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ORIGINAL PAPER

Simplified MPN method for enumeration of soil naphthalene degraders using gaseous substrate

Kaisa Wallenius · Kaisa Lappi · Anu Mikkonen · Annika Wickström · Anu Vaalama · Taru Lehtinen · Leena Suominen

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Abstract We describe a simplified microplate most-probable-number (MPN) procedure to quantify the bacterial naphthalene degrader population in soil samples. In this method, the sole substrate naphthalene is dosed passively via gaseous phase to liquid medium and the detection of growth is based on the automated measurement of turbidity using an absorbance reader. The performance of the new method was evaluated by comparison with a recently introduced method in which the substrate is dissolved in inert silicone oil and added individually to each well, and the results are scored visually using a respiration indicator dye. Oil-contaminated industrial soil showed slightly but significantly higher MPN estimate with our method than with the reference method. This suggests that gaseous naphthalene was dissolved in an adequate concentration to support the growth of naphthalene degraders without being too toxic. The dosing of substrate via gaseous phase notably reduced the work load and risk of contamination. The result scoring by absorbance measurement was objective and more reliable than measurement with indicator dye, and it also enabled further analysis of cultures. Several bacterial genera were identified by cloning and sequencing of 16S rRNA genes from the MPN wells incubated in the presence of gaseous naphthalene. In addition, the applicability of the simplified MPN method was demonstrated by a significant positive correlation between the level of oil contamination and the number of naphthalene degraders detected in soil.

Keywords Hydrocarbon degraders · MPN · Naphthalene · Optical density · Phase partitioning · Soil

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K. Wallenius (✉) · K. Lappi · A. Mikkonen · A. Wickström · A. Vaalama · L. Suominen
Department of Food and Environmental Sciences, University of Helsinki, PO Box 56, FI-00014 Helsinki, Finland
e-mail: kaisa.wallenius@helsinki.fi

T. Lehtinen
Department of Environmental Sciences, Agricultural University of Iceland, Keldnaholt 112, Reykjavik, Iceland

Introduction

The potential of a soil to degrade oil contaminants can be evaluated by quantifying bacterial populations that are capable of degrading different petroleum hydrocarbons. This approach has been shown to be especially useful in the monitoring of bioremediation treatments (e.g. Kirk et al. 2005; Piskonen et al. 2005; Powell et al. 2006; Mikkonen et al. 2010). The degraders of aromatic hydrocarbons are of special interest because aromatics comprise a highly toxic

and widespread group of hydrocarbon contaminants with bioaccumulative properties.

Quantification of specific microbial populations can be performed both by means of nucleic acid based molecular methods and cultivation based procedures e.g. most-probable-number (MPN) technique. Both of these approaches have advantages and drawbacks. We chose the MPN approach for enumeration of naphthalene degraders in soil because we did not want to restrict the assay to bacteria with known metabolic pathways of naphthalene degradation.

The present-day MPN methods for hydrocarbon degraders generally employ a 96-well microplate set-up. Aromatic substrates are typically dissolved in a suitable volatile and added to each well individually (Wrenn and Venosa 1996; Johnsen et al. 2002; Kirk et al. 2005). Johnsen and Henriksen (2009) mixed the aromatic substrates with inert mineral oil in order to reduce the toxicity of pure aromatics to the degrader community. Distinguishing growth-positive wells from negative wells appears to be tricky as there are several methods published for this purpose (reviewed by Johnsen 2010). The most common way of detecting growth is to use respiration indicators (INT and WST-1) which change colour upon reduction by respiratory active cells (Haines et al. 1996; Wrenn and Venosa 1996; Johnsen et al. 2002; Johnsen and Henriksen 2009). The detection of growth by INT indicator is based on visual estimation where objectivity is hard to accomplish. WST-1 has the advantage of non-subjective scoring by automated absorbance measurement. However, the reliability of WST-1 based detection may also be questioned as it is not sensitive enough to demonstrate growth on aromatics by itself but needs to be used in combination with easily degradable carbon sources (Johnsen et al. 2002). In addition to the problems with interpretation, respiration indicators are toxic and expensive.

