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Biodegradation Kinetics Testing of Hydrophobic Organic Chemicals in Mixtures at Low Concentrations

Rikke Hammershøj

PhD Thesis
February 2020

DTU Environment
Department of Environmental Engineering

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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk.

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Preface

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-IV**.

I Birch, H.; **Hammershøj, R.**; Mayer, P. Determining Biodegradation Kinetics of Hydrocarbons at Low Concentrations: Covering 5 and 9 Orders of Magnitude of K_{ow} and K_{aw}. *Environ. Sci. Technol.* 2018, 52, 4, 2143-2151.

II **Hammershøj, R.**; Birch, H.; Redman, A.D.; Mayer, P. Mixture Effects on Biodegradation Kinetics of Hydrocarbons in Surface Water: Increasing Concentrations Inhibited Degradation whereas Multiple Substrates Did Not. *Environ. Sci. Technol.* 2019, 53, 6, 3087-3094.

III **Hammershøj, R**.; Birch, H.; Sjøholm, K.K.; Mayer, P. Accelerated passive dosing of hydrophobic complex mixtures – controlling level and composition in aquatic tests. *In revision for Environ. Sci. Technol.*

IV **Hammershøj, R**.; Sjøholm, K.K.; Birch, H.; Brandt, K.K.; Mayer, P. Investigating the effect of concentration on biodegradation kinetics for two hydrophobic complex mixtures. *Manuscript draft.*

In this online version of the thesis, paper **I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

In addition, the following publication that was initiated during the MSc study was also concluded during this PhD study:

> Birch, H; **Hammershøj, R**.; Comber, M.; Mayer, P. Biodegradation of hydrocarbons in surface waters at environmentally relevant levels – Effects of inoculum origin on kinetics and sequence of degradation. *Chemosphere*, 2017, 184, 400-407

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First of all, I want to thank my supervisors, Prof. Philipp Mayer, Heidi Birch, PhD, and Karina Knudsmark Sjøholm, PhD, for all our interesting scientific discussions and for your commitment to the project. I would like to say a special thank you to Philipp for giving me this opportunity and for your enthusiasm and good humour, which has made it a pleasure to work under your guidance; to Heidi for always being helpful and stepping in to support me when I needed it the most; and to Karina for your positivity and encouragement and all the fun we have. A big thank you also to Hanne Bøggild and Susanne Kruse for all their help with anything and everything in the lab, and to Prof. MSO Henrik Rasmus Andersen for being my co-supervisor during the first two years of the PhD study.

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The PhD study was funded primarily by Concawe, and additional funding was provided by the Research Institute for Fragrance Materials and CEFIC LRI. I am very thankful to these organisations for making this research project possible. During my study I also had the privilege of collaborating with Assoc. Prof. Kristian K. Brandt at Department of Plant and Environmental Sciences, University of Copenhagen, and being hosted by Prof. Pim Leonards at Faculty of Science, Environmental Bioanalytical Chemistry, Vrije Universiteit Amsterdam in the autumn of 2018. Thank you for taking time out of your busy calenders.

Finally, an immense amount of gratitude goes to my friends, my family and Rune.

Summary

Biodegradation is the most important pathway for removal of organic chemicals from the environment. If a chemical is degraded at a slow rate it can accumulate in the environment, where it can potentially cause harmful effects. Yet, standard biodegradation data are lacking for more than half of the chemicals on the European market and there is a need for new methods to generate biodegradation data faster. Today, regulatory biodegradability tests are conducted almost solely on single chemicals though many chemical substances are mixtures, and ultimately all chemicals that enter the environment will end up in mixtures at low concentrations. Conducting biodegradation testing of chemicals in mixtures could accelerate the generation of environmentally relevant biodegradation data.

Chemical mixtures pose new challenges for biodegradation testing as the physicochemical properties and biodegradability can vary widely between chemicals. Mixtures of hydrophobic organic chemicals (HOCs) pose additional challenges due to their low water solubility and potential volatility. **The primary aim** of this PhD study is to explore, discuss and further develop the application of a novel experimental and analytical platform for aquatic biodegradation testing of HOCs in mixtures. This research includes well-defined mixtures of a limited number of chemicals as well as complex mixtures containing hundreds or even thousands of chemicals.

Biodegradation is a highly variable process that depends not only on substance specific properties but also on the microbial communities and the specific experimental conditions. Currently, there is a knowledge gap concerning how the presence of multiple substrates affect the biodegradation kinetics of individual chemicals when testing at environmentally relevant concentrations. **A second aim** of this PhD thesis is to study the effect of multiple substrates and chemical concentration on biodegradation kinetics of chemicals in mixtures.

Testing of mixtures was found to be a practical way to generate primary biodegradation kinetic data for a large number of chemicals in one test. Biodegradation data obtained from chemicals tested in mixtures are generated with the same inoculum and at the same test conditions and are thus directly comparable. This is a clear advantage when studying how different variables affect biodegradation of chemicals, as it reduces the number of confounding factors, and can for instance also be used to generate training set data for

biodegradation models. Testing of complex mixtures may be the most environmentally relevant choice but it also increases the complexity of a study and thus the analytical challenges. The presented biodegradation platform can readily be used for mixtures with a limited number of chemicals while testing of complex mixtures requires additional studies.

Many previous studies have observed distinct effects of multiple substrates on the biodegradation kinetics of individual chemicals when testing with pure or simple mixture bacterial cultures and at concentrations near the water solubility of the chemicals. However, in this thesis it was observed that the number of mixture components had only limited effects on the biodegradation half times (DT_{50}, lag phase + one half-life) for hydrocarbons when tested at environmentally relevant concentrations. The biodegradation half times were affected more by the substrate concentrations than by the number of mixture constituents, at least for mixtures of up to sixteen hydrocarbons. Further research is needed to address the difference in biodegradation kinetics for chemicals tested alone and as part of a complex mixture at environmentally relevant concentrations.

When conducting biodegradation tests with complex microbial communities and a complex mixture of substrates, many different interactions can take place, and it is not straightforward to predict the effect of changing the substrate concentration. Two complex mixtures, diesel oil and lavender oil, were tested at concentrations near water solubility and two orders of magnitude lower. Diesel oil was observed to stimulate the microbial activity at both test concentrations and no marked concentration effect was observed on biodegradation kinetics for the diesel oil constituents. Lavender oil on the other hand stimulated the microbial activity at low concentration but inhibited the microbial activity at higher concentration, which coincided with a delayed and limited biodegradation that was best explained by toxicity near the solubility limit. This underlines the importance of conduction biodegradation kinetics testing of HOCs, tested alone or in mixtures, at low concentrations.

The findings of this PhD study are a step towards improved biodegradation kinetics testing of HOCs at low concentrations and emphasize the importance of controlling the exposure in aquatic biodegradation testing of HOC mixtures.

Dansk sammenfatning

Bionedbrydning er en vigtig proces, der er med til at fjerne mange organiske kemikalier fra miljøet. Hvis et kemikalie bliver langsomt nedbrudt, kan det ophobe sig i miljøet, hvor det potentielt kan have skadelige effekter. Derfor er det vigtigt at undersøge, hvor hurtigt et kemikalie kan blive bionedbrudt (dvs. nedbrudt af f.eks. bakterier). Lige nu mangler der bionedbrydningsdata for over halvdelen af kemikalierne på det europæiske marked, og der er derfor brug for nye metoder, der hurtigere kan generere de manglende data. Én løsning kan være at lave bionedbrydningsforsøg med kemikalier i blandinger. Det vil kunne fremskynde genereringen af bionedbrydningsdata, som samtidig vil have en højere miljømæssig relevans, da kemikalier i miljøet næsten altid findes i blandinger ved lave koncentrationer.

Bionedbrydningsforsøg med blandinger giver nye udfordringer, da forskellige kemikalier kan have vidt forskellige fysisk-kemiske egenskaber, samt forskellig nedbrydelighed. Hydrofobe organiske kemikalier (HOC) har lav opløselighed i vand og kan være flygtige, og blandinger af HOCs er derfor særligt svære at teste. **Det primære formål** med denne ph.d. afhandling er at undersøge, diskutere og videreudvikle anvendelsesområdet af en ny eksperimentel og analytisk platform til at bestemme bionedbrydningen af HOC blandinger i vand ved lave, miljørelevante koncentrationer. Denne forskning omfatter både veldefinerede blandinger af et begrænset antal kemikalier og komplekse blandinger indeholdende hundreder eller tusinder af kemikalier.

Bionedbrydning er en variabel proces, der afhænger af tre faktorer; de kemikalie-specifikke egenskaber, mikroorganismerne, der foretager nedbrydningen, samt af specifikke eksperimentelle forhold, som f.eks. den temperatur forsøget foretages ved. Lige nu er der en grundlæggende mangel på viden om, hvordan tilstedeværelsen af flere kemikalier vil påvirke bionedbrydningen af de individuelle kemikalier, når man tester i blandinger og ved miljørelevante koncentrationer. **Et andet formål** med denne ph.d. afhandling er derfor at undersøge effekten af blandinger og af testkoncentrationer på bionedbrydningshastigheden af kemikalier i blandinger.

Laboratorieforsøg med blandinger viste sig at være en praktisk måde at bestemme bionedbrydningshastigheden for et stort antal kemikalier i én test. En generel fordel ved at teste kemikalier i blandinger er, at det giver bionedbrydningsdata, som er lavet under de samme forhold og derfor er direkte sammenlignelige. Det kan for eksempel bruges til at lave datasæt til at bygge

matematiske bionedbrydningsmodeller samt til at studere, hvordan forskellige faktorer, såsom testkoncentrationen, påvirker bionedbrydning. Mens det er meget miljørelevant at teste komplekse blandinger, øger det også kompleksiteten af et forsøg betydeligt. Der er stadig brug for flere undersøgelser før den præsenterede bionedbrydningsplatform kan anvendes mere bredt for komplekse blandinger, hvorimod den umiddelbart kan bruges til blandinger med et begrænset antal kemikalier.

Tidligere studier har vist tydelige effekter af tilstedeværelsen af flere kemikalier på nedbrydningshastigheden for individuelle kemikalier. Mange af de studier brugte dog rene bakteriekulturer eller simple kombinationer af bakterier, samt koncentrationer af kemikalierne tæt på deres maksimale vandopløselighed. Denne afhandling viste, at antallet af stoffer i en blanding (op til 16 kemikalier) havde meget begrænset indflydelse på, hvor hurtigt halvdelen af et kemikalie blev nedbrudt (DT_{50} = lag phase + én halveringstid), når forsøget blev udført ved lave, miljørelevante koncentrationer. DT_{50} blev mere påvirket af koncentrationsniveauet, hvor en stigning i koncentration førte til langsommere nedbrydning. Der er brug for flere studier, der undersøger forskellen i nedbrydningshastigheden for kemikalier testet alene og i en kompleks blanding ved miljørelevante koncentrationer.

