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Biodegradation of aliphatic and aromatic hydrocarbons at high temperatures

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Abstract In this paper, the high temperature (65–75°C) biodegradation of aliphatic and aromatic hydrocarbons is investigated and kinetic parameters are derived. The shift of the physico–chemical system properties with rising temperature will be discussed in detail. For example, the solubility of naphthalene is increased by a factor of about ten if the temperature is increased from 20 to 75°C. This effect is essential to increase the bioavailability of sparingly soluble hydrocarbons. It is also demonstrated in experiments that very high oxygen transfer rates can be obtained at high temperatures in the presence of hydrocarbons.

It is shown that efficient phenol biodegradation is essential for high temperature hydrocarbon degradation because some microorganisms tend to transform phenols into polyphenols which are very inhibitory for microbial growth. A defined mixed culture adapted to phenol converted more than 90% of a mixture of phenol, hexadecane and pyrene and a very high maximal growth rate of 0.19 h^{-1} was determined. A yield coefficient $Y_{X/S}$ of about 0.8 g (biomass)/g (hydrocarbons) was calculated in this experiment. In a separate experiment the influence of the hydrocarbon droplet size on the biodegradation is investigated at 70°C using a newly isolated *Thermus* sp. In this case, the growth on a hexadecane/pyrene mixture was described by a model based on the Monod equation and the corresponding kinetic parameters are derived.

A mixed culture was used for the bioremediation of soil in a slurry reactor. The initial contamination of 11 g/kg was lowered to about 2 g in a reactor inoculated by an immobilized culture of extreme thermophilic microorganisms, while 9 g/kg remained in a sterile control.

Keywords Aliphatic hydrocarbons; bioavailability; biodegradation; extreme thermophilic microorganisms; PAH; phenol

Introduction

In this paper we report on the degradation of hydrocarbon compounds and well known mixtures of hydrocarbons at high temperatures using cultures of newly isolated aerobic microorganisms. The most important groups of pollutants are investigated using a selected pollutant from each group: phenolic compounds; phenol, polycyclic aromatic hydrocarbons; pyrene, aliphatic hydrocarbons; hexadecane. Important kinetic parameters will be derived and the oxygen supply to microbial cultures at high temperatures in the presence of hydrocarbons are investigated. An example demonstrates the feasibility of high temperature soil bioremediation. In the following, the physico–chemical fundamentals of hydrocarbon biodegradation in two or three phase systems will be discussed in detail. For the biochemical details of aerobic hydrocarbon degradation the reader may refer to Britton (1984) for aliphatic hydrocarbons) and Cerniglia (1992) for aromatic hydrocarbons.

Bioavailability of hydrophobic pollutants in treatment systems

For efficient biodegradation the pollutants must be transferred to the bacteria before degradation, e.g from a droplet or a contaminated soil particle (Volkering, 1996). The mass transfer $\dot{m}$ can be modeled by adapting the well known mass transfer Eq. (1) for transport of a sparingly soluble gas to the medium. In this case a sparingly soluble hydrocarbon has to

be transported to the medium and the main resistance is assumed to be in the boundary layer around the contaminated particle/oil droplet:

$$\dot{m} = k_L a\,(c_{eq} - c_t) \tag{1}$$

In order to obtain shortest possible treatment times in cases where the bioavailability is the bottleneck for bioremediation, each factor in this equation must be maximized:

k_L The mass transfer coefficient k_L value can be considered to be proportional to the square root of the diffusion coefficient of the hydrocarbon in water. Thus, by increasing the diffusion coefficient, the mass transfer will be enhanced. The diffusion coefficient of, for example, PAH generally increases with rising temperature, typical by a factor of five if the temperature is shifted from room temperature to about 70°C as for example with naphthalene (Figure 1).

a The surface area per volume: in *in-situ* bioremediation processes the limited access of bacteria to contaminant clusters and hence long transport distances for contaminants might considerably slow down the biodegradation. Kleijntjens (1992) discusses the general advantages of slurry processes for the accessibility of contaminants, or, translated to the letters of the equations, the advantages of high a values. High a values cannot be reached in static reactors. In principle the same goes for all two or three phase hydrocarbon degradation systems.

c_{eq} The equilibrium concentration of the contaminant in water: for PAH this solubility increases with rising temperature. The solubility of, for instance, naphthalene is increased by a factor of about ten if the temperature is increased from 20 up to about 75°C (Figure 1).

c_t The concentration actually found in the medium in which the microorganisms grow. The highest mass transfer rate is achieved in the case $c_t = 0$. To achieve this goal, a sufficiently high concentration of adapted bacteria must be present in the vicinity of the pollutants. One possibility to keep large amounts of bacteria within the reactor is the use of immobilized biomass.

