group of compounds within the mixture would not be possible.

Investigation of the composition of water and tissue samples on a molecular level was difficult due to the extremely low amounts of hydrocarbons in many samples. In general, this applied to mussels with a total body burden below 30 µg/g wet weight and in particular to those mussels which were exposed to low concentrations of aromatic compounds. In the experiments with aqueous solutions of monoaromatic hydrocarbon fractions, compounds were not accumulated in sufficient amounts for analysis.

6.1 Apolar natural compounds in control mussels

Some compounds in the mussel tissue extracts are natural constituents of the organism itself. The total organic extracts of the tissue from untreated control mussels were dominated by high amounts of cholesterol. Most prominent in the apolar fractions used for quantification and identification of accumulated compounds was squalene which is a typical component in mussel tissue (de Zwaan and Mathieu, 1992). Squalene was also present in the procedural blank (*cf.* Section 3.2.4.8), indicating a source for this component in addition to the biogenic squalene from the mussel tissue. The varying abundance of squalene in different tissue samples is likely to be due to natural differences in lipid content and composition, which are known to change according to season and stage of the life cycle of *Mytilus edulis* (de Zwaan and Mathieu, 1992).

6.2 Distribution of aromatic compounds in aqueous solutions of total aromatic hydrocarbon fractions and in exposed blue mussels

Aqueous solution samples of the two degraded oils (see Table 4-5) and mussel tissue samples from the exposure experiments with the total aromatic hydrocarbon fractions (experiments 1, 3, 5 and 6) were analysed by GC-MS. The composition of alkylnaphthalenes, -phenanthrenes and sulphur-containing compounds was examined and is described for selected samples in the following sections. Ratios of phenanthrene/dibenzothiophene and their methylated homologues were calculated and presented here. The ratios derived from the oil, aqueous solution and tissue samples are discussed in Section 7.

6.2.1 Aqueous solutions of the degraded Monterey Formation oil fraction

The actual concentration of the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil in aqueous solution increased proportionally to its nominal concentration. Nominal aqueous concentrations of 50, 100, 200 and 500 µg/L correspond to measured concentrations of 23, 37, 79 and 93 µg/L, respectively. In the reconstructed ion current chromatograms of the solutions, compounds eluted until 60 minutes and small UCMs occurred between 25 and 50 minutes. The retention time range of eluted compounds does not change with increasing aqueous concentration of the same oil fraction, implying that the composition of the aqueous solutions did not differ in an obvious way.

In the original oil fraction, compounds elute as late as 90 minutes in the gas chromatogram, i.e. beyond those present in the aqueous solution samples. Thus, compared to the original oil fraction, the retention time range of compounds in the aqueous solution is reduced after dissolution in water. Obviously, the high-molecular-weight compounds did not dissolve in the water, owing to their relatively low aqueous solubility (*cf.* Section 2.5). This at least partly accounts for the difference in nominal and actual aqueous concentrations.

GC-MS analysis of extracts from the solutions at the higher nominal concentrations of 200 µg/L and 500 µg/L revealed that mostly alkylated naphthalenes, phenanthrenes, benzothiophenes and dibenzothiophenes were present. Phenanthrene/dibenzothiophene ratios are lower than the methylphenanthrene/methyl-dibenzothiophene ratios (Table 6-1) and quite similar to those in the original oil (see Table 5-8).

Table 6-1 Phenanthrene/dibenzothiophene ratios and ratios of methylphenanthrenes (MP) to methyldibenzothiophenes (MDBT) of aqueous solutions of the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil.

Nominal concentration	Measured concentration	Phen/DBT	MP/MDBT
50 µg/L	23 µg/L	0.35	1.10
100 µg/L	37 µg/L	0.77	1.07
500 µg/L	93 µg/L	0.77	1.00

GC-MS analysis also showed that only very few monoaromatic compounds from the original oil fraction were dissolved in the water. For example, in the mass chromatograms the key ions of typical monoaromatic compounds extend only to a retention time of 18 minutes, which is appromixately the retention time of C_7-substituted benzenes. In addition, the regular pattern of alkylbenzenes and alkyltoluenes, which was visible in the original oil fraction (see Figure 5-3), is replaced by an unresolved mixture. Probably only the polysubstituted and branched alkylbenzenes were dissolved since they are more soluble in water than the straight-chain isomers (e.g. Sherblom et al., 1992; Smith, 2002). This depletion of monoaromatic compounds can be explained by the fact that alkylated polyaromatic hydrocarbons are more polar than alkylated monoaromatics of similar molecular weight, which results in a higher aqueous solubility of the former (Booth, 2004).

6.2.2 Mussel tissue samples after exposure to solutions of the Monterey Formation oil fractions

Distribution of aromatic hydrocarbons in blue mussels from experiment 1

Two mussel tissue extracts from the exposure to the undegraded (samples Ply-uM-200 and Ply-uM-500) and three extracts from the exposure to the degraded oil fraction (samples Ply-dM-200a, Ply-dM-200b and Ply-dM-500) contained sufficient amounts of material to be analysed by GC-MS (see Table 6-2 for explanation of sample labels).

The mussels in sample Ply-uM-200, which were exposed to the aqueous solution of the total aromatic hydrocarbon fraction of the undegraded Monterey Formation oil at the nominal concentration of 200 µg/L, accumulated a total tissue concentration of 43 µg/g wet weight. The total reconstructed ion current chromatogram from GC-MS analysis shows accumulated compounds eluting at 20 to 60 minutes, with the majority of compounds coeluting between 30 and 50 minutes (Figure 6-1 c). The mass spectra are dominated by fragments of coeluting alkylated naphthalenes, phenanthrenes and dibenzothiophenes. This indicates that a high

percentage of the compounds accumulated in the mussels are polyaromatic. As a consequence of their low concentration in the aqueous solutions used, monoaromatic compounds are obviously not highly abundant in the mussel tissue either.

Table 6-2 Labels of mussel tissue samples and their meaning.

Sample label	Meaning
Ply or Whv-I or Whv-II	Location of experiments: Plymouth (experiments 1 and 2) or Wilhelmshaven (experiments 3 and 4) or Wilhelmshaven (experiments 5 and 6)
u or d	Undegraded or degraded oil
M or V	Monterey Formation or Vienna basin oil
100 or 200 or 500	Nominal aqueous concentration of the solution of the total aromatic hydrocarbon fraction
a or b	If both duplicates were analysed, they are distinguished with a and b

Alkylated naphthalenes and phenanthrenes are present in the tissue in a high diversity (Figure 6-1 a and b). 1,2,8-Trimethylphenanthrene, for example, which is the most abundant C_3-phenanthrene in the original oil (see section 5.2.1), is also the most abundant C_3-phenanthrene in the mussel tissue. The C_0- to C_2-phenanthrenes are present in high abundance (Table 6-3). The isomer 3-ethylphenanthrene, which is present in the original oil, was not detected in the tissue extract, indicating its lower aqueous solubility and bioavailability. Sulphur-containing compounds in the tissue are not present in the same diversity and abundance as in the original oil fraction. Individual thiolanes, thianes and thiophenes were not identified. Isomers of C_3- to C_6-benzothiophenes and polymethylated dibenzothiophenes are present in the tissue.

The average feeding rate of the mussels in this sample (Ply-uM-200) was reduced by 39% compared to control mussels (Table 6-3). C_0- to C_2-phenanthrenes and -dibenzothiophenes are present in similar amounts (0.13 and 0.15 µg/g wet weight). Compared to the TEC_{50} values of the parent compounds, which are 31 µg/g wet weight for phenanthrene and 14 µg/g wet weight for dibenzothiophene (Donkin et al., 1989), the amounts present make up approximately 1% each of the TEC_{50} values. There are no literature data for the toxicity of alkylated debenzothiophenes or phenanthrenes but the known narcotic toxicity of the parent compounds in the context of Concentration Addition suggests that the alkylated homologues are also narcotically toxic. The calculated concentrations were derived from GC-MS analysis and should be called semi-quantitative (*cf*. 3.2.4.7), but they still demonstrate that many other compounds accumulated in the tissue must be responsible for the reduction of feeding rates, as well, as predicted by the mixture toxicity concept of Concentration Addition.

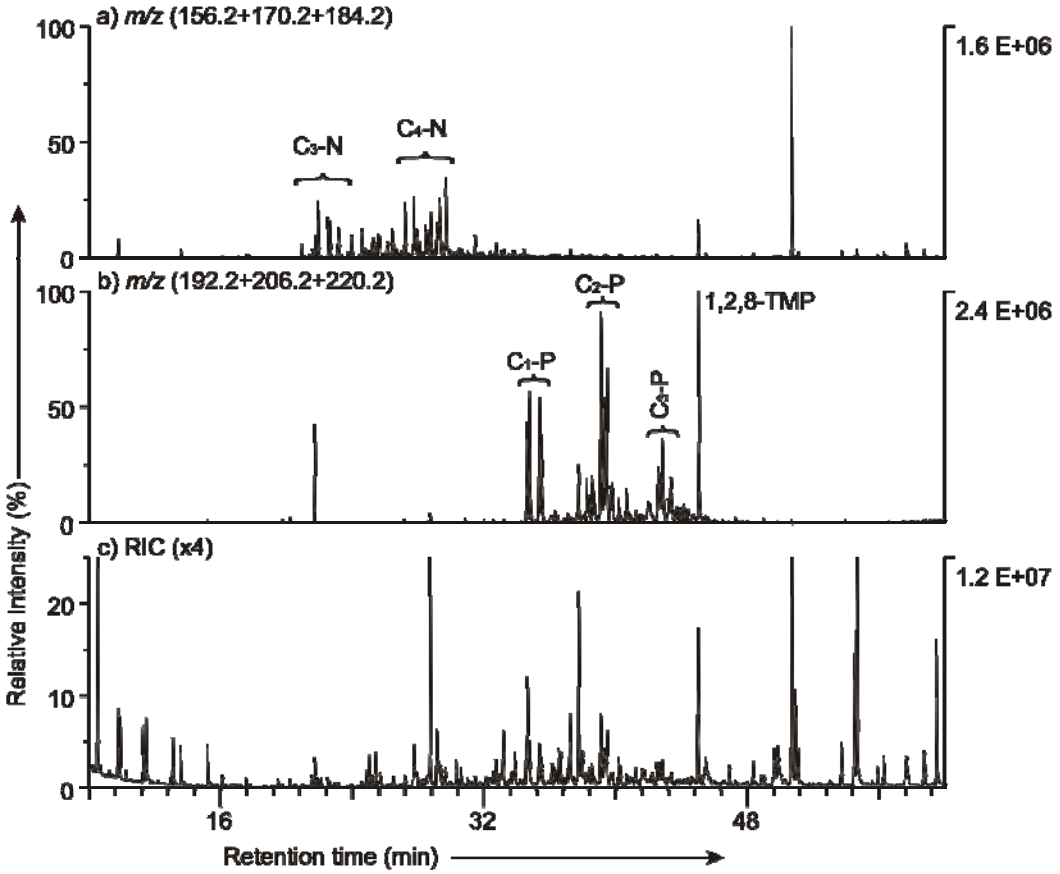


Figure 6-1 Partial chromatograms of (a) molecular ions of C_2- to C_4-naphthalenes, (b) molecular ions of C_1- to C_3-phenanthrenes and (c) total ion current of accumulated compounds in mussels sample Ply-uM-200; N = naphthalene, P = phenanthrene, TMP = trimethylphenanthrene.