Når man laver forsøg med en kompleks blanding af kemikalier og en kompleks sammensætning af mikroorganismer taget fra miljøet, kan der ske mange forskellige interaktioner mellem kemikalierne og bakterierne. Det er derfor ikke simpelt at forudse, hvad effekten af at ændre testkoncentrationen vil være. To komplekse blandinger, dieselolie og lavendelolie, blev testet ved en koncentration tæt ved vandopløselighed samt to størrelsesordener lavere. Dieselolie stimulerede væksten af bakterier ved begge testkoncentrationer, og der var minimal forskel i bionedbrydningshastigheden for bestanddele i dieselolie ved de to koncentrationer. Lavendelolie, derimod, stimulerede væksten af bakterier ved lav koncentration, men hæmmede den ved høj koncentration. Det korrelerede med en langsommere og begrænset bionedbrydning ved den høje koncentration, hvilket bedst kunne forklares ved, at den høje koncentration af lavendelolie var giftig for mikroorganismerne. Det understreger vigtigheden af at foretage bionedbrydningsforsøg med HOCs, testet alene eller i blandinger, ved lave, miljørelevante koncentrationer.

Denne afhandling er et skridt på vejen til at forbedre bionedbrydningstests med HOCs ved lave koncentrationer og viser vigtigheden af at kontrollere koncentration og komposition af HOC blandinger i bionedbrydningstests.

Table of contents

Preface ..... **i**

Acknowledgements ..... **iii**

Summary ..... **v**

Dansk sammenfatning ..... **vii**

Table of contents ..... **ix**

Abbreviations ..... **xi**

1 Background and aims **1**

2 Considerations for biodegradation testing and experimental design **4**

2.1 Relevance versus reproducibility 4

2.2 Factors affecting biodegradation 5

2.3 Challenges for testing hydrophobic organic chemicals in water 8

2.4 Challenges for testing mixtures of hydrophobic chemicals in water 10

3 Biodegradation testing of chemicals in mixtures **12**

3.1 Platform for biodegradation testing of hydrophobic organic chemicals in mixtures 12

3.2 Dosing methods & exposure scenarios 13

3.3 Mixtures with a limited number of chemicals 16

3.4 Complex mixtures 19

3.5 Discussion and applications 22

4 Mixture and concentration effects **24**

4.1 Effect of multiple substrates 24

4.2 Test concentration 28

5 Conclusions **31**

6 References **33**

7 Papers **41**

Abbreviations

HOC	Hydrophobic Organic Chemical
OECD	Organisation for Economic Co-operation and Development
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
UVCB	Unknown or Variable composition Complex reaction products or Biological materials
WAF	Water Accommodated Fraction
WWTP	WasteWater Treatment Plant

1 Background and aims

The global chemical production is ever-increasing, and sales of chemicals are forecasted to almost double from 2017 to 2030 (OECD, 2008; UNEP, 2019, 2013). While not all chemicals are hazardous, some have adverse effects when released into the environment. Chemicals that are persistent (P), bioaccumulative (B) and toxic (T), or very persistent (vP) and very bioaccumulative (vB) are of particular concern (ECHA, 2017). Recently, it was also suggested that high persistency alone should be a cause for concern, the reason being that a continuous release of a persistent chemical can lead to accumulation of that chemical in the environment (Cousins et al., 2019). This can take decades to reverse in case adverse effects of the chemical are later identified (Cousins et al., 2019; McLachlan, 2018). In line with this, the first PMT (Persistent, Mobile and Toxic) substance was recently added to the candidate list of substances of very high concern for authorisation under the European Chemicals regulation REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) (ECHA, 2019).

Persistence of a chemical is determined by its low rate of removal by abiotic and biotic processes. For many chemicals, biodegradation is a main pathway for removal from the environment and thus an important factor for the persistence of a substance. However, there are major deficiencies in biodegradation kinetics data for chemicals on the European market. In 2015, the German Federal Environment Agency published a report on data compliance for chemicals with an annual production above 1000 tons registered under REACH (Springer et al., 2015). For biodegradation they concluded that only 45% out of 1814 screened dossiers were compliant with the standard data requirements. A number of other studies have similarly concluded that data insufficiency is a major challenge, and perhaps one of the biggest challenges that researchers and regulators are currently facing (Altenburger et al., 2018; Aronson et al., 2006; Kortenkamp and Faust, 2018; OECD, 2001). The number of chemicals for which biodegradation data are lacking is immense, and it is imperative to develop new efficient and cost-effective methods for biodegradation testing. One approach could be based on biodegradation testing of chemicals in mixtures.

Today, regulatory biodegradation tests are conducted almost solely on single chemicals (OECD, 2006). By contrast, most natural environments are exposed to complex mixtures of chemicals (Kümmerer et al., 2019; Schwarzenbach et al., 2006), hence biodegradation of a chemical in the environment will almost always take place together with other chemicals. Also numerous substances on

the European market, such as petroleum products and essential oils, are multi-constituents substances or UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials). Some biodegradation research on complex mixtures has been conducted in the last decades (see e.g. Brillet et al., 2018 and review in Prince et al., 2017), but there is no well-established approach to assess the biodegradation of chemicals in mixtures. In fact, there is a fundamental lack of knowledge and consensus on how mixtures affect the extent and kinetics of biodegradation at environmentally relevant concentrations (Hammershøj et al., 2019b Paper II).

Biodegradation kinetics of a chemical depends not only on substance specific properties, but also on the microbial community and the specific environmental or experimental conditions (Boethling et al., 2009). It is thus essential to understand how biodegradation of chemicals is affected by different variables, and also how the factors that drive variability in biodegradation in natural environments can be managed in experimental studies. The effect of multiple substrates on biodegradation kinetics of the individual substrates has so far almost solely been studied using pure or simple mixed bacterial cultures or cultures isolated from contaminated sites (e.g. Desai et al., 2008; Dimitriou-Christidis and Autenrieth, 2007; Knightes and Peters, 2006; Reardon et al., 2002). Furthermore, many studies have used chemical concentrations near or even above the chemicals' water solubility (e.g. Bouchez et al., 1995; Stringfellow and Aitken, 1995), which is a potential problem since the concentration can itself affect the biodegradation kinetics (Edwards, 1970; Prince et al., 2017). While the causality between concentration and biodegradation kinetics is rather well studied for single chemicals, very limited knowledge is available on this relationship for chemical mixtures.

The overall aim of this PhD thesis is to explore and discuss a novel approach to biodegradation testing of chemicals in mixtures. This research includes well-defined mixtures of a limited number of chemicals as well as complex mixtures containing hundreds or even thousands of chemicals. **A second objective** is to study and discuss the effect of multiple substrates and chemical concentration level on biodegradation kinetics of chemicals in mixtures.

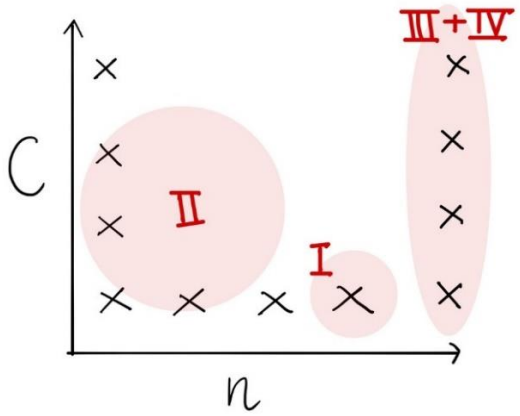


Figure 1: The four studies in this PhD thesis (Paper I – IV) focus on chemical mixtures of different complexity based on the number of mixture constituents (*n*) and at different concentration levels (*C*). The focus area of each paper in terms of *n* and *C* is marked with its roman numeral.

This PhD thesis makes use of an experimental platform for determining primary biodegradation kinetics of hydrophobic organic chemicals in mixtures and at environmentally relevant concentrations. The platform employs partitioning-based methods such as passive dosing and automated solid-phase microextraction, and surface water samples with native microorganisms are used as inoculum. It was first introduced in 2017 where it was applied to a mixture of nine (semi)volatile petroleum hydrocarbons (Birch et al., 2017a). In Paper I (Birch et al., 2018), the applicability domain of this partitioning-based platform is explored and expanded, and biodegradation kinetics were determined for 53 petroleum hydrocarbons. These hydrocarbons belonged to eleven different structural classes and covered five orders of magnitude of octanol-water partition coefficients (K_{ow}) and nine orders of magnitude of air-water partition coefficients (K_{aw}). Paper II (Hammershøj et al., 2019b) explore the effect(s) of concentration level on kinetics of single hydrocarbons and of multiple substrates on kinetics of chemicals in mixtures with up to sixteen hydrocarbons. Paper III (Hammershøj et al., 2019a) is a methodological study showing how accelerated passive dosing can be applied to make defined and reproducible aqueous solutions and dilution series of hydrophobic complex mixtures. In Paper IV (Hammershøj et al., 2019c), the improved passive dosing method is combined with the biodegradation platform to determine biodegradation kinetics of 104 diesel oil constituents and 13 lavender oil constituents, and to study the effect of mixture concentration on biodegradation kinetics. Figure 1 shows the focus areas of Paper I – IV in terms of test concentration and mixture complexity.

2 Considerations for biodegradation testing and experimental design

2.1 Relevance versus reproducibility

Biodegradation of chemicals is a complex process depending on many different factors. The outcome of a biodegradation study is, for instance, dependent on the type of media or compartments that are used for testing (i.e. soil, water, sediment, air), since these will affect the composition of the microbial community and the extent and type of sorption and other partitioning processes that will take place during the experiment (Honti et al., 2016; Junker et al., 2019; Mackay et al., 2014). While more complex multimedia testing will likely result in more realistic and relevant data, complexity comes at a price. Calow (1997) described how there will always be trade-offs between relevance, reproducibility, reliability, robustness and repeatability/sensitivity when designing an ecotoxicity test. The same applies for biodegradation tests that may have different purposes depending on whether they are conducted in a regulatory or scientific context.

