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Article

Determining the Temperature Dependency of Biodegradation Kinetics for 34 Hydrocarbons while Avoiding Chemical and Microbial Confounding Factors

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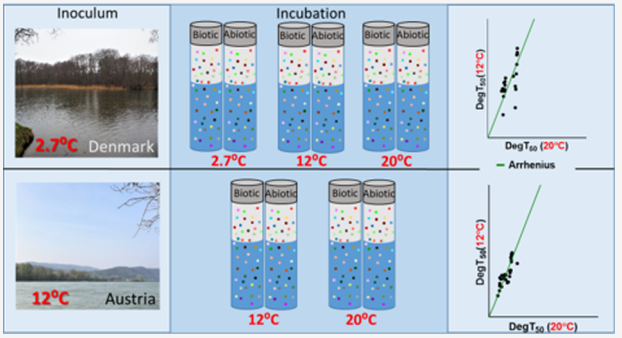
ABSTRACT: Biodegradation kinetics data are keystone for evaluating the environmental persistence and risk of chemicals. Biodegradation kinetics depend highly on the prevailing temperature, which influences microbial community structures, metabolic rates, and chemical availability. There is a lack of high-quality comparative biodegradation kinetics data that are determined at different test temperatures but with the same microbial inoculum and chemical availability. The present study was designed to determine the effect of test temperature on the biodegradation kinetics of hydrocarbons while avoiding confounding factors. We used inocula from a Northern river (2.7 °C) and a Central European river (12.5 °C). Aqueous stock solutions containing 45 individual hydrocarbons were generated by passive dosing and added to river water containing the native microorganisms. Compound-specific biodegradation kinetics were then determined at 2.7, 12, and 20 °C based on substrate depletion. Main findings comprise the following: (1) Degradation half-times ($DegT_{50}$) of 34 test chemicals were determined at different test temperatures and were largely consistent with the Arrhenius equation (activation energy, 65.4 kJ/mol). (2) Differences in biodegradation kinetics between tested isomers were rather limited. (3) The recent lowering of standard test temperature from 20 to 12 °C results typically in a doubling of $DegT_{50}$ values and can lead to a stricter persistency assessment.



KEYWORDS: *Arrhenius equation, Danube, isomer-specific degradation, OECD 309, simulation biodegradation test*

INTRODUCTION

Biodegradation is the primary removal process for petroleum hydrocarbons in the environment.[1] Therefore, biodegradation kinetics data are essential for persistency and risk assessment of these chemicals. In freshwater environments, biodegradation kinetics are influenced by microbial diversity and abundance, the level of pre-exposure of the microorganisms, substrate concentrations,[2,3] availability of electron acceptors and nutrients, pH, and temperature.[4] On the biological side, temperature affects the rate of microbial metabolism[5] and therefore biodegradation rate constants of individual hydrocarbons. In addition, local, seasonal, and climatic temperature regimes can select for microbial populations adapted to ambient temperature.[6] On the chemical side, the effect of temperature on degradation kinetics is also rather complex, especially for mixtures of petroleum hydrocarbons and in cases where a free phase of the mixture exists in the environment or the test system.[7,8] Diffusion coefficients, oil viscosity, partition coefficients, solubility in water, and volatilization affect the bioavailability of hydrocarbon constituents, and all of these factors change with temperature.[9−12] Temperature is thus a highly important factor for biodegradation of petroleum hydrocarbons in test systems and in the environment.

The influence of test temperature on biodegradation kinetics of hydrocarbons has been widely studied.[5,10,13−17] However, most of these studies were conducted on petroleum hydrocarbon mixtures (crude or products) and included a free oil phase in the test system. The observed differences in biodegradation kinetics at different temperatures were then explained by a combination of biological and chemical factors including differences in metabolic rates, differences in oil viscosity, and differences in volatilization rates of the components in the oil. The temperature effect on biodegradation kinetics could thus not be distinguished from the temperature effect on the chemical availability in terms of

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Table 1. Potential Confounding Factors and the Applied Strategies to Avoid Them

confounding factor	strategy
inoculum origin (location, season, and temperature)	Temperature effects were determined based on parallel tests with the same inoculum, i.e., same initial microbial composition, but different test temperatures.
inoculum viability (transport and storage)	The inoculum was transported and stored at the origin temperature (<24 h). Tests at different temperatures were initiated and incubated in parallel.
test substance concentration	Passive dosing at 20 °C was used to achieve uniform initial concentrations in all tests.
oxygen status	Aerobic conditions during testing were confirmed at all temperatures.
cosolvent	No cosolvent was added.
abiotic loss processes	(1) Losses minimized by testing in gastight vials and (2) peak area ratios between biotic and abiotic vials incubated at the same temperature were used to account for losses.
partitioning of test compounds into the headspace	Air-water partitioning is highly temperature-dependent. Compounds with significant headspace partitioning were removed from the data analysis (K_{aw} > 1 L/L at 20 °C).

concentration and composition. As an example, in biodegradation testing conducted with neat oil mixtures, a low temperature will not only slow down the biodegradation kinetics but also reduce the substrate availability due to an increased viscosity of the oil[18] and a decrease in water solubilities. The obtained temperature dependency might then be very meaningful in the context of oil spill remediation but is less suited in the context of environmental persistency assessment of chemicals, where the focus should be on the biodegradation at environmentally relevant concentrations well below water solubility.

Under the EU chemical regulation REACH, simulation biodegradation tests (OECD 307, 308, and 309) are, since 2017, preferably conducted at 12 °C.[19] For degradation kinetics determination, the recommended test temperature was lowered from 20 to 12 °C, defined as the average surface water temperature in the EU, while tests for metabolite identification are still generally requested at 20 °C.[20] The recommendation by the European Chemicals Agency (ECHA) is to use the Arrhenius equation (eq 1)[21] to extrapolate biodegradation rates from one temperature to another and for tests previously performed at 20 °C to the now preferred 12 °C.[19,22]

The Arrhenius equation is defined as:

$$t_{1/2}(T_{ref}) = t_{1/2}(T)\exp^{(E_a/R)[(1/T_{ref})-(1/T)]} \quad (1)$$

where $t_{1/2}$ is the half-life at test temperature T, E_a is the activation energy (kJ/mol), R is the gas constant (0.008314 kJ/mol K), and T_{ref} is the reference temperature for the extrapolation. Applying first-order kinetics, the half-life, $t_{1/2}$, is defined as $\ln(2)/k$ and represents the time required to reduce the concentration during the degradation phase by 50%. The dissipation half-time, DT_{50}, is defined as the time until half of the initial concentration has dissipated, while the degradation half-time, $DegT_{50}$, is the time until half of the initial concentration has been transformed by degradation processes.[23] The original Arrhenius equation is based on rate constants (k) that are inversely proportional to half-lives, as presented in eq 1. The equation has also been applied to other biodegradation metrics such as $DegT_{50}$,[24,25] which has been reported as a more robust parameter obtained from biodegradation studies than rate constants.[26] By doing so, it is assumed that temperature does not only affect the biological factors determining the rate constant but also those determining the lag phase.

