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Biodegradation of hydrocarbon mixtures in surface waters at environmentally relevant levels – effect of inoculum origin on kinetics and sequence of degradation

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Abstract

Biodegradation is a dominant removal process for many organic pollutants, and biodegradation tests serve as tools for assessing their environmental fate within regulatory risk assessment. In simulation tests, the inoculum is not standardized, varying in microbial quantity and quality, thereby potentially impacting the observed biodegradation kinetics. In this study we investigated the effect of inoculum origin on the biodegradation kinetics of hydrocarbons for five inocula from surface waters varying in urbanization and thus expected pre-exposure to petroleum hydrocarbons. A new biodegradation

method for testing mixtures of hydrophobic chemicals at trace concentrations was demonstrated: Aqueous solutions containing 9 hydrocarbons were generated by passive dosing and diluted with surface water resulting in test systems containing native microorganisms exposed to test substances at ng-µg/L levels. Automated Headspace Solid Phase Microextraction coupled to GC-MS was applied directly to these test systems to determine substrate depletion relative to abiotic controls. Lag phases were generally less than 8 days. First order rate constants were within one order of magnitude for each hydrocarbon in four of the five waters but lower in water from a rural lake. The sequence of degradation between the 9 hydrocarbons showed similar patterns in the five waters indicating the potential for using selected hydrocarbons for benchmarking between biodegradation tests. Degradation half-times were shorter than or within one order of magnitude of BioHCwin predictions for 8 of 9 hydrocarbons. These results showed that location choice is important for biodegradation kinetics and can provide a relevant input to aquatic exposure and fate models.

Keywords

Biodegradation kinetics, volatile petroleum hydrocarbons, composed mixtures, SPME, Passive dosing

1. Introduction

Biodegradation is a dominant removal process for many organic pollutants in the aquatic environment, and quantitative knowledge and data on biodegradation kinetics are therefore needed in water research, environmental risk assessment and aquatic fate models. Petroleum products are complex mixtures of varying composition consisting of a large number of chemical structures and degraded by equally complex microbial communities in the environment (Abbasian et al., 2015; Hazen et al., 2016; Head et al., 2006; Van Hamme et al., 2003). Experimental biodegradation data are not available for most of the

37 constituents of petroleum products. Therefore a biodegradation prediction model (BioHCwin) has been
38 developed (Howard et al., 2005) and shown to perform well for classifying persistence conservatively
39 (Prosser et al., 2016). However, there is a lack of high-quality experimental kinetic data useful for
40 optimization of this and other prediction models (Rücker and Kümmerer, 2012).

41 Suitable testing methods are important for generating high-quality experimental data at environmentally
42 realistic concentrations (Prince et al., 2017). Most of the constituents in petroleum products are
43 hydrophobic and have very low solubilities. Passive dosing from a silicone polymer has earlier been used
44 to improve and simplify test methods for hydrophobic organic chemicals in toxicity tests (Smith et al.,
45 2010) and distribution measurements (Birch et al., 2012) by producing stable concentrations of freely
46 dissolved chemicals and avoiding the use of solvents in the test systems. Passive dosing has also been
47 introduced for biodegradation tests of single chemicals (Smith et al., 2012) and mixtures (Comber et al.,
48 2012). In this study we designed and included a passive dosing method in biodegradation tests to
49 investigate mixtures composed of potential petroleum hydrocarbons at environmentally relevant
50 concentrations, 2-4 orders of magnitude below the solubility of the test chemicals.

51 Although the physical and chemical test conditions which can affect biodegradation (Leahy and Colwell,
52 1990) are largely standardized in tests such as the OECD test series (OECD 301, 1992; OECD 303, 2001;
53 OECD 306, 1992; OECD 309, 2004), the biological conditions of the inoculum is poorly defined and
54 variable with respect to cell density, species, origin and history of the sample (Kowalczyk et al., 2015;
55 Thouand et al., 2011). Pre-exposure to the tested chemical or similar chemical structures has been seen
56 to enhance degradation by adaptation of the microbial community (Bauer and Capone, 1988; Leahy and
57 Colwell, 1990; Marchal et al., 2003), and lag phases have been seen to depend on cell density in tests
58 (Caparello and Larock, 1975), pre-adaptation of inoculum (Toräng and Nyholm, 2005) and total amount

