Biodegradation (2009) 20:581–589
DOI 10.1007/s10532-008-9236-9

ORIGINAL PAPER

Microplate MPN-enumeration of monocyclic- and dicyclic-aromatic hydrocarbon degraders via substrate phase-partitioning

Anders R. Johnsen · Sidsel Henriksen

Received: 12 August 2008 / Accepted: 12 November 2008 / Published online: 29 November 2008

Abstract The high aqueous solubility of monoaromatic and some diaromatic oil components may hinder classical growth-based MPN enumeration of bacterial mono- and di-aromatics degraders because these aromatics are toxic in high concentrations. We developed a microplate MPN method for the enumeration of toluene-, xylene-, naphthalene-, biphenyl- and benzothiophene-degraders on the basis of phase-partitioning of substrate between a biologically inert organic phase and an aqueous mineral salt medium. This way, it was possible to maintain non-toxic, aqueous concentrations in the microplate wells. Depletion of aqueous aromatics by growth of the degraders was prevented by the continuous solubilization of aromatics from the silicone phase. The method was validated by MPN enumerating degrader cultures both with phase-partitioned aromatics and with tryptic soy broth as carbon sources. The applicability of the method was demonstrated by MPN-enumerating mono- and di-aromatic degraders in soils of varying hydrocarbon pre-exposure.

Keywords Benzothiophene · Biphenyl · MPN · Naphthalene · Toluene · Xylene

A. R. Johnsen (✉) · S. Henriksen
Department of Geochemistry, Geological Survey of Denmark and Greenland, Øster Voldgade 10, 1350 Copenhagen K, Denmark
e-mail: arj@geus.dk

Introduction

Oil pollution of soil and sediments often induce tremendous changes in the numbers of oil degrading organisms. The total number of oil degraders can be determined by routinely applied microplate MPN methods (Brown and Braddock 1990; Haines et al. 1996), but these methods do not discriminate between degraders of different oil components, and often underestimate population sizes due to substrate toxicity (Haines et al. 1996). In bioremediation studies, it is often of interest to enumerate degraders of specific oil aromatics, however, in the literature, we have not been able to find well-documented procedures for MPN-enumeration of mono- and di-aromatic degraders. In this article we therefore propose a new microplate method for the MPN-enumeration of toluene-, xylene-, naphthalene-, biphenyl- and benzothiophene-degraders.

Petroleum monoaromatics of relatively high aqueous solubility like benzene, toluene, ethylbenzene and xylenes have long been recognized as bacteriocidal agents (Walbum 1920) and have for instance been used to neutralize bacteria in soil enzyme assays (e.g., Kanazawa and Miyashita 1986; Deng and Tabatabai 1994; Boschker et al. 1995). Diaromatics also have antimicrobial effects. Biphenyl, for instance, has been used as a fungicide to control mould on citrus fruits, and naphthalene pucks have been used as disinfectants of urinals to reduce odour. Naphthalene concentrations as low as 5 mg l^{-1} may

be toxic for PAH-degraders (Shuttleworth and Cerniglia 1996). However, due to low solubility, naphthalene and biphenyl are generally regarded as less toxic towards degraders compared to the monoaromatics.

For volatile oil aromatics (e.g., toluene and xylenes), growth-based enumeration has been achieved in previous studies by providing the aromatics in the vapor phase for instance by pouring petroleum compounds into the lids of inverted petri dishes (Bushnell and Haas 1941). Liquid cultures can be placed in closed champers containing a beaker of water saturated with toluene (Holt and Krieg 1994, Andreoni et al. 1997), or a vial containing toluene can be suspended above the medium inside shake-flasks (Gibson et al. 1970). It is evidently difficult to control the dosage in such systems.

Alternatively, the aromatics have been provided in a separate phase, for instance by circulating the aromatics in silicone tubing that was immersed in the aqueous medium (Choi et al. 1992). Kaufmann et al. (2006) demonstrated that degradation of volatile oil constituents in soil could be estimated by using a two-phase microplate set up, where aromatics were added in an inert organic phase (heptamethylnonane) to control the dosage. Soil was placed on top of the organic phase, and increased respiration was detected as CO_2-mediated color change in agar-plugs placed above the microplate wells.

It has previously been shown that phase-partitioning between water and an organic phase is an efficient way of maintaining constant aqueous concentrations of hydrophobic compounds in toxicological studies (Mayer et al. 1999). For MPN enumeration of mono- and di-aromatics degraders, we suggest to control the bacterial exposure to the aromatic substrates by such substrate phase-partitioning between a biologically inert organic phase (silicone oil) and the aqueous mineral salt medium of the MPN plates. This way, it may be possible to maintain a low aqueous concentration of the aromatics in the microplate wells, and at the same time add enough aromatics to detect growth of the degrader cells. Depletion of aqueous aromatics in the microplate wells by the degraders will be prevented by the continuous solubilization of aromatics from the silicone phase. Growth is then detected after incubation by adding the tetrazolium respiration indicator INT.