Table 6-3 Feeding rates (FR) [%], concentrations of C_0- to C_2-phenanthrenes (∑Ps) and C_0- to C_2-dibenzothiophenes (∑DBTs) [both µg/g wet weight], ratios of phenanthrene/dibenzothiophene (n.p. = not possible due to the absence of dibenzothiophene) and of methylphenanthrenes/methyl-dibenzothiophenes in mussels exposed to the total aromatic hydrocarbon fractions of the Monterey Formation oils (M), arranged with increasing body burdens (BB) [µg/g ww]. Chromatograms of the underlined samples are displayed in Figures 6-1 and 6-2.

Oil	Sample	FR [%]	BB [µg/g ww]	∑ Ps [µg/g ww]	∑ DBTs [µg/g ww]	P/DBT	MPs/MDBTs
Undegraded M	Ply-uM-200	61	42.9	0.13	0.15	1.22	1.11
	Ply-uM-500	102	177.5	0.21	0.17	1.30	1.42
Degraded M	Ply-dM-200b	61	32.8	0.03	0.03	n.p.	1.12
	Ply-dM-200a	67	43.6	0.03	0.03	n.p.	0.77
	Ply-dM-500	82	47.6	0.19	0.19	1.78	0.66

Sample Ply-uM-500 yielded an outlying value with high feeding rates in spite of an extremely high body burden (see Section 4.1.1). The composition of the target compound classes was compared to that in the tissue of mussels in sample Ply-uM-200 to see if differences in composition were responsible for the different biological response. The total reconstructed ion current chromatogram of sample Ply-uM-500 (not shown) contains

compounds eluting in the retention time range between 20 and 60 minutes and a UCM is clearly visible with a retention time range of 30 to 50 minutes, as in sample Ply-uM-200. No differences were found concerning the compound groups investigated, e.g. C_0- to C_2-phenanthrenes and -dibenzothiophenes are present in high abundance (Table 6-3). The concentrations of the quantified phenanthrenes and dibenzothiophenes are actually higher in the outlier than in sample Ply-uM-200. All in all, there was no significant difference observed in the accumulated hydrocarbons which could explain why the feeding rate of the mussels with the extraordinarily high body burden was not reduced, i.e. why this sample is an outlier. There must be unknown biological reasons for the lack of toxic response of the mussels.

The mussels in samples Ply-dM-200a and Ply-dM-200b were exposed to the aqueous solution of the total aromatic hydrocarbon fraction of the degraded Monterey Formation oil at the nominal concentration of 200 µg/L and sample Ply-dM-500 to 500 µg/L of the same oil fraction. The total body burdens aquired range from 32.8 to 47.6 µg/g wet weight (Table 6-3). The accumulated compounds in sample Ply-dM-500 elute in the retention time range between 20 and 60 minutes and the majority of compounds can be found between 30 and 50 minutes, where they form a small “hump“” in the chromatogram (Figure 6-2). Those mussels exposed to the lower aqueous concentration contain less compounds and no visible “hump“”. In all three samples many seemingly resolved peaks are visible which are actually coeluting compounds judging by their mass spectra and therefore could not be identified.

As in the mussels exposed to the undegraded oil fraction described above (sample Ply-uM-200), coeluting polyaromatic compounds dominate the mass spectra of these three samples so that the mass chromatograms of typical monoaromatic hydrocarbons have too low concentrations to identify them. The mass chromatogram of the key fragment for monoaromatic steroids (*m/z* 253) suggests that several of these compounds elute between 50 and 60 minutes (not shown). This is the same retention time range as in the original oils but intensities were too low for identification.

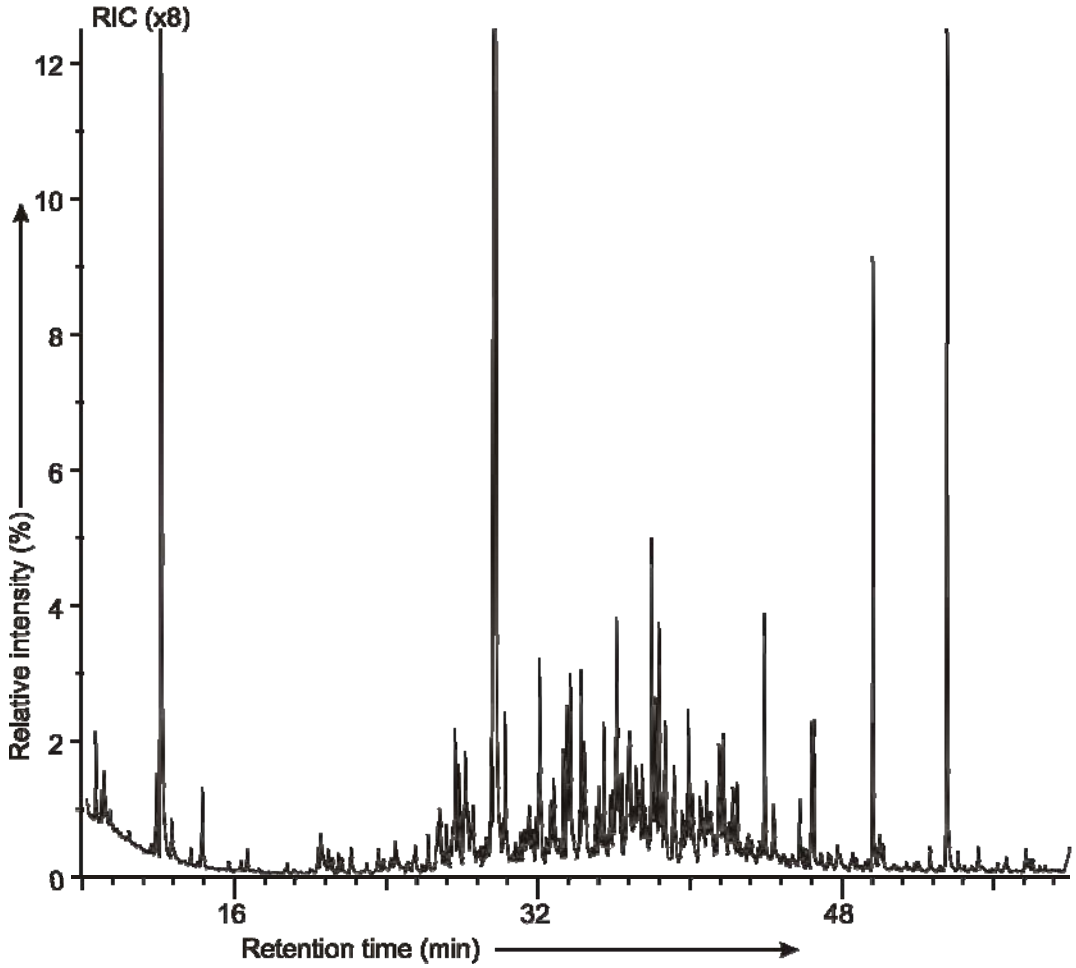


Figure 6-2 Partial reconstructed total ion current chromatogram (8-times magnified) of sample Ply-dM-500.

The tissue contains a number of alkylated naphthalenes and phenanthrenes and, as for the blue mussels exposed to the undegraded Monterey Formation oil, 1,2,8-trimethylphenanthrene was accumulated to a high extent. The three samples do not contain monoaromatic sulphur compounds. Only sample Ply-dM-500 contains substances which produce an *m/z* 161 fragment and were tentatively identified as C_3- to C_6-benzothiophenes. These compounds were also found in sample Ply-uM-200 but fragments of coeluting alkylnaphthalenes mask the mass spectra.

In samples Ply-dM-200a and Ply-dM-200b, the total amount of C_0- to C_2-phenanthrenes and C_0- to C_2-dibenzothiophenes is very low (Table 6-3). The determined concentrations do not correlate well with the total body burdens. Dibenzothiophene was not detected in two samples so that the ratio of the parent compounds could not be calculated. The other ratios are quite scattered (Table 6-3). The quantified phenanthrenes and dibenzothiophenes make up a small proportion of the total aromatics. As outlined above, other compounds taken up by the mussels must also be responsible for the toxic effect observed.

Distribution of aromatic hydrocarbons in blue mussels from experiment 5

In experiment 5, mussel feeding rates were not reduced with increasing concentrations of aromatic compounds from the degraded Monterey Formation oil. GC-MS analysis of the four samples revealed very similar total reconstructed ion current chromatograms. Compounds elute between 15 and 60 minutes retention time and a “hump“ is visible between 25 and 50 minutes. GC-MS chromatograms of sample Whv-II-dM-100a (for explanation of sample labels see Table 6-2) are shown as a representative of all four samples (Figure 6-3).