of specifically degrading bacteria (Ingerslev et al., 2000). Previous studies reported the difference in biodegraded percentages of gasoline, diesel oil, crude oil and kerosene in soil samples (Hamamura et al., 2013; Marchal et al., 2003), hexadecane in surface water samples (Caparello and Larock, 1975), crude oil constituents in sea water (Kristensen et al., 2015), diesel and lubricating oil in sediments (Powell et al., 2007) and selected hydrocarbons in water-sediment mixtures from lakes (Cooney et al., 1985). A concept has been proposed recently to target the environmental microbial variability from a probability perspective in which 10 different inocula are used in ready biodegradability tests to evaluate the probability of biodegradation in the environment (Thouand et al., 2011). However, the kinetics of the biodegradation was not targeted in the above mentioned studies. Given that diverse microbial communities of hydrocarbon degraders are generally present in the aquatic environment (Hazen et al., 2016; Van Hamme et al., 2003), the lag phase, being the time during which the bacteria adjust to the substrate and multiply, is potentially the main parameter influenced by pre-exposure of the inoculum. Once degradation is initiated, the rates could therefore be similar and independent of the level of pre-exposure of the sampling locations. The aim of this study was therefore to investigate the effect of the inoculum origin on biodegradation kinetics. The surface water sampling locations were thus selected to represent different degrees of pre-exposure to petroleum hydrocarbons. Pre-exposure to petroleum hydrocarbons was assumed for locations receiving rainwater runoff discharges (Göbel et al., 2007). Locations ranging from rural locations with no known point sources to urban locations receiving rainwater runoff discharges were selected based on point source information from the Danish river basin management plans (The Danish Nature Agency, 2011a, 2011b). We tested the degradation in natural surface water samples without addition of sediment to ensure a high bioavailable fraction and corrected for partitioning to headspace (Birch et al., 2017).

For petroleum hydrocarbons, a general sequence of susceptibility towards biodegradation has been reported as *n*-alkanes > *iso*-alkanes > low-molecular weight aromatics > cyclic alkanes > PAHs, although exceptions are seen (Leahy and Colwell, 1990). This sequence has also been observed in the field, where after oil spills, the abundance of specialist alkane degraders generally show a bloom followed by a bloom of specialist aromatic hydrocarbon degraders (Head et al., 2006). A further aim of the study was therefore to determine whether the sequence in which the hydrocarbons were degraded remained the same across the sites. If this was the case, then the design would not only facilitate comparisons of biodegradation results from different water sampling locations but also provide a new basis for extrapolating biodegradation results.

The focus of this study was therefore to see whether (1) using water from locations with higher urbanization would result in shorter lag phases in the biodegradation test compared to locations with less urbanization, (2) biodegradation rate constants would be less affected than lag phases by the sampling location and (3) the sequence of degradation of the hydrocarbons would be consistent across different locations. Additionally, we compared the obtained biodegradation kinetic data from the present study to BioHCwin predictions.

2. Materials and methods

2.1. Materials

Test chemicals included potential petroleum hydrocarbons with 8 to 12 carbon atoms: *n*-decane, tetralin, biphenyl, *trans*-decalin, bicyclohexyl, 1,2,4-trimethylbenzene (tmbenzene) and naphthalene (Sigma-Aldrich, Copenhagen, Denmark, purity ≥ 98%), 2,3-dimethylheptane (dmheptane) and 1,3,5-trimethylcyclohexane (tmcyclohexane) (TCI chemicals, Zwijndrecht, Belgium, purity ≥ 98%). 1-octanol (Sigma-Aldrich, Copenhagen, Denmark, ≥ 99%) was included as a reference chemical (positive reference

for microbial activity in the test). Passive dosing systems were prepared using translucent silicone rods (custom-made by Altec Products Ltd., Victoria, UK, product code 136-8380) in 100 mL amber Wheaton glass serum bottles with crimp seals and PTFE-coated silicone septa. Ethyl acetate (Sigma-Aldrich, Copenhagen, Denmark, ≥ 99.7%) and ethanol (VWR chemicals, Søborg, Denmark, 99.8%) were used for cleaning of silicone rods. LaboStar^{TM} 1-DI ultrapure water system from SGwater (Hamburg, Germany) was used to produce ultrapure water.

2.2 Generating stock solution by accelerated passive dosing

Silicone rods were cleaned, loaded with test chemicals and equilibrated with ultrapure water to produce a stable and reproducible low concentration stock solution for the biodegradation tests. The cleaning procedure included washing three 20 g silicone rods (diameter: 3 mm, approx. length: 2.6 m) in a dishwasher without the use of cleaning agents, soaking in ethyl acetate for 24 hours, soaking in ethanol for 24 hours and heating to 120 ˚C for 2 hours. Equal mass of the 9 test chemicals were mixed and the two solid hydrocarbons readily dissolved in the liquid hydrocarbons. This mixture was added to the rods (200 µL each) and was completely absorbed into the silicone by swelling (bottles were rolled horizontally for > 24 hours). Subsequently, each silicone rod was equilibrated with 65 mL of ultrapure water on a roller at room temperature for 1 hour. Without knowing the exact partition ratio between silicone and water for each chemical, adding an equal mass of chemical to the silicone results in water concentrations that are roughly the same fraction of the solubility for each chemical. Equilibration times of < 10 minutes were confirmed for all test chemicals prior to the biodegradation tests. The resulting stock solution was diluted 10 times when preparing test systems and test concentrations were later measured for three abiotic controls to approximately 0.03 µg/L bicyclohexyl, 0.2 µg/L *n*-decane and 2,3-dimethylheptane, 0.5 µg/L *trans*-decalin and 1,3,5-trimethylcyclohexane, 30 µg/L biphenyl and tetralin, 60 µg/L 1,2,4-