According to the Arrhenius equation, an increase in temperature will result in an exponential increase in the metabolic rate. The magnitude of this increase is dependent on the activation energy determined on an empirical basis. The applicability of the Arrhenius equation for extrapolation of biodegradation rates between temperatures is debated,[22,24,27] and for example, which activation energy to use in a given situation is still discussed by authorities and scientists. Based on 99 data sets of $DegT_{50}$, DT_{50}, and half-lives for biodegradation of pesticides in soil pore water, the European Food Safety Authority (EFSA) identified a generic activation energy of 65.4 kJ/mol with a 90% probability that the median value is within the range of 45.8−93.3 kJ/mol.[28] The activation energy of 65.4 kJ/mol is now recommended by ECHA if no compound-specific activation energies are available.[19] From the same data sets, EFSA found that extrapolation between temperatures using the Arrhenius equation is appropriate within the temperature range of 0−30 °C.[28] These conclusions were, however, reached for pesticides and their applicability for other chemical groups was not demonstrated.

Another way to express the Arrhenius relationship is through Q_{10} values.[13,29] The Q_{10} is the multiplier by which the rate of a reaction increases upon a 10 °C temperature increase. The Q_{10} value can be calculated from the ratio of rate constants (k_2/k_1) or other biodegradation metrics, here, degradation half-time, $DegT_{50}$, determined at temperatures T_1 and T_2:

$$Q_{10} = (DegT50_1/DegT50_2)^{10/(T_2-T_1)} \quad (2)$$

As a rule of thumb, Q_{10} values of 2−3 are being applied for converting hydrocarbon biodegradation rates and times from one temperature to another.[29]

Bagi et al.[29] evaluated the Q_{10} approach on published petroleum hydrocarbon data from tests of natural seawater inoculum exposed to crude oil. They found that biodegradation rate conversions by the Arrhenius equation were questionable and recommended not to use a generic Q_{10} value across compound classes and microbial communities. They further found that under non-nutrient-limiting conditions, indirect temperature effects influenced the reported Q_{10} values more than the intrinsic temperature effect on metabolic processes. These indirect temperature effects were attributed to the abundance and metabolic activity of degrader bacteria and the substrate availability without the possibility to distinguish between them.

Very recently, Brown et al.[24] reported a meta-analysis of published biodegradation kinetics data from "temperature-adapted tests", which are tests conducted at temperatures near the inoculum origin temperature. Brown et al. found only a limited temperature effect on biodegradation kinetics when comparing biodegradation data obtained with different types of inocula, chemical concentrations, and chemical availabilities. The present study is fundamentally different since it focuses on determining biodegradation kinetics data with the same inoculum, chemical concentration, and chemical availability

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but at different test temperatures. The temperature effect on biodegradation kinetics can, in this manner, be isolated and quantified while avoiding the chemical and microbial confounding factors that make it difficult to compare data from different biodegradation studies performed under various conditions (Table 1). These data are imperative for a thorough assessment of the test temperature effect on biodegradation kinetics and for evaluating the applicability of the Arrhenius equation to extrapolate biodegradation kinetics between test temperatures for hydrocarbons.

The specific aims of this study were as follows:

1) Quantify the test temperature effect on the biodegradation kinetics of a large number of hydrocarbon test substances using two different inocula and to assess the suitability of the Arrhenius equation for extrapolation between test temperatures.
2) Determine whether testing at standard test temperatures, 12 and 20 °C, with the inoculum collected at low temperatures leads to a systematic over- or underestimation of biodegradability.
3) Investigate substance-specific differences in the temperature dependency of biodegradation kinetics by covering a large number and diversity of petroleum hydrocarbons also including isomers.

To fulfill these aims, two surface water samples were collected across Europe and tested at the origin temperature (2.7 and 12 °C) and standard test temperatures (12 and 20 °C). The applied biodegradation testing approach was further tested and validated by parallel incubations of large volume vessels (240 mL vs 20 mL in the main setup) and additional abiotic controls (poisoned river water).

Several potential confounding factors were identified and their impact was minimized, as shown in Table 1. The experimental and analytical platform developed by Birch et al.[30,31] was then used to determine compound- and temperature-specific biodegradation kinetics for the hydrocarbons using inocula from a Northern river and a Central European river.

MATERIALS AND METHODS

Materials. The following model substances were included in the study to cover a large number and diversity of petroleum hydrocarbons, also including isomers 9,10-dihydroanthracene, phenanthrene, hexachloroethane, 2,3-dimethylheptane, bicyclohexyl, butyldecalin, tetralin, biphenyl, 1,2-dimethylnaphthalene, ethylcyclopentane, 2,6-diisopropylnaphthalene, dibenzothiophene, 3-phenyl-1,1′-bi(cyclohexane) (Sigma-Aldrich), 2,4-dimethylheptane, 2,5-dimethylheptane, 3,3-dimethyloctane, 3,5-dimethyloctane, decalin (TCI), dodecylbenzene, dihexyl disulfide, 2,6,10-trimethyldodecane (Chiron), and a mixture obtained from LGC (Middlesex, UK) containing indane, 1,4-diethylbenzene, 1,2-diethylbenzene, 1,2,4,5-tetramethylbenzene (durene), 1,2,3,5-tetramethylbenzene (isodurene), 1-ethyl-2-methyltoluene, 1-ethyl-3-methyltoluene, benzene, *n*-butylbenzene, ethylbenzene, 4-ethyltoluene, iso-octane, cumene, 1-methylnaphthalene, 2-methylnaphthalene, naphthalene, *n*-propylbenzene, toluene, 1,2,3-trimethylbenzene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, *m*-xylene, *o*-xylene, and *p*-xylene (more information on model substances in S5).

A translucent silicone rod (3 mm in diameter) was ordered custom-made from Altec (altecweb.com, product code 136-8380) and used for passive dosing. Solvents used for cleaning and loading of the silicone included ethyl acetate (Merck), ethanol (VWR Chemicals), and methanol (Sigma-Aldrich). Ultrapure water was produced on an Elga Purelab flex water system from Holm & Halby (Denmark).

Sterile 1 L PET bottles from Bürkle (Mikrolab Aarhus, Denmark) were used for sampling of river water.