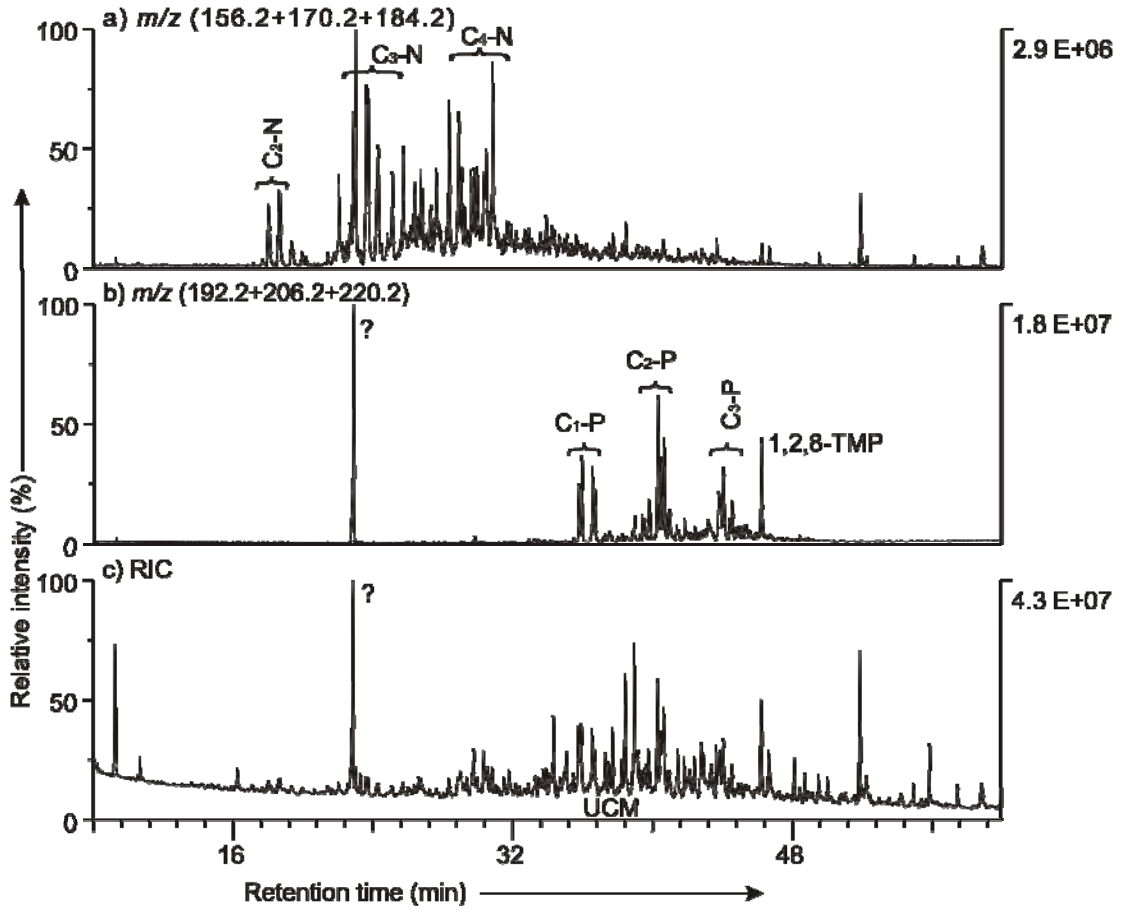


Figure 6-3 Partial chromatograms of (a) molecular ions of C_2- to C_4-naphthalenes, (b) molecular ions of C_1- to C_3-phenanthrenes and (c) reconstructed total ion current of accumulated compounds in sample Whv-II-dM-100b; N = naphthalene, P = phenanthrene, TMP = trimethylphenanthrene, ? = unknown compound.

The composition of summed C_2- to C_4-naphthalenes (Figure 6-3 a) and C_1- to C_3-phenanthrenes (Figure 6-3 b) was similar in the four tissue samples of the exposed mussels. 1,2,8-Trimethylphenanthrene is highly abundant in all four samples. The mass chromatograms of typical fragments of monoaromatic hydrocarbons show slight differences between the four samples (not shown). For example, sample Whv-II-dM-100b contains a substance which is not present in the other three samples (labelled as ? in Figure 6-3).

The mass chromatograms of typical fragments of monoaromatic sulphur compounds are masked by fragments of polyaromatic compounds. Regarding benzothiophenes, sample

Whv-II-dM-100b contains several peaks in the *m/z* 161 mass chromatogram, which are absent or much less abundant in the other three samples but again overlying triaromatic compounds mask the spectra. C_1- to C_3-dibenzothiophenes are present in all samples. All four samples contain the isomers of dibenzothiophenes and phenanthrenes, which were used for quantification, in the same relative abundances (Table 6-4). The ratios of hydrocarbons to sulphur-containing compounds are also very similar for the four samples (Table 6-4).

The sums of phenanthrenes and dibenzothiophenes neither increase proportionally to the total body burden nor to the reduction of feeding rates. Sample Whv-II-dM-100a contains more phenanthrenes and dibenzothiophenes than the other three samples but the mussels had a very high feeding rate. Although the mussel tissue of sample Whv-II-dM-200b contains the lowest amount of aromatic compounds, the mean feeding rate is reduced most strongly. This may be due to unknown compounds present only in this sample.

Table 6-4 Feeding rates (FR) [%], concentrations of C_0- to C_2-phenanthrenes (∑Ps) and C_0- to C_2-dibenzothiophenes (∑DBTs) [both µg/g wet weight], ratios of phenanthrene/dibenzothiophene and of methylphenanthrenes/methyldibenzothiophenes in mussels exposed to the total aromatic hydrocarbons of the degraded Monterey Formation oil, arranged with increasing body burdens (BB) [µg/g ww]. The underlined sample is the one in Figure 6-3.

Sample	FR [%]	BB [µg/g ww]	∑ Ps [µg/g ww]	∑ DBTs [µg/g ww]	P/DBT	MPs/MDBTs
Whv-II-dM-200b	67	22.2	0.58	0.56	0.96	1.15
Whv-II-dM-100a	114	34.3	0.80	0.83	1.08	1.17
Whv-II-dM-100b	115	51.5	0.50	0.49	1.06	1.19
Whv-II-dM-200a	72	52.4	0.44	0.44	0.91	1.09

Comparison of experiments with the Monterey Formation oil fractions

The distribution patterns of the analysed compound groups in the mussel tissue extracts after exposure to aqueous solutions of the total aromatic fractions of the Monterey Formation oils in experiments 1 and 5 are similar to each other. Some compounds differ in relative intensities (*cf.* Figures 6-1 and 6-3) but the ratios of phenanthrenes and dibenzothiophenes are in a similar range (Tables 6-3 and 6-4).

Looking at the exposures to the degraded Monterey Formation oil, one can see that the Cornish mussels accumulated 0.03-0.2 µg/g summed phenanthrenes and dibenzothiophenes and showed reduced feeding rates. In contrast, the German mussels took up 0.4-0.8 µg/g summed phenanthrenes and dibenzothiophenes but feeding rates were reduced to a lesser extent. One the one hand, this supports the hypothesis that the German mussels were less sensitive than the Cornish ones. On the other hand, it shows that the summed concentrations of 17 alkylated phenanthrenes and 11 alkylated dibenzothiophene do not suffice to explain the joint toxic effect caused by the thousands of compounds in the mixture.

6.2.3 Aqueous solutions of the degraded Vienna basin oil fraction

The actual concentration of the aqueous solution of the total aromatic fraction of the degraded Vienna basin oil at a nominal concentration of 500 µg/L from experiment 3 was 244 µg/L. The unresolved complex mixture elutes between 15 and 65 minutes (not shown). The corresponding constituents in the original oil fraction cover retention times up to 90 minutes, indicating that the high-molecular-weight components did not dissolve in the water, as was also observed for the Monterey Formation oil aqueous solution. Ratios of phenanthrene/dibenzothiophene and methylphenanthrene/methyldibenzothiophene of the aqueous solution at 200 µg/L nominal concentration are 3.1 and 9.9, respectively.

GC-MS analysis revealed that alkylbenzenes (*m/z* 91) were present in the aqueous solution up to a retention time of 40 minutes (approximate retention time of C_{13}-benzene). This implies that more long-chain monoaromatic compounds were dissolved in this solution than in the solutions of the Monterey Formation oil fraction (see above). Solutions with nominal concentrations of 50 µg/L and 200 µg/L, which were especially prepared for the determination of the actual concentration, contained small amounts of hydrocarbons with 21 and 34 µg/L, which elute between 4 and 55 minutes retention time (not shown).

6.2.4 Mussel tissue samples after exposure to the Vienna basin oil fractions

Distribution of aromatic hydrocarbons in blue mussels from experiment 3

Mussels exposed to the aqueous solution of the total aromatic hydrocarbon fraction of the undegraded Vienna basin oil at the nominal concentration of 500 µg/L (sample Whv-I-uV-500, for explanation of sample labels see Table 6-2) accumulated a total tissue concentration of 107 µg/g wet weight. The mussels exposed to nominal concentrations of 200 and 500 µg/L of the degraded Vienna basin oil fraction (sample Whv-I-dV-200 and Whv-I-dV-500) accumulated total tissue concentrations of 31 and 84 µg/g wet weight, respectively. The composition of the three samples will be described together.

As an example for all samples from the experiment, the reconstructed total ion current chromatogram from GC-MS analysis of sample Whv-I-uV-500 is shown in Figure 6-4 c. The accumulated compounds elute in the retention time range between 20 and 60 minutes. Many compounds coelute between 30 and 50 minutes, preventing identification of the seemingly resolved peaks.

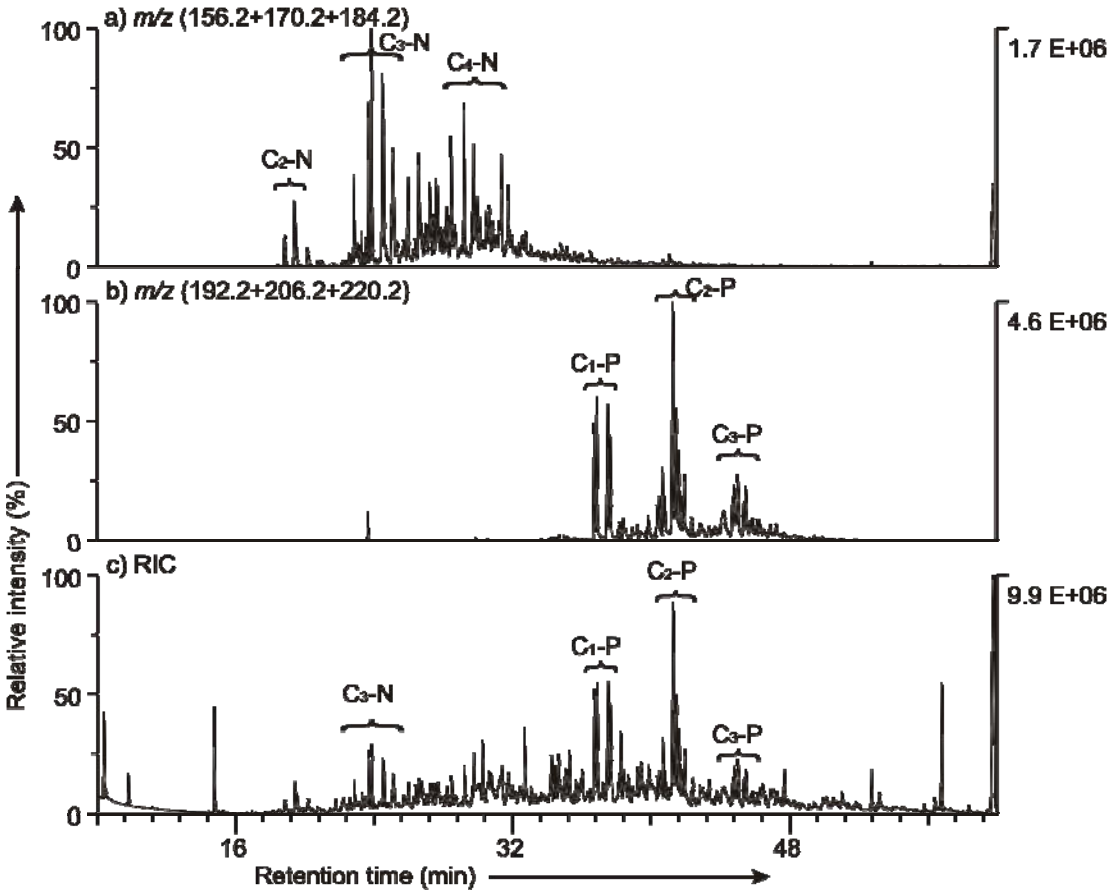


Figure 6-4 Partial chromatograms of (a) molecular ions of C_2- to C_4-naphthalenes, (b) molecular ions of C_1- to C_3-phenanthrenes and (c) total ion current of accumulated compounds in sample Whv-I-uV-500; N = naphthalene, P = phenanthrene.