trimethylbenzene and 70 µg/L naphthalene, which was 2-4 orders of magnitude below the water solubility of each test chemical. Transfer of stock solution to test systems was done using gas tight syringes (glass, PTFE). The loaded silicone rods were used multiple times without reloading and mass balance calculations showed < 3 % depletion of the silicone.

2.3 Surface water inocula

Five surface water grab samples, collected from three Danish streams and two Danish freshwater lakes, were used as inocula (Supporting information S1). Samples were collected from the 12th to 21st October 2015 and used within 24 hours after collection. A description of the locations can be seen in Table 1.

Temperature, pH, dissolved oxygen and conductivity were measured immediately after sampling using portable meters. Indicative levels of nitrate, ortho-phosphate and ammonium were measured with manufactured test kits from Merck Millipore. Initial bacterial density was measured as heterotrophic plate counts using a non-selective agar (Fluka® Analytical 17209 R-2A agar) within 24 hours of sampling. H_3PO_4 was added to a subsample from each location (to conserve the sample at pH 2) and nonvolatile organic carbon (NVOC) was measured on a Shimadzu Total Organic Carbon Analyzer TOC-Vwp. Background concentrations of test chemicals were below detection limits in all surface waters.

144 Table 1: Surface water sampling locations

Characteristics	Location name	Catchment	Possible petroleum hydrocarbon pre-exposure	Ecological status*
Rural lake	Maglesø	Woods, shrubbery and field	No known point sources	High
Urban lake	Lyngby lake	Urban and rural areas through a tributary stream	Stormwater runoff from paved areas and combined sewer overflows	Bad
Rural stream	Fønstrup stream	Woods	No known point sources	High
WWTP impacted stream**	Gadevangs stream	WWTP** outlet, Meadow and copse	WWTP** outlet and combined sewer overflow	Moderate
Urban stream	Harrestrup stream	Urban areas	Stormwater runoff from paved areas	Poor

145 *according to the criteria detailed in the European Water Framework Directive, Annex V, High = no or
146 only very minor anthropogenic alterations to the water body relative to those normally associated with
147 that type under undisturbed conditions, Good = low levels of anthropogenic alterations, Moderate =
148 moderate alterations, Poor = major alterations, Bad = severe alterations (European Parliament, 2000), as
149 evaluated by The Danish Nature Agency (2011a, 2011b) **WWTP= wastewater treatment plant
150
151 **2.4 Biodegradation testing**
152 The biodegradation experiments were designed to measure biodegradation by substrate depletion in
153 test systems relative to abiotic controls. Each biodegradation experiment was therefore composed of a
154 series of test systems and an equal number of abiotic systems that were incubated in parallel. All
155 systems were prepared in 20 mL amber glass vials closed with screw caps and PTFE-coated silicone
156 septa. To prepare the test systems, 1.5 mL stock solution was diluted 10 times into surface water spiked
157 with 30 µg/L of 1-octanol. Abiotic systems were prepared with ultrapure water instead of surface water.
158 Blank controls were included. The test- and abiotic systems were incubated at 20 °C on a bench top
159 laboratory roller at ≈ 30 rpm. At different time points (e.g. day 0, 1, 2, 3, 4, 5, 7, 10, 14, 21, 28 and 56)

triplicate test systems, triplicate abiotic systems and a blank control were sacrificed for chemical analysis.

2.5 Chemical analysis

Automated Headspace Solid Phase Micro Extraction (HS-SPME) (PAL RSI 85 auto sampler) coupled to Gas Chromatography – Mass Spectrometry (GC-MS) (Agilent Technologies 7890B/5877A GC/MSD) was applied to measure the 9 hydrocarbons. Rather than measuring on sub-samples of the test system, automated HS-SPME was applied directly to test systems and abiotic controls to optimize the analytics, minimize losses and minimize the risk for contaminations. HS-SPME sampling was done for 10 minutes at 35 °C directly in the 20 mL test systems using a 100 µm PDMS fiber (SUPELCO, Bellefonte) with shaking at 250 rpm alternating 5 seconds on and 2 seconds off. Desorption was performed at 250 °C in the injection port using a 2:1 split for 3 min followed by a 33:1 split for 2 minutes. Separation was done on a 30 m DB-1ms column of 0.25 mm inner diameter and 0.25 µm film thickness using Helium as carrier gas at 1.2 mL/min. Oven temperature was 35 °C for 6 minutes followed by a ramp of 15 °C/min to 300 °C. The GC signal was split between the MS and a FID, however, only the MS signal was used. Transfer line temperature was 270 °C and the MS was run in SIM mode using a quantifier and qualifier ion for each chemical. The method was operational down to typically 10 ng/L concentrations, depending on the properties of the chemicals.