Surface Water Inoculum. Sampling sites were chosen based on the following criteria: (1) minimizing the potential pre-exposure of petroleum hydrocarbons at the sites (i.e. no refineries, industry, or WWTP discharges upstream close to the sampling site and no large vehicle roads close to the sampling site), (2) availability of information on water quality for the site (good ecological status according to the Water Framework Directive definitions and measurement of pollutants and nutrients), and (3) possibility to transport the samples to Lyngby in Denmark to start the experiments within 24 h of sampling.

To represent the inoculum adapted to "lower than standard test temperature", surface water was sampled in Gudenaa in Denmark on January 29, 2019. The water temperature at sampling was 2.7 °C, and during transportation, the water was kept at 0−3 °C. The average water temperature in the Gudenaa was 5.2 °C that winter.[32] To represent the inoculum adapted to the new standard test temperature (12 °C), a sample from Danube in Austria was collected on April 9, 2019. The Danube at Vienna has an average spring temperature of 10.6 °C.[33] The water temperature at sampling was 12.5 °C, and during transportation, the temperature was kept at 11−12.5 °C. Exact coordinates for the sampling locations are included in Table 2.

Table 2. Sampling Location Information and Parameters Characterized (Average ± Standard Deviation)

parameter	Gudenaa, Denmark (2.7 °C; 56°6′25″N, 9°43′19″E)	Danube, Austria (12.5 °C; 48°23′48″N, 15°30′59″E)
O_2 [mg/L]	11.0	10.1
pH	8.3	6.3
conductivity [μS/cm]	371	364
CFU [mL^{-1}], 24 h, 20 °C	58	123
CFU [mL^{-1}], 72 h, 20 °C	1.6×10^3	3.7×10^3
total dissolved solids (TDS) [mg/L]	140	107
total suspended solids (TSS) [mg/L]	0−0.4	15.8
non-volatile organic carbon (NVOC) [mg/L]	4.1 ± 0.05	2.0 ± 0.2
PO_4^{3-} [μg/L]	49 ± 0.5	1.7 ± 0.4
NO_2^- [μg/L]	12 ± 0.1	6.6 ± 2
NH_3 [μg/L]	57 ± 2	20 ± 8
NO_3^- [mg/L]	2.35 ± 0.02	1.36 ± 0.15

Sampling was performed in the free-floating current of the rivers 5−20 cm below the surface. At the Gudenaa location, sampling was performed in Waders, walking out to 1 m depth in the river, and then 1 L was sampled at the time with a precleaned stainless steel beaker at the end of a 3 m stick. The sampled water was distributed in three 1 L PET bottles and sampling continued until 12 L was collected. The Danube sample was taken from a boat and 12 L was sampled at once in an autoclaved glass container and then distributed to sterile 12

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× 1 L square bottles. The bottles were packed in an insulated aluminum suitcase, which was filled with the appropriate amount of cooling elements to maintain the sampling temperature during transportation by car and airplane. The temperature in the suitcase was logged during transportation. Water temperature at sampling, pH, oxygen content, and conductivity of water were measured in the field. Values for both sites are included in Table 2.

Upon arrival to the laboratory, the sample was stored at the sampling temperature in a temperature-controlled incubator. Within 24 h, total suspended solids (TSS), total dissolved solids (TDS), nonvolatile dissolved organic carbon (NVOC), and dissolved nitrogen and phosphate contents were determined. Microbial abundance was determined as the heterotrophic plate count on R2A agar after 24 and 72 h of incubation at 20 °C.[34] Procedures for determining the sample characterization parameters are described in the Supporting Information (S1) and parameters are given in Table 2. Both inocula were analyzed for the included test substances upon arrival to the laboratory using the gas chromatography mass spectrometry (GC−MS) method described in Chemical Analysis, and none of the substances were found at measurable concentrations.

The total suspended solid contents were for Gudenaa below and for Danube within the ECHA PBT guidance requirements for inocula used in biodegradation testing under REACH (10−20 mg/L).[35]

Passive Dosing to Generate Test Solution. The initial substrate level in terms of concentration and composition was for all treatments set by passive dosing at 20 °C and subsequent dilution with surface water. The passive dosing donor was not included in the test, which is fundamentally different from other studies where passive dosing was an integral part of the biodegradation test.[36,37] Passive dosing was used to introduce the chemicals into the test without the addition of a cosolvent, neat mixture, or microdroplets. The passive dosing method used here was modified from Birch et al.[30] and Hammershøj et al.[38] It included three steps: (1) loading of precleaned silicone with the test substances, (2) equilibration of the loaded silicone with pure water (passive dosing), and (3) addition of this passive dosing solution (PD solution) to the surface water inoculum to prepare biodegradation test systems.

Loading of Silicone with Test Chemicals. Eight passive dosing systems were prepared: one for the solid substances and seven identical systems for the liquid substances. Each consisted of 20.0 g of precleaned silicone rod (precleaning procedure described in S4) in a 100 mL amber glass serum bottle closed with an aluminum crimp lid with a PTFE liner.

The solid test chemicals (9,10-dihydroanthracene, phenanthrene, and hexachloroethane) were loaded to the silicone by partitioning from a methanol solution. A total of 0.5 g of each chemical was dissolved in 50 mL of methanol using sonication. This loading solution was added to the silicone rod and the bottle was rolled for 20 h at 20 °C. The remaining methanol was discarded and the silicone rod was rinsed twice with ultrapure water by vigorous shaking. As the final rinsing step, 60 mL of ultrapure water was added to the bottle and it was rolled overnight. One system was prepared and reused for all experiments. Between experiments, the passive dosing system was emptied and stored at 4 °C.

The liquid test chemicals were loaded to the silicone by full absorption.[38] A mixture of neat liquid substances was prepared by mixing 1900 μL of LGC mix, 950 μL of 2,6,10-trimethyldodecane, and 250 μL of each of the remaining 17 liquid substances in a 10 mL glass vial closed with a gas tight septa lid. A total of 400 μL of this neat mixture was added to each of the seven passive dosing systems using a gas-tight Hamilton syringe. Each passive dosing system was shaken thoroughly and rolled for 48 h at 20 °C in order for the silicone to absorb the test chemicals. For final cleaning, ultrapure water was added to the bottles and exchanged twice and the bottles were then rolled overnight. The seven identical passive dosing systems were reused in all experiments. Between experiments, the passive dosing systems were emptied and stored at 4 °C. Comparable loading levels between experiments were checked prior to starting the experiments by the chemical analysis of passive dosed water.

Passive Dosing of Ultrapure Water. A total of 60 mL of ultrapure water was added to the passive dosing system containing the solid test chemicals and the seven passive dosing systems containing the liquid test chemicals. The PD solutions were then equilibrated for at least 2 h at 20 °C before use in the biodegradation experiments. The concentrations of the test substances included in the study were at the start of the test in the ng/L to μg/L range (0.09−200 μg/L) (Supporting Information, S5).