Alkylated naphthalenes and phenanthrenes are highly abundant in the tissue extract and even visible in the RIC (Figure 6-4). As in the original Vienna basin oil fraction, 1,2,8-trimethylphenanthrene is not present. Aromatic C_{26}-C_{29} steroid hydrocarbons were not detected in the three tissue samples but the triaromatic analogs of pregnanes and methylpregnanes were present. All mass chromatograms of fragments typical for monoaromatic hydrocarbons are very low in intensity in relation to the RIC as was also observed for the Monterey Formation oils. As in the original Vienna basin oil, there are no mono- and diaromatic organic sulphur compounds. Homologues of dibenzothiophene are present in low amounts but dibenzothiophene was not detected so that the P/DBT ratio could not be determined (Table 6-5).

In this set of experiments, there was no significant reduction of feeding rates with increasing total tissue concentrations. The low feeding rate of mussels in sample Whv-I-dV-200 may be linked to the relatively high amount of phenanthrenes present in that sample. However, the quantified phenanthrenes constitute only a small proportion of the whole mixture and thus cannot be solely responsible for the reduction. The dibenzothiophenes are present in much smaller amounts than the phenanthrenes.

Table 6-5 Feeding rates (FR) [%], concentrations of C_0- to C_2-phenanthrenes (∑Ps) and C_0- to C_2-dibenzothiophenes (∑DBTs) [both µg/g wet weight], ratios of phenanthrene/dibenzothiophene (n.p. = not possible due to absence of dibenzothiophene) and of methylphenanthrenes/methyl-dibenzothiophenes in mussels exposed to the total aromatic hydrocarbon fractions of the Vienna basin oil (V), arranged with increasing body burdens (BB) [µg/g ww]. The chromatogram of the underlined sample is shown in Figure 6-4.

Oil	Sample	FR [%]	BB [µg/g ww]	∑ Ps [µg/g ww]	∑ DBTs [µg/g ww]	P/DBT	MPs/MDBTs
Undegraded V	Whv-I-uV-500	85	106.5	0.27	0.07	n.p.	3.04
Degraded V	Whv-I-dV-200	64	30.6	0.52	0.06	n.p.	8.46
Degraded V	Whv-I-dV-500	88	83.5	0.15	0.01	n.p.	15.70

Distribution of aromatic hydrocarbon in blue mussels from experiment 6

In the reconstructed ion current chromatograms of all four tissue extracts from experiment 6, compounds elute between 15 and 60 minutes retention time and a “hump“ of unresolved compounds is visible between 25 and 50 minutes. Sample Whv-II-dV-200b (for explanation of sample labels see Table 6-2) is shown representatively for all four samples (Figure 6-5).

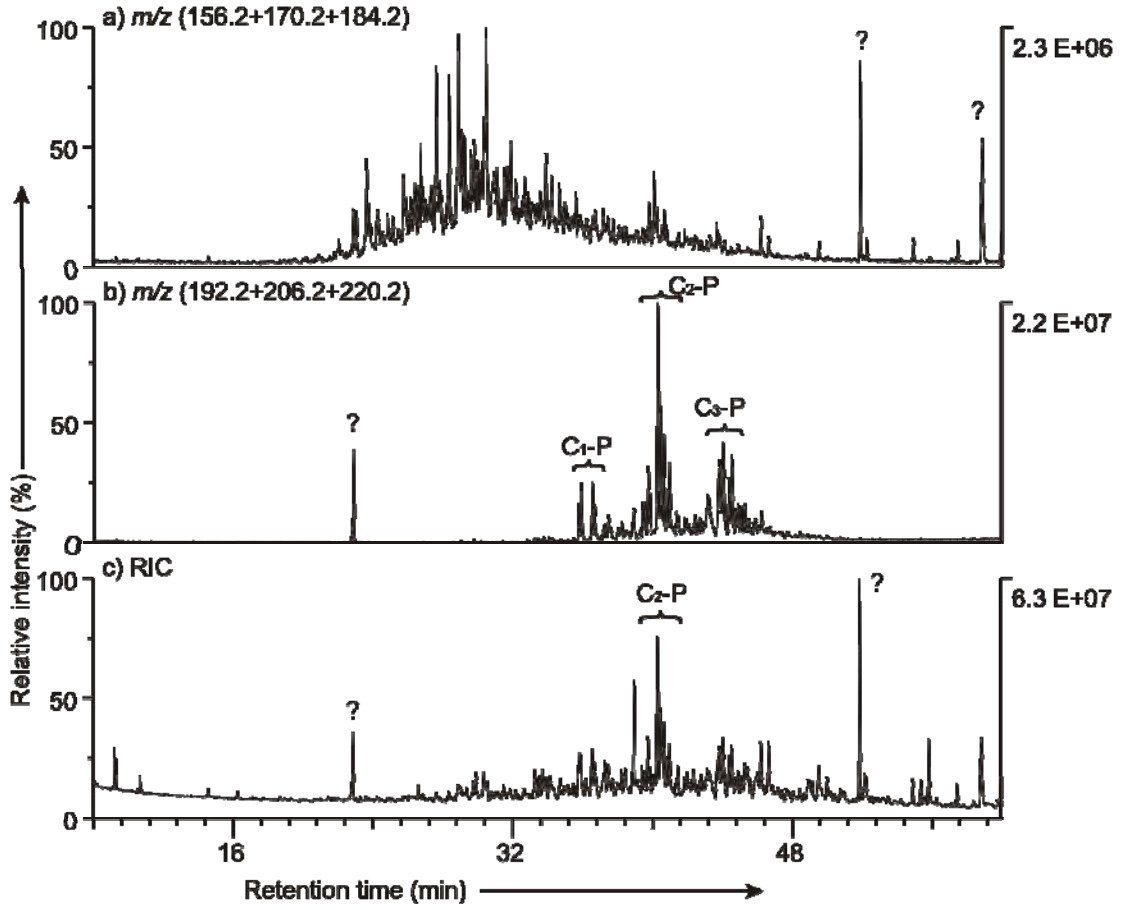


Figure 6-5 Partial chromatograms of (a) molecular ions of C_2- to C_4-naphthalenes, (b) molecular ions of C_1- to C_3-phenanthrenes and (c) total ion current of accumulated compounds in sample Whv-II-dV-200b; P = phenanthrene, ? = unknown compounds.

The added mass chromatogram of C_2- to C_4-naphthalenes (Figure 6-5 a) shows a complex mixture without the typical pattern of resolved naphthalenes, which are perceived in other samples (see e.g. sample Whv-I-uV-500 in Figure 6-4). The relation of the intensity of the added fragments in Figure 6-5 a to the intensity of C_1- to C_3- phenanthrenes (b) and the RIC (c) is lower than in Figure 6-4. In the RIC (Figure 6-5 c), less compounds are visible around a retention time of 20 minutes compared to the tissue extract in Figure 6-4. This indicates that the typically present C_2- to C_4-naphthalenes are less abundant in sample Whv-II-dV-200b, so that other compounds giving the respective fragments dominate the mass chromatogram (Figure 6-5 a). This virtual absence of naphthalenes was similar for all four tissue samples of this experiment. Several unidentified peaks are present in high abundance.

None of the four tissue samples contained phenanthrene or dibenzothiophene. The concentrations of the methyl- and dimethyl-isomers were quantified (Table 6-6). The concentrations of the phenanthrenes are relatively high which is also reflected in the MP/MDBT ratio. For the four tissue samples, concentrations of phenanthrenes increase with increasing total body burdens (Table 6-6).

Table 6-6 Feeding rates (FR) [%], concentrations of C_0- to C_2-phenanthrenes (∑Ps) and C_0- to C_2-dibenzothiophenes (∑DBTs) [both µg/g wet weight], ratios of phenanthrene/dibenzothiophene (n.p. = not possible due to absence of dibenzothiophene) and of methylphenanthrenes/methyl-dibenzothiophenes in mussels exposed to the total aromatic hydrocarbon fraction of the degraded Vienna basin oil (V), arranged with increasing body burdens (BB) [µg/g ww]. The RIC of the underlined sample is shown in Figure 6-5.

Sample number	FR [%]	BB [µg/g ww]	∑ Ps [µg/g ww]	∑ DBT [µg/g ww]	P/DBT	MPs/MDBTs
Whv-II-dV-100a	201	5	0.16	0.03	n.p.	7.07
Whv-II-dV-100b	124	5	0.19	0.03	n.p.	8.52
Whv-II-dV-200b	176	29	0.44	0.06	n.p.	9.61
Whv-II-dV-200a	147	45	0.49	0.07	n.p.	9.54

Comparing the distribution of accumulated compounds in the mussels exposed to the Vienna basin oil fractions in experiments 3 and 6, the patterns are similar except for the absence of alkylnaphthalenes in samples from experiment 6. All samples contain relatively high amounts of phenanthrenes but very low amounts of dibenzothiophenes as can be expected for sulphur-lean oils. All four groups of mussels have higher mean feeding rates than the control mussels. The distribution of investigated aromatic compounds does not help to elucidate reasons for the lack of feeding rate reduction with increasing tissue concentrations of aromatic compounds. Apparently, the high feeding rates are not directly linked to the high body burdens in the Jade Bay mussels.

6.3 The composition of mussel tissue extracts as a means to explain differences in feeding rate reduction during the toxicity tests

In general, all extracts contain mainly derivatives of naphthalene and phenanthrene and very low concentrations of monoaromatic compounds. Samples from experiments with the Monterey Formation oils also contain derivatives of dibenzothiophene in similar amounts to phenanthrenes. As model compounds, C_0- to C_2-phenanthrenes and C_0- to C_2-dibenzothiophenes were quantified in the tissue extracts. Their concentrations are not necessarily proportional to the total tissue concentrations. In Vienna basin oil exposed mussels, the quantified amounts of phenanthrenes are approximately ten times higher than the amounts of dibenzothiophenes. Compared to the experiments with the sulphur-rich Monterey Formation oil fractions, mussels accumulated much smaller amounts of dibenzothiophenes. Figure 6-6 shows summed mass chromatograms of C_1- to C_3-phenanthrenes (*m/z* 192+206+220) and C_1- to C_3-dibenzothiophenes (*m/z* 198+212+226) from the tissue of mussels exposed to fractions of the undegraded Monterey Formation (sample Ply-uM-500, top) and Vienna basin oil (sample Whv-I-uV-500, bottom). The relative amounts of sulphur compounds (lower trace) to pure hydrocarbons (upper trace) in the mussel tissue is much higher after exposure to the sulphur-rich Monterey Formation oil.

As mentioned previously, mussel feeding rates were most strongly reduced in experiment 1. Two possible explanations were proposed in Chapter 4, either differences in the composition of the body burdens or different sensitivities of the blue mussels from the two North Sea sites.