Our aim was to develop an improved MPN method for enumeration of soil naphthalene degraders with three essential requirements: (1) reproducible and objective quantification, (2) simple protocol and (3) a possibility for further characterization of degrader strains. The new method introduced in this paper differs from the alternative MPN methods by the techniques used for substrate dosing and scoring of results. We combined the old techniques of providing the aromatic substrate in vapour-phase (Bushnell and Haas 1941; Gibson et al. 1970; Hanzel et al. 2011) and measuring the growth of bacterial cultures by optical density (Monod 1949) into quantification of microorganisms with microplate-MPN enumeration.

Materials and methods

Experimental set-up

The validity and applicability of the new MPN method was tested in two experiments. The first experiment was a method comparison between our method referred to as 'the vapour method' and the method of Johnsen and Henriksen (2009) referred to as 'the silicone oil method'. In the second experiment we investigated the effect of increasing concentration of oil contamination in soil on the number of naphthalene degraders detected with the vapour method.

Soil samples

Sampling for the method comparison was carried out in January 2010. A contaminated soil sample with an approximate total petroleum hydrocarbon (TPH C_{10}–C_{40}) concentration of 3.8 mg g^{-1} was collected from the frozen topsoil of an old landfarming bioremediation field situated in the southern Finland. The field had received approximately 30,000 tons of moderately oil-contaminated refinery waste sludges per 3.5 ha during 1980–2005. Soil texture was fine sand. A non-contaminated soil sample was collected in a similar manner from an agricultural field with clay loam texture. Due to the wintry conditions during sampling, both soil samples were pre-incubated at room temperature for 7 days before preparing the MPN plates. Samples were analysed with four replicates.

Sampling for the oil gradient experiment was performed in May from the same landfarming bioremediation field as described above. Six samples, with the TPH concentrations ranging from 1.5 to 3.8 mg g^{-1}, were taken from the depth of 0–20 cm. The samples were sieved (Ø 5 mm) on the sampling day and stored at 4°C until the MPN plates were prepared the next day. The TPH concentration of the samples was analysed according to the ISO 16703 (2004) standard procedure as described by Mikkonen et al. (2010) except that the acetone was removed

using the centrifugation method (Jørgensen et al. 2005). Soil total microbial biomass carbon (mic_c) was determined by the chloroform-fumigation extraction method (Vance et al. 1987) as described by Guicharnaud et al. (2010).

MPN

Vapour method

Five grams of fresh soil was mixed with 45 ml of phosphate buffered saline, pH 7.2 (Lauderdale et al. 1999), and shaken for 10 min on a horizontal shaker with the speed of 400 rpm. The soil suspension was left to stand for 20 min in order to let soil particles sediment, after which four-fold serial dilutions of the supernatant were prepared in Bushnell Haas broth (Difco™, USA). Eight sequential dilutions, starting from 1/400 dilution, were used for a six-tube MPN (method comparison) or a 12-tube MPN (oil gradient experiment) in 96-well microplates (Nunc™, Denmark). Two identical plates were prepared for each dilution series. One of the plates was placed in a sealed but not fully air-tight polypropylene plastic box with the dimensions 19 × 14 × 5 cm^3 (originally meant for storage of food) that contained 200 mg of solid naphthalene (substrate plate) and the other plate in a similar box but without naphthalene (negative control plate). The plates in the boxes were incubated for 3 weeks at room temperature (21–24°C) in a fume cupboard (due to evident naphthalene smell) and detected for growth using an absorbance reader (Labsystems iEMS Reader MF) with the wavelength of 540 nm.