Biodegradation Testing. Biodegradation experiments were carried out at 12 °C (origin temperature) and 20 °C for the Danube sample and at 2.7 °C (origin temperature), 12 °C, and 20 °C for the Gudenaa sample. Preparation of test systems followed the procedure described by Birch et al.[30] A total of 100 μL of PD solution containing the solid test chemical and 650 μL of PD solution containing liquid test chemicals were transferred to 14.25 mL of surface water (i.e., inoculum) in 20 mL amber glass auto sampler vials, leaving a 5 mL headspace in the vial. Passive dosing solution was transferred with gas-tight Hamilton syringes, and the inoculum was transferred with a dispenser. To avoid volatilization losses, test systems were closed with gas-tight lids immediately after the addition of the PD solution containing liquid test chemicals. Triplicate biotic test systems were generated for each of seven incubation time points (approximately days 1, 4, 8, 10, 14, 21, and 28). Abiotic test systems were generated the same way, with ultrapure water instead of the surface water inoculum. Each biotic/abiotic pair was prepared from the same passive dosing bottle. Test systems were incubated in temperature-controlled incubators for up to 28 days. At seven time points, three biotic and three abiotic test systems were transferred from the incubators to the GC autosampler.

Chemical Analysis. Primary biodegradation was determined based on peak areas of single constituents in biotic test systems relative to abiotic test systems (substrate depletion) as described by Birch et al.[31] Automated headspace solid-phase microextraction (HS-SPME) was performed with a PAL3 autosampler (CTC Analytics, Zwingen, Switzerland) mounted on a gas chromatography mass spectrometer (Agilent Technologies 7890B/5977A GC/MSD). An Agilent 122-5562 DB-5 ms Ultra Inert 60 m × 0.25 mm, 0.25 μm column was used for separation. HS-SPME was performed with a 30 μm PDMS fiber for 20 min at 50 °C and 250 rpm. Analytes were desorbed for 10 min at 225 °C (splitless), succeeded by thermal cleaning of the fiber for 1 min at 250 °C. The GC oven temperature was 40 °C during the 10 min desorption and increased then by 15 °C/min up to 310 °C, where it was held for 7 min. Helium with a flow of 1.2 mL/min was used as the

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carrier gas. The compounds were analyzed in selected ion mode (SIM), and quantifier and qualifier m/z's are listed in Supporting Information (S5). Peak area ratios between biotic test systems and abiotic control were determined for consecutive samples. Sterilized abiotic controls were analyzed after the abiotic controls. Test systems were continually added to the autosampler, ensuring a maximum of 4 h before analysis if they originated from test temperatures below 20 °C. The average acquisition time for the three biotic/abiotic pairs of an analysis point was used as the time point in the further data treatment.

Data Treatment. The analytical method was successfully developed for 42 of the 45 test substances. The isomers 1,4-diethylbenzene and 1,2-diethylbenzene could not be separated and were thus analyzed together. Dodecylbenzene was excluded from the data analysis due to insufficient reproducibility at low concentrations. The LCG mix solvent iso-octane was also excluded from the analysis.

The peak area was integrated by MassHunter Quantitative Analysis (Agilent Technologies). Each integration was manually checked. Relative concentrations were calculated as:

$$C_{\text{relative}} = \frac{\text{peak area}_{\text{biotic}}}{\text{peak area}_{\text{abiotic}}} \quad (3)$$

The relative concentrations were plotted against time and fitted with the "plateau followed by one phase decay" model (4) using GraphPad Prism ver. 8.1.2, with the constraints $t_{\text{lag}} \geq 0$, $k_{\text{system}} \geq 0$, and $C_{\text{relative}}(0) = 1$ and data not being weighted.

$$C_{\text{relative}}(t) = \begin{cases} 1 & \text{for } t < t_{\text{lag}} \\ e^{-k(t-t_{\text{lag}})} & \text{for } t \geq t_{\text{lag}} \end{cases} \quad (4)$$

The pseudo-first-order half-life ($t_{1/2}$) for a compound was calculated as $\ln(2)/k$. As in a previous biodegradation work, t_{lag} was constrained to positive values; no weighting of the data was used, and the number of and scatter among replicates were accounted for in the fit.[30] The biodegradation half-time (DegT_{50}) was calculated as the sum of lag phase and half-life.

Regressions with a goodness-of-fit $R^2 > 0.70$ were used in further data analysis. The lag phase, rate constant, and half-life were reported when at least two measurements of C_{relative} between 0.9 and 0.1 were observed during the degradation phase.

Uncertainties in the data are reported as confidence intervals (95%, asymmetrically) on $T_{1/2}$. Confidence intervals for DegT_{50} were determined by adding T_{lag} values to the confidence interval of $T_{1/2}$ in order not to include the uncertainty on setting the transition point between the lag phase and the degradation phase in the uncertainty on the DegT_{50} values (S6).

Data were also fitted to a logistic model (S9 and S10). The logistic model is a simplification of Monod-based biodegradation kinetics that can be applied at low substrate concentrations and low initial biomass.[39] For biodegradation kinetics, the equation for the logistic model was applied as in the study of Birch et al.:[40]

$$Y = \frac{Y_0 + a}{1 + (a/Y_0)\exp[k(Y_0 + a)X]} \quad (5)$$

where Y is the relative substrate concentration, a is the amount of substrate required to produce the population density present at time 0, Y_0 is the relative substrate concentration at time 0 (set to 1), $k = \mu\text{max}/K_s$ or maximum specific growth rate divided by the half saturation constant for growth (K_s), and X is the time (days). $Y_0 \ll K_s$. DegT_{50} values (listed in S11) were obtained from the logistic model by isolating X at $Y = 0.5$ with the obtained model parameters.

Generally, a quantification of the test temperature effect on biodegradation kinetics can be accomplished for each of the above-described biodegradation kinetics parameters. The original Arrhenius equation is based on rate constants or half-lives. However, in the present study, determination of the rate constant and half-life was based on 0−3 data points, whereas determination of DegT_{50} was based on all 7 data points. DegT_{50} values were determined for more water-chemical-test temperature combinations and with higher certainty compared to the other parameters, since DegT_{50} values rely less on setting the transition point between the lag phase and the pseudo-first-order degradation phase. DegT_{50} was thus a more robust parameter for the data evaluation, and the main analysis and assessment were therefore based on DegT_{50}. Data and analyses of the temperature dependency of lag phases and half-lives are shown in the Supporting Information.