After analysis of body burdens, the organic sulphur compounds are the main difference and therefore may be responsible for the different biological response. The low TEC_{50} value of dibenzothiophene (14 µg/g wet weight) (Donkin et al., 1989) shows that this compound is toxic to blue mussels. The quantified dibenzothiophenes make up about 1% for the sulphur-rich oil experiments and 0.05% for the sulphur-lean oil experiments. Even if these values are only semi-quantitative, there is a difference of two orders of magnitude between them and the TEC_{50} value from the literature. This indicates that only a minor part in the overall toxicity can be attributed to this compound group but also that the high abundance of sulphur aromatics in sulphur-rich oils increases their share in the overall toxicity of the oil fraction and thus may well enhance the overall oil toxicity. Seeing that even in the sulphur-rich oil fraction sulphur-containing compounds do not account for a major part of the toxic effect, the lack of toxic effect in the experiments with the Vienna basin oils cannot solely be attributed to the low concentration of sulphur aromatics. From this follows that dibenzothiophene and its methyl- and dimethyl-derivatives cannot alone be made responsible for the differences in feeding rate reduction observed between experiments 1 and 3.

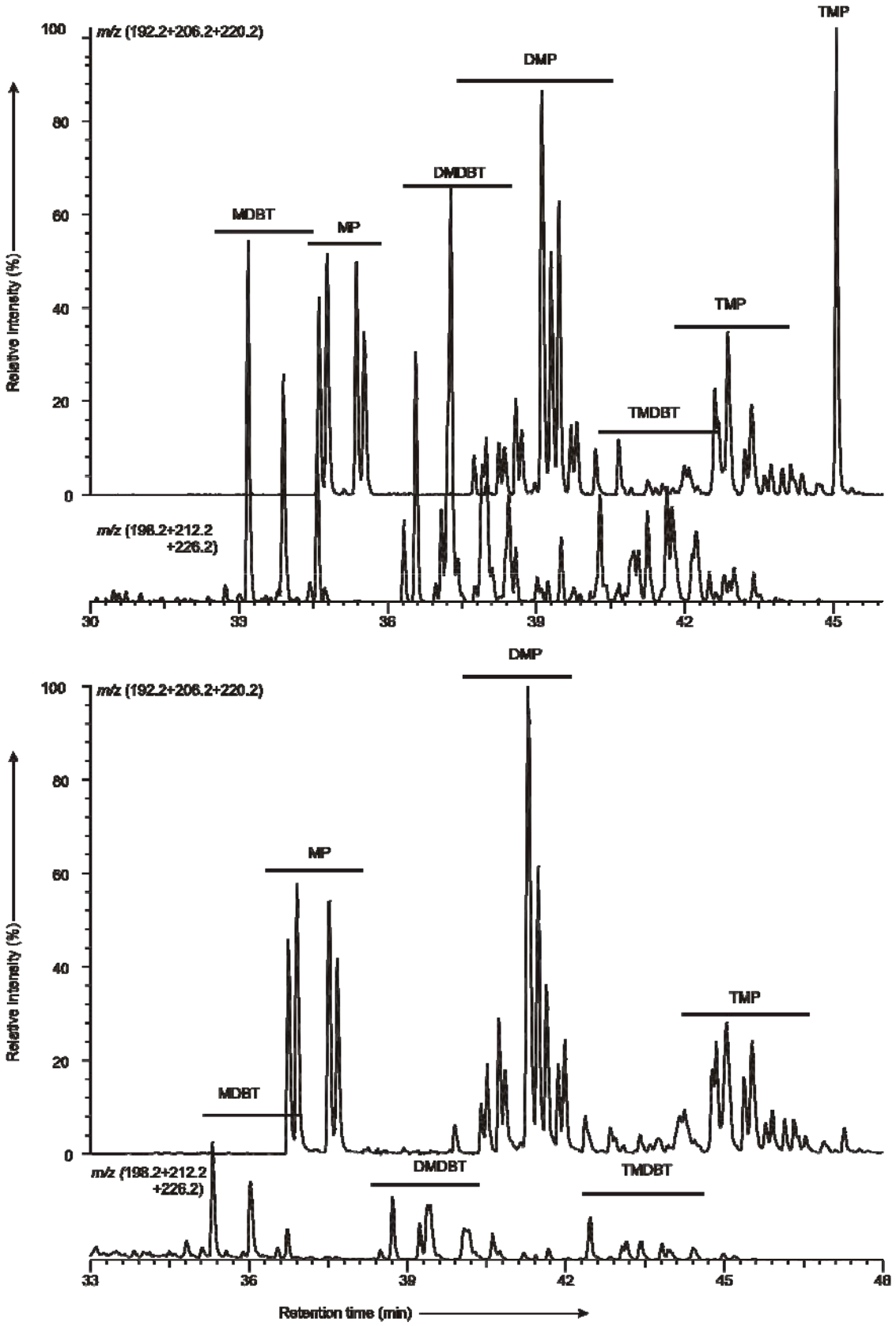


Figure 6-6 Partial chromatograms of summed mass chromatograms of C_1- to C_3-phenanthrenes (*m/z* [192.2+206.2+220.2]) and C_1- to C_3-dibenzothiophenes (*m/z* [198.2+212.2+226.2]) in mussels exposed to the total aromatic hydrocarbon fractions of the undegraded Monterey Formation oil (sample Ply-uM-500, top) and of the undegraded Vienna basin oil (Whv-I-uV-500, bottom).

Since in general the amounts of body burden and the concentrations of phenanthrenes and dibenzothiophenes are in a similar range for all mussels exposed to the Monterey Formation oil (experiments 1 and 5), the high feeding rates of the German mussels (experiments 3, 5 and 6) very probably have biological reasons and are not directly connected to the toxicity of the oil fractions. Thus, the different biological responses of the mussels used in this study are more likely due to a difference in sensitivity of the mussels rather than a difference in toxicity of the oil fractions used, as was suggested in Chapter 4.

6.4 Discussion – accumulated aromatic compounds and toxic effects

6.4.1 Depletion of monoaromatic compounds

Field studies have shown that blue mussels from hydrocarbon-contaminated environments usually contain mostly monoaromatic compounds (Wraige, 1997; Rowland et al., 2001; Donkin et al., 2003). In these studies, mussel tissue of *Mytilus edulis* sampled at the UK coast was analysed and fractionation of the aromatic UCMs in the tissue revealed that the hydrocarbons present were mainly monoaromatic. Blue mussels in the present study, though, did not accumulate monoaromatic hydrocarbons to a high extent, neither from the solutions of the monoaromatic hydrocarbon fractions nor from the total aromatic hydrocarbon fractions. Analysis of selected aqueous solutions of hydrocarbons indicated that they did not contain many monoaromatic compounds, either.

The poor water solubility of high-molecular-weight monoaromatic hydrocarbons has often been reported. For example, analysis of water-accommodated fractions of crude oils showed that already the C_4- and C_5- benzenes were absent in aqueous solutions (Hokstad et al., 2000; Neff et al., 2000). Aqueous solutions of weathered and biodegraded oils were observed to contain mainly naphthalenes, phenanthrenes and dibenzothiophenes (Carls et al., 1999). In the aqueous solutions of aromatic hydrocarbons analysed in the present study, monoaromatics larger than C_7-benzenes were not present whereas naphthalenes and phenanthrenes predominated. Consequently, if high-molecular-weight monoaromatic compounds were not present in the solutions used for exposure, the mussels could not take them up.

One reason for the low accumulation of monoaromatic compounds in mussel tissue in the present study is their low aqueous solubility in connection with the experimental setup used. For example, filtered seawater was used for the preparation of solutions of hydrocarbons and the exposure of blue mussels. Sediment was not added to the exposure vessels. Poorly water soluble aromatic hydrocarbons, however, are known to adsorb on particles (Ahrens and Depree, 2004). Frysinger et al. (2003) detected octylbenzene and longer-chain homologues in oil-contaminated sediments. In a study with contaminated sediments, C_6- to C_{20}-alkylbenzenes were taken up by exposed flounders (*Pseudo-*

pleuronectes americanus), indicating that these poorly soluble monoaromatic hydrocarbons are bound to particulate material (Hellou and Upshall, 1995). The lack of particles during the preparation of the aqueous solutions in the present study probably reduced the amount of poorly soluble aromatic compounds available to the mussels in the exposure vessels, leading to a limited uptake. The particulate algal food was added to the exposure vessels after transfer of the aqueous solutions, which by then were already depleted in monoaromatics.

Another aspect of the experiments, which differed from natural conditions, is the lack of changing water level with tides, which may bring the mussels into contact with an existing oil film in the marine environment. This can result in the uptake of poorly water soluble hydrocarbons such as high-molecular-weight monoaromatic hydrocarbons, which can also be toxic. In addition, exposure time is of relevance here since the poorly soluble compounds may dissolve slowly but steadily, similar to poorly water soluble PAHs as reported by Carls et al. (2001). Dissolved and adsorbed aromatic compounds are probably taken up via different routes (Mackay et al., 1992).

6.4.2 Joint toxic effects of oil fractions and their attribution to certain compounds and compound groups

Several field studies, in which blue mussels with reduced scope for growth (SFG) were investigated, report tissue concentrations of various compound classes but the main reason for the poor state of health of the collected mussels were the high tissue concentrations of aromatic compounds (Widdows et al., 1995; 2002). However, the compounds present in the mussels tissue usually could not fully explain the extent of reduction of SFG. Thus, the authors concluded that there must have been unknown and unidentified components in the tissue, which reduced the scope for growth in addition to the investigated compounds and compound groups (Widdows et al., 1995; 2002). It is very likely that this is also the case in the experiments in this study. For example, there are several cases in which mussels accumulated similar total body burdens and similar concentrations of phenanthrenes and dibenzothiophenes but exhibited different feeding rates (e.g. Table 6-4). Due to coelutions, many compounds which may be toxic remained unindentified. It is likely that the Monterey Formation and Vienna basin oils contain different unidentified compounds, which may also affect the mussels differently.

In toxicity tests with water-accommodated fractions of fresh and weathered crude oils, the higher toxicity of the fresh oils was attributed to the high aqueous concentration of acutely toxic compounds such as the volatile BTEX (Fuller et al., 2004). In another study with water-accomodated fractions of fresh crude oils, the high aqueous concentrations of target analytes such as C_0- to C_4-benzenes and C_0- to C_3-naphthalenes were sufficient to explain approximately 60% of the toxic effects observed. The remaining toxic effect was attributed to unidentified polar compounds and in particular the toxic potential of the unresolved complex

mixture as a cause for unexplained toxicity was pointed out (Neff et al., 2000). In the C_{11+} fractions of aromatic compounds used in this study, volatile and polar compounds were largely absent and the whole UCM acted as the toxicant, implying that no compound-specific attribution would be possible.

