Biodegradation (2008) 19:621–633
DOI 10.1007/s10532-007-9167-x

ORIGINAL PAPER

Method for measuring substrate preferences by individual members of microbial consortia proposed for bioaugmentation

Martin Hesselsoe · Marianne L. Bjerring · Kaj Henriksen · Per Loll · Jeppe L. Nielsen

Received: 13 October 2006 / Accepted: 22 November 2007 / Published online: 13 December 2007

Abstract In this study we used the assimilation of isotope labeled CO_2 to measure the substrate preferences by two different bioaugmentation mixtures proposed for bioremediation of diesel oil contamination. All active microorganisms assimilate CO_2 in various carboxylation processes involved in growth. The CO_2 assimilation by the two mixtures was measured upon addition of glucose, diesel oil or specific compounds present in diesel oil (naphthalene, toluene, hexadecane, and octane). It was shown that within short term incubations with diesel oil (<5 h), one bioaugmentation mixture was superior to the other regarding the assimilation of CO_2. This observation was confirmed in a labor-intensive long term microcosm study (60 days). The applied method open various possibilities for fast pre-testing of substrate-preferences by microbial-bioaugmentation mixtures without microcosm experiments, onsite tests, and complicated chemical analysis. This study also demonstrates the possibility to obtain further information on the substrate preferences at a single cell level of phylogenetically defined microbial subgroups in bioaugmentation mixtures, based on combined analyses of microautoradiography and fluorescence in situ hybridization.

Keywords Bioremediation · Bioaugmentation · Microautoradiography · CO_2 assimilation · Fluorescence in situ hybridization

Abbreviations

AI	CO_2 assimilation Index
BS and JR	Microbial-bioaugmentation mixtures tested in this study (see material and methods)
FISH	Fluorescence in situ hybridization
$HetCO_2$-MAR	Microautoradiography of heterotrophic bacteria on the basis of assimilation of $^{14}CO_2$
MAR	Microautoradiography

M. Hesselsoe (✉) · M. L. Bjerring · K. Henriksen · J. L. Nielsen
Department of biotechnology, chemistry and environmental engineering, Aalborg University Denmark, Aalborg, Denmark
e-mail: mh@amphi.dk
URL: http://www.bio.aau.dk

Present Address:
M. Hesselsoe
Amphi Consult, Aalborg, Denmark

P. Loll
DMR A/S, Jerslev, Denmark

Introduction

Bioremediation of contaminated soil or groundwater based on addition of non-indigenous microorganisms is called bioaugmentation. Many scientific studies have questioned the value of bioaugmentation using

lab-cultured bacterial mixtures (Mueller et al. 1992; Bouchez et al. 2000; Smith et al. 2005). The most successful application of bioaugmentation is generally seen in confined systems rather than in natural habitats such as soil and water (El Fantroussi and Agathos 2005). Often poor results from bioaugmentation can be explained by poor survival of the inoculated organism (van Veen et al. 1997; Hesselsoe et al. 2001) or low bioavailability of the compounds subjected to remediation (Alexander 1995). However, in some cases bioaugmentation has been reported as a relevant supplement or prerequisite to obtain improved removal of xenobiotic compounds such as di-(2-ethylhexyl)phthalate (Roslev et al. 1998), pentachlorophenol (Miethling and Karlson 1996), atrazine (Rousseaux et al. 2002), methyl-tert-buthyl ether (Salanitro et al. 2000) and tetrachloroethene (Major et al. 2002). Further development of e.g. encapsulated bioaugmentation cells or rhizosphere bioaugmentation may increase the efficiency and effect of bioaugmentation technology in the near future (Gentry et al. 2004).

Large numbers of different microbial mixtures have been proposed for bioaugmentation of organic contaminants. Quantitative comparison of such products or functional verification of a given mixture selected for a specific remediation task can only be inadequately determined with existing techniques. Hence, tools for verification of the claimed properties and the efficiency of bioaugmentation mixtures are somehow missing.

The purpose of this study was to develop a new quantitative tool to describe the substrate preferences of bacteria in bioaugmentation mixtures prior to application in the field. The proposed method is based on substrate responsive isotope assimilation in active bacteria. Heterotrophic organisms assimilate CO_2 during biosynthesis in various carboxylation reactions induced by enzymes such as pyruvate carboxylase, phosphoenolpyruvate carboxylase, CoA carboxylase etc. (Barker 1941; Werkman and Wood 1942; Dijkhuizen and Harder 1985). This phenomenon, often described as "heterotrophic CO_2 assimilation", has been used previously for quantification of microbial activity in environmental samples (Romanenko 1964; Saralov et al. 1984), as a measure of perturbations by xenobiotic compounds (Johnson and Romanenko 1984), and for autoradiographic detection of growing bacteria (Romanenko 1961). However, in the last decades heterotrophic CO_2 assimilation has received somewhat less attention in microbial ecology. We recently applied heterotrophic CO_2 assimilation in combination with modern techniques in microbial ecology and obtained a method for detection of the survival of organisms in the environment (Roslev et al. 2004) and a tool for single cell detection of active bacteria based on microautoradiography (MAR), the so called $HetCO_2$-MAR approach (Hesselsoe et al. 2005). The $HetCO_2$-MAR approach enables isotope labeling of active organisms in samples, which respond to unlabeled substrates in the presence of $^{14}CO_2$. Complex substrates which are not available as radiochemicals can thus be applied and numerous substrates can be tested simultaneously, using only one radiolabeled compound ($^{14}CO_2$).

In this study we quantify the assimilation of $^{14}CO_2$ in two different bioaugmentation mixtures as a response to addition of glucose, diesel oil and relevant components present in diesel oil. Based on these data the degradation capacity and substrate preferences of the two bioaugmentation mixtures were evaluated. We also applied the $HetCO_2$-MAR approach in combination with FISH to describe the substrate preferences by different phylogenetic subgroups of bacteria in the bioaugmentation mixtures. To our knowledge, this is the first time the combined MAR-FISH technique has been applied to examine enrichment cultures of microorganisms involved in the metabolism of xenobiotic compounds.

Materials and methods

Growth of bioaugmentation mixtures

Two different test mixtures were applied in this study. The mixtures were treated differently as described below. Different treatment was applied to obtain bioaugmentation mixtures with different physiological and phylogenetic properties.

JR mixture

The JR mixture represents a generic enrichment culture for biodegradation of diesel oil and was enriched from leachate collected at a soil remediation

plant in Aalborg, Denmark (Soilrem). Enrichment was carried out at ambient room temperature (18–25°C) in tap water supplemented with a commercial blend of nutrients and trace elements (NPK 6:1:5, summed concentration 6.5 mg/l) and diesel oil (5–20 mg/l). The culture was supplemented with nutrients and diesel oil every 1–2 days and re-inoculated weekly. The enrichment culture was continuously aerated.