We optimized the method with regard to incubation time. The method was then validated by comparing MPNs of reference cultures grown both with aromatics and tryptic soy broth as substrates. Finally, we used the method to estimate numbers of aromatics degraders in environmental samples with different histories of aromatics exposure.

Materials and methods

Chemicals and reagents

All reagents were of analytical grade. Bushnell-Haas minimal medium (BH-medium, per liter: magnesium sulfate, 0.2 g; calcium chloride, 0.02 g; monopotassium dihydrogen phosphate, 1.0 g; diammonium hydrogen phosphate, 1.0 g, potassium nitrate, 1.0 g; ferric chloride, 0.05 g (Bushnell and Haas 1941)) was purchased from Becton, Dickinson and Company (Le Pont de Claix, France), and adjusted to pH 6.8 with HCl. Iodonitrotetrazolium violet (INT) was purchased from ICN Biomedicals Inc. (Eschwege, Germany). Commercial xylene contained *o*-xylene, *m*-xylene, *p*-xylene and ethylbenzene (Aldrich, cat. no. 24764-2). Silicone oil AR20 (polyphenylmethylsiloxane) was purchased from Fluka (cat. no. 10836).

Soils

Bioremediated subsoil from a tar and asphalt production plant was obtained from a previous project (Hestbjerg et al. 2003). Arctic topsoil polluted with Jet A1 was sampled next to a jet fuel storage tank at Longyearbyen airport (Svalbard, August). A pristine sample was taken 100 m from the storage tank at a site which is protected from snowmobile traffic and has no known history of fuel contamination. The Arctic soils were stored at minus 18°C and preincubated 7 days at 5°C before use. Subsoil containing 2, 34 and 650 mg kg^{-1} of PAHs (Σ7PAHs) were obtained from a Danish bioremediation facility (RGS90, Copenhagen, Denmark, March). Motorway topsoil (Vejenbrød, Denmark) was sampled 1- and 24-m from the asphalt pavement (November). Diffusely polluted topsoils (road-side and park soil, central Copenhagen) were sampled in January for enumeration of mono- and di-aromatics degraders, in

July for enumeration of xylene isomer degraders, and in October for toxicity tests.

MPN-enumeration of mono- and di-aromatic degraders in soil

Soil samples (10 g) were mixed with 90 ml tetrasodium pyrophosphate (2 mM, pH 7.0), and placed on a horizontal shaker at 200 rev min^{-1} for 15 min. Then, ten-fold dilution series (10^{-2}–10^{-9}) were prepared in BH-medium, and four 200-µl aliquots from each dilution were transferred to sterile, polystyrene, flat-bottom, 96-wells microplates. About 10 µl of silicone oil containing aromatics (1%, v v^{-1} for liquid substrates, w v^{-1} for solid substrates) was added to each well, starting with the highest dilutions, and lids were placed on the microplates. Parallel plates were prepared for each aromatic. The microplates were placed in air-tight, polypropylene plastic boxes (9.5 × 15.0 × 8.5 cm) normally used for storage of food, and incubated for at 20–22°C in a fume cupboard. Controls included inoculated microplates with BH-medium and silicone oil to distinguish between unspecific growth and growth of specific aromatics degraders.

The plates were tested for growth after 1, 2, 3, 4, and 5 weeks using the respiration indicator INT. About 50 µl INT-solution (3 g l^{-1} dissolved in MilliQ water) was added to each well, and the plates were incubated overnight at 20–22°C in their polypropylene boxes. Metabolically active bacteria reduce INT to the corresponding formazan that forms a purple, intracellular precipitate. The number of growth-positive wells at each dilution was scored by visual inspection of the plates. MPNs were calculated using the DOS program "Most Probable Number Calculator" version 2.70 (Klee 1993).

MPNs of xylene isomer degraders were determined for three city soils as described above except that three-fold dilution series were prepared in stead of ten-fold dilution series to increase precision. Xylene degraders were enumerated with *o*-xylene, *m*-xylene, *p*-xylene or a commercial isomer mixture as carbon sources.