Assessment of biodegradability in the aquatic environment under REACH follows a tiered approach (OECD, 2006). The purpose is to screen chemicals for biodegradability ideally before they can cause harm to humans and the environment. The first tier is a conservative screening test for ready biodegradability designed to determine whether a chemical can be rapidly degraded or not (OECD, 2014, 1992a). If the outcome is negative, the chemical is passed on to more elaborate second tier (inherent biodegradation, e.g. OECD, 1992) or third tier (simulation, e.g. OECD, 2004) testing. Since biodegradation kinetic parameters such as half-lives can vary more than 2 orders of magnitude based on the specific experimental conditions (Boethling et al., 2009), regulatory tests are standardized and often simplified to enhance reproducibility, reliability and robustness. Especially first and second tier tests may, however, have low environmental relevance as the test conditions are far from being environmentally realistic (Kowalczyk et al., 2015). For example, test concentrations are much higher than what is found in most natural environments (Berg and Nyholm, 1996; Kowalczyk et al., 2015).

In a scientific context, the aim is often to investigate the influence of certain factors or processes on biodegradation, sometimes in relation to a certain en-

vironment. Biodegradation research studies will thus often deviate from standardized test methods and some will increase the complexity of the test conditions to increase the environmental relevance. Still, reproducibility, reliability and robustness are arguably factors that should be given weight in all tests to ensure credibility of the test results (Calow, 1997).

The studies that form the basis of this PhD thesis are based on simulation tests but do not use standardized test setups as the aim was to develop and explore new methods with a different applicability. This research thus has a scientific focus, while at the same time aiming to provide a technical and scientific foundation for future regulatory testing.

2.2 Factors affecting biodegradation

The biodegradation kinetics of a substance depend on both substance specific properties, the microbial community and the conditions of the environment or experiment where biodegradation takes place (Boethling et al., 2009). Understanding how biotic and abiotic factors can affect the biodegradation of a given substance, and whether or how to deal with these factors, is paramount when setting up a biodegradation test. Some of the more important factors are briefly discussed in the following.

Physical and chemical conditions like pH, temperature, nutrient availability and dissolved oxygen concentration and redox conditions can all affect (and be affected by) the biodegradation of organic chemicals (Leahy and Colwell, 1990; Schwarzenbach et al., 2005). It is desirable that these factors are kept at environmentally relevant levels in experiments and tests, though it is not always clear cut what such levels should be (Boethling et al., 2009). For example, the incubation **temperature** used in regulatory biodegradation studies is still a matter of debate. This is related to how a biodegradation half-life obtained at a given temperature can be extrapolated to other temperatures, e.g. for exposure modelling, and also to a lack of consensus on which experimental temperature that is the most relevant for comparison with regulatory persistency cut-off values (Matthies and Beulke, 2017). Lower temperatures lead to lower microbial activity and delayed degradation, which in turn lead to slower (but overall similar) changes in microbial community composition with time (Vergeynst et al., 2018). This has for example been observed to affect the persistence of chemicals in a Swedish lake as the water temperature changed with the seasons (Zou et al., 2015).

Partitioning to organic matter can influence the persistence of a chemical in the environment where sediments and particulate organic matter can act as a sink and limit the **bioavailability** of the chemical (Kästner et al., 2014; Schäffer et al., 2018). According to Ortega-Calvo et al. (2015), the bioavailable fraction includes the freely dissolved and the rapidly desorbing chemical fractions, while a bioaccessible fraction also comprises the more slowly desorbing fraction of a chemical that can become available over time. Another definition is that the bioavailability of a substance comprises the chemical activity and the bioaccessible fraction, where the latter includes the chemical fraction that can become available with time under given environmental conditions (Reichenberg and Mayer, 2006). Whichever definition is chosen, bioavailability limitations can delay biodegradation if mass-transfer is the rate limiting step (Gharasoo et al., 2015).

In addition to the bioavailable and bioaccessible fractions, all chemicals will form non-extractable residues (NER) when in contact with organic matter, and these NER can be divided into three types (Kästner et al., 2014; Schäffer et al., 2018; Trapp et al., 2018): Type I NER is parent compound or metabolites adsorbed or sequestered into organic matter, which are not available for biodegradation, but could be released again if the environmental conditions change and thus potentially constitute a risk. Type II NER is parent compound or metabolite covalently bound to organic matter, which in many cases is non-reversible and as such may sometimes be considered a 'safe sink' (Schäffer et al., 2018). Type III NER is carbon from the chemical incorporated into microbial biomass that will not constitute a risk (Kästner et al., 2014). How to quantify, differentiate between and account for different NER types in persistence assessment is currently a very active area of research (Kästner et al., 2018). Chemical mixtures pose additional challenges as current methods only allow NER quantification for single constituents or blocks of constituents with a common chemical structure (Schäffer et al., 2018).

The **microbial community** is one of the most important factors affecting the extent and kinetics of biodegradation. Inoculum volume and density of degraders (and thus total amount of degraders) will, for instance, affect the length of the lag-phase, which is the time it takes for the biomass to acclimate, adapt and grow prior to the on-set of detectable degradation (Ingerslev et al., 2000; Schwarzenbach et al., 2005). Similarly, the inoculum density is related to the likelihood of including specific degraders into a test system (Martin et al., 2017). While testing with pure or simple mixed cultures can be useful to study basic principles and concepts, these will eventually have to be confirmed by

test with mixed microbial communities (Egli, 2010), which are also more relevant for biodegradation research within a persistence and hazard assessment context. The physiological state of microorganisms and community composition are affected by physical and chemical conditions such as temperature but also by (pre)exposure to chemicals (Van Hamme et al., 2003; Winding et al., 2019). There can be associations between the biodiversity of a microbial community and the rate of biodegradation for specific micropollutants (Johnson et al., 2015), but most natural microbial communities are believed to exert a functional redundancy (Brandt et al., 2004). Community function and dynamics are still very active areas of research (Mansfeldt et al., 2019; Widder et al., 2016).

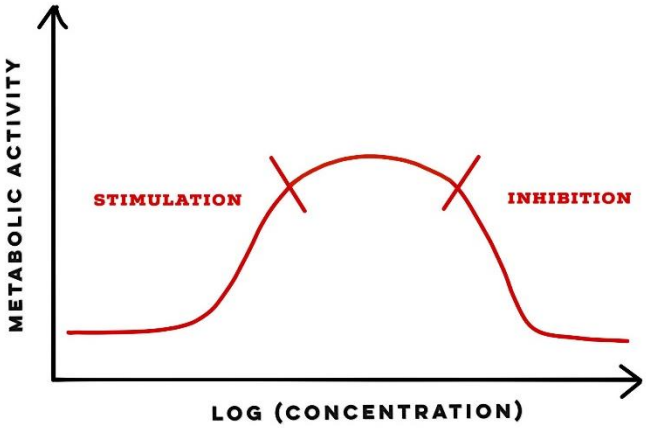


Figure 2: The typical effect of major changes in substrate concentration on e.g. metabolic activity of a pure bacterial culture. Modified from Edwards (1970).

In general, the metabolic activity of the degrading microorganisms is closely related to the **concentration** of the test chemical (Figure 2) (Edwards, 1970). At particularly low concentrations, the limited substrate availability may limit biodegradation (Boethling and Alexander, 1979; Toräng et al., 2003), whereas an increase in substrate concentration will lead to an increased degradation activity of the microorganisms and potentially growth, which again result in faster degradation kinetics, but only up to a certain point. If increasing the concentration above this point, the degradation activity will stagnate and eventually decrease due to increasing toxicity (Edwards, 1970; Gharasoo et al., 2015). While the causal relationship between test concentration and biodegradation is rather well established for single chemicals, limited literature is available on the concentration effect for mixtures. The significance of the test concentration for mixtures is a topic in Paper II and Paper IV and is discussed in more details in chapter 4.

Not only the concentration level of a test substance but also the way test substances are introduced into a test system is important. A common way to introduce hydrophobic organic chemicals (HOC) in aquatic tests is solvent spiking. However, in biodegradation testing the solvent can have some unfortunate side effects since it can act as a substrate and enhance microbial growth, or it can have a toxic effect if used at a too high concentration (Hutchinson et al., 2006; Weyman et al., 2012). For example, Shrestha et al. (2020) observed that co-solvent addition reduced the levels of dissolved oxygen in a closed sediment-water test with petroleum hydrocarbons, which again affected the microbial activity and thus potentially the biodegradation. In another recent study, dissimilar but remarkable differences were observed in biodegradation behaviour for six chemicals tested at their natural concentration in unspiked surface water and spiked into surface water at a concentration 10-1000 times higher than in the unspiked test (Li and Mclachlan, 2019). While the test concentration and solvent-spiking may have been confounding factors, they concluded that 'spiking can strongly influence biodegradation, reducing the environmental relevance of test results'. The importance of the dosing methods was the focus of Paper III (Hammershøj et al., 2019a), and is discussed more detailed for chemicals in mixtures in Chapter 3.

2.3 Challenges for testing hydrophobic organic chemicals in water

Hydrophobic organic chemicals (HOC) are poorly soluble in water, and the hydrophobicity entails a higher potential for sorption and evaporative losses (Shrestha, 2019). Thus, the first challenge in biodegradation testing of HOCs is to establish a well-defined initial test concentration, and for (semi)volatile chemicals it is necessary to employ a method with closed test systems (Birch et al., 2017a; CONCAWE, 2012; Shrestha et al., 2020). Furthermore, bioavailability of a chemical will be related to the test compartments included in an experimental setup (e.g. sediment, soil, water), which thus requires careful consideration for HOCs due to the high potential for sorption. In this PhD study, a closed test design is used, and all biodegradation tests are aquatic to help disentangle the biodegradation process from other dissipation processes such as sorption.

The low water solubility of HOCs can conflict with some biodegradation endpoints that require a minimum concentration level. For example, test methods

to assess ready biodegradability recommended for poorly soluble chemicals include the OECD (Organisation for Economic Co-operation and Development) technical guidelines 301B (measurement of CO_2 formation), 301C/D/F (oxygen consumption) and 310 (measurement of inorganic carbon formation) (OECD, 2014, 1992a). The required concentrations of test chemical for these tests are 2-40 mg/L (310), 50-100 mg/L (301C/F), 2-5 mg/L (301D), and 10-20 mg DOC/L (301B). Many HOCs have a water solubility lower than 1 mg/L, and would thus be present as free phase in all of these tests, which affects the rate of biodegradation and reduces the reproducibility (Prince et al., 2017; Singer et al., 2000). Furthermore, HOCs are known to exert baseline toxicity when reaching a critical concentration in the lipid membrane of bacteria, which typically requires aqueous concentrations in the range 1-100% of solubility (Schmidt and Mayer, 2015; Winding et al., 2019).