BS mixture

The BS mixture (DSC BS03) represents a commercialized culture proposed for bioaugmentation of diesel oil contamination. The culture was supplied by the company bioREM (www.biorem.dk), and was grown under constant aeration at a constant temperature of 23°C. The culture was grown from a lyophilized form in tap water with addition (-according to the supplier-) of glucose (100 mg/l), diesel oil (20 mg/l) and a mixture of mineral nutrients as supplied from bioREM. Substrates and nutrients were added every 3 days and the mixture was cultivated for up to 4 weeks without re-inoculation. New cultures were started with the original lyophilized inoculum as described above.

Fluorescence in situ hybridization (FISH)

Samples from JR and BS mixtures were fixed directly with freshly defrosted paraformaldehyde (PFA) for 1 h and subsequently stored in phosphate buffered saline and 50% ethanol at −20°C as described previously (Amann et al. 1990). PFA fixed samples were subjected to FISH according to earlier descriptions (Manz et al. 1992) using a broad collection of previously published oligonucleotide probes targeting various bacterial subgroups: ALF968 (Neef 1997), BET42a (Manz et al. 1992), GAM42a (Manz et al. 1992), BONE23a and, BTWO23a (Amann et al. 1996), ARCH915 (Stahl and Amann 1991), CREN499 and EURY498 (Burggraf et al. 1994), HGC69a (Roller et al. 1994), LGC354A (Meier et al. 1999), PLA46 and PLA886 (Neef et al. 1998), SRB385 and EUB338 (Amann et al. 1990), EUB338-II and EUB338-III (Daims et al. 1999). Sequences, target organisms, stringency conditions and other information on the applied probes are available from "probeBase" (Loy et al. 2003). Thermo Hybaid (Ulm, Germany) supplied all probes. A mixture of EUB338, EUB338-II and EUB 338-III ("EUB-mix") was used simultaneously in all samples with the same fluorescent modification (FLUOS) for detection of most *Eubacteria* (Daims et al. 1999). All other probes were used with Cy3 fluorescent modification and hybridized together with the "EUB-mix" to visualize the target group and all *Eubacteria* on the same slide.

Isotope labeling

Both bacterial mixtures were harvested by centrifugation. After harvest the cells were resuspended and washed twice in fresh liquid mineral (LM)-medium to a final optical density measured at 600 nm of approximately 0.3 and stirred gently in the dark for 1 h prior to the simultaneous addition of isotope and electron donor. This preincubation was carried out to remove residual substrates before adding isotope. An optical density of 1 was equivalent to approximately 1×10^9 cells ml^{-1} as derived from microscopic enumeration.

LM-medium was modified from McDonald and Spokes (1980) with the following composition (l^{-1}): $(NH_4)_2SO_4$, 0.13 g; KH_2PO_4, 0.2 g; $CaCl_2 \cdot 2H_2O$, 20 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; FeNaEDTA, 3.8 mg; *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, 4.8 g; trace element solution (Donaldson and Henderson 1989), 1 ml. The medium was adjusted to pH 7.5 with 10 M NaOH and autoclaved.

$NaH^{14}CO_3$ (58 mCi $mmol^{-1}$, Amersham Phamacia Biotech, Buckinghamshire, UK) was added to 1 ml cell suspension (above) in 9.5 ml glass vials (between 10 and 30 μCi ml^{-1}). Immediately after addition of isotope, the growth was initiated by addition of electron donor. Glass vials were sealed with Teflon-coated gas-tight rubber stoppers.

Different electron donors were tested in the isotope labeling experiments: Glucose (5000 mg/l), diesel oil (2000 mg/l), toluene (10 mg/l), naphthalene (10 mg/l), hexadecane (50 mg/l) and octane (50 mg/l). Different concentrations were applied to ensure that an excessive amount of substrate was present during the incubation without reaching toxic levels of the applied substrates. Control samples without any substrate addition were always included.

Quantification of radioactive incorporation (filter-count)

Subsamples of suspensions (100 µl) from radioactive incubations were filtered through a 0.2 µm mixed cellulose filter (Advantec MFS Inc., Pleasanton, CA, USA); 5 ml 0.1 N HCl was subsequently added to the filtration unit. After 3 min of acidification, the acid was washed through the filter and the filter was immediately transferred to a scintillation vial (20 ml), and dissolved in 10 ml scintillation fluid (Filter-Count™, Packard, Groningen, NL). Radioactivity was subsequently quantified by liquid scintillation counting and corrected for quench using external standards (Packard 1600 TR, Groningen, NL).

Microautoradiography (MAR) and FISH

The combined MAR-FISH procedure includes incubation with radiolabeled compounds under well defined incubation conditions, fixation and hybridization with oligonucleotide probes, and subsequent coverage by a radiosensitive film emulsion, exposure, processing and microscopic investigation of the combined FISH signal (recorded by fluorescence microscopy) and the MAR signal (light microscopy). The entire procedure was described previously (Nielsen and Nielsen 2005). Modifications of the MAR-FISH procedure applied in the current study are briefly summarized: fixed samples were washed thoroughly in 0.1 N HCl and distilled water to remove precipitated radioactive carbonate, which would potentially interfere with the MAR signal. Prior to hybridization, small subsamples were smeared in a monolayer onto gelatin-coated coverslips (24 × 60 mm) and immobilized by drying at 50°C. The samples were briefly rinsed with distilled water to remove precipitates and hybridized with fluorescent-labeled oligonucleotide probes. After hybridization the slides were covered with radiosensitive film emulsion, exposed in the dark and processed following standard procedure (Nielsen and Nielsen 2005). A series of pre-experiments was used to determine the optimal exposure time. Enumeration of MAR-positive cells was carried out with a combination of light microscopy and epifluorescence microscopy (Axioskop 2 plus, Carl Zeiss, Oberkochen, Germany). Images applied for presentation were recorded on a confocal laser scanning microscope (LSM510, Carl Zeiss, Oberkochen, Germany).

The percentage of FISH-positive cells that assimilated $^{14}CO_2$ was estimated by enumeration of MAR-positive cells among at least 100 FISH-positive cells on at least two different specimens. Standard deviations presented in Fig. 4 are calculated from the percentage variation in MAR-positive cells observed in each quantified field of view. At least 10 different fields of view were enumerated on each specimen.