The toxicity of light aromatics in systems that are not phase-partitioned was tested by carrying out classical MPN-enumerations in closed, 10-ml pyrex tubes containing 1-ml aliquots of a three-fold dilution series of traffic-polluted topsoil from central Copenhagen. Eight dilutions and three replicates per dilution were used for each treatment. Toluene- and naphthalene-MPNs were determined when the substrates were added to the soil dilutions as pure compounds and when dissolved in silicone oil (50 µl per tube). The total substrate concentration was in al treatments 1 mg ml^{-1}. For the non-phase-partitioned count, naphthalene was added in hexane solution to empty tubes followed by evaporation of the hexane. The tubes were vortexed and incubated 4 weeks at 20–22°C before addition of INT (250 µl, 3 g l^{-1}).

Isolation and identification of xylene- and benzothiophene-degrading bacteria

One µl from the bottom of positive wells of xylene- and benzothiophene-MPN-plates (Ringe soil) was streaked on R2A agar plates. Single colonies were tested for growth on either benzothiophene or the commercial xylene mix. This was done by inoculating 5-ml glass test tubes containing 2 ml BH-minimal medium and 200 µl silicone oil with aromatics (0.25%). The tubes were incubated on a Whirley-mixer at 20–22°C, and growth determined as increased turbidity. Four isolates were identified by partial 16S rRNA gene sequencing. Approximately 530 bp of the 16S rRNA genes were amplified by PCR as described previously (Willumsen et al. 2005). The PCR products were sequenced by MWG-Biotech (Ebersberg, Germany).

MPN-enumeration of reference strains

Each strain was MPN enumerated on the relevant aromatic and on TSB (tryptic soy broth, Becton, Dickinson and Company, Le Pont de Claix, France). Cultures were grown in 5-ml glass test tubes containing 1 ml BH-minimal medium and 50 µl silicone oil with the relevant aromatic (1%). Ten-fold dilution series were prepared in BH medium followed by MPN enumeration with aromatics as sole carbon sources as described above. Silicone oil with aromatics was substituted with 10 µl TSB for MPN-enumeration of total cell numbers.

Steady-state aqueous aromatic concentrations

The steady-state, aqueous concentration of lipophilic compounds in phase-partitioned systems can be

calculated from distribution coefficients according to Eq. 1. Distribution coefficients are not available for the type of silicone oil used in this study (polyphenylmethylsiloxane), instead we have used values for solid silicone of the cross-linked polydimethylsiloxane (pdms) type.

$$C_w = \frac{m}{\left(1 + \frac{V_o}{V_w} \times K_{pdms/w}\right) \times V_w} \quad (1)$$

$K_{pdms/w}$ is the silicone–water partition coefficient, C_w is the aqueous aromatic concentration at steady state, m is the total amount of aromatic, V_w is the volume of water, and V_{pdms} is the volume of silicone. Rough estimates of the steady-state aqueous concentrations of the aromatics in the microplate wells were calculated from Eq. 1. $K_{pdms/w}$ for biphenyl and benzothiophene was estimated from literature values of $\log(K_{ow})$ according to Xia et al. (2005).

Results and discussion

Enumeration of degraders of specific oil components by growth-dependent methods is not trivial because the relatively high solubility of light, aromatic oil components, when present in growth media as pure compounds, may make them toxic to degrader organisms.

In our set up, the total aromatic concentrations in the microplate wells were 450–500 mg l^{-1}, which were close to or above the aqueous solubilities (Table 1). However, by using a silicone phase we could reduce the estimated aqueous equilibrium concentrations of the more soluble compounds (Table 1). When aqueous aromatics in the microplate wells were metabolized by the degraders, more aromatic would be released from the silicone phase thereby providing the degrader cells with more substrate for growth.

We used a constant amount of both substrate and silicone oil in each well leading to varying aqueous concentrations (Table 1), but the aqueous concentration may be adjusted by adding more or less silicone oil (Eq. 1). Also, the total amount of available substrate may be increased without affecting the aqueous concentration by adding more of both silicone oil and growth substrate (Eq. 1).

The aqueous concentrations indicated in Table 1 are maximum estimates because the actual concentrations were reduced by sorption to the microplate surfaces and evaporation of especially the monoaromatics. Aqueous concentrations were estimated from pdms/water partition coefficients, however, the silicone oil has aromatic side-chains, whereas the pdms has methyl side-chains, probably leading to stronger sorption to the silicone oil. Together with the salts present in the minimal medium this would shift the equilibrium concentrations towards higher concentrations in the silicone phase and lower aqueous concentrations. The aqueous concentrations of naphthalene, benzothiophene and biphenyl were further overestimated because they were based on partition coefficients determined at 25°C whereas the temperature in our experiment was only 20–22°C.