The duration of the incubation period (the maximum turbidity reached) and the initial quantity of naphthalene crystals in the box (some crystals remaining after incubation, guaranteeing continuous dosing) were chosen on the basis of preliminary optimization experiments for the vapour method. The naphthalene concentration reaching the buffer in the wells was quantified by UV-spectroscopic measurement (280 nm; Schwarz and Wasik 1976) to be approximately in the range 2–8 mg l^{-1}. The aqueous concentration depended inversely on the distance from the spot source of solid naphthalene (4–14 cm) (Online resource 1). However, the concentration could relatively easily be modified and made more even across the plate by placing several spot sources around the plate. We also tested double-sided tape to attach the naphthalene crystals in the lid of the incubation box but had to give up on this as some crystals dropped to the MPN wells during incubation.

Silicone oil method

The plates for the silicone oil method were prepared in parallel from exactly the same soil dilution series as the ones for the vapour method. Naphthalene was dissolved to Silicone oil AR20 (Sigma-Aldrich) to yield a concentration of 1% (w v^{-1}) and added individually to each well (10 µl/well) as described by Johnsen and Henriksen (2009). Using silicone-water partition coefficient, Johnsen and Henriksen (2009) estimated the maximum aqueous concentration of naphthalene to be 18 mg l^{-1} in their system. Silicone oil without naphthalene was added to negative control plates. The incubation time and temperature were the same as for the vapour method. The results were scored in two ways: (1) by the absorbance measurement as described above for the vapour method and (2) visually using iodonitrotetrazolium violet (INT) indicator dye (Sigma-Aldrich) as described by Johnsen and Henriksen (2009).

Genetic identification of naphthalene degraders

Several positive MPN wells from the vapour method showing different visual appearance (colour, turbidity) were selected for genetic identification by cloning and sequencing. Tenfold dilutions of the cell suspensions in the incubated MPN wells were prepared in water and used as the templates for PCR with no prior DNA extraction. The first third of the ribosomal 16S rDNA was amplified with general bacterial primers fD1 (AGAGTTTGATCCTGGCTCAG) and PRUN518r (ATTACCGCGGCTGCTGG) adopted from Tiirola et al. (2003). PCR was performed in 50 µl reaction volume containing 1 × Phusion HF buffer (Finnzymes, Finland), 0.2 mM of each dNTP (Finnzymes, Finland), 0.5 µM of both primers (Oligomer, Finland), 1 U of Phusion DNA Polymerase (Finnzymes, Finland), and 1 µl of the template. Peltier Thermal Cycler DNA Engine (MJ Research) was used for PCR cycling with the following programme: initial denaturation (98°C, 3 min) followed by 30 cycles of 98°C 20 s, 57°C 45 s, 72°C 30 s, and final elongation at 72°C for

5 min. The PCR products were purified with MinElute PCR Purification Kit (Qiagen, Germany), after which A overhangs (required for the TA cloning procedure) were added to 9 µl of the product in 20 µl reaction volume containing 1 × PCR reaction buffer with 2 mM $MgCl_2$ (Biotools, Spain), 0.2 mM dATP (Finnzymes, Finland) and 0.5 U of DNA Polymerase (Biotools, Spain) by 20 min incubation at 72°C. The 16S rDNA fragments of approximately 500 bp were cloned into TOP10 cells with TOPO-TA Cloning kit (Invitrogen) according to the manufacturer's instructions. The clones were screened with LH-PCR fingerprinting (Suzuki et al. 1998) as described in Wallenius et al. (2010) to identify and select different amplicon lengths. The plasmids were extracted with GeneJET Plasmid Miniprep Kit (Fermentas) and the inserts were sequenced from both directions at the DNA Sequencing and Genomics Laboratory, Institute of Biotechnology, University of Helsinki using the cloning vector primers UP (M13–20) and RP.