Microcosm experiments

Sandy soil (4% sand, 4% silt; 91% sand, $pH_{(H2O)}$ 6.1, soil organic matter 2.2% of dry wt.) was homogenized, sieved ($\varnothing = 2$ mm), and air dried to a water content of 2% relative to dry weight. The soil was mixed thoroughly with 3 mg diesel oil g^{-1} dry soil, mineral nutrient solution (0.3 mg ammonium N and 0.03 mg phosphate P g^{-1} dry soil), and 4×10^7 cells g^{-1} dry soil from the bioaugmentation mixtures suspended in water after thoroughly washing the cells twice by centrifugation. Pure water substituted the cell suspension in the controls. After mixing the soil water content was adjusted with pure water to obtain a final water content approximately equivalent to the field capacity of the soil (12% water of dry weight). Soil mixture (samples of 80 g dry soil) was transferred to rubber sealed glass vials (500 ml) and incubated aerobically in the dark. Headspace samples (0.3 ml) were collected for CO_2 analysis on gas chromatograph (Chrompack CP9002, Delft, NL) equipped with a thermal conductivity detector. CO_2 is considered the most important respiration product for aerobic organisms, hence representing the gross microbial metabolic activity in the microcosms. The first measurement of CO_2 in the headspace was carried out after one day, followed by repetitive analysis twice a week for 60 days of incubation. Subsamples from each treatment (control, JR mixture and BS mixture) from the first day of the experiment and after 64 days of incubation was frozen and shipped for analysis of total hydrocarbon content in a certified commercial laboratory (AnalyCem A/S, Fredericia, Denmark).

Statistical evaluation of data

All experiments were carried out in duplicates or triplicates. Data are shown in all figures with error

bars indicating standard deviation between replicates. Only exception is Fig. 1 showing all single observations available. Data derived from manually enumeration by microcopy (Fig. 4) are shown with standard deviations between two parallel microscopy slides enumerated by the same person.

Results

Microbial composition

Thirteen group-specific FISH probes were tested on the two microbial mixtures. Three probes representing the most abundant subgroups were selected for further quantitative FISH analysis: ALF968 (targeting *Alphaproteobacteria)*, BET42a (targeting *Betaproteobacteria)*, and GAM42a (targeting *Gammaproteobacteria)*. The FISH results were quantified relative to the number of cells hybridized with the general *Eubacteria* probe combination EUB-mix. It was found that the JR mixture contained approximately 19% *Alphaproteobacteria*, 23% *Betaproteobacteria* and less than 2% *Gammaproteobacteria*. The BS mixtures contained 8% *Alphaproteobacteria*, 6% *Betaproteobacteria* and 71% *Gammaproteobacteria*. The rest of the biomass was not covered by the applied FISH probes.

Based on morphological evaluation of the FISH-stained cells, it was clear that several morphotypes (mainly rod and coccoid shapes) were present in each of the groups covered by the three probes applied for FISH quantification.

Isotope labeling

Incorporation of $^{14}CO_2$ as a response to addition of diesel oil or glucose as organic substrates was monitored by filtration of biomass samples. The isotope assimilation was continuously compared with the isotope assimilation in parallel samples without any addition of substrate. The cell specific gross assimilation of isotope was clearly stimulated by addition of the tested substrates compared to the control without substrate addition (Fig. 1). Cell specific assimilation was calculated on the basis of the initial cell density measured as optical density before addition of isotope. The data in Fig. 1 are all single observations based on samples with the same isotope addition (10 $\mu Ci^{14}C\ ml^{-1}$). No statistical evaluation of the single observations has been made.

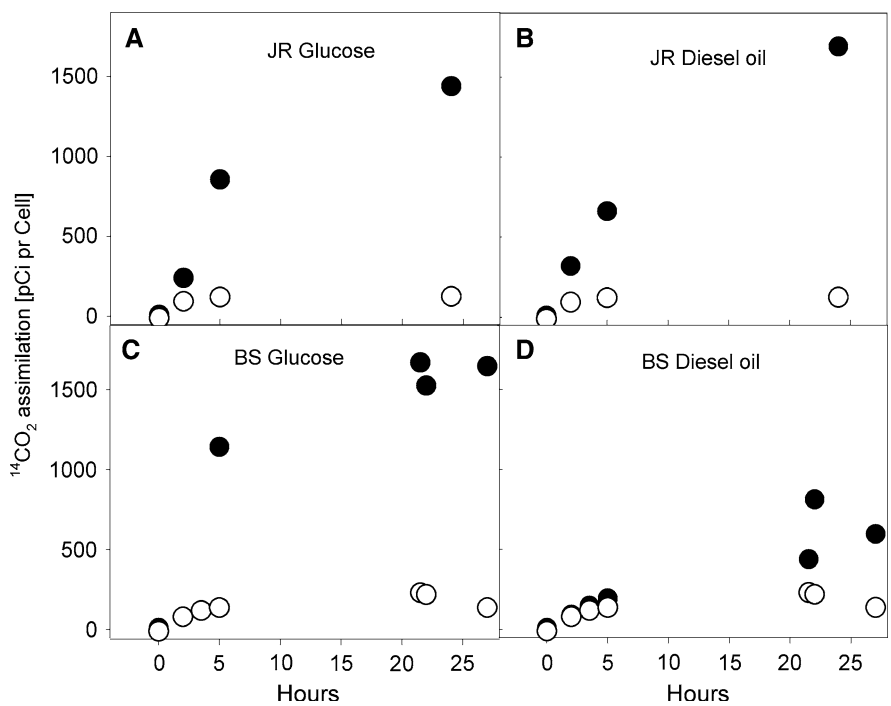

Fig. 1 Gross assimilation of $^{14}CO_2$ in bioaugmentation mixture JR (Panel **A** and **B**) and BS (Panel **C** and **D**). Filled symbols indicate assimilation of $^{14}CO_2$ in the presence of organic substrate (glucose or diesel oil). Open symbols shows $^{14}CO_2$ assimilation in parallel control without any substrate addition. The radioactivity is expressed per cell based on the cell density at the beginning of incubation

The isotope labeling clearly indicated that the BS mixture responded considerably less to the addition of diesel oil than the JR mixture. This difference was further evaluated by adding selected compounds present in diesel oil (naphthalene, toluene, hexadecane and octane). To illustrate the comparative effect of the different substrates we calculated an assimilation index (AI) of $^{14}CO_2$ defined as follows:

$$AI_{Treatment} = (A_{Treatment} - A_{Control})/A_{Control}$$

where "A" is the absolute assimilation of radioactivity and "Treatment" is the different substrates tested after a specified incubation time.

The $^{14}CO_2$ assimilation index after 5 h of labeling showed a considerably greater response from the JR mixture after addition of diesel oil and typical oil components than the BS mixture (Fig. 2).