Table 1 Solubility, silicone–water partition coefficient ($K_{pdms/w}$), and estimated aqueous maximum concentration of mono- and di-aromatics in microplate wells used for MPN enumeration of aromatics degraders

	Solubility (mg l^{-1})	Partition coefficient, $K_{pdms/w}$	Estimated aq. concentration in MPN plates (mg l^{-1})
Toluene	526[a]	330[d] (22°C)	29
Benzothiophene	192[b]	331[e] (25°C)	28
Naphthalene	31[c]	524[f] (25°C)	18
m + p-Xylene	162[a]	885[d] (22°C)	11
Biphenyl	6.9[c]	1,580[g] (25°C)	6

[a] Sanemasa et al. (1982)

[b] Meylan and Howard (1995)

[c] Pearlman et al. (1984)

[d] Nardi (2003, pp. 39–45)

[e] Estimated from $\log K_{ow}$ (Andersson and Schräder 1999) according to Xia et al. (2005)

[f] Calculated from $\log K_{pdms/w}$ (Xia et al. 2005)

[g] Estimated from $\log K_{ow}$ (Klein et al. 1988) according to Xia et al. (2005)

We prepared identical MPN-plates, containing dilutions of bioremediated gasworks soil. The development in MPN-estimates over time is depicted in Fig. 1. Biphenyl MPN was relatively constant after 2 weeks incubation. Xylene MPN increased the first 3 weeks, whereas benzothiophene-, naphthalene-, and toluene-MPNs increased for 4 weeks. Four weeks incubation gave acceptable MPNs for all five compounds, and was applied in the subsequent experiments.

Ten-fold dilution MPNs are inherently imprecise, so small variations, for instance naphthalene and

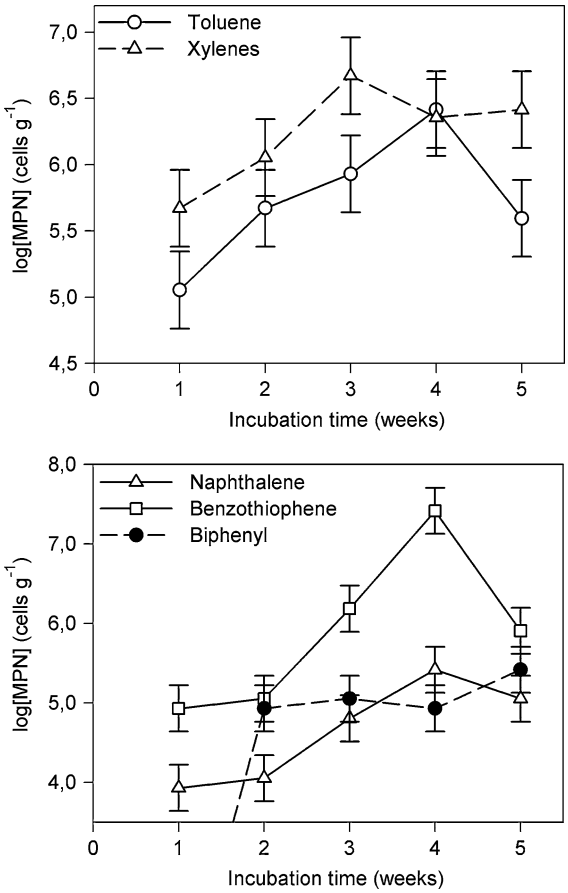

Fig. 1 Development in most probable numbers (log[MPN] ± one standard error) of monoaromatic degraders (*upper panel*) and diaromatic degraders (*lower panel*) as a function of the incubation time of the MPN plates. The carbon sources were added as 10 µl silicone oil containing 1% aromatics per microplate well. Ten-fold dilution factor, four wells per dilution

biphenyl from 4 to 5 weeks, were non-significant (Cochran 1950; Niemelä 1982). The toluene and benzothiophene estimates, however, significantly decreased when incubation was extended to 5 weeks. For toluene, this may have been caused by depletion of substrate in growth-positive wells combined with evaporation, leading to low conversion of INT. For benzothiophene, coloration of growth-positive wells indicated that accumulation of toxic metabolites may have inhibited the cells.

The dilution factor and the number of replicates per dilution used for the MPN-enumeration determine the standard error of the log(MPN) estimate (Cochran 1950). The standard errors of Fig. 1 therefore describe the systematic uncertainty of the method, they are not determined by the likelihood of the observed pattern of positive and negative microplate wells.

Best color development was seen for biphenyl, naphthalene and toluene, whereas the color of xylene and benzothiophene growth-positive wells was weaker. Occasionally, a false positive well was observed in low dilutions of the negative controls containing silicone oil only. Particulate organic matter or hydrocarbons present in the soils may have caused this.