Testing at environmentally relevant concentrations is thus necessary for HOCs to avoid confounding factors, such as a free phase or toxicity, and more relevant but also more challenging. The simulation test OECD 309 for aerobic mineralization in surface water is designed for low concentrations (less than 1 µg/L to 100 µg/L) (OECD, 2004). In this test method, ^{14}C labelling is recommended to determine (ultimate) biodegradation, potentially in combination with specific chemical analysis to measure primary degradation, though it is highlighted that the latter is frequently impossible at concentrations < 100 µg/L. However, recent advancements in analytical chemistry are enabling biodegradation kinetic studies without labelled substances even in the sub µg/L concentration range, at least for evaluating primary degradation (Birch et al., 2017a; Li and Mclachlan, 2019). This is important because the concentration required for a given test method is correlated with the chemical applicability domain in terms of logK_{ow}, taken that the test substance should be fully dissolved (Figure 3). The sensitivity of the analytical set up is especially important when conducting biodegradation tests with chemicals in mixtures since techniques such as ^{14}C labelling are not readily transferable to mixtures.

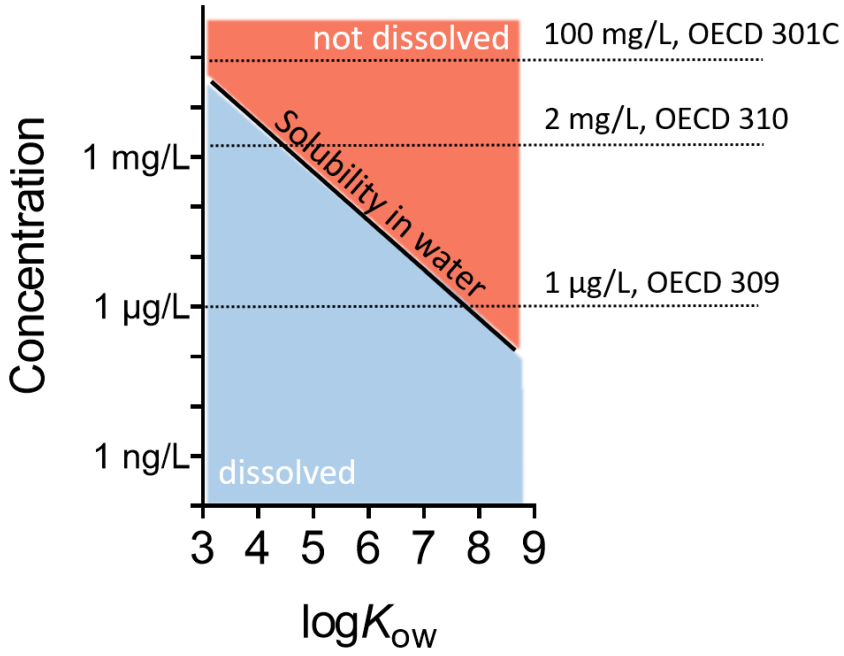


Figure 3: Stylised relationship between concentration level (C) and octanol-water partition coefficient (K_{ow}), both in log scale, and the water solubility of a hydrophobic organic chemical. The approximate concentration level required or recommended for three regulatory biodegradability tests (OECD 301C, OECD 309 and OECD 310) are marked with dotted lines.

2.4 Challenges for testing mixtures of hydrophobic chemicals in water

Mixtures of HOCs pose new challenges for experimental biodegradation research and testing. Each chemical in the mixture will have its own degradation and physicochemical properties and be subject to varying degrees of sorption and evaporative losses (OECD, 2019; Prince et al., 2013; Prosser et al., 2016). Further, the difference in water solubility between constituents necessitates special consideration when selecting a test method (Figure 3). Biodegradation testing of mixtures thus requires a combination of smart approaches and novel analytical methods while ideally still being simple and robust.

Again, not all biodegradation end-points are suitable for determining biodegradation (kinetics) of mixtures, and especially not of hydrophobic complex mixtures (Brillet et al., 2018). Ultimate biodegradation (mineralization) of a mixture could be determined, for example, by measurements of biological oxygen demand (Brillet et al., 2018). However, there are the same constraints on

biodegradation end-points due to solubility for hydrophobic mixtures as for single HOCs. Furthermore, determining the mineralization of the mixture will not provide information on potentially persistent constituents, even if a pass criteria like 60% of the theoretical oxygen demand is reached. While Brillet et al. (2018) state that "the assessment of complex mixture biodegradability can be limited by technical issues and/or difficulties to rule on ready or inherent biodegradability", one could argue that ready and inherent biodegradability tests cannot stand alone for mixtures. Today the primary regulatory solution to this is to identify and assess all constituents present above a certain threshold in the mixture individually (ECHA, 2017), which is time-consuming and costly. Two new ways forward could be to test a number of representative chemicals in a mixture (Paper I & II), or to test an entire complex mixture while following the biodegradation kinetics of a number of representative mixture constituents (Paper IV).

3 Biodegradation testing of chemicals in mixtures

3.1 Platform for biodegradation testing of hydrophobic organic chemicals in mixtures

In this PhD study a recently developed experimental and analytical platform was used for biodegradation tests of volatile and hydrophobic organic chemicals in mixtures (Birch et al., 2017a). In this platform, surface water grab samples mainly from lakes and streams, or filtered samples from a wastewater treatment plant, are used as inoculum. As a rule, the tests are aquatic. Passive dosing from a pre-loaded silicone rod is used to introduce the chemical mixtures into the tests without using a co-solvent (Figure 4a). Test systems are made with 15 mL inoculum with test chemicals added in 20 mL amber glass vials closed with gas-tight screw caps and polytetrafluoroethylene (PTFE) coated silicone septa to minimize sorption and evaporative losses (Figure 4b). This leaves 5 mL of headspace to maintain aerobic conditions. Abiotic test systems are made with ultrapure water or poisoned inoculum instead of active surface water inoculum. Sampling is performed by automated solid-phase microextraction (SPME) directly on the test systems, coupled to gas chromatography – mass spectrometry (GC-MS) (Figure 4c). Automated SPME provides significant enrichment of the hydrophobic organic chemicals which in turn allows testing at environmentally relevant concentrations. Using autosampler vials for test systems minimizes losses as the test systems are not opened before sampling and the analysis is done directly on the test system with no extraction steps in between. Primary biodegradation kinetics are then based on a ratio between biotic and abiotic test systems, which makes it possible to distinguish between biodegradation and other dissipation processes such as sorption and evaporation. As default, a (pseudo-)first-order kinetic model is fitted to the data to obtain estimates of lag phase, first-order degradation rate constant and half-life, as this is a simple model that requires a minimum of assumptions compared with more complex modelling approaches.

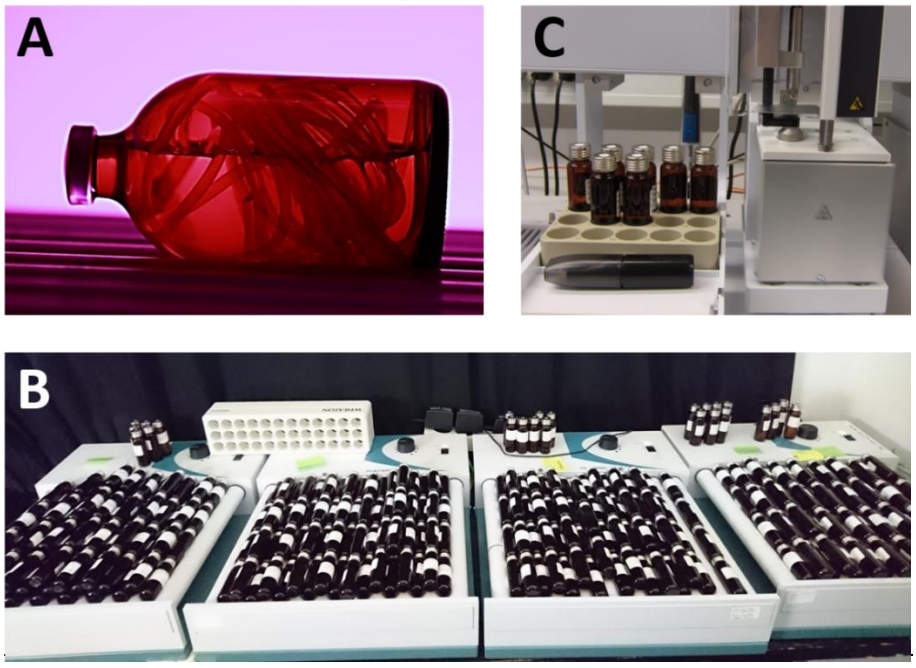


Figure 4: A) A passive dosing system where ultrapure water or inoculum is equilibrated with a pre-loaded silicone rod to create a stock solution containing a mixture of hydrophobic organic chemicals. B) A large number of biotic and abiotic test systems, with 15 mL inoculum/ultrapure water with test chemicals added in 20 mL amber glass vials, are prepared for each biodegradation experiment and incubated at 20 °C with horizontal rolling. C) Automated SPME applied directly on the test systems is coupled to a GC-MS for specific chemical analysis to measure the level of each chemical in the test mixture.

Most aspects of the biodegradation method employed here are similar and in compliance with the OECD 309 simulation test, as both tests are designed for environmentally relevant concentrations (ng-µg/L range), and use surface water inoculum and closed test systems. The test method employed here is primarily different in that it was designed for mixtures of hydrophobic and potentially volatile chemicals, and only targets primary degradation.