2.6 Data analysis

Aqueous standard solutions were used to determine the initial concentration level in the experiments. For the rest of the experiment, biotic test systems were always referenced against abiotic test systems: The measurement of each biotic test system was normalized by the mean of the three abiotic controls

that were measured on the same GC-run. This yielded the relative response R for each biotic test system, which was plotted as a function of incubation time and fitted to a first order degradation model with lag phase (Equation 1) using no weighting and considering each replicate as an individual point in GraphPad Prism 5.00.

(1) $$R = \begin{cases} R_0\,, & if\ t < t_{lag} \\ R_0 \cdot \exp\left(-k_{system} \cdot \left(t - t_{lag}\right)\right), & if\ t > t_{lag} \end{cases}$$

R_0 is the relative response at time 0, t is the incubation time (days), t_{lag} is the lag phase (days) and k_{system} is the first order degradation rate constant of the test system (days^{-1}).

Additional to the kinetic model, the 10% degradation time, DT_{10} and the degradation half-time, DT_{50}, which describe the time at which 10% and 50% of the test chemical was degraded in the test, respectively, was found by equation (2) and (3).

(2) $$DT_{10} = \frac{\ln(10/9)}{k_{system}} + t_{lag}$$

(3) $$DT_{50} = \frac{\ln(2)}{k_{system}} + t_{lag}$$

As described in Birch et al. (2017) some of the chemicals distribute into the headspace. The first order water phase degradation rate constant, k_{water}, was calculated using the dimensionless Henry's laws constant, K_H^*, volume of water, V_w, and headspace, V_h, in the test system according to equation (4) (Birch et al., 2017). The K_H^* and fraction in headspace are shown in Supporting Information (S3)

(4) $$k_{water} = k_{system} \cdot \frac{V_w + K_H^* V_h}{V_w}$$

3. Results and discussion

3.1 Surface water sample characteristics

The reference compound, 1-octanol, was added to demonstrate the microbial activity of the inocula (positive reference), and were degraded within one day in all five inocula.

Bacterial densities (heterotrophic plate counts) were higher in the streams than in the lakes, and the urban lake and stream had higher densities than the rural lake and stream respectively (rural lake: $1.4 \cdot 10^3$ CFU/mL, urban lake: $4.6 \cdot 10^3$ CFU/mL, rural stream: $1.2 \cdot 10^4$, wastewater treatment plant (WWTP) impacted stream: $1.5 \cdot 10^4$, urban stream: $8.0 \cdot 10^4$ CFU/mL). The differences are probably caused by the regular discharges of organic matter into urban streams compared to rural streams. The bacterial densities are in the same range as the number of cells recommended in the standard OECD test guideline 'ready biodegradability' (OECD 301, 1992). The legends in Figures 1-3 are ordered from the highest to lowest bacterial densities.

The five surface water samples had a pH between 7.7 and 8.0, organic carbon (NVOC) of 6 - 10 mg/L and temperatures between 8 - 11 °C (see Supporting Information S2). These parameters were thus quite similar between test locations. Earlier studies have shown that uncharacterized dissolved organic carbon in test systems leads to higher degradation rates and shortening of the acclimatization phase of microorganisms (Kovarova-Kovar and Egli, 1998). However, because the amount of NVOC is similar in the investigated surface waters, this parameter is not considered to be important for the differences in biodegradation in this study.

The sample from the WWTP impacted stream showed slightly elevated concentrations of phosphate and nitrate (1.8 mg/L NO^{3-} and 0.14 mg/L PO_4^{3-}) compared to the level in the other samples (0.7-1.0 mg/L

NO^{3-} and 0.01 – 0.06 mg/L PO_4^{3-}). These nutrient levels are sufficient for the total degradation of the test chemicals, which were added in the µg/L concentration level.

3.2 Biodegradation

Figure 1 shows the fit of the first order degradation model with lag phase to biodegradation data for *n*-decane, biphenyl and *trans*-decalin. *n*-Decane is an example of a chemical which was quickly degraded in water from all test locations. Biphenyl was, similar to the other aromatic chemicals, quickly degraded after a lag-phase. *trans*-Decalin is an example of a chemical with a slower degradation. Degradation curves for the remaining chemicals are shown in Supporting Information (S4).