Some substrate unavoidably evaporated and sorbed to the inside of the plastic boxes that were used for incubation. It is therefore important to use airtight boxes, and to use each box only for one compound.

The phase-partitioning method was validated with pure cultures of aromatic degraders. Because we could not readily obtain xylene- and benzothiophene-degraders, we isolated degraders from the MPN-plates of the bioremediated ringe soil. Four isolates were identified by partial 16S rRNA gene sequencing (~440 bp). The xylene degraders Xyl-2 and Xyl-8 showed 100% similarity both to *Rhodococcus opaqus* and to *R. wratislaviensis*. The benzothiophene degrader She-3 was 99.5% similar to *Williamsia muralis*, and She-4 was 99.8% similar to *R. fascians*.

The aromatic-MPNs of the reference cultures were not significantly different from the TSB-MPNs ($P = 0.49$, two-tailed, paired *t*-test), indicating that the degraders were not intoxicated by the aromatics. Another way of comparing the two methods is to test the aromatic-MPN against the TSB-MPN for each strain as described by Cochran (1950) and Niemelä (1982). Using this test, *Williamsia* sp. She-3 showed a significantly lower MPN on benzothiophene than on TSB, whereas the other strains all gave similar MPNs on the two substrates. This suggests that toxicity of the aromatics was only a problem for *Williamsia* sp. She-3.

For the MPN-method to be accurate, one degrader-cell in wells of the highest positive dilution must be able to multiply to a detectable level. The number of cells needed to give a positive response can be estimated from the data in Table 2 by calculating the (TSB-MPN)/(aromatic-MPN) ratios. The average ratio (excluding *Williamsia* sp. She-3) was 1.4 ± 1.4, demonstrating that about one culturable

Table 2 Validation of the phase-partition MPN method (ten-fold dilution factor, four wells per dilution) by comparison of reference strains counted simultaneously on aromatics and tryptic soy broth (TSB)

Degrader strain	Reference	Aromatic	Aromatic MPN (cells ml^{-1})	TSB MPN (cells ml^{-1})
Rhodococcus sp. Xyl-2[a]	This study	Xylene	4.7×10^6	1.1×10^6
Rhodococcus sp. Xyl-8[a]	This study	Xylene	2.6×10^4	1.1×10^5
Pseudomonas putida mt-2/pWWO, DSM3931	Yamamoto and Harayama (1998)	Toluene	8.5×10^8	2.3×10^8
Pseudomonas putida F1/pTOD, DSM6899	Zylstra et al. (1988)	Toluene	2.6×10^6	2.6×10^6
Sphingomonas yanoikuyae B1, DSM6900	Gibson et al. (1973)	Naphthalene	4.7×10^8	8.5×10^8
Pseudomonas putida OUS82	Takizawa et al. (1994)	Naphthalene	2.6×10^7	8.5×10^6
Sphingomonas yanoikuyae B1, DSM6900	Gibson et al. (1973)	Biphenyl	1.1×10^9	2.5×10^9
Burkholderia xenovorans LB400 DSM17367	Bopp (1986)	Biphenyl	2.6×10^7	8.5×10^6
Williamsia sp. She-3[a]	This study	Benzothiophene	1.1×10^5	4.7×10^6
Rhodococcus sp. She-4[a]	This study	Benzothiophene	2.6×10^7	6.4×10^7

[a] Tentative identification based on partial 16S rRNA gene sequences

cell per well was sufficient to initiate a growth-positive response. The inocula were pre-grown on media containing the aromatics, and it is possible that inocula not pre-exposed to aromatics may be more vulnerable. The (TSB-MPN)/(aromatic-MPN) ratio was 43 for *Williamsia* sp. She-3, suggesting that for this strain, it takes about 40 cells per well to overcome the toxic effects of benzothiophene in spite of phase-partitioning. Presumably, this strain would not be detected in traditional MPN procedures where aqueous benzothiophene concentrations would be much higher.

We carried out a comparison of phase-partitioned and non-phase-partitioned MPN counts of naphthalene degraders and toluene degraders. Traditional 10-ml reagent tubes were chosen in favor of microplates because much naphthalene would evaporate from the wells when crystals were formed by evaporation of the hexane solvent. The MPNs were not statistically different for naphthalene degraders (phase-partitioned: 5,800 cells g^{-1}; crystals: 6,600 cells g^{-1}), whereas saturated conditions turned out to be highly toxic for toluene degraders (phase-partitioned: 17,000 cells g^{-1}; liquid: 290 cells g^{-1}). This shows that the method has its' main advantage when enumerating degraders of oil aromatics with relatively high solubility like toluene, ethylbenzene, xylenes and heterocyclic N-, S- or O-substituted diaromatics. We suspect that the method might also be adapted for enumeration of chloroaromatics degraders which cannot be detected with the standard Biolog MT plates (Fulthorpe and Allen 1994). Another application may be enumeration of total hydrocarbon degraders where creosote or diesel fuel could be diluted with silicone oil to avoid toxicity.