The situation is more difficult if the contaminant is absorbed into the particle or sticks in a pore within the particle. Rulkens *et al.* (1996) demonstrate that the diffusion in the particle or in a pore might be the limiting factor for biodegradation in some situations. Since diffusivities of pollutants in the pores of soil particles are positively affected by higher temperature, elevated temperatures are also advantageous in this case. (A more detailed investigation of other temperature dependent parameters is given in Feitkenhauer, 1998.)

An important aspect for the application of aerobic thermophilic microorganisms in bioremediation is the oxygen transfer into the slurry. Measurements in laboratory-scale stirred bioreactors (Boogerd *et al.*, 1990; Krahe *et al.*, 1996) filled with medium or water demonstrate that the supply of oxygen to an aerobic culture increases, or is at least constant, at thermophilic conditions compared to mesophilic conditions. In a soil slurry the prediction of the possible oxygen transfer rate is further complicated, for instance by the influence of the soil particles. Furthermore, the oily contaminants change the coalescence of gas bubbles in the reactor. Measurements of the $k_L a$ values will be presented to clarify the influences of the oily contaminants on the oxygen transfer.

Materials and methods

Biomass content and kinetic parameters

Samples containing carrier material or soil slurry were treated with 1 N NaOH for 15 minutes at 95°C and the supernatant after centrifugation (5,000 g, 15 minutes) cell proteins were measured using the Lowry method (Süssmuth *et al.*, 1987).

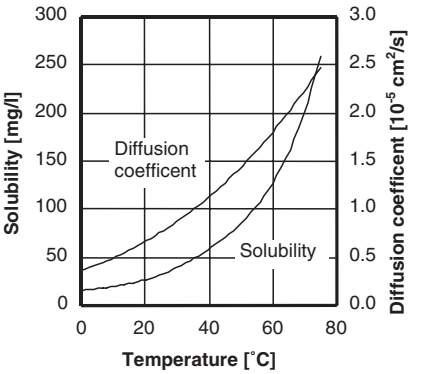

Figure 1 The solubility of naphthalene in water (IUPAC solubility data series, 1989) and the diffusion coefficient of naphthalene in water (Yaws *et al.*, 1995) increase with rising temperature

The specific growth rates μ were calculated from the protein content Pr (t = time, $\overline{\text{Pr}}$ = average protein content in the investigated interval):

$$\mu = \frac{\Delta(\text{Pr})}{\Delta t} \times \frac{1}{\overline{\text{Pr}}} \tag{2}$$

The yield coefficient for microbial growth $Y_{X/S}$ [g/g] was calculated from the increase in cell dry weight, Δ(cdw), divided by the decrease in hydrocarbon content, Δ (hydrocarbon), in the investigated period of time:

$$Y_{x/s} = \frac{\Delta(\text{cdw})}{\Delta(\text{hydrocarbon})} \tag{3}$$

The cell dry weight was calculated from the protein measurements, assuming 50% of the cell dry weight consists of protein (van den Akkere *et al.*, 1992).

The degree of substrate conversion α was calculated from the measured hydrocarbon contents:

$$\alpha = \frac{c_0 - c_i}{c_0} \tag{4}$$

where c_i is the current hydrocarbon concentration and c_0 is the start hydrocarbon concentration.

Microbial cultures and determination of hydrocarbons

A mixed culture of 11 extreme thermophilic, aerobic microorganisms was used where a mixed culture was employed. For soil biodegradation this culture was immobilized on Aquacel (Biomaterials, Japan) regenerated carrier material (Feitkenhauer, 1998). Ten isolates were *Bacillus* sp., while one was identified as a *Thermus* sp. For this mixed culture a phenol degrading *Bacillus thermoleovorans* sp. was kindly provided by Dr A. Muzzle and a naphthalene and two benzoic acid degrading strains by Dr S. Hebenbrock (both with Biotechnolgie I, TUHH).