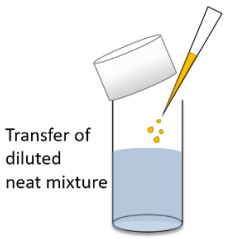
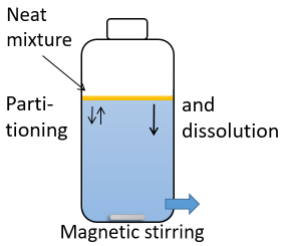
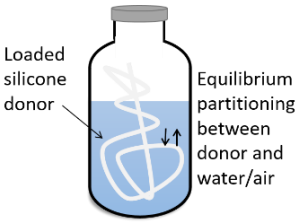
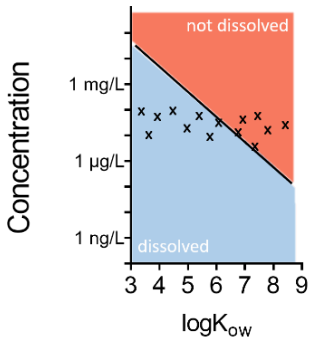
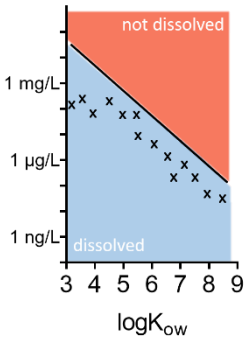
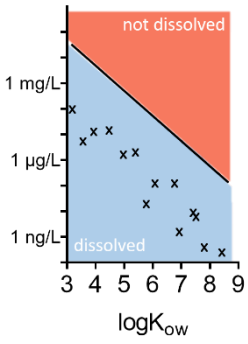
3.2 Dosing methods & exposure scenarios

Since the concentration and composition of a mixture can affect the outcome of a biodegradation study, it is imperative to have reliable and well defined methods for dosing mixtures into water. This can be challenging. Table 1 provides a brief overview of some characteristics for three commonly used dosing methods, solvent spiking, WAF and passive dosing. Solvent spiking is often used to introduce single HOCs into aquatic tests, but this method is generally not recommended for HOC mixtures (OECD, 2019). The water accommodated

fraction (WAF) method can be used to introduce complex mixtures containing HOCs, such as petroleum products, into water (OECD, 2019; Singer et al., 2000), but while the WAF is very relevant when conducting tests within an oil spill context it also has some limitations as discussed in Hammershøj et al. (2019a Paper III). Lastly, passive dosing is used more and more to establish the exposure of HOCs in water (Hammershøj et al., 2019a Paper III). The relevance of the dosing method depends on the emission and exposure scenario, and particularly whether the HOC mixture is completely transferred to water (e.g. lavender oil emitted via toilet cleaner) or the mixture rather enters water via phase partitioning from an organic phase (HOCs sorbed to organic matter in sediments or WWTP sludge) or even the free phase mixture (e.g. marine oil spills, Non Aqueous Phase Liquids (NAPL) in Aquifers). However, as discussed in Section 2.1 reproducibility and reliability should often be given more emphasis than relevance, especially in regulatory biodegradation tests.

Hammershøj et al. (2019a Paper III) showed that passive dosing can be used to establish reproducible and tightly controlled aqueous exposures of hydrophobic complex mixtures, and to linearly change the aqueous concentration level of each constituent of a complex mixture while preserving the composition, at least when working at low concentrations (roughly two orders of magnitude below water solubility and lower). Furthermore, the dosing kinetics were very fast, with equilibrium times in the minute range. Thus, passive dosing was used to establish the initial concentration in all biodegradation studies included in this PhD study. This was chosen based on the criteria that the mixtures should be dosed without using a co-solvent and i) at low environmentally relevant concentrations, ii) below solubility (i.e. all mixture constituents dissolved) and iii) below toxicity. These criteria are generally recommended for aquatic biodegradation research and testing on HOCs and HOC mixtures.

Table 1: Overview of three common methods for dosing HOCs into water. The logK_{ow} vs concentration figures are based on idealized data. Table is modified from Hammershøj et al. (2019a Paper III).

	Solvent spiking	**Water accommodated fraction**	**Passive dosing**
			
Physico-chemical principle	Nominal concentration is set by quantitative transfer of mixture.	Partitioning and dissolution from mixture to water.	Partitioning of each mixture constituent from dominant donor to water.
Composition in water	Composition of mixture transferred to water .	Composition is modified by partitioning and dissolution and can vary with concentrations due to depletion. Figure shows idealized low energy WAF.	Composition is modified by partitioning. Constant composition at varying loading within Henry's law regime.
			
Precipitation of pure phase	Prone to precipitation of least soluble constituents at high concentration additions	Common for high energy WAFs, very limited for low energy WAFs	Very limited, but can happen close to saturation

3.3 Mixtures with a limited number of chemicals

The new partitioning-based biodegradation platform was first used to study the biodegradation of nine (semi)volatile petroleum hydrocarbons in a mixture (Birch et al., 2017a). It has also been used to study the influence of inoculum origin on biodegradation kinetics of a different mixture of petroleum hydrocarbons (Birch et al., 2017b). Paper I sets out to explore the applicability domain of the platform by applying it to a broader range of chemicals in terms of hydrophobicity and volatility than used in the previous studies (Birch et al., 2018 Paper I). A total of 53 petroleum hydrocarbons were selected based on their structure and physicochemical properties. These hydrocarbons have between eight and twenty carbon atoms and cover twelve structural classes as well as a broad chemical space (five orders of magnitude of octanol-water partition coefficients, K_{ow}, and nine orders of magnitude of air-water partition coefficients, K_{aw}) (Figure 5). All 53 hydrocarbons can be found in various petroleum products but are together not representative of any particular petroleum product.

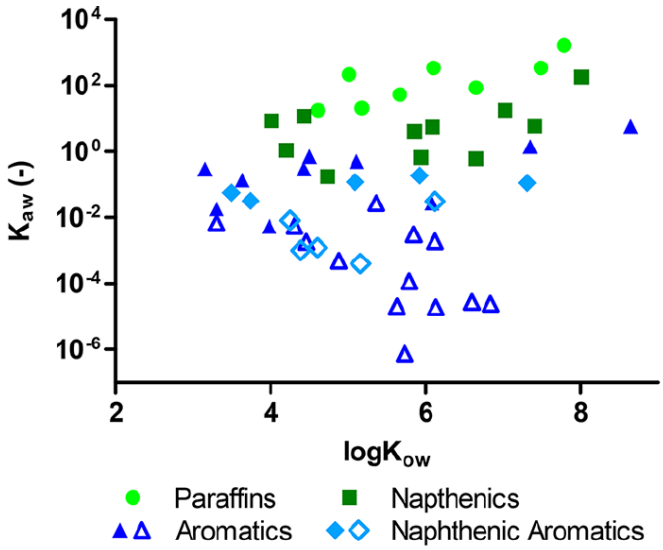


Figure 5: Chemical space covered by the 53 petroleum hydrocarbons in terms of log K_{ow} and K_{aw}. Open/closed symbols indicate compounds that were tested together. Paraffins include *n*-paraffins and *i*-paraffins, Naphthenics cover mono-, di and polynaphthenics, Aromatics comprise mono-, di-, tri- and polyaromatics, and Naphthenic Aromatics include naphthenic mono-, di- and triaromatic. From Birch et al. (2018 Paper 1).

Based on how they had to be loaded into the passive dosing donor, the 53 hydrocarbons were divided into two mixtures. One group contained 35 hydrocarbons and one 19 (see Birch et al., 2018 Paper I). Naphthalene was included in both mixtures. Biodegradation of the two mixtures was studied separately with three different inocula: lake water, sea water and an activated sludge filtrate. Initial test concentrations were in the range 0.004 to 170 µg/L, and each compound was between ten times and 400 times below its solubility (Figure 6).

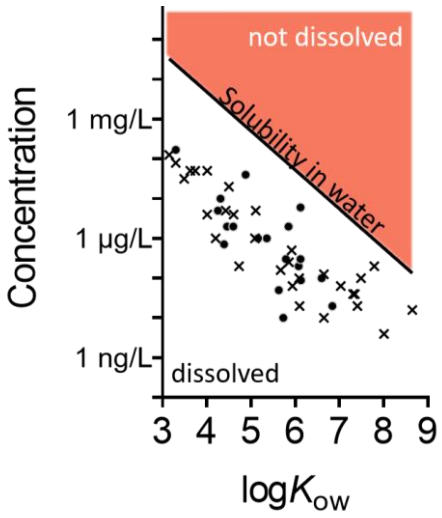


Figure 6: Test concentration vs log K_{ow} for petroleum hydrocarbons in biodegradation experiments presented in Paper I. x and • indicate the compounds that were tested together.

A first-order degradation model fitted to experimental data are shown for three hydrocarbons in each of the three inocula (Figure 7).

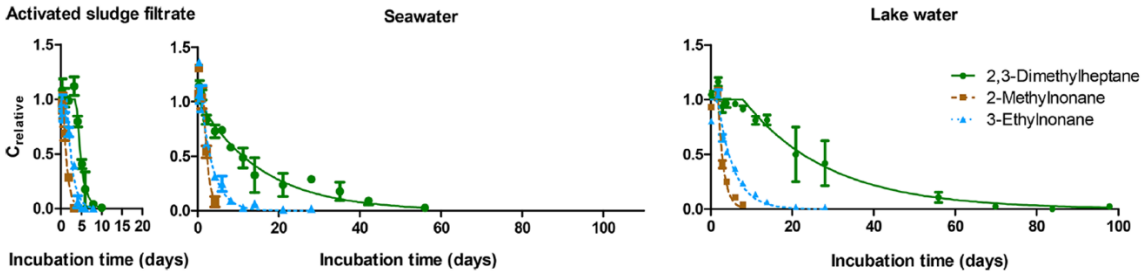


Figure 7: First-order degradation model fitted to experimental biodegradation data for three petroleum hydrocarbons tested with three different inocula. The remaining amount of test substance is given as relative concentration, that is, the ratio between peak areas in the biotic test systems and the peak areas in the abiotic test systems. Error bars show standard error of the mean, *n*=3. From Birch et al., (2018 Paper I).

The testing approach with defined mixtures of up to 35 chemicals enabled the determination of biodegradation data for the hydrocarbons covering a large chemical space. Birch et al. (2018 Paper I) thus demonstrate that biodegradation kinetics can indeed be determined for HOCs in mixtures at environmentally relevant concentrations. This approach is more time efficient than testing one chemical at a time. The close alignment of the test systems with the analytical method, and the minimized number of experimental steps, is a clear advantage when testing hydrophobic and volatile chemicals. Further, the consistent use of biotic/abiotic ratios to determine the degree of biodegradation makes it easier to differentiate biotic from abiotic losses, and provides a good basis for biodegradation studies on mixtures of chemicals with different physicochemical properties.

The inoculum had an influence on the biodegradation kinetics of the tested petroleum hydrocarbons (figure 7), and thus on the number of hydrocarbons that were degraded in each study. This is consistent with the findings of Birch et al. (2017b) showing that the source of inoculum had an influence on the biodegradation kinetics (lag phase and half-life) for nine petroleum hydrocarbons. The inoculum volume (15 mL) was chosen to facilitate the practical biodegradation set up with a high throughput of samples and a close alignment to the analytical measurements. In most cases this inoculum volume should be sufficient, but with one inoculum (a clean lake with a small catchment area and no major point sources, and a relative low culturable bacterial density based on heterotrophic plate count) the inoculum volume may have been too small (Birch et al., 2018 Paper I).