The MPN-method was tested with eight soils of varying degree of aromatic contamination (Table 3). The size of the total, culturable populations (TSB-MPNs) were comparable for all soils, showing that the differences in specific degrader populations were not caused by differences in the general size of the bacterial populations.

Highest variation was seen for naphthalene and biphenyl MPNs which ranged four to five orders of magnitude depending on pollution level. In the PAH-polluted city soils, the naphthalene- and biphenyl-MPNs varied two orders of magnitude, and were closely linked to the total PAH concentrations. Heavy jet fuel contamination of the arctic Svalbard soil increased the naphthalene- and biphenyl-MPN counts by three orders of magnitude.

Xylene- and toluene MPNs varied only two orders of magnitude. This probably reflected that monoaromatics are common in the environment because of the high consumption of gasoline, diesel and heating oil in industrialized areas, combined with high vapor pressures, which promote evaporation from polluted topsoil and redeposition in unpolluted soils. It is especially interesting that increased PAH concentrations in the city soils had only negligible effects on the toluene- and xylene MPN counts. The reason is probably that the PAHs are of pyrogenic origin, and thus rich in high molecular weight compounds, but not enriched in monoaromatics.

Table 3 MPN estimates (cells g^{-1}, ten-fold dilution factor, four wells per dilution) of hydrocarbon degrader populations in soil with varying degrees of hydrocarbon contamination

Sample	Growth substrate					
	Naphthalene	Biphenyl	Toluene	Xylene	Diesel	TSB
Svalbard, pristine	8.5×10^2	1.1×10^2	2.6×10^3	$<1.1 \times 10^2$	$<1.1 \times 10^2$	1.1×10^7
Svalbard, jetA1	2.6×10^5	1.1×10^5	1.1×10^5	8.5×10^4	4.7×10^3	1.1×10^7
Motorway, 24 m	2.6×10^2	$<1.1 \times 10^2$	8.0×10^4	1.1×10^3	1.1×10^4	1.1×10^8
Motorway, 1 m	4.7×10^4	1.1×10^3	8.0×10^4	5.3×10^4	1.1×10^5	1.1×10^8
Street	3.7×10^4	2.7×10^3	2.7×10^3	2.5×10^3	2.7×10^4	2.7×10^7
City, 2 mg kg^{-1} PAH	4.7×10^4	6.4×10^4	8.5×10^4	4.7×10^4	2.6×10^4	6.4×10^7
City, 34 mg kg^{-1} PAH	2.6×10^5	2.6×10^5	8.5×10^4	8.5×10^4	2.3×10^5	4.7×10^7
City, 650 mg kg^{-1} PAH	8.5×10^6	8.5×10^6	2.3×10^5	8.5×10^4	8.5×10^5	4.7×10^7

TSB Tryptic soy broth

MPNs on specific aromatics were in many cases of the same magnitude as MPNs counted on the complex mixture of aliphatics, mono- and di-aromatics in diesel. In the jet fuel polluted soil, the MPNs on defined aromatics were significantly higher than on diesel (Cochran 1950; Niemelä 1982), suggesting that the diesel mixture inhibited growth of some of the aromatic degraders.

Set-ups with ten-fold dilution and four replicates per dilution are well-suited for screening purposes because they cover eight orders of magnitude, but they are also inherently imprecise. To increase precision one can either increase the number of replicates per dilution or decrease the dilution factor. We reduced the dilution factor from ten to three to investigate the effect on MPN of using different xylene isomers as carbon sources. We MPN-enumerated degrader populations from three city soils on *m*-xylene, *o*-xylene and *p*-xylene individually, and on a commercial xylene mixture (Table 4). MPNs with *o*-xylene or *m*-xylene were all lower than the corresponding MPNs on the commercial mixture, whereas MPNs on *p*-xylene were greatly increased. In the initial method development, we used the commercial mixture. However, it seems that many xylene degraders may grow only on *p*-xylene, and that *o*- and *m*-xylene are toxic to *p*-xylene degraders when enumerated on the isomer mixture.