Data analysis

The threshold absorbance value for naphthalene-induced growth (A_{th}) was determined separately for the different methods and soil matrix types using the absorbances measured from negative control plates (incubated for 3 weeks without naphthalene). Calculations were made using the formula $A_{th} = A_{neg} + 3\ s$, where A_{neg} is the average absorbance value of the second lowest dilution (1/1600) on the negative control plate and s its standard deviation. The second lowest dilution of the soil suspensions was chosen for determining the A_{th} because the lowest dilution (1/400) showed notably higher background turbidity than all the other dilutions.

MPNs were calculated with a Windows based MPN calculator downloaded from the internet (Curiale 2000).

All basic statistical analyses (t-test, Pearson correlation) were performed using Statistix for Windows 8.0 (Analytical Software, Tallahassee, USA).

The DNA sequences were analysed using BioNumerics 6.0 (Applied Maths, Sint-Martens-Latem, Belgium). The quality of the chromatograms was checked, the consensus sequence constructed, and primer regions excluded. The nearest neighbours for the trimmed sequences were retrieved with SINA Webaligner of the SILVA rRNA database project (Pruesse et al. 2007; Schloss 2009). The similarity of the sequences with their nearest neighbours was calculated in BioNumerics 6.0 using the Standard Pairwise alignment algorithm with the default settings.

Results

Method comparison

We carried out a comparison of the vapour and silicone oil methods with regard to the MPN counts of naphthalene degraders in oil-contaminated and non-contaminated soils. The set-up comprised four replicate soil suspensions, four-fold dilution series and six wells per dilution (six-tube MPN).

Results from both the methods were scored with an absorbance reader. The use of respiration indicator INT had to be rejected because the negative control plates showed extensive purple colour formation making it impossible to differentiate visually between the negative and positive wells (Online Resource 2).

Growth-positive wells were easily distinguished from negative wells by absorbance measurement at 540 nm. Difference in absorbance between the negative and positive wells was clear-cut with both the methods: With the vapour method, the average absorbances (standard deviation in parentheses) of positive and negative wells on the substrate plates were 1.03 (±0.37) and 0.051 (±0.032), respectively. The same figures for the silicone oil method were 0.554 (±0.18) and 0.0460 (±0.025). With the vapour method, no sporadic positive wells were detected on the negative control plates whereas a few false positive wells were observed with the silicone oil method.

The average MPN count of four replicates obtained from the oil-contaminated soil by the vapour method ($2.1\ [\pm 0.13] \times 10^5$ cells g^{-1}) was slightly higher than the count obtained by the silicone oil method ($1.1\ [\pm 0.63] \times 10^5$ cells g^{-1}). The difference of methods was statistically significant ($P = 0.028$).

Neither of the methods detected naphthalene degraders from non-contaminated agricultural soil. The theoretical detection limit of the used protocol, calculated by assuming one positive well at the lowest dilution, was 270 cells g^{-1} with the 95% confidence interval between 38 and 1,900 cells g^{-1}.

The absorbance values measured from the lowest dilution of non-contaminated soil on the negative control plates were slightly higher than the corresponding values measured from the substrate plates of the vapour method. With the silicone oil method, no such difference was observed between the negative control and substrate plates of non-contaminated soil. This indicates that naphthalene provided from gaseous phase inhibited heterotrophic soil bacteria that grew on soil-derived carbon in the lowest dilutions.

Oil gradient experiment

The density of naphthalene degraders increased slightly towards the more contaminated end of the landfarming field being between 2.2×10^3 and 1.5×10^4 cells g^{-1} (Fig. 1).