The total hydrocarbon contents (IR-method) and pyrene (HPLC method) were measured as described previously (Feitkenhauer, 1998). The mean droplet size of emulsions was determined from slides taken from the emulsion and displaying a defined grid (microscope: Zeiss Axioplan, Germany).

Substrates

All substrates (pollutants) were purchased from Sigma-Aldrich, Deisenhofen, Germany and had a quality of at least "for synthesis".

Results and discussion

Oxygen transfer at high temperatures in the presence of hydrocarbons

The transfer of oxygen to supply the needs of the microbial culture was often described as a bottleneck for high temperature biodegradation as the solubility of oxygen in water decreases with rising temperature. To investigate the effect of hydrocarbon pollutants on the oxygen transfer to the microbial growth medium at high temperatures, $k_L a$ values were measured by a dynamical method in the presence and absence of diesel oil in a 2 L stirred bioreactor. 0.5 g/l diesel oil was added to a mineral salt medium. The resulting $k_L a$ values were considerably higher in experiments with diesel oil compared to experiments without diesel oil in the medium, especially at lower stirrer speeds (Table 1).

These measurements were supported by a visible change in the bioreactor. Smaller bubble diameters were observed after the addition of the diesel oil to the bioreactor and hence higher $k_L a$ values were measured. It has been previously shown that the oxygen transfer rate in bioreactors did not decrease with rising temperature despite the lower solubility of oxygen in water at higher temperatures (Boogerd *et al.*, 1990; Krahe *et al.*, 1996). The effect of the lower solubility is more than compensated by the faster diffusion of oxygen (higher diffusion coefficient). For example, the $k_L a$ value at 30°C and a stirrer speed of 1,500 rpm (in the same reactor) was determined to be 380 h^{-1} (Krahe *et al.*, 1996). The corresponding oxygen transfer rate of 68 mmol l^{-1} h^{-1} is slightly below the corresponding rate at 70°C (82 mmol l^{-1} h^{-1}) and well below the rate at 70°C in the presence of diesel fuel (139 mmol l^{-1} h^{-1}). The above presented results demonstrate that the air-supply in bioreactors for high-temperature hydrocarbon biodegradation is even easier because the obtainable oxygen transfer rates increase considerably in the presence of hydrocarbons.

Influence of phenol on hexadecane and pyrene degradation

Hydrocarbons in wastewater or contaminated sites are often mixtures of phenolic compounds and aliphatic and aromatic hydrocarbons. Therefore, the phenol degradation of a mixture of hexadecane, pyrene and phenol by a mixed culture was investigated (Table 2 and Figure 2).

Mixed cultures that are not adapted to phenol need a longer period of time before phenol degradation starts. Growth inhibiting phenol resins (visible by an increasingly dark-brown color) were formed during the long adaptation time (curve I in Figure 2). The conversion of contaminants was only 22% and the increase in biomass was negligible. Growth took place in the shake flask containing phenol and adapted microorganisms (curve II in Figure 2). The specific growth rate of 0.19 h^{-1} during the exponential growth phase was higher compared to the control experiment (0.11 h^{-1}) without phenol (curve III in Figure 2). The yield coefficient $Y_{X/S}$ of 0.8 g/g substrate and the degree of conversion (0.9) were virtually the same in both cases where growth took place.

Table 1 $k_L a$ values in a 2 L bioreactor measured in the presence and absence of 0.5 g/l diesel oil in mineral salts medium. The experiment was performed at 70°C and at an aeration rate of 1 vvm air

Stirrer speed [rpm]	$k_L a$ [h^{-1}] in medium	$k_L a$ [h^{-1}] in medium + diesel
1,000	349	658
1,500	451	767
2,000	631	772

Table 2 Compositions of the shake flasks used for the biodegradation of mixed contamination (Figure 2) and the specific growth rate during the exponential growth phase. Additionally, the degree of conversion and the yield coefficient are listed