3.4 Complex mixtures

Birch et al. (2019 Paper I) studied defined mixtures with a limited number of chemicals. For chemicals that naturally occur in a complex mixture, it can be more relevant to test this mixture while obtaining biodegradation kinetics for the individual constituents. A fair amount of research has been conducted on aquatic biodegradation of petroleum products such as crude oil (Prince et al., 2017, 2013), gasoline (Marchal et al., 2003; Prince et al., 2007; Solano-Serena et al., 2000, 1999), diesel oil (Marchal et al., 2003) and biodiesel (Prince et al., 2008). Prince et al. (2017) reviewed twenty-two papers published between 1995 and 2016 that reported half-lives of oil biodegradation in seawater ranging from 1 to 276 days. They observed that one major variable between these studies is the chemical test concentration, which was between 2 and 10 000 ppm, and they further derived that the majority of the hydrocarbon constituents must have been in a free phase in many of the studies. While this can be highly relevant for an oil spill situation, it is favourable to use concentrations below water solubility in biodegradation tests with a focus on prospective risk assessment since this is the more common scenario in most natural waters. However, ensuring that all constituents are below solubility is a general challenge when testing complex mixtures of HOCs in water (Section 3.2). Furthermore, such low concentrations require high analytical sensitivity (see Section 2.3 and 2.4). One solution to this is to use partitioning based techniques such as passive dosing and SPME.

The technical aim of Paper IV was to explore the possibility of applying the partitioning-based biodegradation platform to complex mixtures of HOCs to obtain well-defined biodegradation data for the individual mixture constituents when testing at concentrations below solubility (Hammershøj et al., 2019c Paper IV). To this end, parallel biodegradation tests with diesel oil and lavender oil were conducted at concentrations near water solubility and two orders of magnitude lower. The passive dosing method was optimized to set the initial concentration of each mixture constituent directly in the inoculum (Hammershøj et al., 2019a Paper III), which was then combined with the partitioning-based biodegradation platform. The composition of the two test mixtures were unknown prior to the biodegradation experiment, and all samples were thus analysed by full scan MS. In the ensuing data treatment, biodegradation data were obtained for 104 diesel oil and 32 lavender oil constituents based on a number of quality criteria. Tentative identifications were found for 50 diesel oil and 27 lavender oil constituents. In Figure 8 is shown the chemicals space covered by these constituents in terms of $\log K_{ow}$ and K_{aw}.

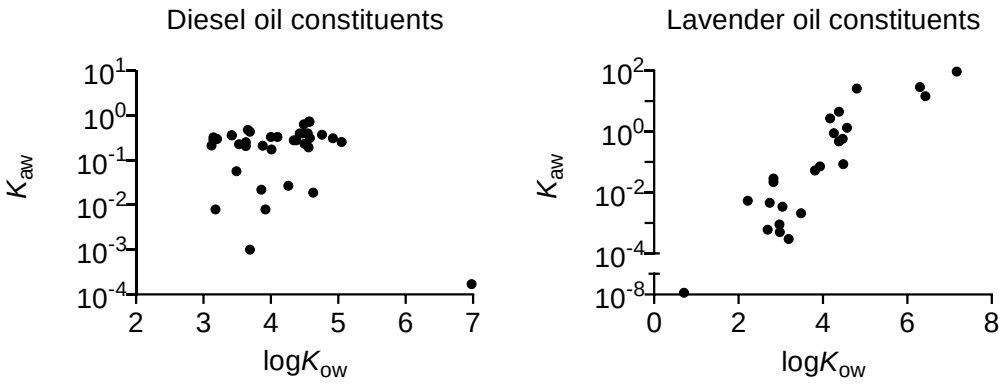


Figure 8: The chemical space, in terms of log K_{ow} and K_{aw}, covered by 48 diesel oil constituents (data was unavailable for two constituents) and 27 lavender oil constituents based on their tentative identifications. Note the different scale of the axes.

Biodegradation curves are shown for the three diesel oil and three lavender oil constituents that had the best fit (R^2) at the low test concentration (Figure 9).

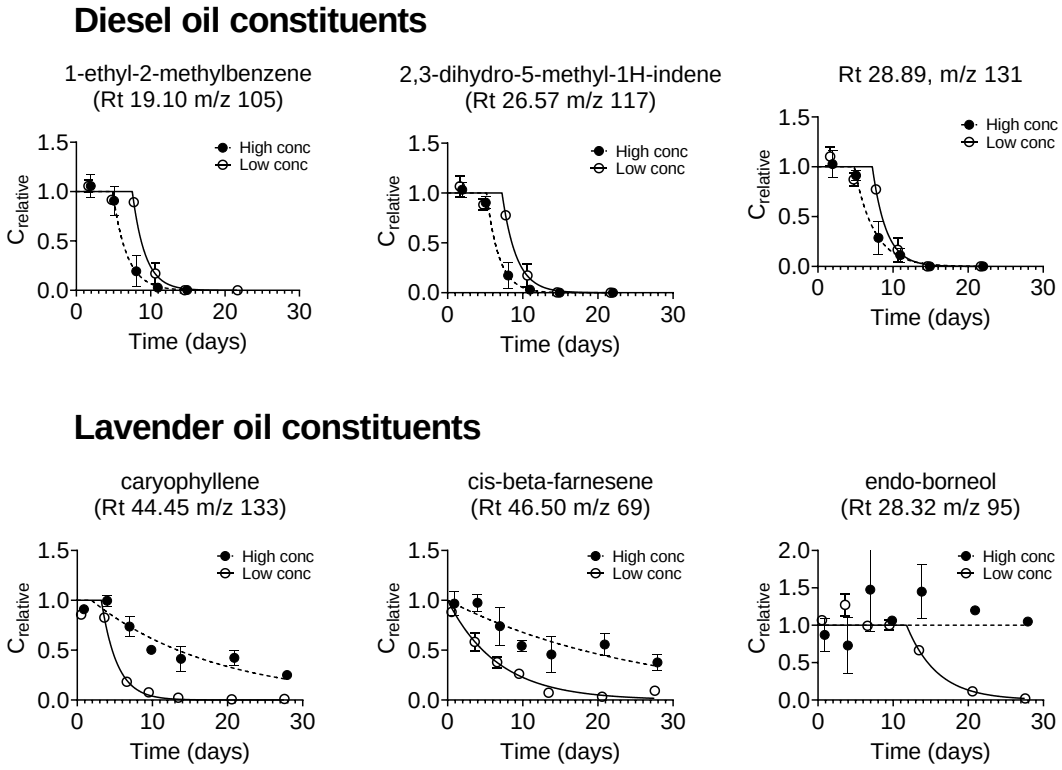


Figure 9: First-order degradation model fitted to experimental biodegradation data for three diesel oil and three lavender oil constituents at the low and the high test concentration. The

constituents were chosen as they had the best fit (based on R^2) at the low test concentration. Error bars represent standard error of the mean, $n = 3$. Figure modified from Hammershøj et al. (2019c Paper IV).

Hammershøj et al. (2019c Paper IV) is the first study applying the new biodegradation platform to complex mixtures. Generally, the approach is promising for determining biodegradation kinetics of the individual constituents in complex mixtures but some obstacles were also identified.

The diesel oil that was tested has an unknown composition but is likely to contain hundreds of constituents. Biodegradation data were collected for 104 diesel oil constituents at high test concentration and for 62 at low test concentration since some constituents were below the quantification limit at the lower concentration. This reflects a trade-off between testing at environmentally relevant concentrations and having sufficiently high concentrations of a complex mixture to ensure that many (relevant) constituents are detectable. Under REACH, a relevant constituent is generally defined as one present at a concentration above 0.1% in the mixture (ECHA, 2017). However, very complex UVCB substances can consist solely of constituents that are each at a concentration below 0.1% in the mixture, which may require different testing strategies altogether (ECHA, 2017). Overall, Hammershøj et al. (2019c Paper IV) show that the concentration of complex mixtures in biodegradation testing is something that requires particular consideration, especially as it can also have an impact on the resulting biodegradation kinetics (Chapter 4).

The consistent pairing of biotic and abiotic test vials is a focal feature of the partitioning-based biodegradation platform. However, for some lavender oil constituents, the pairing was not adequate to separate biodegradation from abiotic processes (see Hammershøj et al., 2019c Paper IV). Essential oil components can undergo abiotic transformations and be converted into each other by, for example, autoxidation and isomerization processes, which are impacted primarily by light, temperature and oxygen availability and can take place rapidly even at room temperature (Turek and Stintzing, 2013). In general, very few studies are available on biodegradation of essential oils or essential oil constituents (Jenner et al., 2011), and this is an area where further research is needed.

3.5 Discussion and applications

Testing of chemicals in mixtures can provide primary biodegradation kinetics for a large number of chemicals in one test, potentially covering a large chemical space. This was shown here for mixtures with a limited number of HOCs (Birch et al., 2018 Paper I) and for hydrophobic complex mixtures (e.g. diesel oil, Hammershøj et al., 2019c Paper IV), and has similarly been shown for polar organic chemicals by Achermann et al. (2018), who determined biotransformation rate constants for 42 micropollutants based on mixtures of 77 and 93 chemicals tested in activated sludge. In fact, biodegradation research on polar organic chemicals in mixtures is widely used and accepted (e.g. Li et al., 2015; Li and Mclachlan, 2019; Stravs et al., 2019). With the newest developments in experimental and analytical chemistry the same is now possible for mixture of HOCs at environmentally relevant concentrations. Biodegradation testing of chemicals in mixtures at low concentrations result in data that are often more environmentally relevant since the presence of multiple substrates at low concentrations is the most common situation in the environment. This approach can therefore accelerate the generation of environmentally relevant biodegradation data, also for hydrophobic organic chemicals.

Biodegradation data are the basis for building, refining and testing predictive models (Howard et al., 2005; Rücker and Kümmerer, 2012). Models that predict biodegradability based on chemical structures will for instance need training set data with a minimal variability in inoculum and test conditions, as natural variability in biodegradation data is a significant complication when creating persistence models (Prosser et al., 2016; Rücker and Kümmerer, 2012). Biodegradation testing of chemicals in mixtures will produce data that are generated with the same inoculum and at the same test conditions, which minimises the number of confounding factors. This is an excellent basis to systematically study and compare the variables that affect biodegradation processes.

The main limitation of the presented approach is that it is limited to primary biodegradation. Partly related to this, biodegradation testing of mixtures entails a potential for co-metabolism, which is when a chemicals is degraded simultaneously with another substrate but without supporting growth (Horvath, 1972). Co-metabolism alone may not lead to full mineralization (Horvath, 1972), but this distinction cannot be made when only primary degradation is measured. Co-metabolism may, however, be the initial step that leads to ultimate degradation through other processes (Horvath, 1972). Thus, one argument against biodegradability testing of chemicals in mixtures that has repeatedly been put

forward is the risk of false positives due to the presence of other 'favourable' substrates. But with this line of reasoning, a counterargument is that all chemicals should ideally be tested in the mixtures where they occur rather than alone to avoid false negatives or false positives, since the presence of co-substrates can enhance but also inhibit the degradation of the individual chemicals (see Section 4.1).