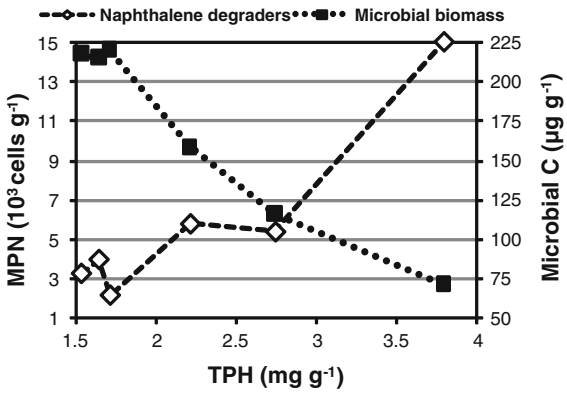

Fig. 1 MPN of naphthalene degraders and microbial biomass C as a function of soil TPH content

A significant positive Pearson correlation (0.93) between the TPH concentration (C_{10}–C_{40}) and the density of naphthalene degraders was found. By contrast, mic_c showed significant negative correlation (−0.98) with the TPH concentration (Fig. 1). Thus, the number of naphthalene degraders was not dependent on the size of the total soil microbial community but reflected the level of hydrocarbon contamination.

Identification of naphthalene degraders

Three clone libraries were constructed from the partial 16S rRNA genes amplified from the positive wells of the vapour method: libraries I and II from coloured wells with high and low absorbance, respectively, and library III from non-coloured wells with absorbance only slightly above the positive threshold. Libraries I and II contained various *Pseudomonas* species, whereas *Acidovorax* and *Aeromonas* where identified in library III (Table 1).

Discussion

The aim of this study was to develop a simple but sound most-probable-number (MPN) procedure to quantify the bacterial naphthalene degrader population in soil samples.

Firstly, we wanted to test whether naphthalene can be provided to growth medium via gaseous phase without being toxic to growing naphthalene degraders.

Table 1 Genera according to the SILVA taxonomy and the closest database matches of the clones sequenced from positive MPN wells with different appearances (vapour method)

Appearance	Genus (number of clones sequenced)	Nearest neighbours[a] (% identity), putative species
Coloured high turbidity	Pseudomonas (4)	AJ244724 (100-99.8), *Pseudomonas stutzeri*
		DQ536514 (100), *Pseudomonas trivialis*
		EU534727 (99.8), uncultured
Coloured low turbidity	Pseudomonas (9)	AY940128 (100), *Pseudomonas migulae*
		AJ441004 (100-99.8), *Pseudomonas syringae*
		AB021382 (99.8), *Pseudomonas meliae*
Non-coloured low turbidity	Acidovorax (2)	EF550172 (100), *Acidovorax* sp.
		AY853667 (99.6), uncultured
	Aeromonas (1)	AB027546 (91.8), *Aeromonas salmonicida*

[a] In order of frequency

The optimal concentration of hydrocarbon substrate in MPN assays is such that it maximally supports the growth of its degraders but is not toxic to the more sensitive members of the degrader community. For mono- and di-aromatics, this ideal might be difficult to accomplish as these substrates are known to have antimicrobial effects already in relatively low aqueous concentrations (Johnsen and Henriksen 2009). For example Shuttleworth and Cerniglia (1996) noticed naphthalene to be toxic to some degraders already at 5 mg l^{-1}. Concerned about this, Johnsen and Henriksen (2009) introduced a new MPN method in which the aqueous concentration of aromatic substrates was adjusted to a non-toxic level by using biologically inert silicone oil as a carrier material. In order to evaluate the toxicity of gaseous naphthalene on the degrader community, we chose the method of Johnsen and Henriksen (2009) for method comparison.

The results of method comparison performed in this study promote the use of gaseous naphthalene: the average number of naphthalene degraders obtained from the oil-contaminated soil by the vapour method was slightly but significantly higher than the number obtained by the reference method. This suggests that naphthalene provided from gaseous phase was equally good or slightly better than naphthalene provided from inorganic oil phase (on the bottom of each well) at supporting the growth of naphthalene degraders. However, comparing the absorbances measured from the positive and negative plates of non-contaminated soil revealed that gaseous naphthalene inhibited to some extent the growth of heterotrophic bacteria whereas no inhibition was observed for the silicone oil method. Thus, the aqueous concentration of naphthalene was most likely higher in our system than in the silicone oil method.