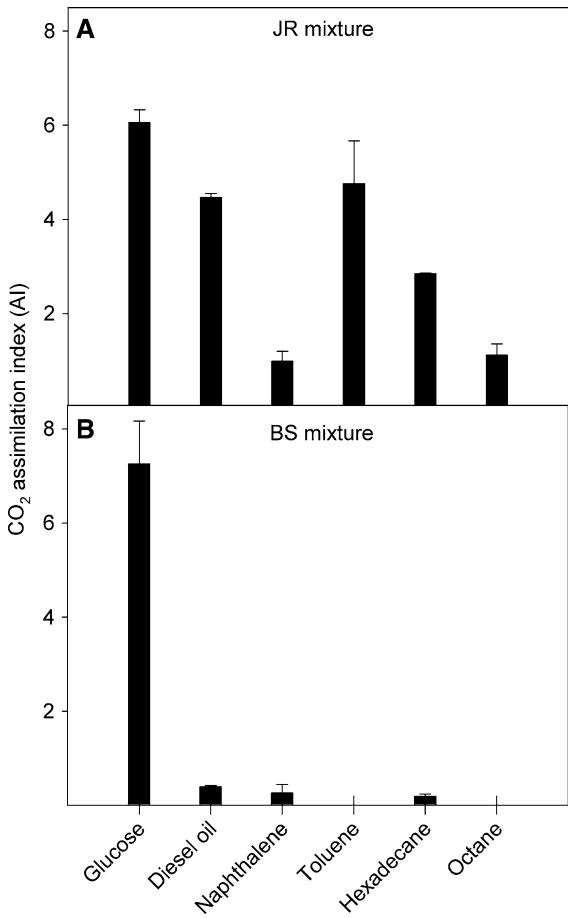

Fig. 2 $^{14}CO_2$ assimilation index (AI) for bioaugmentation mixture JR (Panel **A**) and BS (Panel **B**) after exposure to selected relevant substrates for 5 h (BS and JR). "AI" is defined in the text. Error bars indicate standard deviation

HetCO$_2$-MAR-FISH imaging

The MAR approach was used to quantify the relative fraction of cells in the analyzed mixtures which assimilated $^{14}CO_2$ during incubation. After isotope labeling the bacterial cells were hybridized with general and group-specific oligonucleotide probes. Immobilized FISH-stained cells on glass slides were covered with a photographic film emulsion. The exposure time was optimized to 10 days before processing, fixation and MAR-FISH visualization by microscopy. Multiple layers and clustering of cells under the photographic film were avoided by carefully smearing the sample into a monolayer on the glass slide, to obtain a MAR signal with a single cell resolution. Black silvergrains precipitated in the photographic film above cells which assimilated the isotope (see Fig. 3). Silvergrains were detectable up to 3 μm from the cells.

It was clearly seen that background assimilation of $^{14}CO_2$ without addition of organic substrate was very low with the applied MAR-FISH approach, and almost no cells were MAR positive (Fig. 3a). With the exposure time and radioactive labeling applied in this study, MAR-positive cells were defined as single cells having four or more silver grains in the closest sphere around the cell (3 μm radius from the cell sphere). In the JR mixture exposed for 2 h to glucose, only a few cells assimilated a lot of radioactivity as seen in Fig. 3b, whereas more cells were MAR positive when the JR mixture was exposed to diesel oil (Fig. 3c).

The relative abundance of MAR-positive cells as compared with the total number of *Eubacteria* was manually quantified in all samples through a combination of epifluorescence microscopy (FISH signal) and light microscopy (MAR signal). The cells responding to glucose as the substrate made up a considerably larger fraction of all bacteria in the BS mixture than the JR mixture (Fig. 4). On the other hand, diesel oil was the preferred substrate among the organisms in the JR mixture (Fig. 4a). As seen in Fig. 4, relatively few MAR-positive cells were observed in the control samples without any substrate addition. These were carefully enumerated to emphasize the difference between the absence and addition of potential energy substrates.

The probes ALF968, BET42a, and GAM42a were used for further quantitative analysis of the

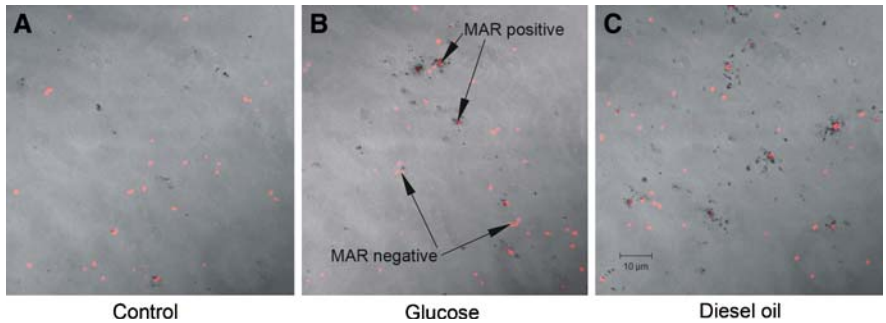

Fig. 3 Superimposed microautoradiography (MAR) and fluorescence in situ hybridization (FISH) of JR mixture after short incubation (2 h) with $^{14}CO_2$ and no organic substrate added (**A**), glucose (**B**) and diesel oil (**C**). FISH signal from a general mixture of probes targeting all *Eubacteria* is shown in red pseudocolor. Positive MAR signals are defined as 4 or more silver grains within 3 μm around each single cell

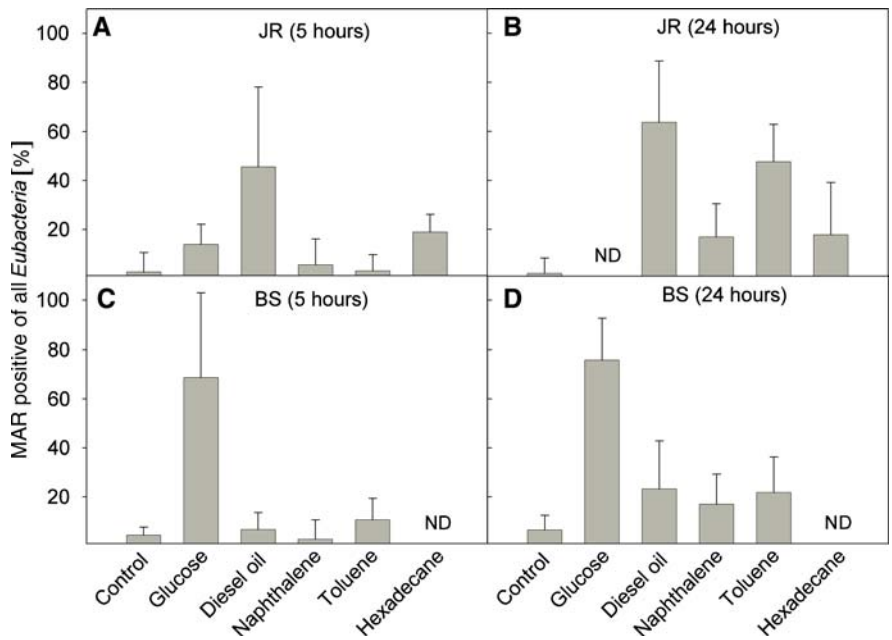

Fig. 4 Enumeration of MAR positive cells relative to all FISH stained *Eubacteria* after 5 and 24 h of $^{14}CO_2$ labeling in the presence of test substrates. Bioaugmentation mixture JR (Panel **A** and **B**) and BS (Panel **C** and **D**). Error bars indicate standard deviation. ND: Not determined