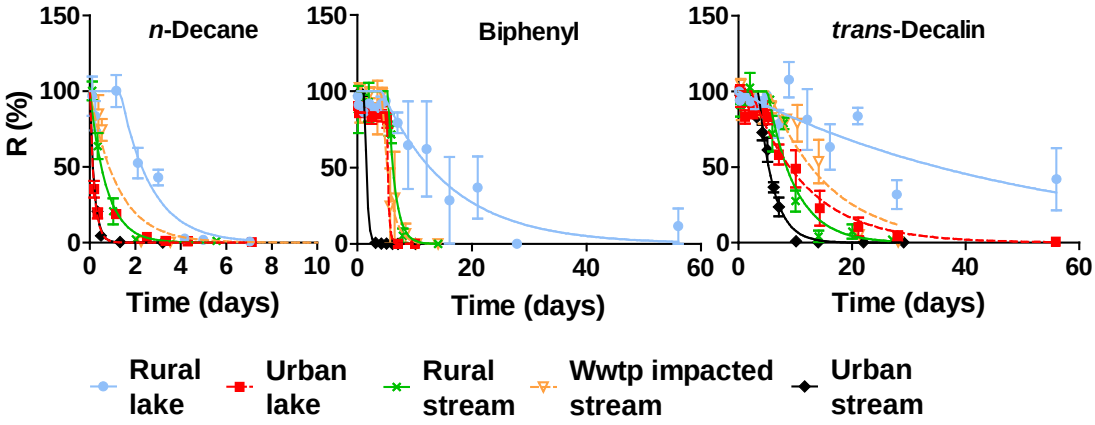


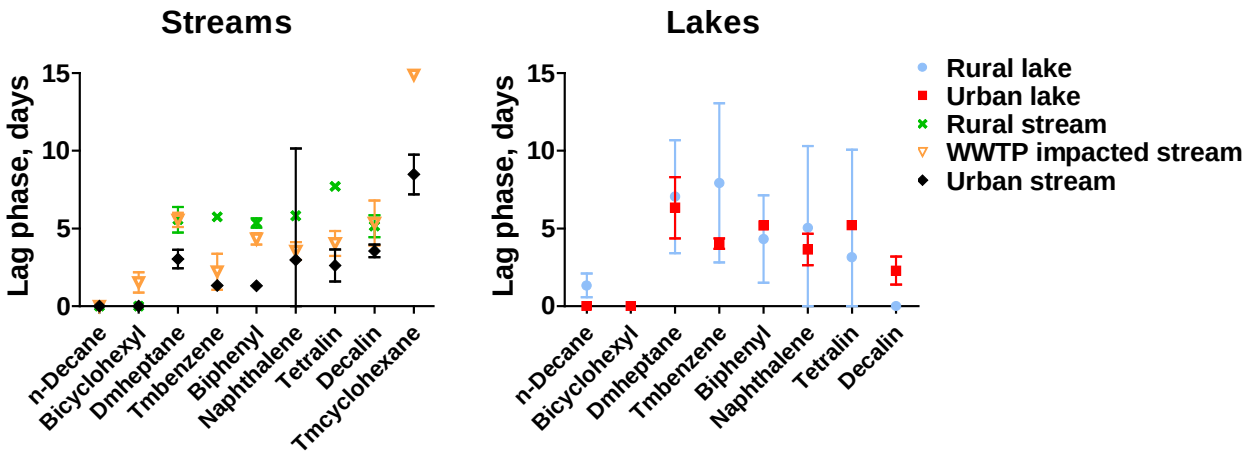
Figure 1: First order degradation curves for *n*-decane, biphenyl and *trans*-decalin in surface water from three streams and two lakes. Legend order indicates increasing initial bacterial density. Error bars show standard error of mean for the biotic test systems.

1,3,5-trimethylcyclohexane was the least degraded of the nine tested hydrocarbons. In the urban stream sample degradation started after 8 days, and it was degraded according to 0^{th} order kinetics after day 18

238 in the WWTP impacted stream. However, no degradation of 1,3,5-trimethylcyclohexane was seen in the
239 two lake samples, and degradation was only detected in two test systems in the rural stream sample
240 (one of three on day 14 and day 56). An explanation for the absence of biodegradation in some of the
241 samples could be that only rare specific degraders are able to degrade this hydrocarbon. The higher
242 microbial population in the urban stream and WWTP impacted stream would then increase the
243 probability of such degraders to be present in the test systems (Thouand et al., 2011). The fact that
244 1,3,5-trimethylbenzene was degraded in two test systems from the rural stream, which had the third
245 highest bacterial density, supports this explanation. The higher pre-exposure in the WWTP impacted
246 stream and the urban stream compared to the other samples, would further increase the probability of
247 specific degraders to be present in these samples. This structure is less susceptible to microbial
248 degradation than the other eight hydrocarbons tested in this experiment.