Choosing the right dosage of aromatic substrate in the buffer is challenging. Lowering the naphthalene concentration to a level which is not toxic to any soil bacteria might restrict the growth of naphthalene degraders due to shortage of carbon substrate. Naphthalene degraders belonging to the genus *Acidovorax* have been reported to be especially sensitive to high naphthalene concentrations (Shuttleworth and Cerniglia 1996; Jeon et al. 2003). Their presence in one clone library of this study provides support for the argument that the aqueous concentration of naphthalene in the vapour method was not too high for most of the naphthalene degraders.

Secondly, we wanted to test whether automated turbidity measurement can be used for reliable scoring of results. Hitherto, mainly respiration indicator reagents (Haines et al. 1996; Johnsen and Henriksen 2009) and naturally coloured intermediate degradation products (Stieber et al. 1994; Wrenn and Venosa 1996) have been used for the visual estimation of growth-positive wells of aromatic hydrocarbon degraders. However, it is well recognised that visual scoring systems are always subjective to some degree. Johnsen et al. (2002) reported that different persons examining the same MPN plates treated with INT came to different conclusions. Also Wrenn and Venosa (1996) discovered that INT was not suitable for detecting growth on PAH compounds because it was reduced poorly in positive wells. Automated measurement of turbidity with an absorbance reader is free from subjective interpretation errors but problems may arise from possible interference of insoluble hydrocarbons present as liquids or crystals with the optical density measurements (Johnsen 2010).

The results of this experiment support the earlier findings of the subjectivity of INT-based visual detection of growth. We found it practically impossible to distinguish a positive well from a negative well based on the colour change of INT. The use of naturally coloured intermediate products for scoring of naphthalene degrader results was also shown to be inadequate as not all positive wells gained colour in our experiment. A simple turbidity measurement using a microplate absorbance reader was found to be a reliable and objective means to detect microbial biomass growth on naphthalene in the wells of the vapour method. In contrast to the earlier assumptions, naphthalene + silicone oil mix did not harmfully interfere with the optical density measurement at 540 nm, making the absorbance measurement a suitable technique for reading of the silicone oil method results as well. With both the methods, the difference in absorbance was clear-cut (over ten-fold) between positive and negative wells. It has to be noted, however that, due to possible differences in the background turbidity of different methods and soil matrix types, the threshold absorbance value for naphthalene-induced growth has to be determined separately for every assay. The threshold level should ideally be set at a point which detects the maximum number of positive wells on the substrate plates but is

above the highest absorbance observed on the negative control plate.

The bacterial 16S rDNA sequences identified from the coloured wells of the vapour method were *Pseudomonas*, the genus likely most frequently cultivated from soils with hydrocarbon contaminants. Three of the seven major *Pseudomonas* subgroups defined by Anzai et al. (2000) were recovered, the most abundant sequences representing *Pseudomonas migulae*. The clone library constructed from a positive but non-coloured well showed a completely different view of the naphthalene degrader diversity. A single clone was related to *Aeromonas*, another gammaproteobacterial genus which is able to utilise naphthalene as the sole carbon source (Kiyohara and Nagao 1978). The *Acidovorax* clones identified from the non-coloured wells showed higher than 98% sequence similarity to the uncultured *Acidovorax* which Yu and Chu (2005) and Huang et al. (2009) found to be responsible for naphthalene uptake and degradation in soil and sediment in situ. These betaproteobacterial naphthalene utilisers have been resistant against culturing efforts but may comprise the effective degrader population in the authentic low substrate concentrations of many contaminated sites (Huang et al. 2009; Jeon et al. 2003; Yu and Chu 2005). The oxygenase enzymes of *Acidovorax* appear to differ from those of pseudomonads, as they have been reported to possess *Comamonas*-type naphthalene dioxygenase genes (*nag* or *Comamonas*-type *nahAc*) (Huang et al. 2009; Jeon et al. 2003; Yagi and Madsen 2009). The different pathway might explain why no coloured intermediates were observed in some of the wells.