As discussed in Section 2.1, there is a trade-off between relevance, reproducibility, reliability and robustness in biodegradation testing. While biodegradation testing with complex mixtures is certainly more environmentally relevant than testing the constituents individually, this will introduce uncertainty in a test due to the large number of (potentially unknown) constituents, some of which will not even be detectable but may still serve as substrates. Furthermore, the composition of some complex mixtures (e.g. essential oils) can vary from batch to batch and/or over time, which increases the complexity and potentially hampers reproducibility. One way to get around this issue is to test mixtures made of a defined number of chemicals. Testing of defined mixtures may thus be a good compromise between the simplicity of testing single chemical testing and complexity of testing complex mixtures.

Currently, there are some crucial data and knowledge gaps related to how the presence of multiple substrates influence the biodegradation kinetics of the individual mixture components when testing at environmentally relevant concentrations, as well as a need to better understand the factors that govern the extent and kinetics of biodegradation for chemicals in mixtures. This is in spite of the fact that many biodegradation studies conducted with single test chemicals are actually multi-substrate studies due to a natural content of assimilable organic carbon in e.g. surface waters and wastewater (Helbling et al., 2014).

4 Mixture and concentration effects

4.1 Effect of multiple substrates

As outlined in Paper II (Hammershøj et al., 2019b), the presence of multiple substrates can lead to either enhancement or inhibition of biodegradation of the individual substrates. Interactions that can enhance degradation include co-metabolism and increased biomass growth (Chang et al., 1993; Knightes and Peters, 2006). Inhibiting interactions include competitive inhibition and toxicity at elevated concentrations (Bouchez et al., 1995; Stringfellow and Aitken, 1995). The mixture interactions can also reinforce or counteract each other (Knightes and Peters, 2006), and there is no agreement in the literature on whether the combined effects of multiple substrates are more likely to enhance or inhibit biodegradation (Hammershøj et al., 2019b Paper II). One factor that can affect the extent and direction of the mixture effect on biodegradation kinetics is the composition of the microbial community partaking in the degradation and, most importantly, whether a pure or mixed bacterial culture is used (Ellis et al., 1998; Reardon et al., 2002). Other key variables are the concentration of the test chemicals and the mixture complexity, that is, the composition and number of mixture components (Desai et al., 2008; Dimitriou-Christidis and Autenrieth, 2007; Guha et al., 1999; Knightes and Peters, 2006; Stringfellow and Aitken, 1995).

All of the above mentioned studies on mixture effects, except Ellis et al. (1998) and Hammershøj et al. (2019b Paper II), used pure or simple mixed cultures or pre-exposed inocula. Furthermore, many previous studies on mixture effects, especially for petroleum hydrocarbons, were related to bioremediation of contaminated sites, and test concentrations were thus near or even above water solubility (e.g. Bouchez et al., 1995; Desai et al., 2008; Stringfellow and Aitken, 1995). In most natural aquatic environments, the concentration of HOCs will be governed by natural distribution processes and thus often be several orders of magnitude below water solubility. When Ellis et al. (1998) studied the effect of simultaneous biodegradation of multiple substrates on the biodegradation kinetics of individual substrates in activated sludge bioreactors, they found only small stimulatory effects of the multiple substrates in comparison with single substrate tests. They argued that this was due to a low substrate concentration compared to the density of specific degraders, which underlines the need for studies on mixture effects in biodegradation testing at environmentally relevant concentrations and with natural microbial communities.

The aim of Paper II was to study how the number of compounds in a mixture affects the biodegradation kinetics of the individual compounds when testing at low chemical concentrations and using surface water with its native microbial community as inoculum (Hammershøj et al., 2019b). The main hypothesis of this work was that the total substrate concentrations would have a higher impact on biodegradation kinetics than the number of mixture components. Sixteen petroleum hydrocarbons were selected for the study and three of these were used as model compounds (2-methylnonane, 1,2,4-trimethylbenzene and *trans*-decalin). The three model compounds were tested individually and in mixtures with 2, 7 and 15 additional hydrocarbons (Figure 10) at concentrations two to three orders of magnitude below solubility for each mixture constituent (Figure 11).

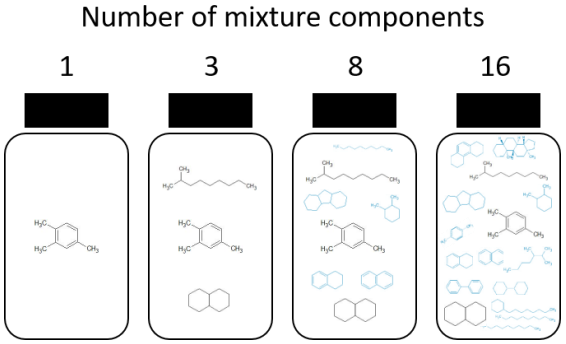


Figure 10: Experimental setup to study the effect of mixture complexity, i.e. number of mixture components, on biodegradation kinetics of chemicals in mixtures. 1,2,4-trimethylbenzene is used as example. Modified from Paper II (Hammershøj et al., 2019b)

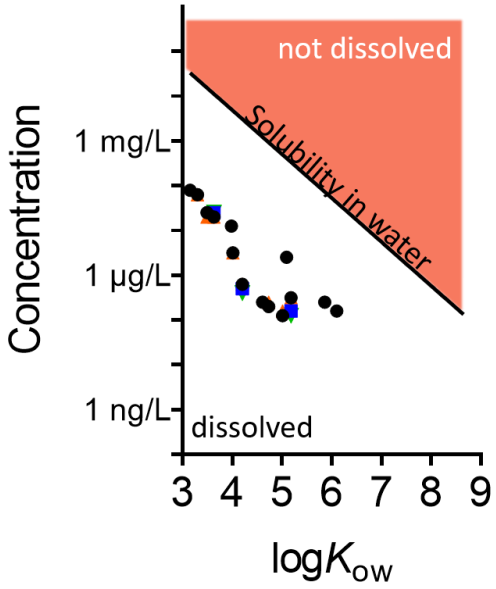


Figure 11: Test concentration versus log K_{ow} for petroleum hydrocarbons in biodegradation experiments presented in Paper II. • indicate the mixture of 16 hydrocarbons, ▲ indicate the mixture of eight hydrocarbons, ■ indicate the mixture of three hydrocarbons and ▼ indicate the three model hydrocarbons tested alone (see figure 10)

In general, biodegradation testing of the model chemicals alone and in the three mixtures revealed limited or no effect of the increasing number of mixture components (Figure 12). One deviation was observed for 1,2,4-trimethylbenzene, which was degraded in the three mixtures but not when tested alone. However, the test with 1,2,4-trimethylbenzene alone was terminated already after 14 days as the test duration was pre-determined based on a simple preliminary test, where 1,2,4-trimethylbenzene was degraded in less than a week (data not shown). The preliminary test was conducted two weeks before the real experiment, and the biomass composition could have been affected by a change in temperature in the time between the tests, resulting in an absence of competent degraders (Hammershøj et al., 2019b Paper II). While this might explain a longer lag phase or lack of degradation for 1,2,4-trimethylbenzene tested alone, it does not explain the disagreement with 1,2,4-trimethylbenzene tested in the mixtures.

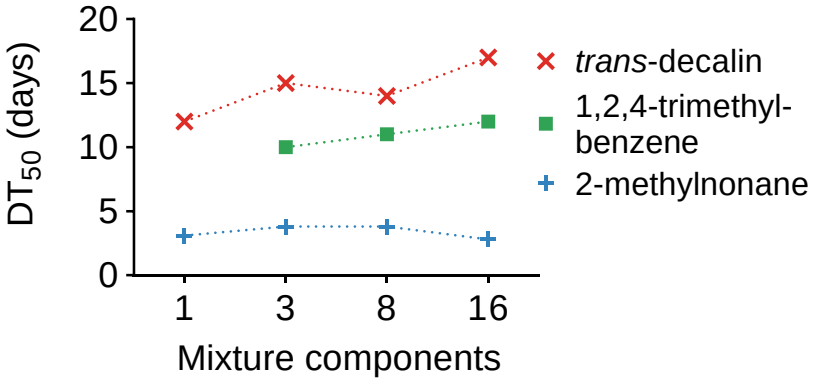


Figure 12. Biodegradation half times for trans-decalin, 1,2,4-trimethylbenzene, and 2-methylnonane tested in mixtures of 1, 3, 8, and 16 hydrocarbons. From Hammershøj et al., (2019b) Paper II.

If leaving 1,2,4-trimethylbenzene tested alone out of account, the biodegradation half times (DT_{50} = lag phase + one half-life) were largely constant between the tests, with the largest relative difference observed for *trans*-decalin tested alone and in the mixture with 16 constituents (factor of 1.4, Figure 12). In comparison, Birch et al. (2017b) found that different sources of surface water inoculum resulted in one order of magnitude differences in biodegradation kinetics for nine petroleum hydrocarbons.

A possible explanation for the limited effect of multiple substrates observed in Paper II is the low test concentration, as it was observed that biodegradation half-times for the three model compounds were affected more by the initial substrate concentration than by the number of components in the mixtures (Hammershøj et al., 2019b). Ellis et al. (1998) also observed only a small stimulating effect by the presence of multiple substrates on biodegradation kinetics of the individual substrates at low substrate concentrations. In general, there is a close link between concentration level and the effect and degree of mixture interactions. For instance, the likelihood of competitive inhibition for enzymatic sites must be higher if the chemical concentration is so high that there is enzyme saturation. Similarly, biomass growth is also related to the concentrations of the substrates.

4.2 Test concentration

The relationship between chemical concentration and biodegradation kinetics is relatively well studied for single chemicals (e.g. Boethling and Alexander, 1979; Hammershøj et al., 2019 Paper II; Smith et al., 2012; Toräng et al., 2003) as briefly outlined in Section 2.2. However, the concentration effect on biodegradation kinetics is less predictable when conducting tests with natural microbial communities and a mixture of substrates. For example, biodegradation has been observed to be limited for chemicals tested alone at low concentration (low ng/L range, Boethling and Alexander, 1979) while bacteria have also been seen to grow on a multitude of carbon substrates that were each present at minuscule concentrations (Egli, 2010). Biodegradation kinetics can be affected by competitive inhibition, as discussed in the paragraph above, but also by toxic inhibition. Hydrophobic organic chemicals (HOCs) typically exert baseline toxicity at concentration above 1% of solubility, but mixture interactions can result in lower or higher toxicity than expected (Schmidt et al., 2013; Schmidt and Mayer, 2015; Smith et al., 2013). At the same time, not all microorganisms are equally sensitive to HOCs (Bouchez et al., 1995; Winding et al., 2019).