	(I): non-adapted microorganism	(II) adapted microorganism	(III) adapted microorganism
Substrate	750 mg/l hexadecane 40 mg/l pyrene 190 mg/l phenol	750 mg/l hexadecane 40 mg/l pyrene 190 mg/l phenol	750 mg/l hexadecane 40 mg/l pyrene
Growth rate μ [h^{-1}]	0. 00	0.19	0.11
Degree of conversion ([–]	0.22	0.92	0.90
Yield coefficient $Y_{X/S}$ [–]	–	0.79	0.77

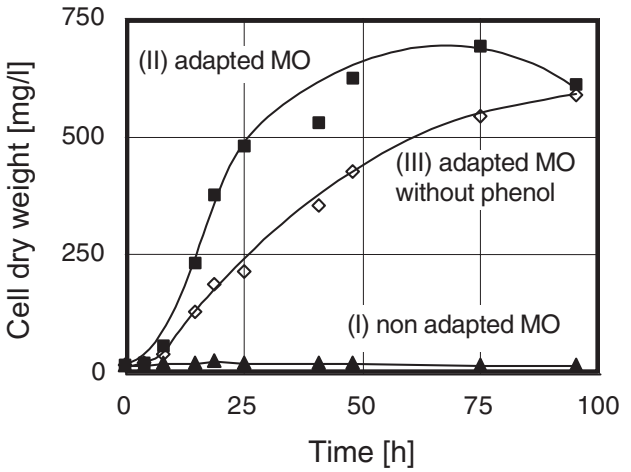

Figure 2 Growth of a mixed culture of microorganisms (MO; MC 11) on a mixture of 750 mg/l hexadecane, 40 mg/l pyrene and 190 mg/l phenol at 65°C and pH 6.5 in baffled shake flasks. In the first case (I) the mixture was not adapted to phenol and growth was nearly completely inhibited. In the second case (II) the culture had been adapted to phenol. In the third case (III) the mixed preculture was grown only on hexadecane and pyrene

Hexadecane biodegradation

Organic supplements or vitamins were not essential for growth and do not influence the degradation rates of hexadecane. Applying the same conditions, a maximal growth rate of $\mu = 0.13\ h^{-1}$ was observed whether vitamins were present or not in the medium. Alternatively, the described media cells could be grown in *Thermus* medium (DSMZ-Catalogue of strains, 1993) with similar growth rates. The nitrogen source in this medium is nitrate, which hence can replace ammonium as the nitrogen source. The growth rate was $0.12\ h^{-1}$ using the same substrates and conditions.

Pyrene biodegradation

Pyrene dissolved in heptamethylnonane (HMN; Table 3) was converted to a higher percentage compared to crystalline pyrene or pyrene solubilized with a synthetic emulsifier. The growth on pyrene and phenanthrene was not sustained over more than three transfers.

Biodegradation of hexadecane/pyrene mixtures

The degradation of hexadecane/pyrene mixtures was investigated in shaker flasks filled with a previously prepared emulsion. The hydrocarbon phase consisted of 800 mg/l hexadecane and 40 mg/l pyrene. The reactor was filled with sterile mineral salts medium. Depending on the stirrer speed and treatment time different mean droplet diameters were measured in the emulsions (Table 4).

Table 3 Degradation of pyrene and growth of *Thermus brockii* Hamburg at pH 6.25 and 70°C in closed shake flasks during ten days of incubation. Each flask initially contained 100 mg/l pyrene and 5 mg cell dry weight. Additionally, the effects of the emulsifier ET 5 or the non-degradable heptamethylnonane (HMN) were tested in separate shake flasks. (The cell weight increase caused by ET 5 alone was subtracted from the ET 5/pyrene value)

Description	Pyrene degraded [mg/l]	Cell dry weight increase [mg/l]
Pyrene	10	4
Pyrene + 50 µl ET 5	9	5
Pyrene + 1 g/l HMN	34	14

Table 4 Preparation of emulsions in a 2 L reactor. The hydrocarbons were added and the reactor operated for the indicated time with the indicated stirrer speed. After this time, the stirrer speed was set to 500 rpm and the emulsion was filled immediately into sterile shake flasks. The right row displays the resulting mean droplet diameters (d_{50})

Time	Stirrer speed [rpm]	d_{50} [µm]
1 minute	2,000	3.6
1 minute	3,000	2.1
15 minute	3,000	1.5