249 **3.3 Surface water origin effect on lag phase**

250 Lag phases were between < 1 day and 8 days for eight of the nine tested hydrocarbons (see Figure 2). In
251 samples from all five locations very short lag phases (<2 days) were observed for *n*-decane and
252 bicyclohexyl, which have the lowest aqueous solubilities and were added at very low concentration
253 levels (<1 µg/L). This absence of lag phase shows that bacterial growth and adaptation was not needed
254 for initiating the biodegradation of these two compounds and that microbial populations capable of
255 degrading these two chemicals were widely present in the surface waters in numbers sufficient for
256 degradation at low concentrations levels.



257
258

259 **Figure 2**: Lag phases for the test chemicals in five surface water samples. 95% confidence limits are
260 shown except for *trans*-decalin in the rural lake and tetralin in the urban lake, for which confidence limits
261 were wide.

262

263 Microbial activity and pre-exposure can affect lag phases. These two factors are, however, not
264 independent because pre-exposure can lead to higher microbial activity and because of the likely
265 correlation between pre-exposure and other anthropogenic pollution parameters such as eutrophication
266 and input of microorganisms. The streams with higher urbanization were found to have higher bacterial
267 densities. The microbial population in the urban stream is considered extensively pre-exposed to
268 petroleum hydrocarbons because it receives stormwater runoff discharges (occasionally thin oil films are
269 visible despite treatment in oil separators before discharge to the stream), and the WWTP impacted
270 stream is considered medium pre-exposed to petroleum hydrocarbons since most hydrocarbons are
271 removed in the treatment plant before discharge, but bacteria from the treatment plant are discharged
272 with the effluent. Generally shorter lag-phases were seen in the urban stream than in the WWTP

impacted stream and rural stream. This can be attributed to an effect of either the pre-exposure or the higher bacterial density in the samples.

The only comparison in which pre-exposure can be separated from bacterial density is the rural stream, which had a higher heterotrophic plate count than the more pre-exposed urban lake. The sample from the urban lake showed shorter lag phases than the sample from the rural stream for 4 of the chemicals and overlapping confidence limits for two chemicals even though the initial bacterial density was lower. This finding indicates that higher pre-exposure of test locations shortened the biodegradation lag phase compared to less pre-exposed locations, although only one pair of test locations could be used for this comparison.

For most of the chemicals, confidence limits for the lag phase were much wider in the sample from the rural lake than in the remaining samples. The reason for the wide confidence limits for the lag phase in the rural lake is the lower degradation rates observed in the rural lake (see Figure 1), which makes it difficult to determine the length of the lag phase with high accuracy and precision. An alternative method to find lag-phases is to determine the 10% degradation time, DT_{10} (see equation 2), the time at which the concentration is 90% of the initial concentration in the test systems. This method will primarily affect determination of lag phases if the degradation is slow, and in this case primarily the rural lake. The DT_{10} for the rural lake was between 5-10 days for the degraded chemicals except the fast degrading n-decane and bicyclohexyl, and thus decalin did not show a shorter lag-phase in the rural lake than in water from the other sites.

3.4 Comparison to BioHCwin predictions and sequence of degradation

Degradation half-times (lag phase + one first order half-life) and BioHCwin half-life predictions for the test chemicals are shown in Figure 3 (and listed in Supporting Information S5). For four of the chemicals, the BioHCwin predicted half-lives were higher than the observed degradation half-times in all five surface waters. For four chemicals, the BioHCwin predictions were within the range of observed half-times. For 1,3,5-trimethylcyclohexane, all the observed degradation half-times were longer than the BioHCwin prediction. The measured degradation half-times were thus shorter or within one order of magnitude of the BioHCwin predictions for 8 of 9 tested chemicals.

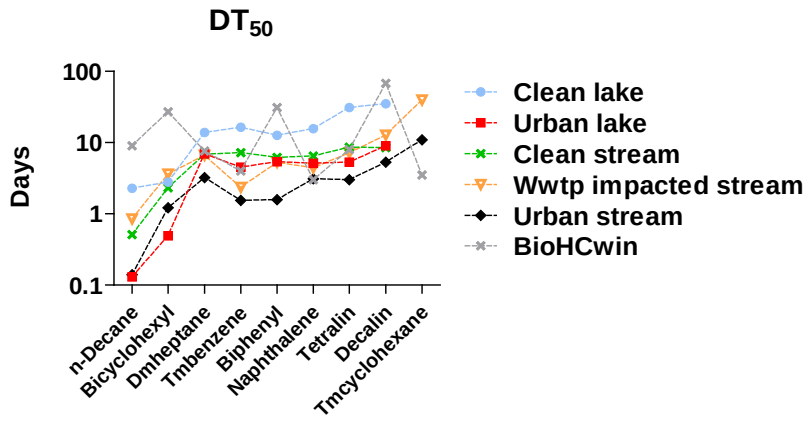


Figure 3: Degradation half-times (lag phase + one half-life) of test chemicals in surface water from five locations and BioHCwin predictions.