bioremediation mixtures in combination with MAR and the general eubacterial probe mixture (EUB338, EUB338-II and EUB338-III). The purpose was to describe substrate preferences by phylogenetic subgroups in the bioremediation mixtures. Figure 5 demonstrates the strength of the applied $HetCO_2$-MAR-FISH approach exemplified by the BS mixture after 21 h of incubation with diesel oil. Figure 5a shows the FISH result which consist of a superimposed signal from the general EUB probes targeting all bacteria and the GAM42a probe targeting the *Gammaproteobacteria*. Cells with a positive signal from the GAM42a and EUB probes simultaneously (i.e. all *Gammaproteobacteria*) are yellow.

Figure 5b shows the FISH signal (as Fig. 5a) but superimposed on the MAR signal. Finally, Fig. 5c presents the MAR signal alone with only the GAM42a positive cells visible, showing that most GAM42a positive cells did not assimilate radioactive CO_2 in the presence of diesel oil as a substrate. The quantitative distribution of the MAR-positive cells in the probe-defined taxonomic subgroups was evaluated as presented in Table 1.

Quantification showed that only 9% of the *Gammaproteobacteria* in the BS mixture were MAR positive after 21 h of exposure to diesel oil (Table 1). On the other hand 98% of *Gammaproteobacteria* in the BS mixture were MAR positive in the alternative

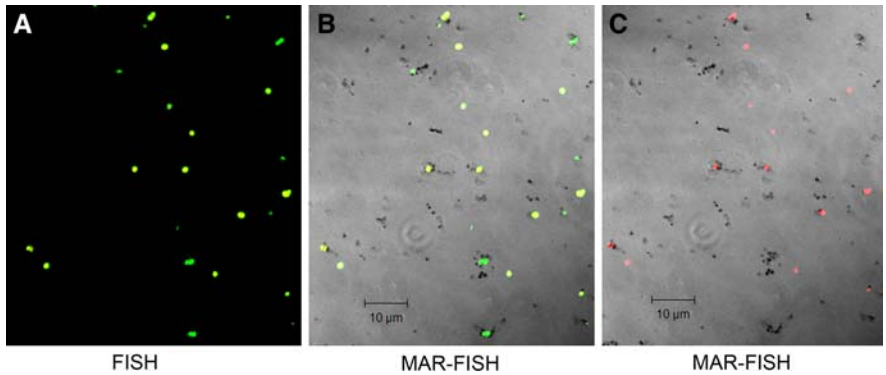

Fig. 5 Bioaugmentation mixture BS after 21 h of incubation with $^{14}CO_2$ and diesel oil. Panel **A**: Superimposed fluorescence in situ hybridization (FISH) of general Eubacterial probe mixture (green) and a group specific probe (GAM42a) targeting *Gammaproteobacteria* (yellow). Panel **B**: As Panel **A** but further superimposed with image of microautoradiography (MAR). Panel **C**: MAR signal superimposed with FISH signal from a group specific probe (GAM42a) targeting *Gammaproteobacteria* (red)

Table 1 Relative abundance (%) of microautoradiography (MAR) positive cells in three phylogenetic subgroups of bacteria

% MAR positive	Probe name		
	Alphaproteobacteria ALF968	*Betaproteobacteria* BET42a	*Gammaproteobacteria* GAM42a
JR mixture			
Control (no substrate added) (%)	0	10	Absent
Glucose (%)	75	12	Absent
Diesel oil (%)	34	40	Absent
BS mixture			
Control (no substrate added) (%)	5	7	2
Glucose (%)	0	69	98
Diesel oil (%)	50	18	9

exposure to glucose (Table 1). Analysis of the initial phylogenetic composition of the BS mixture showed that approximately 70% of all cells in the mixture belonged to the *Gammaproteobacteria*. The *Betaproteobacteria* comprised more than 50% of the MAR-positive cells when the BS mixture was fed on diesel oil, whereas about 20% of the MAR-positive cells belonged to the *Alphaproteobacteria* when feed on oil (data not shown). Quantification of MAR-positive cells in the probe-defined phylogenetic subgroups of the JR mixture (after only 2 h of incubation with test substrates) showed that *Betaproteobacteria* were stimulated more than *Alphaproteobacteria* when exposed to diesel oil. It was also observed that members of the *Alphaproteobacteria* in the JR mixture were stimulated with glucose in contrast to the BS mixture, whereas no *Alphaproteobacteria* were MAR positive after exposure to glucose (Table 1).

Microcosm experiment

The microcosm experiments showed that diesel oil-contaminated soil inoculated with the JR mixture mineralized four times more organic material within 18 days than a parallel control sample inoculated with the same amount of BS mixture. No difference was observed between inoculation with BS mixture and a parallel diesel-oil contaminated control, which was only enriched with mineral nutrients solution. The observed pattern did not change during the prolonged incubation period (up to 60 days). Measurement of

total hydrocarbon content in the microcosms showed 17% removal in the control within 64 days, whereas 26% of the hydrocarbon was degraded after augmentation with JR mixture. Bioaugmentation with the BS mixture gave only 14% removal of oil components.

Discussion

CO_2 assimilation index (AI) as a screening method

Heterotrophic organisms generally assimilate CO_2 when metabolic active, but the quantity of assimilation may vary considerably from substrate to substrate (Romanenko 1964; Doronina and Trotsenko 1984; Roslev et al. 2004; Hesselsoe et al. 2005). Additionally, the incorporation of isotope labeled CO_2 will depend on the background concentration of inorganic carbon and the dilution of the inorganic carbon pool during the metabolization of available substrates. Assimilation of $^{14}CO_2$ as a response to the added substrate will indicate, that the substrate is metabolized, but quantitative interpretation of the CO_2 assimilation must be done with caution.