Table 4 Comparison of xylene-degrader MPNs (cells g^{-1}, three-fold dilution factor, four wells per dilution) of city soils when enumerated on *m*-xylene, *o*-xylene, *p*-xylene and a commercial xylene mixture

	o-xylene	*m*-xylene	*p*-xylene	Xylene mix
Park soil	160	260	51,000	390
Road-side soil 1	82	390	14,000	1,400
Road-side soil 2	760	710	8,000	2,300

A drawback of the method is that visual scoring of growth-positive wells is subjective to some degree. When aromatics degraders of highly polluted soils were enumerated, the color change was generally clear cut, whereas the color change was ambiguous for some wells containing dilution series of non-polluted or slightly polluted soil. Different persons inspecting the same plate may thus come to slightly different conclusions when some wells are weakly colored. It has also been shown that not all environmental isolates metabolize tetrazolium compounds (McCluskey et al. 2005), which obviously is a general draw-back of all methods that rely on tetrazolium reduction.

The Wrenn and Venosa (1996) method for enumerating PAH-degraders relies on accumulation of colored dead-end metabolites, probably from co-metabolization of fluorene and dibenzothiophene. For toluene, xylene, naphthalene and biphenyl, this is not an option when present as pure compounds. The coloration from metabolites was weak or absent in many INT-positive wells suggesting complete degradation of these compounds. Benzothiophene degradation, on the other hand, showed accumulation of colored metabolites which helped distinguishing growth-positive from growth-negative wells.

INT, which is insoluble when reduced to the formazan, is sometimes substituted with modern

tetrazolium compounds that are soluble both in the tetrazolium and the formazan forms, thus allowing non-subjective absorbance measurements using a microplate photometer. In preliminary experiments, we tested the modern tetrazolium compound WST-1 which forms a yellow, soluble formazan when metabolized by bacteria (Johnsen et al. 2002). The color changes in positive wells were, however, weak when MPN-enumerating total oil degraders in contaminated soil (data not shown). A possible explanation may be that the higher hydrophilicity of WST-1 compared to INT may reduce the uptake into some hydrocarbon degraders because these cells often have very hydrophobic cell surfaces.

Acknowledgment We want to thank Trine Henriksen (GEUS) for deriving Equation 1.

References

Andersson J, Schräder W (1999) A method for measuring 1-octanol–water partition coefficients. Anal Chem 7:3610–3614. doi:10.1021/ac9902291

Andreoni V, Origgi G, Colombo M et al (1997) Characterization of a biofilter treating toluene contaminated air. Biodegradation 7:397–404. doi:10.1007/BF00056423

Bopp LH (1986) Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J Ind Microbiol 1:23–29. doi:10.1007/BF01569413

Boschker HTS, Bertilsson A, Dekkers EMJ et al (1995) An inhibitor-based method to measure initial decomposition of naturally occurring polysaccharides in sediments. Appl Environ Microbiol 61:2186–2192

Brown EJ, Braddock JF (1990) Sheen screen, a miniaturized most-probable-number method for enumeration of oil-degrading microorganisms. Appl Environ Microbiol 56:3895–3896

Bushnell H, Haas H (1941) The utilization of certain hydrocarbons by microorganisms. J Bacteriol 41:653–673

Choi Y-B, Lee J-Y, Lim H-K et al (1992) A novel method to supply the inhibitory aromatic compounds in aerobic biodegradation process. Biotechnol Tech 6:489–494. doi:10.1007/BF02447819

Cochran WG (1950) Estimation of bacterial density by means of the 'most probable number'. Biometrics 6:105–116. doi:10.2307/3001491

Deng SP, Tabatabai MA (1994) Cellulase activity of soils. Soil Biol Biochem 26:1347–1354. doi:10.1016/0038-0717(94)90216-X

Fulthorpe RR, Allen DG (1994) Evaluation of Biolog MT plates for aromatic and chloroaromatic substrate utilization tests. Can J Microbiol 40:1067–1071

Gibson DT, Hensley M, Yoshioka H et al (1970) Formation of (+)-*cis*-2, 3-dihydroxy-1-methylcyclohexa-4, 6-diene from toluene by *Pseudomonas putida*. Biochemistry 9:1631–1635. doi:10.1021/bi00809a024

Gibson DT, Roberts RL, Wells MW et al (1973) Oxidation of biphenyl by a *Beijerinckia* species. Biochem Biophys Res Commun 50:211–219. doi:10.1016/0006-291X(73)90828-0

Haines JR, Wrenn BA, Holder EL et al (1996) Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable-number procedure. J Ind Microbiol 16:36–41. doi:10.1007/BF01569919