In a study with oil-contaminated sediments, a cause-effect relationship between toxic effects and total PAHs (parent compounds and up to C_4-alkylated homologues) was established but attribution of the effects to individual compounds or compound groups was not sucessful (Colavecchia et al., 2004). Heintz et al. (2000) compared the concentrations of 40 individual parent and alkylated PAHs in several aqueous solutions of a crude oil. Their concentrations were not all enhanced in the solution with a higher total content, e.g. the abundance of phenanthrenes decreased whereas the abundance of naphthalenes was enhanced at higher total concentrations of aromatic hydrocarbons. Correlation of observed mortality to a compound or a compound group was not possible (Heintz et al., 2000). When working with solutions of whole oils, one has to bear in mind that the presence of additional compound groups such as aliphatic and polar compounds influences the composition of the aqueous solution formed and that there are other compounds present than aromatic ones that are toxic (Barron et al., 1999). In this study, C_{11+} fractions of aromatic hydrocarbons were used which still contained a huge number of possible toxicants.

These examples demonstrate that cause-effect relationships are rarely obtained in toxicity tests applying mixtures as complex as oil fractions. If there is a limited amount of dominating compounds such as the low-molecular-weight aromatics the toxic effect can often be attributed to their concentration because they make up a large proportion of the mixture. In a mixture of hydrocarbons, all compounds present add to the overall toxic effect (Hermens et al., 1984). This means that studies investigating mainly parent PAHs and no alkylated derivatives, omit an important group of potential toxicants. In the present study, compound groups which represent many individual compounds were analysed but an attribution of the toxic effects to certain compound groups was not achieved. Thus, it is plausible that cause-effect relationships can be obtained whenever a small number of compounds makes up a sufficient proportion of the whole fraction. However, if each compound group is looked at individually, and the proportion of each group makes up only a small proportion of the whole fraction, as for example the summed phenanthrene in this study, this cannot explain the whole toxic effect. Even less so can the whole toxic effect observed be attributed to an individual compound, even if it is present in relatively high amounts as for example 1,2,8-trimethylphenanthrene in the Monterey Formation oil fractions.

Further fractionation of an aromatic UCM into subfractions of mono- and polyaromatic UCMs may provide a means to attribute the cause of a toxic effect to a less complex mixture. This is also an important aspect in bioassay-directed fractionation and toxicity identification evaluation (TIE) schemes (Schuetzle and Lewtas, 1986) and was the reason for investigating mono- and total aromatic hydrocarbon fractions separately in this study. Unfortunately, compounds from the monoaromatic hydrocarbon fractions were not accumulated by the mussels in sufficient amounts for GC-MS analysis. Donkin et al. (2003) found a UCM isolated from field mussels to be toxic and after HPLC fractionation, the mono- and polyaromatic UCM subfractions were both toxic. This indicates that analysis of subfractions of aromatic UCMs rather than compound classes may be a rewarding approach to correlate toxic effects to less complex mixtures and thereby define subfractions being more or less toxic. For this, larger amounts of sample are needed. For instance, Donkin et al. (2003) used 16 g mussel tissue for extraction and subsequent fractionation as opposed to the 0.5 g available from the experiments in this study (*cf.* Chapter 3). However, Frysinger et al. (2003) reported that in mono-, di-, tri- and polyaromatic hydrocarbon subfractions gained by normal phase HPLC not even two-dimensional GC managed to resolve mixtures of alkylindanes and alkyltetralins because the fractions were still too complex to achieve resolution of previously unresolved compounds.

Another difficulty with subfractionation is that fractionation of complex mixtures containing xenobiotics from diverse chemical compound classes often yields several quite nontoxic subfractions, which counteracts the establishment of new cause-effect relationships (e.g. Reineke, 2003). As Colavecchia et al. (2004) concluded, “the myriad of chemical constituents makes it difficult to correlate toxic effects with specific compounds.“

6.5 Summary – composition of aqueous solutions and tissue extracts

Monoaromatic hydrocarbons are present in low concentrations and in a limited molecular-weight range in the solutions of the total aromatic hydrocarbon fractions and consequently also in the tissue of exposed mussels. This reflects the low aqueous solubility of alkylated monoaromatic compounds. Some monoaromatic compound classes such as alkylated benzenes were depleted compared to the original oil fraction whereas others were not detected at all in tissue samples such as alkylated indanes and aromatic steroid hydrocarbons.

Distribution of derivatives of naphthalene and phenanthrene in aqueous solution and mussel tissue samples was similar to the distribution in the original oil fractions. Origin-specific distribution of isomers such as the occurrence or absence of 1,2,8-trimethylphenanthrene was reflected in the mussel tissue. The aliphatic and monoaromatic organic sulphur compounds present in the original oils were not detected in the aqueous solutions or the mussel tissue samples. Mussels exposed to the fractions from the Monterey

Formation oils accumulated much more organic sulphur compounds than mussels exposed to the ones from the Vienna basin oils. This reflects the content of sulphur compounds of the original oils.

Besides similar total tissue concentrations, the mussels from Jade Bay also took up similar amounts of phenanthrene as the ones from Cornwall. However, they were not affected in the same way. Thus, results of the analysis of the distribution of aromatic hydrocarbons support the suggestion that the mussels from Jade Bay are not as sensitive to the toxicity of aromatic compounds as the Cornish mussels.

The toxic effect of the Monterey Formation oil fractions cannot solely be attributed to the quantified amounts of phenanthrenes or dibenzothiophenes. They make up a small proportion of all accumulated compounds and of the toxic effect observed. The mass chromatograms demonstrate the complexity of the aromatic mixtures used and accumulated by the mussels. It is also possible that some unidentified compounds in the Monterey Formation oils, which added to the overall mixture toxicity were not present in the Vienna basin oils.

7 Discussion and synthesis of results – differences in composition and toxicity of aromatic hydrocarbon mixtures from crude oils

Blue mussels (*Mytilus edulis*) were exposed for 24 hours to aqueous solutions of isolated total aromatic and monoaromatic hydrocarbon fractions from two Monterey Formation and two Vienna basin crude oils at several concentrations.

At first in this chapter, the presence of aromatic compound groups in the different media is compared (7.1). In section 7.2 and 7.3, the results from this study are interlinked. The last section (7.4) summarises the conclusions obtained in this study.

7.1 Overview over the distribution of aromatic compound groups in the four oil fractions, their aqueous solutions and tissue extracts of exposed mussels

The total aromatic hydrocarbon fractions of the four oils were dissolved in water and the solutions subsequently used for the toxicity experiments. After exposure of blue mussels to the aromatic hydrocarbon mixtures, the original oil fractions, several aqueous solutions and mussel tissue samples were analysed by GC-MS for changes in the distribution patterns of various compound classes. Figure 7-1 juxtaposes the appearance of the “hump” in the original oil fraction of the degraded Monterey Formation oil aromatic hydrocarbons, an aqueous solution thereof at 200 µg/L nominal concentration and the apolar tissue extract of mussels exposed to the aqueous solution at a nominal concentration of 500 µg/L. The total aromatic hydrocarbon fraction of the oil contains compounds which elute at 6 to 90 minutes (for chromatograms of the other oil fractions see Chapter 5). In contrast, the retention times of the compounds which were dissolved in water and were present in the tissue extract elute only up to 60 minutes (Figure 7-1). This indicates that the high-molecular-weight compounds, which elute late on the GC, did not dissolve in the water as can be expected from their relatively low aqueous solubility.

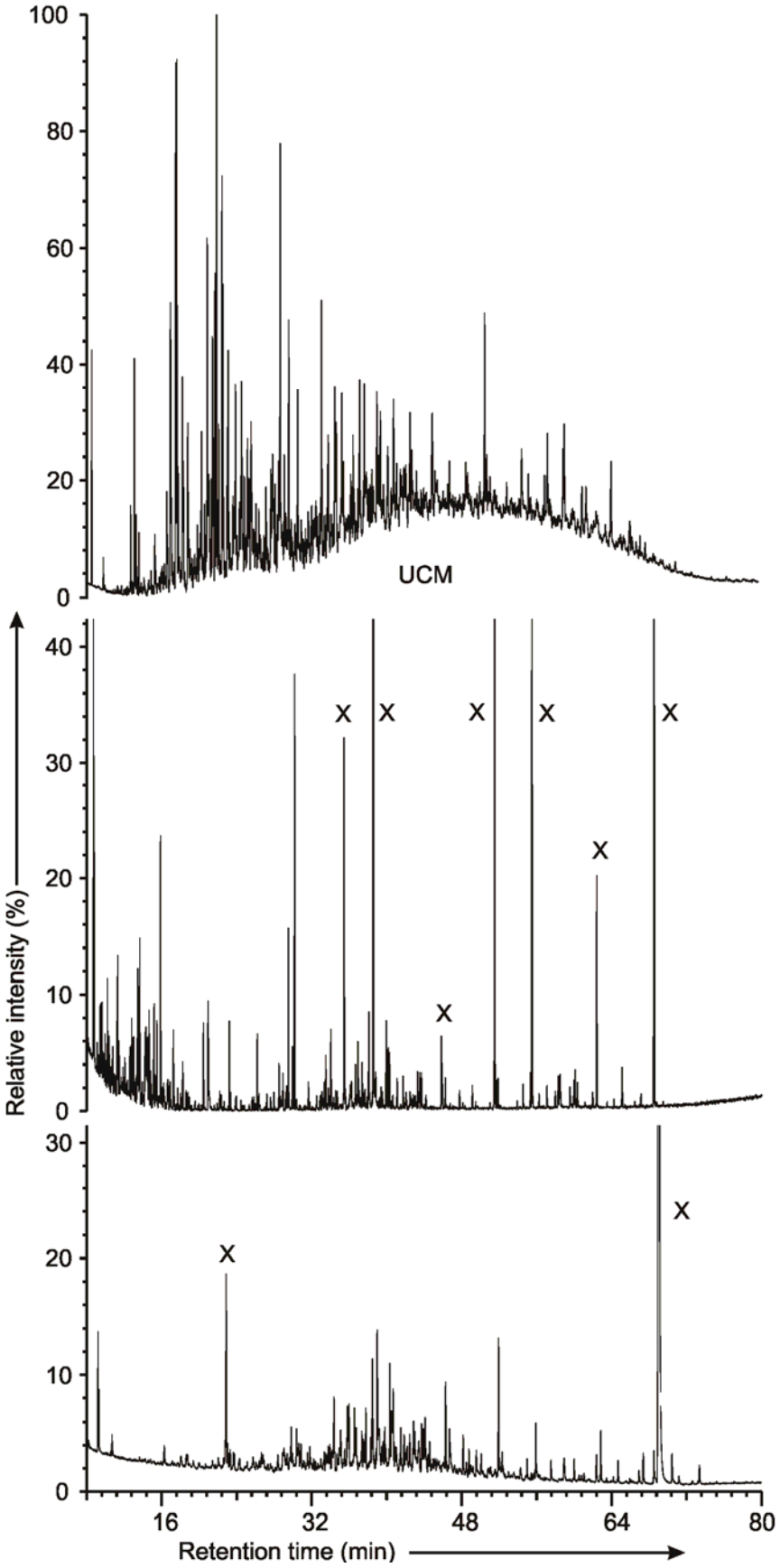


Figure 7-1 Partial reconstructed ion current chromatograms of the degraded Monterey formation oil aromatic hydrocarbon fraction (top), an aqueous solution thereof (middle) and of the unpolar fraction of a tissue extract of mussels exposed to this aqueous solution (bottom). X denotes contaminants and artefacts as identified in Figure 3-6.