The partitioning of test chemicals to the headspace of the test system can lead to an underestimation of the biodegradation kinetics (Birch et al.[30]). This partitioning is highly temperature-dependent, with increasing partitioning to the headspace with increasing temperature.[11] The temperature-dependent headspace partitioning can thus be a potential confounding factor when quantifying the temperature effect on the biodegradation kinetics of test chemicals with a significant partitioning into the headspace (Table 1). In this analysis, data were thus omitted for chemicals (8) with a $K_{\text{aw}} > 1$ L/L (at 20 °C, Table S5), which partition >25% into the headspace of a 20 mL vial filled with 15 mL of water.

Validation of Biodegradation Tests Using Larger Vessels and Sterilized Abiotic Controls. As an extra set of controls, 240 mL test systems were prepared using the Danube water and incubated in parallel with the 15 mL test systems at 20 °C. This parallel biodegradation experiment was conducted with larger vessels to confirm that the 15 mL test volume was sufficient. The larger test systems were prepared in triplicates for three incubation time points, and at each time point, 15 mL aliquots were taken through the septum and transferred rapidly to 20 mL amber glass autosampler vials using gas-tight Hamilton syringes. These vials were then analyzed with the same method as for the 20 mL test system.

To investigate for potential abiotic losses including sorption to dissolve and particulate organic matter, an additional set of abiotic test systems was prepared using a sterilized surface water inoculum. The sterilization was performed by autoclavation followed by poisoning (sodium azide at a concentration of 0.05% (m/v)). The "sterilized abiotic" test systems were prepared in triplicate for four time points and analyzed along with the biotic/abiotic pairs. Sterilized abiotic controls were included for both surface water inoculum test series.

Data from both validation tests are provided in the Supporting Information (S6 and S7), and DegT_{50} values for the large volume test are listed in the Supporting Information (S8). The data obtained in these validation tests were consistent with the main data set, which confirmed that 20 mL was a sufficient test volume and ultrapure water was an appropriate abiotic control.

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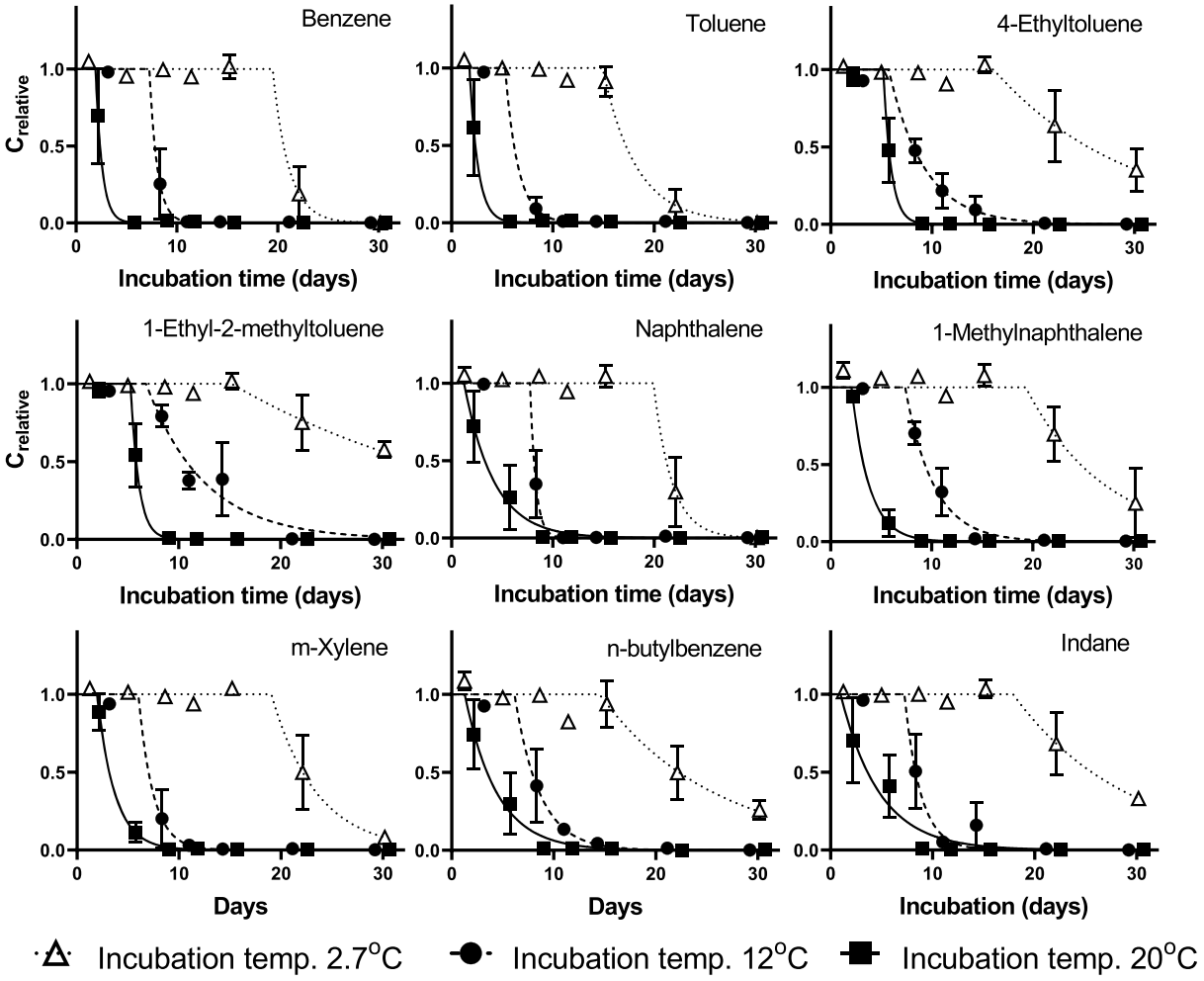

Figure 1. Selected biodegradation curves for the Gudenaa inoculum tested at 2.7, 12, and 20 °C. $C_{relative}$ is calculated from eq 3 and the curves are modeled by eq 4. Symbols represent mean values ± standard error of mean (SEM) (n = 3).

DNA Characterization. DNA extraction and microbial community analysis by 16S rRNA gene amplicon sequencing using Illumina MiSeq were performed on the original inoculum by DNASense (Aalborg, Denmark). Details on DNA materials and methods and the most abundant genera in the Gudenaa sample are included in the Supporting Information (S2 and Table S3). Unfortunately, the DNA sequencing of the Danube sample failed, which is the reason why data for this inoculum are not included in the work.