It is well known, that metabolic activity and growth in some cases may be induced as stress response e.g. when organisms are exposed to toxic compounds. In this study we carefully kept the initial concentrations of potentially toxic compounds on a level, which did not induce any toxicity response. This was confirmed by lack of isotope assimilation in the BS mixture when exposed to diesel oil. Considering additionally, that both tested microbial mixtures were enriched in the presence of diesel oil, it is unlikely that the observed CO_2 assimilation can be interpreted as a toxicity response. Finally, chemical analysis of remaining oil components in the long term microcosm experiments confirmed the results based on CO_2 assimilation, since more degradation of oil was observed after soil augmentation with JR compared to BS. Hence, it is evident, that the observed excessive CO_2 assimilation, as compared to the unamended control sample, was associated with metabolization of the added compounds.

To enable quantitative interpretation of the CO_2 assimilation, we introduced the CO_2 assimilation index (AI). The index expresses the assimilation of radioactive CO_2 after incubation with a given substrate relative to a control sample without substrate addition but with similar initial cell density and isotope addition. Hence, through elimination of variations in specific isotope activity and initial cell density, the AI value can be used when comparing substrate responses from different bioaugmentation mixtures. Since substrate dependent differences in CO_2 assimilation have often been reported, the AI value must be compared for the same substrate between different organisms and mixtures. In the present study we reported AI = 4.5 after 5 h of exposure to diesel oil of the JR mixture and AI = 0.4 for the BS mixture under similar conditions. This indicates significantly more metabolic activity induced by the same substrate (diesel oil) for the JR than for BS mixture (Fig. 2). We suggest that the AI determined within 5 h is a clear indication of superior bioremediation capacity of the JR mixture. Further, all examined single substrates originating from diesel oil also gave a significant higher AI after 5 h for JR compared to BS (Fig. 2). In fact, a negative AI was observed for BS exposed to toluene for 5 h, which indicated less CO_2 assimilation than in the control. It is also important to emphasize the relatively low standard deviations observed, which support the strength of the method as a screening tool (Fig. 2).

MAR-FISH detection of xenobiotic degraders

MAR applications (without FISH identification) for detection of bacteria assimilating different radioactive substrates in the environment have been used for years e.g. in freshwater (Gray et al. 1999) and marine environments (Meyer-Reil 1978; Tabor and Neihof 1984; Grossmann 1994; Paerl et al. 2001) and soil (Romanenko 1961; Sengeløv et al. 2000). However, most applications of the combined MAR-FISH have been carried out in activated sludge from sewage treatment plants (Dedysh et al. 2000; Daims et al. 2001; Nielsen and Nielsen 2002; Nielsen et al. 2003). Recently Yang et al. (2003) investigated a mixture of two pure cultures and applied MAR-FISH to show which of the two species assimilated ^{14}C labeled O-nitrophenol. This study demonstrated in a simple model system the strength of MAR-FISH when studying microbial degradation of xenobiotic compounds. Biological degradation of xenobiotic compounds in natural environments as well as engineered reactors

will always occur in mixed species systems. To our knowledge the work presented here is the first application of MAR-FISH for studying xenobiotic degradation in multi-species microbial enrichment cultures.

From the presented data it is possible to derive new information when comparing the CO_2 assimilation and the obtained MAR results. The assimilation of CO_2 in the JR mixture after 5 h of incubation with glucose was six times higher than the background (AI = 6), and AI was 4.5 after 5 h of incubation with diesel oil (Fig. 2). In contrast to the AI observations, the percentage of MAR-positive cells was less (14%) after incubation with glucose than after incubation with diesel oil (45% MAR positive, Fig. 4). This showed that the average cell-specific CO_2 assimilation with glucose as a substrate was higher than among cells which metabolized diesel oil. This is most likely due to a very fast growth response from the cells in the mixture which are capable of using glucose as a substrate. This is consistent with previous results for pure cultures of *Escherichia coli* and *Pseudomonas putida*, which showed that assimilation of CO_2 was higher after incubation with glucose than with other substrates such as pyruvate, acetate and yeast extract (Hesselsoe et al. 2005).

The quantitatively dominant group in the BS mixture was the *Gammaproteobacteria*, which made up about 70% of the population. Nearly all (98%) the *Gammaproteobacteria* were MAR positive with glucose, whereas only 9% were MAR positive with diesel oil (Table 1). Hence, the phylogenetic information clearly indicates that *Gammaproteobacteria* in the BS mixture are not quantitatively important for the metabolization of diesel oil. The frequency of MAR-positive cells in the *Alphaproteobacteria* group from the BS mixture was considerably higher with diesel oil compared to glucose (Table 1). This indicates that some species in the BS mixture which are involved in the degradation of diesel oil belong to the *Alphaproteobacteria*. However, when looking at all MAR-positive cells in the BS mixture after incubation with diesel oil, 55% of the MAR-positive cells still belonged to the *Betaproteobacteria* (data not shown). Diesel oil clearly increased the fraction of MAR-positive *Betaproteobacteria* in the JR mixture (Table 1), and also in this microbial mixture more than 50% of the MAR-positive cells belonged to the *Betaproteobacteria* after incubation with diesel oil.

In conclusion, the HetCO_2-MAR-FISH approach showed that the dominating populations of *Gammaproteobacteria* in the BS mixture were not actively involved in the metabolization of diesel oil. Hence approximately 70% of the biomass in the BS mixture was not directly relevant for the proposed purpose. The dominant population of *Gammaproteobacteria* was clearly enriched by glucose feeding, which was supplied during the production of the BS biomass. In the JR as well as the BS mixture, the organisms mainly involved in the degradation of diesel oil belonged to the group of *Alpha-* and the *Betaproteobacteria*. The *Gammaproteobacteria*, which responded poorly to the diesel oil in the BS mixture, were nearly absent in the JR mixture. Hence the enrichment procedures applied for the JR mixture produced a biomass which was much more appropriate for the suggested purpose. This is also illustrated in Fig. 4a, c, where 10 times more cells in JR than BS obtained a positive MAR signal within 5 h of incubation with diesel oil. For any bioaugmentation purpose it is important to introduce mainly those organisms which are specialized in the desired purpose. As shown, the approach applied quantitatively describes how many bacteria in the mixture are relevant for the purpose. Obviously it will not be beneficial to apply bioaugmentation cultures with less than 10% of the biomass involved in the degradation of the target compounds as seen in the BS mixture (Fig. 4). This was further confirmed in the microcosm experiment where inoculation with BS induced less degradation of oil compounds than the control.