For several compound classes, the composition was considerably different in the mussel tissue compared to the original oil (Table 7-1). The main reason for this constitutional alteration is the low aqueous solubility of many aromatic compounds, in particular the higher-molecular-weight monoaromatics. Comparing the original oil fraction to the aqueous solution, it becomes obvious that the diversity of compounds is very much reduced. In this dissolution step, most monoaromatic compounds are lost.

Table 7-1 Compound classes in the total aromatic hydrocarbon fractions of the original oils, their aqueous solutions and in the tissue of exposed mussels; xx: highly abundant, x: present, o: depleted, -: absent; M: Monterey Formation oils, V: Vienna basin oils.

Compound class	Original oil fraction	Aqueous solution	Mussel tissue
Monocylic monoaromatics	xx	o	-
Dicyclic monoaromatics	xx	o	-
Naphthalenes	xx	xx	xx
Phenanthrenes	xx	xx	xx
Aromatic pregnanes	xx	x	x
Aromatic steroids	M: xx V: x	M: o V: -	M: - V: -
Organic sulphur compounds (except dibenzothiophenes)	M: xx V: -	M: xx V: -	M: x V: -
Dibenzothiophenes	M: xx V: x	M: xx V: x	M: xx V: x

The original aromatic hydrocarbon fraction of the Monterey Formation oils contain high amounts of aromatic steroids. In the aqueous solutions, they were detected only in low amounts. In contrast, the Vienna basin oils contain low amounts of aromatic steroids to start with and in the aqueous solutions they were not detected at all. This shows that the mole fraction of a component in the original oil fraction determines the mole fraction of the compound in the aqueous solution (Raoult´s Law, see Section 2.5). This, of course, interacts with the solubility of the compound.

The second step from the water into the mussel tissue is less discriminating. This agrees with results from Baussant et al. (2001a) who found that the pattern of polyaromatic compounds accumulated by mussels, which were exposed to aqueous solutions of a slightly weathered crude oil, resembled the pattern of polyaromatic compounds in the aqueous solution. This indicates that dissolved compounds in general are bioavailable, e.g. via passive diffusion into the tissue, as has often been reported (Fent, 1998).

In general, the oil fractions used contain thousands of aromatic compounds. The diversity of compounds was reduced in the aqueous solutions, the monoaromatic compounds being particularly depleted as can be inferred from Table 7-1. GC-MS analysis of mussel tissue extracts showed mainly alkylated naphthalenes, phenanthrenes and dibenzothiophenes as identifiable compounds after exposure to the total aromatic hydrocarbon fractions. The other compounds remain unresolved and unidentified.

7.2 Are differences in composition of hydrocarbons due to origin or biodegradation of the oils a deciding factor for their toxicity?

Differences in composition and toxicity due to origin of the oils

According to Chapter 5, there are many compositional differences between the Monterey Formation and the Vienna basin oils. Most of the investigated compound groups did only dissolve in water in small amounts. Consequently, the composition of the tissue samples does not completely mirror the diverse distribution of aromatic compounds in the oil pairs. Some compositional differences were transferred into the mussel tissue, for example the predominance of the isomer 1,2,8-trimethylphenanthrene and the higher content and diversity of sulphur compounds in the Monterey Formation oils (the sulphur compounds are discussed in Section 7.3). The summed concentration of C_0- to C_2-phenanthrenes constitutes only a small proportion of all compounds acumulated in the mussel tissue, indicating that the presence of the additional trimethylphenanthrene in the Monterey Formation oils does not influence the overall toxicity of the aromatic hydrocarbon fraction considerably.

Differences in composition and toxicity due to biodegradation level of the oils

The reduction of mussel feeding rates caused by the Monterey Formation oil fractions indicate a possible higher toxicity of the undegraded oil (*cf.* Chapter 4). Investigation of the original oil fractions revealed that the majority of differences due to changing level of biodegradation involved monoaromatic compounds, to be more specific the absence of long-chain monoaromatic compounds in the biodegraded oil (*cf.* Chapter 5). These compounds did not dissolve well in the water so that the mussels did not accumulate them (*cf.* Chapter 6). Thus, the compositional differences of the undegraded and the biodegraded oils were not transferred to the aqueous solutions or the mussel tissue. Minor noticable differences due to a changing level of biodegradation e.g. regarding the abundance of individual isomers of trimethylnaphthalenes probably did not influence the toxic potential, considering their small proportion of the whole mixture of aromatics. Thus, the level of biodegradation of the C_{11+} fractions did not significantly influence the toxicity of the total aromatic oil fractions towards the marine mussels.

There are no unequivocal data in the literature as to whether undegraded crude oils are more toxic than degraded crude oils or the other way round. One reason for this is the large amount of toxicological tests available, which come to diverse conclusions depending on the duration of exposure and on the biological endpoints studied. It is widely accepted now that fresh oils can be acutely toxic to marine organisms but chronic effects on the sublethal level are also known to occur regularly after long-term exposure to low concentrations in the marine environment (e.g. Heintz et al., 2000).

Fresh oils are usually regarded as more acutely toxic than weathered oils due to their higher amount of e.g. the highly toxic low-molecular-weight aromatics (BTEX). In weathered and biodegraded oils, low-molecular-weight compounds have been removed. Thus, the composition of weathered oils resembles the composition of biodegraded oils to a certain degree. This also applies to their aqueous solutions, so that a few comparative studies of unweathered and weathered crude oil toxicities are included here.

The lower toxic potential of weathered oils is usually ascribed to the lower content of low-molecular-weight compounds and monoaromatic compounds (Bobra et al., 1983). This leads to a reduced aqueous solubility of the weathered oil. However, the question of higher toxicity can be answered from several antagonistic perspectives, e.g. acute versus chronic toxicity or narcotic (unspecific) versus specific toxicity. Yet another perspective states that on the one hand, a fresh oil is water soluble to a higher extent so that a saturated solution can form. This aqueous solution is at a higher concentration and hence more toxic than a saturated solution of a weathered oil, which produces solutions of a lower concentration (Bobra et al., 1983). On the other hand, when comparing aqueous solutions of the same concentration, more weathered oils have been observed to be relatively more toxic than less weathered oils indicating that it contains more potent toxicants (e.g. polyaromatic and UCM compounds), which require lower amounts to exert a certain toxicity (Hokstad et al., 2000).

In a study with several nonweathered and in-laboratory weathered oils, no consistent trend was found whether nonweathered or weathered oils were more acutely toxic. The toxicity for the oils used varied and the toxicity of the various oil solutions was species-dependent (Neff et al., 2000). This again shows that there is no general trend whether nondegraded/nonweathered or biodegraded/weathered oils are more toxic but rather that the composition of the oils and their respective aqueous solutions is crucial.

In the present study, solutions of aromatic hydrocarbon fractions of the crude oils were applied to the test organisms in contrast to solutions of the whole oil in most studies so that the toxicity of aromatic hydrocarbon fractions was investigated. Analysis of the composition of aromatic compounds in the original oil fractions, the aqueous solutions and the mussels exposed to the undegraded and the biodegraded Monterey Formation oil showed that most of the compositional differences of the original oil fractions are lost with uptake into the mussel tissue. Largely owing to solubility effects, the tissue samples contained similar compounds. This explains the similar toxicity of the two oil fractions. Thus, the different levels of biodegradation (here levels 1 and 5 after Peters and Moldowan, 1993) did not result in ecotoxicologically relevant differences in the composition of the aromatic hydrocarbon fractions as far as can be judged with this short-term toxicity test.

7.3 Dibenzothiophenes – indications for their toxic potential

In the course of this study, sulphur-containing aromatic compounds were found to be a key compositional difference between the sulphur-rich crude oils from the Monterey Formation and sulphur-lean ones from the Vienna basin. Organic sulphur compounds are typical constituents of crude oils with sulphur being one of the main three heteroatoms (NSO) to be found in oils. They enter the environment along with the hydrocarbons during an oilspill. Sulphur-containing aromatics are water soluble to a higher degree due to their higher polarity and more reactive than aromatic hydrocarbons (Beyer and Walter, 1988). This led to the assumption that following the spillage of sulphur-rich oils, sulphur-containing aromatic compounds can damage the respective ecosystem. There are not many studies regarding the ecotoxicity of sulphur-containing organic compounds.

Partitioning of phenanthrene and dibenzothiophene from oil fraction into water and organism

The higher aqueous solubility of organic sulphur compounds may lead to an enhanced uptake from contaminated water by marine organisms. This was investigated by comparing the ratios of phenanthrene to dibenzothiophene in the original oil samples, in selected toxicant solutions and mussel tissue samples (Table 7-2). Several samples of mussels, which were exposed to the sulphur-lean oil fraction, did not contain detectable amounts of dibenzothiophene, so the ratios of methylated derivatives are also included.

The ratios of phenanthrene to dibenzothiophene and methylphenanthrenes to methyldibenzothiophenes in the Vienna basin oils are much higher than the ones derived from the Monterey Formation oils, which mirrors the lower concentration of aromatic sulphur compounds in the former oil. One of the three solutions of the Monterey Formation oil and the solution of the sulphur-lean Vienna basin oil show lower ratios for the parent compounds but higher ratios for the methylated compounds compared to the original oil fractions. Overall, the majority of the ratios in the solutions of aromatic hydrocarbons is higher than for the original oils. Thus, they show that sulphur compounds are not enriched in aqueous solutions compared to the original oil fractions.

Table 7-2 Mean ratios of phenanthrene/dibenzothiophene and of methylphenanthrene/methyldibenzothiophene in samples of the original oil fractions, aqueous solutions and in tissue of exposed mussels (in parenthesis number of samples for mean value), M = Monterey Formation oil, V =Vienna basin oil.

Crude oil fraction	Phenanthrene/dibenzothiophene			Methylphenanthrene/methyldibenzothiophene		
	Original oil	**Aqueous solution**	**Tissue**	**Original oil**	**Aqueous solution**	**Tissue**
Undegraded M	0.64 (1)	--	1.26 (2)	0.71 (1)	--	1.27 (2)
Degraded M	0.72 (1)	0.35 (1), 0.77 (2)	1.16 (5)	0.81 (1)	1.06 (3)	1.02 (7)
Undegraded V	2.03 (1)	--	--	2.99 (1)	--	3.04 (1)
Degraded V	4.69 (1)	3.08 (1)	--	7.31 (1)	9.81 (1)	9.82 (6)

The phenanthrene/dibenzothiophene and methylphenanthrene/methyldibenzothiophene ratios in mussel tissue extracts infer that the sulphur-containing triaromatic compounds are not more abundant in mussel tissue samples than in the original oil. Rather, the higher ratios in the tissue shows that the concentration of phenanthrenes is enhanced compared to the oil. This implies that the sulphur-containing compounds are not favoured in uptake compared to phenanthrene in spite of their higher aqueous solubility and that their potential to penetrate biological membranes is similar if not lower compared to that of aromatic hydrocarbons.