RESULTS AND DISCUSSION

Determination of Biodegradation Kinetics at Different Test Temperatures. Figures showing the fits of both biodegradation models (eqs 4 and 5) to the experimental data, and the obtained kinetic parameters, are included in the Supporting Information (S6, S7, S9, and S10 and Tables S8 and S11). The two models resulted in very similar $DegT_{50}$ values (see Figure S12), and the main data analysis was then performed with the first-order model (eq 4). Biodegradation kinetics in Gudenaa water (i.e., 2.7 °C inoculum) at different test temperatures are presented in Figure 1 for the nine chemicals with the best model fit at 2.7 °C. The effect of test temperature on biodegradation kinetics was similar for most tested compounds; biodegradation half-times ($DegT_{50}$) decreased with increasing test temperature. Biodegradation within 30 days (defined as $C_{relative} \leq 0.5$) was observed for 17, 39, and 41 chemicals when testing at 2.7, 12, and 20 °C, respectively. The test temperature affected the lag phase (T_{lag}), the biodegradation rate constant (k), and consequently, the half-life ($T_{1/2}$) (Figure 1) with the general observation that T_{lag} and $T_{1/2}$ decreased with the test temperature and k increased.

The test temperature effect on biodegradation kinetics was rather similar for Danube water. Within 30 days, 40 compounds were degraded at 12 °C and 39 compounds at 20 °C. In general, biodegradation was faster at 20 °C compared to 12 °C due to a shorter lag phase and higher degradation rate.

Slightly faster degradation was observed for the Danube inoculum than for the Gudenaa inoculum at both 12 and 20 °C, probably due to a higher initial microbial abundance in the Danube sample compared to the Gudenaa sample (Table 2). The $DegT_{50}$ values obtained for the same compound and test temperature were generally within a factor of 3 between Gudenaa and Danube inocula, with an average factor of 1.7 for the 20 °C data and average factor of 3.0 for the 12 °C data. These are modest differences, considering that $DegT_{50}$ values obtained with five different inocula, all from Danish surface waters, were reported to be within a factor of 10.[40] Therefore, Danube and Gudenaa inocula performed remarkably similar despite the considerable geographical distance and temperature difference.

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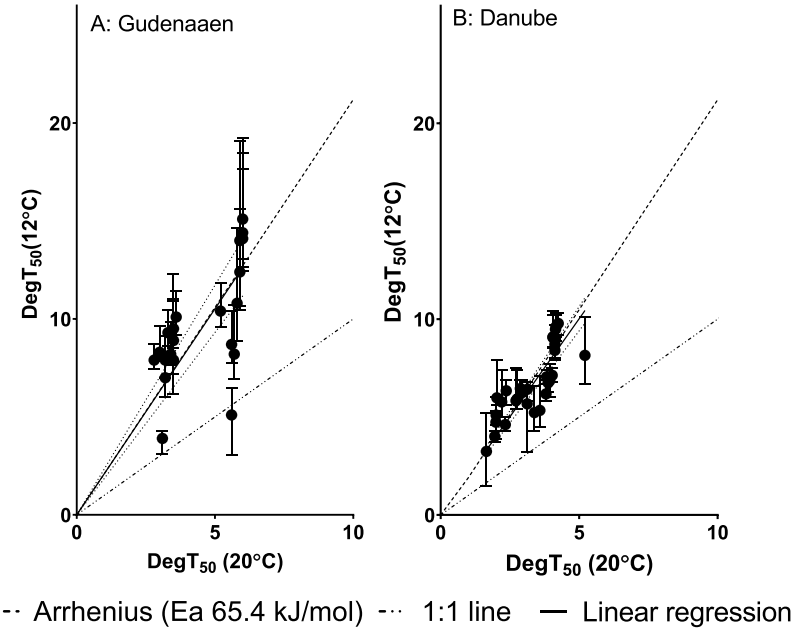

Figure 2. $DegT_{50}$ (12 °C) vs $DegT_{50}$ (20 °C) obtained with Gudenaa water (A) and Danube water (B). Data are modeled by eq 4. $DegT_{50}$ values from model fits with $R^2 > 0.70$ are included. Error bars are T_{lag} + the upper and lower 95% CI on $T_{1/2}$. 1:1 line (dashed and dotted), Arrhenius lines (dashed), and linear regressions to experimental data (solid) are included as visual references. Dotted lines represent 95% CI on the linear regression.

Quantification of the Temperature Effect on Biodegradation Kinetics. For each test chemical, $DegT_{50}$ values obtained at 12 °C were plotted against the corresponding $DegT_{50}$ values obtained at 20 °C (Figure 2). Three visual references were included in these plots: (1) the Arrhenius equation using an activation energy (E_a) of 65.4 kJ/mol as recommended by ECHA, (2) a 1:1 line ($DegT_{50}$ (12 °C) = $DegT_{50}$ (20 °C)), and (3) a linear regression fitted to the experimental values.

In general, there was very good agreement between the fitted linear regression and the Arrhenius equation for both inocula and most test chemicals (Figure 2). The Arrhenius equation with the recommended E_a fitted well in the present study where the test temperature was higher than or at the origin temperature. *p*-Xylene and 1,3,5-trimethylbenzene were exceptions, since they showed temperature-dependent degradation kinetics only in Danube water. In the Supporting Information (S13), $DegT_{50}$ (12 °C) calculated via the Arrhenius equation from the measured 20 °C data is plotted against the measured $DegT_{50}$ at 12 °C for both water samples. These plots also show that the Arrhenius conversion fits well with the experimental data, as all compounds except one (1,3,5-trimethylbenzene) were within a factor 5 deviation from the 1:1 line for Gudenaa data, and in the case of the Danube data, all compounds except one (butyldecalin) were within a factor 2 deviation.

Knowing that biodegradation kinetics depend on several factors in addition to temperature, the strategy of the present study was to vary the test temperature for each of the two inocula while keeping all other factors constant (Table 1). Observed differences within inocula can thus be attributed solely to the test temperature effect, whereas differences between inocula can be attributed to other factors. In Figure 3, the effects of inoculum origin temperature and test temperature are separated by plotting $DegT_{50}$ values from both inocula as a function of test temperature. Six compounds were selected to represent the general picture. In these plots, the slope denotes the activation energy if data are consistent with the Arrhenius equation. A line connecting the two data points from the Danube data set and the three data points in the Gudenaa data set is included solely as a visual reference. The observed parallel lines indicate similar activation energies and therefore similar Q_{10} values for inocula originating from the Northern river and the Central European river. The linear relationship for the Gudenaa data shows consistency with the Arrhenius equation also when including the 2.7 °C data. The biodegradation kinetics of all compounds for which this analysis was possible followed the pattern shown, which in turn suggests similar activation energies and Q_{10} values among the test chemicals.