The shaker flasks were inoculated with *T. brockii* Hamburg. The growth on hexadecane/pyrene emulsions was nearly independent of the droplet diameter and could be described with a growth model based on Monod kinetics (Figure 3). The maximal growth rate of about 0.14 h^{-1} in the exponential phase was similar to the maximal growth rate on the non-emulsified hexadecane. However, one has to be aware that the assumptions of the Monod model are not fulfilled as the substrate mainly forms a second (organic) phase and

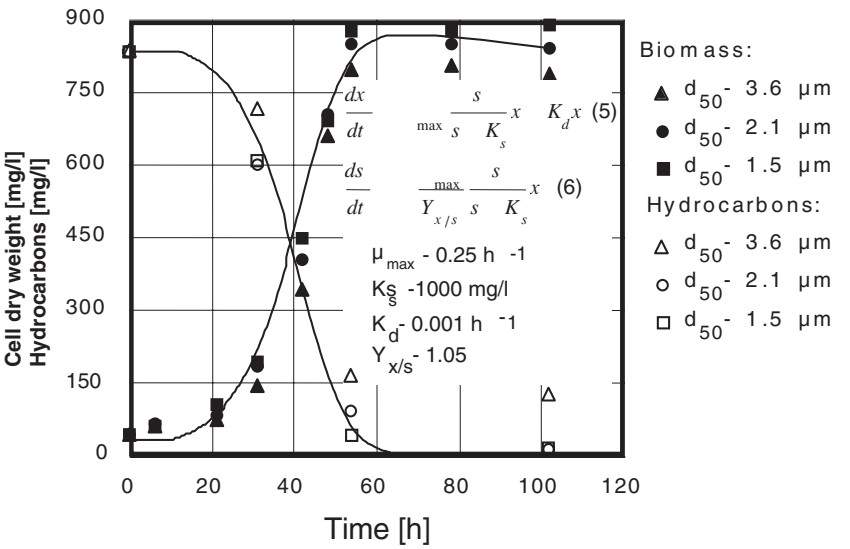

Figure 3 Growth of *T. brockii* Hamburg on hexadecane (800 mg/l)/ pyrene (40 mg/l) emulsions with different mean droplet diameters (d_{50}). Emulsions were produced at different stirrer speeds in a 2 L reactor before the degradation experiment. The degradation took place in baffled shake flasks at 70°C, pH 6.5 and at a shaker speed of 160 rpm. The lines were computed using the growth model (Eqs 5 and 6) and parameters listed above (after a lag-phase of ten hours)

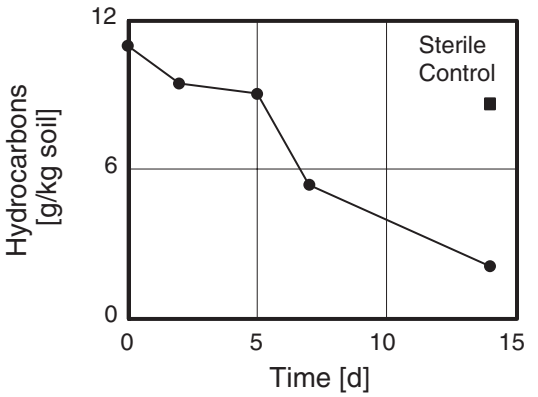

Figure 4 Reduction of the hydrocarbon content at 65°C and pH 6.5 in soil material spiked with defined diesel oil

was not dissolved in water. Therefore, the kinetics of growth on the substrate depends on the accessibility of the organic phase. Hence, different kinetic parameters will be measured in a system with the same concentration of the substrate, but different distribution of the droplet size.

The parameter μ_{max} of 0.25 h^{-1} (Figure 3) was higher than the maximal growth rate of 0.14 h^{-1} for one droplet size because the hydrocarbon concentration of 840 mg/l was lower than the K_s value of 1,000 mg/l.

The measured yield coefficients ranged from 0.95 to 1.03 g cell dry weight per g hydrocarbon for all droplet sizes. Pyrene and hexadecane were simultaneously removed from the medium. The biggest droplet size (mean diameter 3.6 µm) was not completely converted within about 100 hours and a degree of conversion of $a = 0.85$ was measured in the shake flasks with smaller droplets ($d_{50} = 2.2$ and 1.5 µm) nearly all hexadecane ($a = 0.98$ and 1, respectively) as well as nearly all pyrene ($a = 0.99$ and 0.92, respectively), was degraded.