Another possible explanation for the reduced relative concentration of dibenzothiophenes compared to phenanthrenes in the mussel tissue is an improved elimination of sulphur-containing compounds by the mussels. However, it is known that blue mussels have poor metabolism rates of PAHs due to virtual absence of the necessary enzymes (Stegeman, 1985). Metabolic rates of blue mussels for sulphur-containing compounds have not been studied.

Are aromatic sulphur compounds more potent toxicants than pure hydrocarbons?

There are not many studies regarding the ecotoxicity of organic sulphur compounds. In some studies, dibenzothiophene and its alkylated homologues are mentioned when PAHs were analysed but the sulphur-containing aromatic compounds were not investigated in detail (e.g. Carls et al., 1999). Donkin et al. (1989) tested the narcotic toxicity of dibenzothiophene to blue mussels along with other aromatic compounds and the TEC_{50} value of 14 µg/g wet weight was the lowest of all compounds tested (*cf.* Table 2-2). Thus, dibenzothiophene is an extremely potent toxicant. Like other PAHs, it may also exert toxicity via a special mechanism. There is one study indicating that dibenzothiophene and its homologues cause damage via a specific toxic mechanism to the fish medaka (*Oryzias latipes*) during early live stages (Rhodes, 2002 in; Colavecchia et al., 2004).

Analysis of tissue samples in this study showed that mussels exposed to the sulphur-rich oil fractions took up sulphur compounds in elevated amounts and higher diversity compared to the mussels exposed to the sulphur-lean oils. The results of the toxicity tests with oils of different sulphur contents performed in the present study were unfortunately masked by the different sensitivities of the Cornish and German mussels used (experiments 1 and 2 and experiments 3 and 4, respectively). In experiments 5 and 6, German mussels were exposed to fractions from the sulphur-rich and the sulphur-lean oil in order to directly compare their biological responses. Some mussels exposed to the sulphur-rich Monterey Formation oil fraction exhibited reduced feeding rates, albeit without statistically significant differences from the control mussels and without a consistent trend in the four groups of mussels. In contrast, all mussels exposed to the sulphur-lean Vienna basin oil fraction show enhanced feeding rates compared to the control mussels. Thus, some German mussels exposed to the

Monterey Formation oil fraction may have been narcotisised in contrast to those exposed to the Vienna basin oil fraction. These responses were combined with the different compositions of the aromatic hydrocarbon fractions of the oils. Data indicate that the oil fraction containing more sulphur compounds did reduce some feeding rates of insensitive mussels compared to the oil fraction with less sulphur compounds. If the German mussels were insensitive due to previous contamination with hydrocarbons as suggested in Chapter 4, one could speculate that organic sulphur compounds possess an additional toxic potential other than unspecific narcosis.

Similar to the toxicity test applied in the present study, *Mytilus edulis* from Whitby Bay in Cornwall were exposed to a monoaromatic UCM isolated from a North Sea Gullfaks oil (Smith, 2002). The study reports a (nominal) WEC_{50} value for the monoaromatic mixture of 518 µg/L and a TEC_{50} value of 500 µg/g wet weight mussel tissue. This is much higher than the TEC_{50} value of 116 µg/g obtained for the monoaromatic fraction isolated from the undegraded Monterey Formation oil in the present study. Thus, if the Cornish mussels used in the two studies were equally sensitive, the Monterey Formation oil fraction causes a stronger acutely toxic effect to blue mussels than the Gullfaks oil fraction. A possible explanation for this assumed higher acute toxicity of the Monterey Formation oil fraction could be its elevated amount of sulphur compounds. The typical sulphur content of Gullfaks oils is <1%. Its monoaromatic hydrocarbon fraction isolated by Smith (2002) was re-investigated by GC-MS analysis in this study for organic sulphur compounds but none were found. The lower toxicity of the Gullfaks monoaromatic UCM compared to the Monterey Formation monoaromatic UCM may therefore be explained by the absence of sulphur-containing compounds. Analogously, the same may be true for the sulphur-lean Vienna basin oil fractions, which may exert a similarly strong toxicity as the Gullfaks UCM when applied to mussels of equal sensitivity.

Overall, sulphur compounds add to the toxicity of an aromatic oil fraction and probably enhance the toxicity of sulphur-rich oils compared to sulphur-lean oils but obviously more research is necessary.

7.4 Conclusions

The presented work was a research project combining ecotoxicity testing with chemical analysis. It was linked to a previous study which showed narcotic toxicity of a monoaromatic hydrocarbon fraction dominated by an unresolved complex mixture (UCM) isolated from a biodegraded Gullfaks (Norwegian North Sea) crude oil towards blue mussels (*Mytilus edulis*) (Smith, 2002). In the present work, the narcotic toxicity of monoaromatic and total aromatic hydrocarbon C_{11+} fractions dominated by UCMs isolated from two Monterey Formation and two Vienna basin crude oils towards blue mussels was investigated. In particular the biotest with the Monterey Formation oil fractions and blue mussels from Cornwall (see Chapter 4) confirmed the sublethal toxic potential of UCM-dominated aromatic crude oil fractions as the toxic effect was successfully correlated with total tissue concentrations.

One of the main objectives of the present study was to investigate whether the monoaromatic or the polyaromatic hydrocarbon C_{11+} subfraction of a crude oil is more toxic. The toxicity tests revealed a similarly strong toxicity for both subfractions of the two Monterey Formation oils. This is for example shown by the TEC_{50} values of the monoaromatic and the total aromatic hydrocarbon fractions of the undegraded Monterey Formation oil (116 µg/g and 119 µg/g wet weight, respectively, see Chapter 4). The main reason for this probably is the fact that acutely toxic low-molecular-weight aromatics such as the BTEX compounds were absent in the fractions used.

The exposure of blue mussels from Jade Bay, Germany, to several fractions of the two Vienna basin oils and the degraded Monterey Formation oil indicated that the German mussels were less sensitive than the Cornish ones (see Chapter 4). Thus, the possible higher toxic potential of the Monterey Formation oil fractions compared to the Vienna basin oil fractions was not confirmed.

Another aim of this work was to examine the distribution of several compound classes in the different oil fractions (see Chapter 5). For example, alkylindanes and alkyltetralins occur in high diversity in all oils. Their presence confirms previous suggestions about cyclic structures of alkylated monoaromatic hydrocarbons in complex crude oil fractions (Warton et al., 1999).

Polyaromatic hydrocarbons identified in all oils were mainly polymethylated napthalenes and phenanthrenes, of which the Monterey Formation and Vienna basin oils contain several individual isomers in different abundance. For example, 1,2,8-trimethylphenanthrene was not present in the Vienna basin oils whereas it was highly abundant in the Monterey Formation oils. Except for naphthalene, phenanthrene and dibenzothiophene, parent PAHs were not detected in any of the four oils.

The composition of the in-reservoir biodegraded oil of each oil pair was clearly altered compared to the respective undegraded oil, in particular regarding monoaromatic compounds. Investigation of changes in the relative abundance of isomers within several aromatic compound groups revealed that the Vienna basin oil is slightly more biodegraded than the Monterey Formation oil (levels 5 and 4 after Peters and Moldowan, 1993, respectively, see Chapter 5).

One main objective of this work was to clarify whether undegraded or degraded crude oils are more toxic as there is no definite answer available in the literature. With the biotest applied, the acute narcotic toxicity of the different Monterey Formation oil fractions was compared. However, GC-MS analysis showed that the compositional differences between the undegraded and degraded oil from each oil pair were only partly transferred into the contaminated water and into the mussel tissue (see Sections 6 and 7.1). This was due to the fact that the compositional differences had mainly occurred among the poorly-soluble monoaromatic compounds. Thus, these compositional differences did not significantly influence the narcotic toxicity towards blue mussels. In general, the toxicity of the aqueous solutions of the oil fractions was caused by the mixture of components rather than by individual compounds or specific compound groups (see Chapter 6).

This relevance of mixture toxicity emphasises the importance of full-scan GC-MS analysis in contrast to the often applied single ion monitoring GC-MS analysis in environmental monitoring programs, which only reveals the parent PAHs and neglects the alkylated homologues. This applies in particular to oil spill scenarios since alkylated PAHs are extremely abundant in crude oils. In this context it is also crucial to establish a standard method for the quantification of UCMs. Since the biotest applied in this study determined the acute narcotic toxicity of UCMs, further research should include toxicity tests with other organisms and different biological endpoints based on specific toxic mechanisms. In general, studies about the toxic mechanism of alkylated aromatic compounds are scarce.

Another question evolved during this work, when it became apparent that the Monterey Formation oils contain a much higher diversity and abundance of aliphatic and aromatic sulphur-containing compounds in contrast to the Vienna basin oils. The Vienna basin oils contain only dibenzothiophene and its polymethylated homologues and these in much smaller amounts than the Monterey Formation oils (see Chapter 5).

Considering the higher aqueous solubility of organic sulphur compounds compared to pure hydrocarbons, it was investigated whether methyldibenzothiophenes accumulate to a higher extent than methylphenanthrenes in the exposed mussels. Calculated ratios of these compounds in various samples demonstrated that the dibenzothiophenes are not preferentially dissolved and taken up into the organism compared to the phenanthrenes (see

Chapter 7.3). There is not much known about the toxicity of sulphur-containing compounds. Thus, further research should investigate the mechanism of toxicity of sulphur compounds in order to distinguish their narcotic toxicity from their possible specific toxicity.

Overall, the present study illustrates that the whole fraction of aromatic hydrocarbons from a crude oil causes acute toxicity at a sublethal level. Even if not all components dissolve well in seawater, a large proportion of the unresolved complex mixtures can dissolve and pose a threat to marine organisms when taken up. This can have severe implications for an ecosystem. Thus, it is crucial to reduce the amount of aromatic hydrocarbons in marine ecosystems and to prevent further contamination of ecosystems due to spills and discharges of crude oil and oil products. This is particularly true since in spite of new laws and regulations which aim at reducing petroleum hydrocarbon contamination, it is anticipated that due to increased activity by offshore oil industries the discharges of e.g. produced water and associated oil will increase (OSPAR, 2003).

Results from this study imply that threshold values for single components are no safeguard against adverse effects due to the mixture toxicity exerted by hydrocarbons. Thus, environmental monitoring and environmental risk assessment procedures need to routinely take account of alkylated aromatic hydrocarbons and aspects of mixture toxicity. Improving the understanding of the composition of unresolved complex mixtures and of the toxicity they cause will provide further insight into the manifold aspects of oil toxicity.

8 References

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Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zusätzlich erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

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