Biodegradation half-times obtained at different test temperatures were largely consistent with the Arrhenius model (Figures 2 and 3). The same assessment with a smaller data set was also made for those T_{lag} and $T_{1/2}$ values that fulfilled the quality criteria (described in Data Treatment), and the resulting figures are included in the Supporting Information (S14). The uncertainty on these parameters is larger than on $DegT_{50}$, making it more difficult to draw clear and solid conclusions. This is exemplified for $T_{1/2}$ values in Figure S14, where several of the error bars are crossing both the Arrhenius line and the 1:1 line. However, $T_{1/2}$ values for the Gudenaa inoculum were almost evenly distributed above and below the Arrhenius line, whereas most data points were above the 1:1 line. The majority of the $T_{1/2}$ values obtained with the Danube inoculum were at or below the Arrhenius line. For the Danube inoculum, the T_{lag} values generally followed the Arrhenius relationship, whereas for the Gudenaa inoculum, T_{lag} data points were distributed above or below the Arrhenius line. Generally, it was not possible to establish an unambiguous linear relationship between these kinetics parameters obtained at 12 and 20 °C in any of the inocula.

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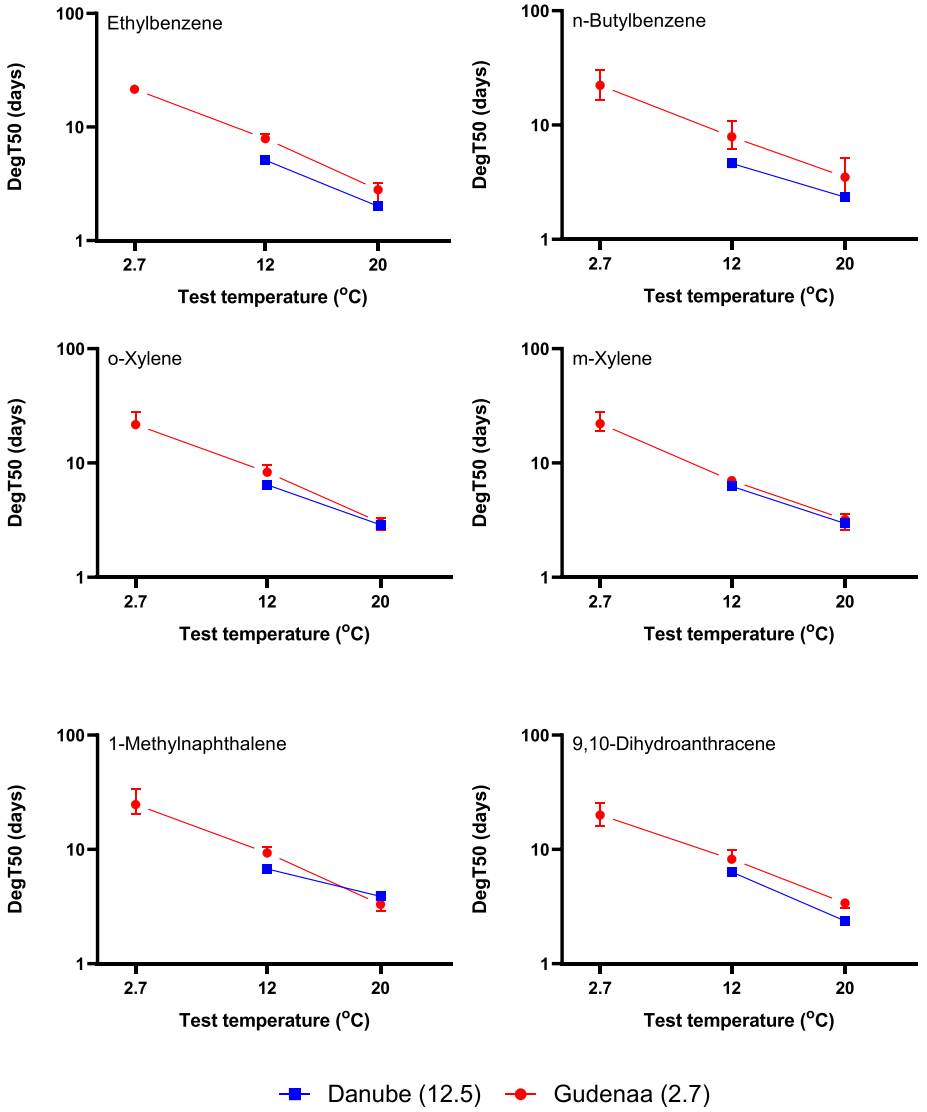

Figure 3. Biodegradation half-times ($DegT_{50}$) (days) of selected compounds versus test temperature for Gudenaa (inoculum sampling temperature, 2.7 °C) and Danube inoculum (inoculum sampling temperature, 12.5 °C). Error bars are T_{lag} + the upper and lower 95% CI on $T_{1/2}$. Agreement with the Arrhenius model is indicated by linearity in the connecting lines (semilog).

Compound-Specific Temperature Effects. To evaluate the compound-specific temperature effect in more detail, Q_{10} values were calculated from $DegT_{50}$ data using eq 2 (Table 3).

With a few exceptions, the Q_{10} values in Table 3 were quite uniform across compounds and inocula and the majority of the values were in the range of 2−3. Both mean and median values of all the Q_{10} values equaled 2.6 and thus were in the center of the rule of thumb range of 2−3. For the Q_{10} values in bold (best model fits), the mean value amounted to 2.6 and the median to 2.7. Overall, the obtained Q_{10} values were consistent with a recent study[15] where biodegradation rates were experimentally determined for component groups in oil in seawater with different temperatures, even though the Q_{10} values in the present study were slightly lower.

The selected model substances included several isomers, with the purpose of investigating how molecular features affect biodegradation kinetics and the temperature effect on the biodegradation kinetics. In Figure 4, the biodegradation half-times for the isomers included are plotted for both inocula tested at 20 °C, and corresponding plots for 12 °C are found in S15. For both inocula, differences observed in $DegT_{50}$ were very limited for the included xylenes, dimethyloctanes, dimethylheptanes, trimethylbenzenes, and tetramethylbenzenes. The only exception from this was 1,3,5-trimethylbenzene that degraded slightly faster in the Danube inoculum than 1,2,4-trimethylbenzene and 1,2,3-trimethylbenzene. The three ethylated toluenes had similar degradation kinetics but were degraded significantly slower than toluene. For the naphthalenes, there was no difference in degradation time obtained with the Danube inoculum, whereas the dimethylated naphthalene was degraded significantly slower in the Gudenaa inoculum than the two monomethylated naphthalenes (Figure 4). These observations support read-across within such isomer groups.