Application in soil slurry bioremediation

To demonstrate the feasibility of high temperature bioremediation using a defined mixed culture the mixed culture MC 11 immobilized on Aquacel carrier material (10 g/l) was used in a 1 L stirred bioreactor (see Figure 4). The initial content of hydrocarbons (11 g/ kg soil) was lowered to about 2 g/ kg soil in the inoculated sample, but only to about 9 g/ kg soil in the sterile control (7 g hydrocarbons/kg soil biodegradation). The biodegradation using the same mixed culture without immobilization was about 1 g/kg soil in the same period of time. Conditions: soil slurry experiment (20% w/v soil) in a 1 L bioreactor, 1,400 rpm, 65°C, pH 6.5, aeration 0.01–0.03 vvm.

Conclusion

Elevating the temperature was shown to be a simple but yet efficient way to increase the bioavailability of sparingly soluble hydrocarbons. The obtainable oxygen transfer rates at 70°C were very high and even increased in the presence of hydrocarbons. Hence, the oxygen supply for active microbial cultures is easier than at lower temperatures. The conversion of PAH (pyrene) and aliphatic hydrocarbons (hexadecane) was very efficient and high growth rates were obtained. The presented data are very encouraging for several future applications of the high temperature hydrocarbon biodegradation, e.g. in soil bioremediation or wastewater treatment.

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References

Britton, L.N. (1984). Microbial degradation of aliphatic hydrocarbons. In: *Microbial Degradation of Organic Compounds*, D.T. Gibson (ed.), Marcel Decker, New York, pp. 89–129.

Boogerd, F.C., Bos, P., Kuenen, J.G., Heijnen, J.J. and vander Lans, R.G.J.M. (1990). Oxygen and carbon dioxide mass transfer and the aerobic, auxotrophic cultivation of moderate and extreme thermophiles: A case study related to the microbial desulfurication of coal. *Biotechnology and Bioengineering*, **35**, 1111–1119.

Cerniglia, C.E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation*, **3**, 351–368.

Hebenbrock, S. (1998). *Isolation and Characterization of Thermophilic Microorganisms and their Application for the Microbial Degradation of Naphthalene and Benzoic acid*. PhD. thesis. Technical University of Hamburg-Harburg, Germany (in German).

IUPAC solubility data series (1989). *Vol 38: Hydrocarbons with Water and Sea Water, Part II*. International Union of Pure and Applied Chemistry, Pergamon Press.

Feitkenhauer, H. (1998). *Biodegradation of Aliphatic and Aromatic Hydrocarbons at High Temperatures: Kinetics and Applications*. PhD. thesis. Technical University of Hamburg-Harburg, Germany.

Kleijntjens, R.H. (1992). *Biotechnological Slurry Process for the Decontamination of Excavated Polluted Soil*. Dissertation, Delft University of Technology.

Krahe, M., Antranikian, G. and Märkl, H. (1996). Fermentation of extremophilic microorganisms. *FEMS Microbiology Reviews*, **18**, 271–285.

Rulkens, W.H., Grotenhuis, J.T.C. and Field, J. (1996). Effect of the physical state of PAH pollutants on clean-up possibility of soil. In: *Biologische und Chemische Behandlung von PAK-haltigen Böden und Abwässern*, M. Cuno (ed.), Schriftenreihe Biologische Abwasserreinigung 7, TU Berlin, pp. 99–122.

Süssmuth, R., Eberspächer, J., Haag, R. and Springer, W. (1987). *Biochemisch-Mikrobiologisches Praktikum*, Thieme Verlag, Stuttgart.

van den Akkere, H.E.A., Heijnen, J.J., Leach, C.K. and Mudde, R.F. (1992). *Bioprocess Technology: Modeling and Transport Phenomena*. Butterworth-Heinemann, Oxford.

Volkering, F. (1996). *Bioavailability and Biodegradation of Polycyclic Aromatic Hydrocarbons*. Ph.D. Thesis, Wageningen, The Netherlands.

Yaws, C.L., Nijhawan, S. and Li, K.Y. (1995). Diffusion coefficients in water. In: *Handbook of Transport Property Data*, C. Yaws (ed.), Gulf Publishing Company, pp. 141–168.