Further investigations with genus or even species specific 16S rRNA-targeted oligonucleotide probes can be applied in future studies for even more detailed mapping of the substrate preferences by different genera and species in the bioaugmentation mixtures. From an academic point of view, further mapping of the substrate preferences will be interesting. However, from a practical point of view the major achievement is already obtained: the BS mixture is quantitatively dominated by glucose-utilizing *Gammaproteobacteria*, which are irrelevant for the degradation of diesel oil.

Advantages of the HetCO_2-MAR approach

The present study is based on the application of $^{14}CO_2$ as a common source for the isotope labeling of

metabolically active bacteria. The application of $^{14}CO_2$ for isotope labeling of heterotrophs prior to MAR ("HetCO_2-MAR") has been applied previously in a study on ecophysiology of a filamentous bacterium in activated sludge (Hesselsoe et al. 2005). The $^{14}CO_2$ isotope labeling approach has several advantages which are especially relevant when studying the metabolization of xenobiotic compounds in mixed microbial systems:

(1) The CO_2 assimilation will occur in all organisms which respond to a given substrate. Macromolecular or complex substrates which are metabolized in a successive cascade of reactions e.g. involving different organisms will induce CO_2 assimilation in all organisms involved in the metabolization. This fact may be an advantage, depending on the aim of the study. In our data (Fig. 4) we used the HetCO_2-MAR to enumerate all bacteria in the bioaugmentation mixtures, which were actively involved in the metabolization of the tested substrates. This included species which utilize degradation products and metabolites excreted from other organisms in the mixture. In total, the numbers reported in Fig. 4 indicate the fraction of cells which are actively involved in the entire utilization of the added substrate which occur in the mixture. Traditional MAR based on specific isotope-labeled organic compounds identify mainly microorganisms which take up the radiolabeled compound, whereas the HetCO_2-MAR approach indicates metabolic activity. Hence, the two methods are potentially important supplements as described previously (Hesselsoe et al. 2005).

(2) Many xenobiotic compounds, also some of the components in diesel oil, will adhere to surfaces, e.g. bacterial cell walls, because of their hydrophobic properties. Hence, when using radioactively labeled organic compounds for MAR experiments, it may be difficult to distinguish MAR-positive cells, which have assimilated the studied compound from cells which are covered with the radioactive test compound. This problem is not present with the HetCO_2-MAR approach.

(3) When using HetCO_2-MAR, the metabolic response of many different substrates can be tested using only one inexpensive isotope source: $^{14}CO_2$. Additionally, when using HetCO_2-MAR, the experiments are not restricted to available radiochemicals. Compounds, or even mixtures of several compounds (e.g. diesel oil), which are not commercially available as radiochemicals can be tested in combination with $^{14}CO_2$.

(4) Specific radioactivity is normally rather low in commercially available ^{14}C labeled xenobiotic compounds, since only one or few C atoms in the molecules will be isotope labeled. More ^{14}C atoms in the molecule usually make the compound significantly more expensive. When using the inorganic [^{14}C]–HCO_3^- for isotope labeling, the maximum specific radioactivity, with nearly all C-atoms as ^{14}C, is available at a relatively low price. These circumstances often increase the sensitivity of the HetCO_2-MAR approach, since more isotope assimilation occurs when using inorganic ^{14}C as an isotope source rather than ^{14}C labeled organic substrates (Hesselsoe et al. 2005).

Evaluation of bioaugmentation mixtures

The BS mixture was resuscitated from lyophilized samples, and grown on a mixture of glucose and diesel oil. This mixture was largely dominated by cells, which in short term incubations (less than 5 h) practically only assimilated CO_2 when supplied with glucose (Fig. 2b). This was further confirmed by enumeration of MAR-positive cells after incubation for 5 h with diesel oil, which showed that very low numbers of cells in the BS mixture were MAR positive ($7 \pm 7\%$, not significantly different from zero) (Fig. 4c). Hence, it seems that most of the biomass in BS mixture is not capable of degrading diesel oil. In contrast, the JR mixture responded immediately to the addition of diesel oil as a substrate.

The CO_2 assimilation index clearly indicated that the JR mixture was potentially superior to the BS mixture as a bioaugmentation culture for treatment of diesel oil contaminated sites. These results were further confirmed by the microcosm experiment, which showed increased mineralization and degradation of diesel oil in soil after inoculation with the JR mixture. Our results indicate that augmentation with bacterial biomass grown with glucose addition (the BS

mixture) induce less effective remediation compared to biomass grown on diesel oil (JR mixture).

To our knowledge the work presented here is the first application of MAR-FISH for studying xenobiotic degradation in multi-species microbial enrichment cultures.

Acknowledgements The principal funding for this study was provided by Danish Technical Research Council (grants # 26-03-0036 to MH and #26-03-02250 to JLN). Additional generous private support to MH from the R98-foundation is highly appreciated. Maria Grøn and Pia Jensen are acknowledged for their contribution to the microcosm work (M.Sc. thesis at the Department of biotechnology, chemistry and environmental engineering, Aalborg University).

References

Alexander M (1995) How toxic are toxic chemicals in soil? Environ Sci Technol 29:2713–2717

Amann R, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. Appl Environ Microbiol 56:1919–1925

Amann R, Snaidr J, Wagner M, Ludwig W, Schleifer KH (1996) In situ visualization of high genetic diversity in a natural microbial community. J Bacteriol 178:3496–3500

Barker HA (1941) The chemistry and metabolism of bacteria. Annu Rev Biochem 10:553–580

Bouchez T, Patureau D, Dabert P, Juretschko S, Dore J, Delgenes P, Moletta R, Wagner M (2000) Ecological study of a bioaugmentation failure. Environ Microbiol 2:179–190

Burggraf S, Mayer T, Amann R, Schadhauser S, Woese CR, Stetter KO (1994) Identifying members of the domain Archaea with rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 60:3112–3119

Daims H, Bruhl A, Amann R, Schleifer KH, Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 22:434–444

Daims H, Nielsen JL, Nielsen PH, Schleifer KH, Wagner M (2001) In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. Appl Environ Microbiol 67:5273–5284

Dedysh SN, Liesack W, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Bares AM, Panikov NS, Tiedje JM (2000) Methylocella palustris gen. nov., sp nov., a new methane-oxidizing acidophilic bacterium from peat bags, representing a novel subtype of serine-pathway methanotrophs. Int J Syst Evol Microbiol 50:955–969

Dijkhuizen L, Harder W (1985) Microbial metabolism of carbon dioxide. In: Dalton H (Eds), Comprehensive biotechnology: the principles of biotechnology, vol 1. Pergamon Press Ltd., Oxford, pp 409–423

Donaldson JM, Henderson GS (1989) A dilute medium to determine population size of ammonium oxidizers in forest soils. Soil Sci Soc Am J 53:1608–1611