A preferential sequence of degradation of PAHs has earlier been observed using single strains or a mixed bacterial culture (Leblond et al., 2001; Wammer and Peters, 2005). However, it has also been found that in systems in which carbon sources are restricted and available as many different compounds at low concentrations, heterotrophic organisms do not utilize only one carbon source, but assimilate many compounds simultaneously (Kovarova-Kovar and Egli, 1998). The sequence of degradation of test

309 chemicals was in this study largely conserved between the five surface water locations as illustrated in
310 Figure 3. *n*-decane and bicyclohexyl were degraded first, then 2,3-dimethylheptane and the four
311 aromatic test chemicals were degraded simultaneously, then *trans*-decalin and lastly 1,3,5-
312 trimethylcyclohexane (if degraded at all). Overall, the general sequence of degradability of
313 hydrocarbons, *n*-alkanes > *iso*-alkanes > low-molecular weight aromatics > cyclic alkanes (Leahy and
314 Colwell, 1990), was therefore seen to describe the degradation in this experiment well. The exception
315 was the cyclic alkane bicyclohexyl, which was degraded before the low-molecular weight aromatics.
316 However, as will be discussed further below, the degradation rate constant of *trans*-decalin and 1,3,5-
317 trimethylcyclohexane is notably influenced by the test system dimensions, and these chemicals have a
318 shorter half-life in the water phase than in the test system (Birch et al., 2017). The BioHCwin predictions
319 did however not predict the same sequence of degradation.

320 The finding that the overall sequence of degradation was consistent in the samples from the different
321 locations opens the possibility for using benchmark chemicals in biodegradation testing. If a benchmark
322 chemical is included in a number of biodegradation studies it could then be used to relate or rank the
323 biodegradation capabilities of the inocula relative to each other. Furthermore, it could possibly be used
324 to estimate the degradation of a test chemical at one test location where only benchmark chemical
325 degradation was measured using data from another location where both the benchmark and test
326 chemical degradation were measured. In this way, kinetic data from different biodegradation
327 experiments could be compared even though the tests used inocula from different locations. This
328 approach would however be based upon the assumption that the factors that influence the degradation
329 of one chemical would change the degradation of another chemical in the same manner, and that there
330 would be no significant mixture effects on the degradation kinetics. Considering pre-exposure as such a
331 factor, the test chemical and benchmark chemical would have to have similar exposure routes for

locations to be pre-exposed to the chemicals to the same degree. This is a more reasonable assumption for chemicals which are emitted as mixtures (such as petroleum hydrocarbons) than for chemicals which are emitted as single substances (e.g., pesticides). More work needs to be done, to reveal the potential and limitations of the use of benchmark chemicals in biodegradation testing of hydrocarbons.

3.5 Surface water origin effect on first order rate constants

Test system and water phase first order degradation rate constants for the five surface waters are shown in Figure 4.