Implications and Perspectives. The conducted experiments resulted in a large set of well-aligned $DegT_{50}$ values, which allows a rigorous analysis of the test temperature effect on the biodegradation kinetics. In this way, the effect of test temperature on degradation half-times ($DegT_{50}$) was isolated, quantified, and found to be in general agreement with the Arrhenius equation and the recommended activation energy of 65.4 kJ/mol. There was no indication that testing low- and

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Table 3. Compound-Specific Temperature Effect Expressed as Q_{10} for Gudenaa and Danube Inocula[a]

compound	Danube (Q_{10})	Gudenaa (Q_{10})
toluene	3.9	
biphenyl	**3.4**	
9,10-dihydroanthracene	**3.3**	
1,2(1,4)-diethylbenzene	**2.9**	
isodurene	**2.9**	
1-ethyl-3-methyltoluene	**2.8**	
o-xylene	**2.7**	3.6
1,2,3-trimethylbenzene	**2.7**	
1-ethyl-2-methyltoluene	**2.7**	
p-xylene	**2.6**	
tetralin	2.6	
1,3,5-trimethylbenzene	**2.5**	
m-xylene	**2.5**	2.7
2-methylnaphthalene	**2.5**	**3.1**
durene	**2.5**	
4-ethyltoluene	**2.1**	1.6
naphthalene	2.1	
1,2-dimethylnaphthalene	**2.0**	2.4
indane	2.0	
1-methylnaphthalene	**2.0**	3.7
1,2,4-trimethylbenzene	**1.8**	
butyldecalin	1.8	

[a]Calculated from eq 2 using data obtained at 12 and 20 °C. The data are presented in decreasing order for the Danube values. Q_{10} values are calculated on $DegT_{50}$ values from model fits with $R^2 > 0.90$, and Q_{10} given in bold are based on model fits with $R^2 > 0.95$.

medium-temperature inocula at higher temperatures leads to a systematic over- or underestimation of biodegradability. This supports the common practice where environmental water samples often are tested at a higher standard test temperature (12 or 20 °C) than their temperature of origin. The results also support in principle the use of the Arrhenius equation for extrapolating $DegT_{50}$ from one test temperature to another. Such conversion will always introduce a numerical error, meaning that biodegradation tests should preferably be conducted at the environmentally or regulatory relevant temperature. Overall, the differences of $DegT_{50}$ values between structural isomers were limited, which can support read-across within such isomer groups. Further, the obtained biodegradation data can inform future development, optimization, and validation of predictive biodegradation models.

Within the European Union, the standard test temperature was recently reduced from 20 °C to 12 °C, which typically will lead to a doubling of degradation half-times ($DegT_{50}$ values). The experimental data of the present study in combination with the analysis by Matthies and Beulke[22] clearly demonstrate that this reduction of standard test temperatures can lead to a considerably stricter PBT and vPvB assessment, unless persistency time thresholds also are adjusted. The recent reduction of the standard test temperature at unchanged time thresholds will in fact change the P-criterion within REACH and lead to an increase of chemicals that will be labeled PBT and vPvB.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c02773.

S1: method descriptions for inoculum characterization parameters; S2: microbial community analysis through DNASense ApS; S3: five most abundant genera; S4: precleaning of silicone rods; S5: petroleum hydrocarbons tested, structure, retention time, quantifier, qualifier *m*/*z*'s, K_{aw}, and initial concentration; S6: biodegradation kinetics obtained with the Gudenaa inoculum; S7: biodegradation kinetics obtained with the Danube inoculum; S8: table of biodegradation kinetics parameters ($DegT_{50}$, $t_{1/2}$, and t_{lag}) for all inocula and test temperatures; S9: biodegradation kinetics obtained with the Gudenaa inoculum applying the logistic model; S10: biodegradation kinetics obtained with the Danube inoculum applying the logistic model;

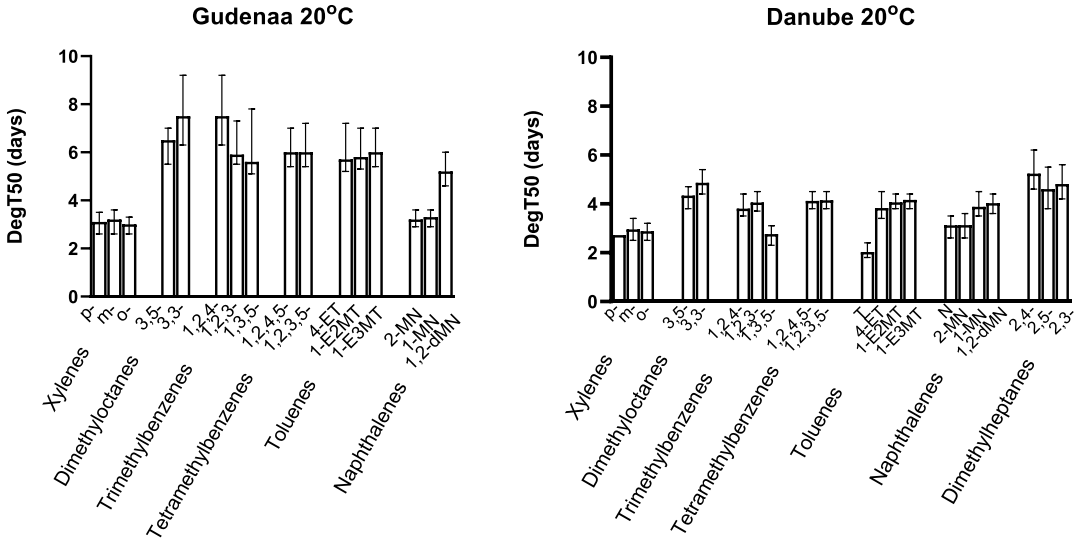

Figure 4. Biodegradation half-times ($DegT_{50}$, days) obtained with Gudenaa and Danube inocula at a test temperature of 20 °C for the isomers included in the study. These plots include biodegradation half-times also for isomers with $K_{aw} > 1$, since all data were obtained at the same temperature. T: toluene; 4-ET: 4-ethyltoluene; 1-E2MT: 1-ethyl-2-methyltoluene; 1-E3MT: 1-ethyl-3-methyltoluene; N: naphthalene; 2-MN: 2-methylnaphthalene; 1-MN: 1-methylaphthalene; 1,2-dMN: 1,2-dimethylnaphthalene.

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S11: table of $DegT_{50}$ obtained with the logistic model for both inocula; S12: $DegT_{50}$ obtained via the logistic model vs $DegT_{50}$ obtained via the first-order model; S13: $DegT_{50}$ (12 °C) calculated via the Arrhenius equation from the obtained 20 °C data is plotted against the measured $DegT_{50}$ at 12 °C; S14: Arrhenius plots of $T_{1/2}$ and T_{lag} for both samples; S15: biodegradation times for isomers included in the study obtained with Gudenaa and Danube inocula at 12 °C (PDF)

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Notes

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