Doronina NV, Trotsenko YA (1984) Levels of carbondioxide assimilation in bacteria with different pathways of C1 metabolism. Mikrobiologiya 53:885–889

El Fantroussi S, Agathos SN (2005) Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Curr Opin Microbiol 8:268–275

Gentry TJ, Rensing C, Pepper IL (2004) New approaches for bioaugmentation as a remediation technology. Crit Rev Environ Sci Technol 34:447–494

Gray ND, Howarth R, Pickup RW, Jones JG, Head IM (1999) Substrate uptake by uncultured bacteria from the genus *Achromatium* determined by microautoradiography. Appl Environ Microbiol 65:5100–5106

Grossmann S (1994) Bacterial activity in sea-ice and open water of the weddell sea, Antarctica- a microautoradiographic study. Microb Ecol 28:1–18

Hesselsoe M, Brandt KK, Sørensen J (2001) Quantification of ammonia-oxidizing bacteria in soil using microcolony technique combined with fluorescent in situ hybridization (FISH). FEMS Microbiol Ecol 38:87–95

Hesselsoe M, Nielsen JL, Roslev P, Nielsen PH (2005) Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of $^{14}CO_2$. Appl Environ Microbiol 71:646–655

Johnson BT, Romanenko VI (1984) Xenobiotic pertubation of microbial growth as measured by CO2 uptake in aquatic heterotrophic bacteria. J Great Lakes Res 10:245–250

Loy A, Horn M, Wagner M (2003) probeBase- an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Res 31:514–516

MacDonald RM, Spokes JR (1980) A selective diagnostic medium for ammonia-oxidizing bacteria. FEMS Microbiol Lett 8:143–145

Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, Starr MG, Payne JA, Buonamici LW (2002) Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. Environ Sci Technol 36:5106–5116

Manz W, Amann R, Ludwig W, Wagner M, Schleifer KH (1992) Phylogenetic oligodeoxynucleotide probes for major subclasses of proteobacteria: problems and solutions. Syst Appl Microbiol 15:593–600

Meier H, Amann R, Ludwig W, Schleifer KH (1999) Specific oligonucleotide probes for in situ detection of a major group of Gram-positive bacteria with low DNA G + C content. Syst Appl Microbiol 22:186–196

Meyer-Reil LA (1978) Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl Environ Microbiol 36:506–512

Miethling R, Karlson U (1996) Accelerated mineralization of pentachlorophenol in soil upon inoculation with *Mycobacterium chlorophenolicum* PCP1 and *Sphingomonas chlorophenolica* RA2. Appl Environ Microbiol 62:4361–4366

Mueller JG, Resnick SM, Shelton ME, Pritchard PH (1992) Effect of inoculation on the biodegradation of weathered Prudhoe Bay crude oil. J Ind Microbiol 10:95–102

Neef A (1997) Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Ph.D. thesis from the Technical University of Munich, Germany

Neef A, Amann R, Schlesner H, Schleifer KH (1998) Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144:3257–3266

Nielsen JL, Christensen D, Kloppenborg M, Nielsen PH (2003) Quantification of cell-specific substrate uptake by probe-defined bacteria under in situ conditions by microautoradiography and fluorescence in situ hybridization. Environ Microbiol 5:202–211

Nielsen JL, Nielsen PH (2002) Enumeration of acetate-consuming bacteria by microautoradiography under oxygen and nitrate respiring conditions in activated sludge. Water Res 36:421–428

Nielsen JL, Nielsen PH (2005) Advances in microscopy: microautoradiography of single cells. Method Enzymol 397:237–256

Paerl HW, Steppe TF, Reid RP (2001) Bacterially mediated precipitation in marine stromatolites. Environ Microbiol 3:123–130

Roller C, Wagner M, Amann R, Ludwig W, Schleifer KH (1994) In situ probing of Gram-positive bacteria with high DNA G + C content using 23S rRNA- targeted oligonucleotides. Microbiology 140:2849–2858

Romanenko VI (1961) Use of the autoradiographic method for a quantitative assay of methane-oxidizing bacteria. Mikrobiologiya 30:292–293

Romanenko VI (1964) Heterotrophic assimilation of CO2 by bacterial flora of water. Mikrobiologiya 33:610–614

Roslev P, Larsen MB, Jørgensen D, Hesselsoe M (2004) Use of heterotrophic CO2 as a measure og metabolic activity in planctonic and sessile cells. J Microbiol Methods

Roslev P, Madsen PL, Thyme JB, Henriksen K (1998) Degradation of phthalate and di-(-2-ethylhexyl)phtalate by indigenous and inoculated microorganisms in sludge-amended soil. Appl Environ Microbiol 64:4711–4719

Rousseaux S, Hartmann A, Lagacherie B, Piutti S, Andreux F, Soulas G (2002) Inoculation of an atrazine-degrading strain, Chelatobacter heintzii Cit1, in four different soils: effects of different inoculum densities. Chemosphere 51:569–576

Salanitro JP, Johnson PC, Spinnler GE, Maner PM, Wisniewski HL, Bruce C (2000) Field-scale demonstration of enhanced MTBE bioremediation through aquifer bioaugmentation and oxygenation. Environ Sci Technol 34:4152–4162

Saralov AI, Krylova IN, Kuznetsov SI (1984) A modification of Sorokińs method for proportional determination of bacterial chemosynthesis and organotrophic CO2 assimilation in lakes. Mikrobiologiya 53:989–996

Sengeløv G, Sørensen S, Frette L, Kroer N (2000) A new method for determining the metabolic activity of specific bacterial populations in soil using tritiated leucine and immunomagnetic separation. Biol Fertil Soils 32:202–208

Smith AE, Hristova K, Wood I, Mackay D, Lory E, Lorenzana D, Scow KM (2005) Comparison of biostimulation versus bioaugmentation with bacterial strain PM1 for treatment of groundwater contaminated with methyl tertiary butyl ether (MTBE). Environ Health Perspect 113:1–9

Stahl DA, Amann R (1991) Development and application of nucleic acid probes. In: Stackebradt E, Goodfellow M (eds), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichester, England, pp 205–248

Tabor PS, Neihof RA (1984) Direct determination of activities for microorganisms of chesapeake bay populations. Appl Environ Microbiol 48:1012–1019

van Veen JA, van Overbeek LS, van Elsas JD (1997) Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol Rev 61:121–135

Werkman CH, Wood HG (1942) On the metabolism of bacteria. Bot Rev VIII:1–68

Yang YR, Zarda A, Zeyer J (2003) Combination of microautoradiography and fluorescence in situ hybridization for identification of microorganisms degrading xenobiotic contaminants. Environ Toxicol Chem 22:2840–2844