Prince et al. (2017) studied the degradation of a dispersed crude oil in seawater at different initial crude oil concentrations (between 2.5 and 2500 ppm_V), and observed that biodegradation slowed significantly as the oil concentration increased. However, some constituents were present as oil droplets in all of these tests, as the least soluble constituents were less than 0.1 % dissolved even at the lowest test concentration. Reviewing the literature, no studies were identified that systematically studies the effects of test concentration on biodegradation kinetics of hydrophobic complex mixtures at concentrations below solubility. Thus, this was the focus of Paper IV (Hammershøj et al., 2019c). Here, the influence of test concentration on primary biodegradation kinetics for constituents in two complex mixtures, diesel oil and lavender oil, was studied. The two mixtures were tested at concentrations near saturation and approximately two orders of magnitude lower. Incorporation of radiolabelled leucine, as described in Brandt et al. (2004), was conducted in parallel to the biodegradation experiments as a measure for microbial productivity. The hypothesis was that biodegradation kinetics of mixture constituents will be delayed at high concentration of the mixture compared to the low concentration (i.e. longer lag phase and half-life) and that this delay is caused by toxicity of the mixture (Hammershøj et al., 2019c Paper IV).

As described in Section 3.4, fewer constituents were detectable at the low than at the high test concentrations. Biodegradation kinetics were obtained at both test concentrations for 62 diesel oil and 10 lavender oil constituents. Figure 13 shows biodegradation half times (lag phase + one half-life) obtained for some of these constituents (only data from fits with $R^2 > 0.8$ included) at high versus low concentration.

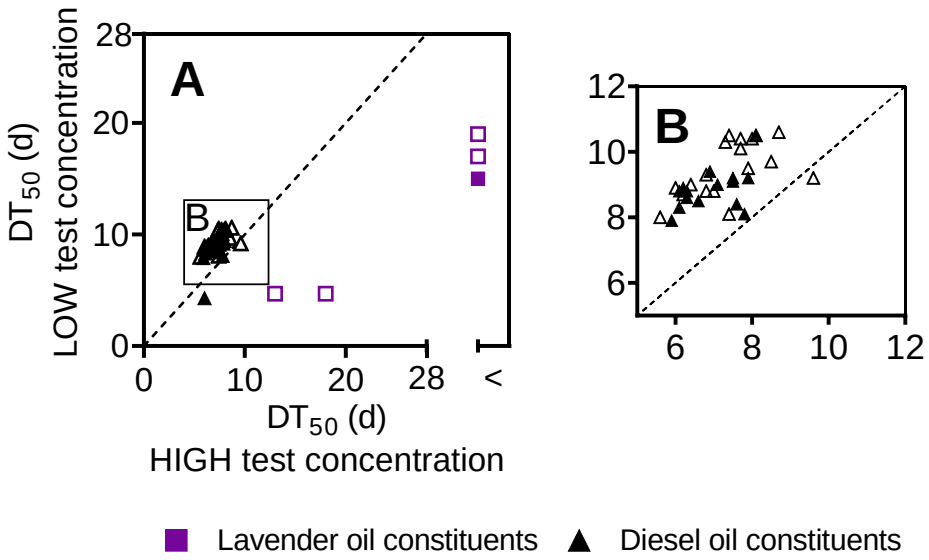


Figure 13: A) Biodegradation half times (DT_{50}) for diesel oil and lavender oil constituents at low test concentration vs at high test concentration. The dotted line is unity. <: Degradation not initiated within test duration. Filled symbols indicate the data with the best fits. B) is a zoom of data in A. From Hammershøj et al. (2019c Paper IV)

All but four diesel oil constituents were degraded within the 28 day test duration. The four diesel oil constituents that were only partly degraded at the end of the biodegradation study are not included in Figure 13 as they were only quantifiable at the high test concentration (Hammershøj et al., 2019c Paper IV). The diesel oil constituents that were degraded had very similar half times at the two test concentrations (Figure 13), with only marginally longer half times at the low concentration due to longer lag phases. Contrary to this, not all lavender oil constituents were degraded. For those that were, biodegradation was slower or even absent at the high test concentration compared to the low test concentration (Figure 13A).

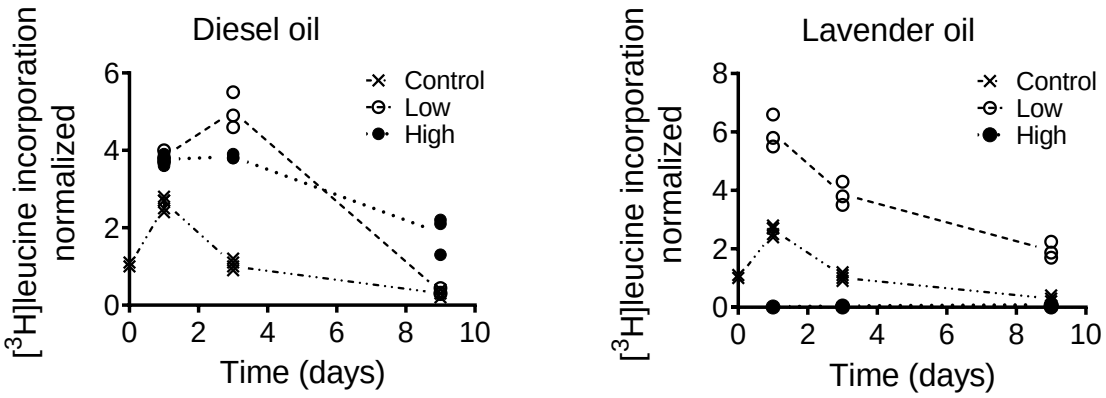


Figure 14: [^{3}H]Leucine incorporation measured in parallel with the biodegradation experiment (first nine days). The amount of leucine incorporated at the low test concentration (Low) and high test concentration (High) is normalised against the uptake of [^{3}H]leucine in five pure inoculum controls (Control) measured at day 0. Modified from paper IV (Hammershøj et al., 2019c).

The biodegradation behaviour of diesel oil and lavender oil constituents was quite consistent with the observation for leucine incorporation. Diesel oil stimulated the microbial productivity at both low and high test concentration relative to the pure inoculum controls (Figure 14). Lavender oil stimulated the productivity at low concentration whereas a strong inhibition was observed at high concentration (Figure 14), which was best explained by toxicity near the solubility limit (Hammershøj et al., 2019c Paper IV). The dissimilar concentration effect observed for diesel and lavender oil may be explained by the inoculum containing degraders that were not very sensitive to diesel oil. This underlines the complex relationship between a mixed microbial community, a complex mixture of substrates and substrate concentrations. It also underlines how crucial it is to consider the substrate concentration in biodegradation studies, for single chemicals as well as for mixtures (Hammershøj et al., 2019b Paper II, 2019c Paper IV).

5 Conclusions

Recent developments in analytical chemistry provide an excellent basis for biodegradation testing of chemicals in mixtures at environmentally relevant concentrations. A novel biodegradation platform was used to obtain primary biodegradation kinetics for individual HOCs tested in self-prepared mixtures and natural complex mixtures. The main findings are:

- Using mixtures of chemicals at environmentally relevant concentrations for biodegradation testing is a practical way of obtaining primary biodegradation kinetics for several chemicals in one test, potentially covering a large chemical space. Data for chemicals tested together are generated with the same inoculum and under the same test conditions, which provides an excellent basis for studying how chemical-specific variables affect biodegradation kinetics.
- Little to no effect was observed on biodegradation kinetics for hydrocarbons when increasing the number of constituents from one to sixteen, whereas, higher test concentrations of single hydrocarbons led to longer lag phases and half-lives and even a complete inhibition of degradation at concentrations near saturation. Biodegradation kinetics for individual hydrocarbons were thus affected more by the initial concentration than by the number of hydrocarbons in the test system. A knowledge gap remains regarding the effect of the presence of multiple substrates on biodegradation kinetics at environmentally relevant concentrations, especially on the difference between single chemicals and complex mixtures.
- When increasing the test concentrations, biodegradation kinetics were largely unchanged for the diesel oil constituents while biodegradation of the lavender oil constituents was delayed and limited at the high test concentration. This was likely related to toxic inhibition and the presence of less sensitive degraders. In general, there are still many research questions related to biodegradation testing of hydrophobic complex mixtures at environmentally relevant concentrations.
- This PhD thesis emphasizes the importance of understanding and controlling the chemical exposure (concentration and composition) in biodegradation tests of HOC mixtures. The most important exposure criteria are that the concentrations are below solubility, i.e. all chemicals are dissolved, and below toxic levels, and furthermore preferably also at environmentally relevant levels.

The analytical challenges are one of the main limitations to biodegradation testing of mixtures, and the challenges are bigger for a complex mixture than for a well-defined mixture. The partitioning-based biodegradation platform used in this PhD study can readily be used to generate biodegradation data for well-defined mixtures of a limited number of HOCs while testing of complex mixtures requires additional studies.

Today, persistence assessment of complex mixtures such as UVCB substances is centred on mapping the single constituents or constituent blocks with similar characteristics, which then can be tested. However, it is not always feasible or possible to detect all constituents of a highly complex mixture. Instead, it could be investigated whether a biodegradation test of the whole mixture can be used to screen for persistent components, as these will also not be readily degraded in a mixture. Currently, new research is being directed at whole substance approaches for hazards assessment of UVCB substances, and potentially the development of fate-directed ecotoxicity assessment (Cefic-LRI, 2019). If successful, such an approach could result in more efficient and relevant testing strategies for complex mixtures.

Many of the biodegradability tests used within a regulatory context are more than 20 years old and were not designed for the broad spectrum of chemicals they are applied to today, and especially for hydrophobic and volatile chemicals there is a need for improvements. This PhD study is a step towards improved biodegradation kinetics testing of hydrophobic organic chemicals at low concentrations.

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7 Papers

I Birch, H.; **Hammershøj, R.**; Mayer, P. Determining Biodegradation Kinetics of Hydrocarbons at Low Concentrations: Covering 5 and 9 Orders of Magnitude of K_{ow} and K_{aw}. *Environ. Sci. Technol.* 2018, 52, 4, 2143-2151

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III **Hammershøj, R**.; Birch, H.; Sjøholm, K.K.; Mayer, P. Accelerated passive dosing of hydrophobic complex mixtures – controlling level and composition in aquatic tests. *In revision for Environ. Sci. Technol.*

IV **Hammershøj, R**.; Sjøholm, K.K.; Birch, H.; Brandt, K.K.; Mayer, P. Investigating the effect of concentration on biodegradation kinetics for two hydrophobic complex mixtures. *Manuscript draft.*

In this online version of the thesis, **paper I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from:

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