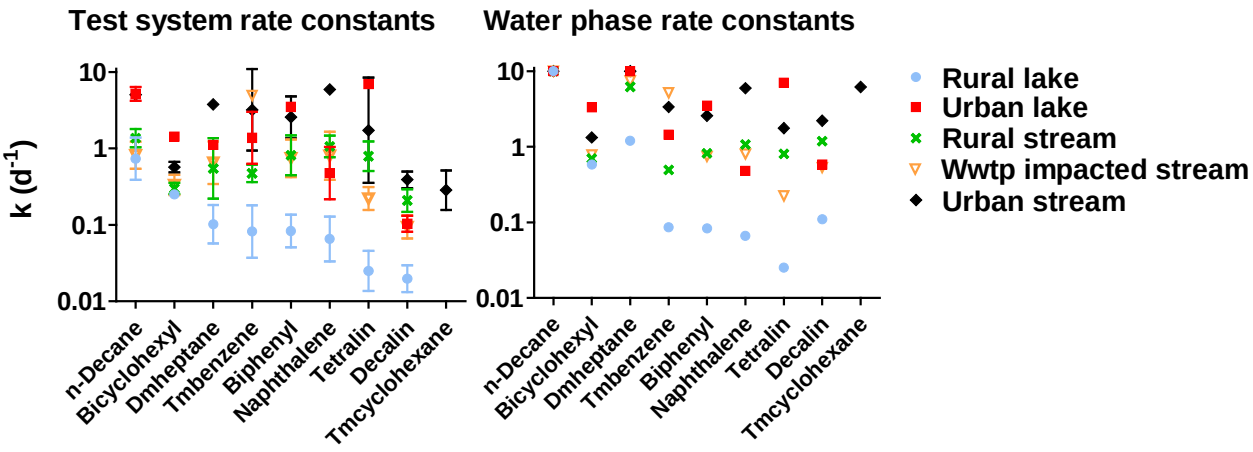


Figure 4: Test system (left) and water phase (right) first order degradation rate constants for nine test chemicals in five surface water samples. Water phase first order rate constants > 10 d^{-1} (5 *n*-decane and 2 dmheptane rate constants) were assigned a value of 10 d^{-1} in this figure. Error bars for test system rate constants show 95% confidence limits except some rate constants above 1, where large error bars were omitted from the graph for visibility reasons.

346 Contrary to our expectations, the biodegradation rate constants were affected to the same extent as the
347 lag phases by the differences in the urbanization of the sampling locations. The rate constants found in
348 samples from four of the test locations spanned approximately one order of magnitude, while lower rate
349 constants were found for the rural lake sample. Generally, the rate constants were highest in the urban
350 stream samples and lowest in water from the rural lake. For five of the chemicals the degradation rate
351 constants were higher in the urban lake sample, which had a lower initial bacterial density, than in the
352 rural stream sample, for two chemicals confidence limits overlapped, and for one chemical the rate was
353 higher in the rural stream sample.

354 When the test system rate constants were corrected for partitioning to headspace in order to determine
355 the water phase rate constants (Figure 3 right), the three first (*n*-decane, bicyclohexyl and 2,3-
356 dimethylheptane) and two last chemicals (*trans*-decalin and 1,3,5-trimentylcyclohexane) on the x-axis of
357 Figure 4 were mainly affected. This correction revealed that the main reason for a slower degradation of
358 *trans*-decalin was the headspace partitioning. The water phase rate constants for all tested chemicals in
359 the sample from the urban stream were above 1 d^{-1}.

360 The difference in biodegradation rates found here shows that choice of inoculum location can be an
361 important consideration for biodegradation testing and can influence biodegradation kinetics. The
362 results show that a rural location without surface water runoff discharges can reveal conservative
363 biodegradation rates. However since these types of locations are not representative of most surface
364 waters, other locations can be more appropriate as input to aquatic exposure and fate models.
365 Independent of the type of location, this study can be used as input to quantify the uncertainty
366 associated with biodegradation kinetic data employed in fate and exposure models.

3.6 Extrapolation from test to environment

Simulation biodegradation testing should ideally be conducted at environmentally realistic conditions in order to obtain data which can be extrapolated to the environment. The focus and emphasis in the present study was to improve environmental realism by testing of (1) composed mixtures rather than single compounds, (2) at environmentally relevant low concentrations (ng/L – µg/L) and (3) by populations of native microorganisms from the different locations. Other factors and aspects were not (yet) environmentally realistic: The tests were conducted at a temperature of 20°C, which is higher than at the sites from where the samples were taken and may lead to an underestimation of lag phases and half-lives. The tests were also conducted in closed systems of 15 mL, which implies a rather limited population of microorganisms and an even more limited population of metabolic potent microorganisms, which can lead to an overestimation of lag phases and half-lives. The latter is particularly critical for surface waters with limited pre-exposure and low bacterial density, where the presence of a sufficient amount of specific degraders may not be ensured in all test systems. This might be part of the explanation for the markedly longer lag-phases and lower degradation rates for several compounds in inoculum from the rural lake.

4. Conclusions

Measurements of the degradation of nine petroleum hydrocarbons in water from five diverse sampling locations, showed biodegradation rate constants ranging a factor 10 for each chemical in water from four of the five locations. Lag phases were comparable but generally slightly shorter in tests with inocula from urban locations compared to rural locations. The first order degradation rate constants were also lower in water from locations with low initial bacterial density and low urbanization. Although biodegradation kinetics were influenced by differences in the level of urbanization of inoculum locations,

389 the sequence of degradation was quite consistent among the five locations. The experiments thus show
390 the importance of the choice of sampling location for biodegradation tests, and open the possibility of
391 using benchmark chemicals in biodegradation testing of hydrocarbons.

392 The study showed the added value of combining partitioning based methods (passive dosing and HS-
393 SPME) with GC-MS analysis for testing biodegradation of mixtures of hydrophobic chemicals with low
394 water solubility to produce biodegradation kinetic data of high technical quality and at environmentally
395 relevant concentrations.

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