Hestbjerg H, Willumsen PA, Christensen M et al (2003) Bioaugmentation of tar-contaminated soils under field conditions using *Pleurotus ostreatus* refuse from commercial mushroom production. Environ Toxicol Chem 22:692–698. doi :10.1897/1551-5028(2003)022<0692:BOTCSU>2.0.CO;2

Holt JG, Krieg NR (1994) Enrichment and isolation. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC, pp 179–215

Johnsen AR, Bendixen K, Karlson U (2002) Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates using the respiration indicator WST-1. Appl Environ Microbiol 68:2683–2689. doi:10.1128/AEM.68.6.2683-2689.2002

Kanazawa S, Miyashita K (1986) A modified method for determination of cellulase activity in forest soil. Soil Sci Plant Nutr 32:71–79

Kaufmann K, Chapman SJ, Campbell CD et al (2006) Miniaturized test system for soil respiration induced by volatile pollutants. Environ Pollut 140:269–278. doi:10.1016/j.envpol.2005.07.011

Klee AJ (1993) A computer program for the determination of most probable number and its confidence limits. J Microbiol Methods 18:91–98. doi:10.1016/0167-7012(93)90025-D

Klein W, Kördel W, Weiss M et al (1988) Updating of the OECD test guideline 107 "Partition coefficient *n*-octanol/water": OECD laboratory intercomparison test on the HPLC method. Chemosphere 17:361–386. doi:10.1016/0045-6535(88)90227-5

Mayer P, Wernsing J, Tolls J et al (1999) Establishing and controlling dissolved concentrations of hydrophobic organics by partitioning from a solid phase. Environ Sci Technol 33:2284–2290. doi:10.1021/es9808898

McCluskey C, Quinn JP, McGrath JW (2005) An evaluation of three new-generation tetrazolium salts for the measurement of respiratory activity in activated sludge microorganisms. Microb Ecol 49:379–387. doi:10.1007/s00248-004-0012-z

Meylan WM, Howard PH (1995) Atom fragment contribution method for estimating octanol–water partition-coefficients. J Pharm Sci 84:83–92. doi:10.1002/jps.2600840120

Nardi L (2003) Determination of siloxane–water partition coefficients by capillary extraction-high-resolution gas chromatography study of aromatic solvents. J Chromatogr A 985:39–45. doi:10.1016/S0021-9673(02)01457-7

Niemelä S (1982) "Most probable number" methods. In Statistical evaluation of results from quantitative microbiological examinations, Nordic Committee on food analysis, pp 8–11

Pearlman RS, Yalkowsky SH, Banerjee S (1984) Water solubilities of polynuclear aromatic and heteroaromatic compounds. J Phys Chem Ref Data 13:555–562

Sanemasa I, Araki M, Deguchi C et al (1982) Solubility measurements of benzene and alkylbenzenes in water by making use of solute vapor. Bull Chem Soc Jpn 55:1054–1062. doi:10.1246/bcsj.55.1054

Shuttleworth KL, Cerniglia CE (1996) Bacterial degradation of low concentrations of phenanthrene and inhibition by naphthalene. Microb Ecol 31:305–317. doi:10.1007/BF00171574

Takizawa N, Kaida N, Torigoe S et al (1994) Identification and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase and polycyclic aromatic hydrocarbon dihydrodiol dehydrogenase in *Pseudomonas putida* OUS82. J Bacteriol 176:2444–2449

Walbum LE (1920) Untersuchungen über die Einwirkung von Petroleumäther und einigen reinen Kohlenwasserstoffen auf die Bakterien der *Typhus-Coli*-Gruppe. Zentralblatt für Bakteriologie Parasitenkunde Infektionskrankheiten 1(69):6–9

Willumsen PA, Johansen JE, Karlson U et al (2005) Isolation and taxonomic affiliation of *N*-Heterocyclic aromatic hydrocarbon transforming bacteria. Appl Microbiol Biotechnol 67:420–428. doi:10.1007/s00253-004-1799-8

Wrenn BA, Venosa AD (1996) Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number method. Can J Microbiol 42:252–258

Xia XR, Baynes RE, Monteiro-Riviere NA et al (2005) Membrane uptake kinetics of jet fuel aromatic hydrocarbons from aqueous solutions studied by a membrane-coated fiber technique. Toxicol Mech Methods 15:307–316. doi:10.1080/15376520590968888

Yamamoto S, Harayama S (1998) Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of gyrB, rpoD and 16S rRNA genes. Int J Syst Bacteriol 48:813–819

Zylstra GJ, McCombie R, Gibson DT et al (1988) Toluene degradation by *Pseudomonas putida* F1, genetic organization of the *tod* operon. Appl Environ Microbiol 54:1498–1503