Johnsen and Karlson (2005) discovered that high MPN counts for phenanthrene degraders reflected high soil PAH concentrations. They also found a clear correlation between the mineralization of phenanthrene and pyrene and soil PAH concentrations. This is in agreement with the results of this study: TPH content and MPN for naphthalene degraders showed a significant positive correlation. The high number of naphthalene degraders did not coincide with the high level of soil total microbial biomass; instead these parameters showed completely opposite trends as a function of TPH content. This observation demonstrates that the potential of a soil to degrade aromatic hydrocarbons cannot be estimated by analysing the size of the total microbial community alone, but more specific measures are needed.

The initial objectives of our MPN method development were well achieved. The new vapour method proved to be reproducible and objective as well as simple and inexpensive to perform. No toxic reagents were needed for the detection of growth, which enabled the recovery of degrader strains for further culture-based or molecular characterization. The dosing of naphthalene as vapour notably reduced pipetting, and the handling of plates, and thereby the risk of contamination. The reduced work load also enables more replicate samples to increase the precision and/or the representativeness of the measurement. The dosage of naphthalene in buffer may be optimised with regard to concentration and evenness between wells by the placement of naphthalene crystals in the incubation box. UV-spectroscopy provides a simple means for measuring the aqueous naphthalene concentration in the wells (Schwarz and Wasik 1976) in preliminary tests with uninoculated plates. However, the naphthalene concentration at different depths of the inoculated wells will not be homogenous, but constitute a dynamically changing system due to the consumption of the substrate by variable degraders and slower diffusive naphthalene mass transfer in water than in air (Mayer et al. 2007). Surprisingly, the heterogeneity of naphthalene concentrations in the wells was not reflected in the MPN counts: No systematic difference was observed between the replicate samples on the different halves of the plate although there was only one spot source of naphthalene at the other end of the plate.

A drawback of the vapour method is that it can only be applied to volatile hydrocarbon substrates. In addition, MPN methods share the basic restriction of cultivation-dependent procedures (Pandey et al. 2009), due to which it is presumable that the size of degrader populations in environmental samples is underestimated. As a whole, soil is a challenging matrix for quantitative measuring of micro-organisms. Soil suspensions, especially those with high clay or organic matter content, often show high initial turbidity in the lowest dilutions even after sedimentation. The detection limit of MPN methods can therefore be relatively high and growth on soil-derived carbon sources add to the uncertainty of measurement. On the other hand, PCR-based

quantification methods also have several disadvantages (e.g. Smith and Osborn 2009). Quantitative DNA extraction and purification are challenging especially from organic and contaminated soils. The design of specific PCR primers which cover the whole target group might as well be problematic. Additional challenges come from impure DNA extracts which cause variable amount of PCR inhibition hampering the preparation of representative PCR standards. Thus, it is clear that both MPN and PCR approaches have limitations. The choice of the method for quantification of hydrocarbon degraders has to be grounded on the research question and the available budget, equipment and know-how.

In conclusion, we introduce here a new practical and sound MPN method for the quantification of naphthalene degrader population in soil samples. The main advantage of the new method over alternative methods is its simplicity, which makes the simultaneous analysis of a large number of samples and replicates feasible. The method comparison indicated that naphthalene may be dosed in vapour-phase without compromising the result. Turbidity measurement by absorbance reader was found to be a suitable method to detect growth-positive wells in naphthalene MPN microplates. As no toxic respiration indicator chemicals are needed, bacteria can be recovered viable from the plates after scoring MPN results. The introduced method enriched both beta- and gamma-proteobacterial naphthalene degraders. In addition, MPNs of naphthalene degraders reflected the soil exposure to petroleum hydrocarbon contamination.

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