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Phenotypic biomonitoring using multivariate flow cytometric analysis of multi-stained microorganisms

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Abstract

A new method for monitoring phenotypic profiles of pure cultures and complex microbial communities was evaluated. The approach was to stain microorganisms with a battery of fluorescent dyes prior to flow cytometry analysis (FCM) and to analyse the data using multivariate methods, including principal component analysis and partial least squares. The FCM method was quantitatively evaluated using different mixtures of pure cultures as well as microbial communities. The results showed that the method could quantitatively and reproducibly resolve both populations and communities of microorganisms with 5% abundance in a diverse microbial background. The feasibility of monitoring complex microbial communities over time during the biodegradation of naphthalene using the FCM method was demonstrated. The biodegradation of naphthalene occurred to differing extents in microcosms representing three different types of aromatic-contaminated groundwater and a sample of bio-basin water. The FCM method distinguished each of these four microbial communities. The phenotypic profiles were compared with genotypic profiles generated by random-amplified polymorphic DNA analysis. The genotypic profiles of the microbial communities described only the microbial composition, and not their functional change, whereas the phenotypic profiles seemed to contain information on both the composition and the functional change of the microorganisms. Furthermore, event analysis of the FCM data showed that microbial communities with initially differing compositions could converge towards a similar composition if they had a capacity for high levels of degradation, whereas microbial communities with similar initial compositions could diverge if they differed in biodegrading ability.

Keywords: Flow cytometry; Principal component analysis; Partial least square; Phenotype; Biomonitoring

1. Introduction

The study of microbial community dynamics is rapidly developing in microbial ecology, but many problems remain. For instance, analysing the microbial communities involved in hydrocarbon biodegradation in situ has been challenging, partly because probably less than 1% of the microorganisms present in the environment can be readily cultured [1]. It has been suggested that the most cost-effective way of remediating organic pollutants in the environment is by natural attenuation [2]. However, more fundamental research into community dynamics and microbial ecology at both the genotypic and phenotypic levels is needed to improve our understanding of the intrinsic processes of bioremediation.

In order to characterise microbial communities in the environment, various methods have been developed that circumvent the need for isolating components of the communities. Many phenotypic and genotypic methods are available, each with specific advantages and limitations including fatty acid methyl ester (FAME) analysis [3–5], phospholipid fatty acid (PLFA) profiling [6,7], amplified ribosomal DNA restriction analysis ([8]), denaturing gradient gel electrophoresis ([9,10]), random-amplified polymorphic DNA (RAPD), terminal restriction fragment length polymorphism [11,12] and length heterogeneity polymerase chain reaction analysis [13,14].

We have previously reported that RAPD is a reproducible and quantitative tool for analysing changes in microbial community composition during biodegradation/bio-

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transformation of different aromatic compounds [15–18]. However, RAPD does not reflect functional changes per se, since it is based on the extraction of total DNA, a property that it shares with the other DNA extraction-based methodologies for microbial community analysis listed above.

The significance of methods depending on the physiological state of the microorganisms, such as FAME and PLFA, may also be limited by the impact of changes in environmental conditions on the microbial physiology. The ability to monitor true changes in microbial composition is diminished when environmental conditions fluctuate, as is often the case in natural systems. Consequently, a combination of both genotypic and phenotypic profiles for characterising microbial communities would be preferable since information on both compositional and functional changes would be obtained. A genotypic profile would give information on compositional change while a phenotypic profile will measure both compositional and functional characteristics of a population of microorganisms.

Flow cytometry (FCM) is a powerful and commonly-used technique for phenotypic studies of both prokaryotic and eukaryotic cells [19,20]. FCM is particularly suitable for studies of microorganisms in water and air, and it has the potential for rapid on-line field measurements. However, the analysis of soil samples is much more demanding due to the problem of adherence between soil particles and microorganisms, and the need to include time-consuming extraction steps [21,22]. Previously, the physiological response of *Escherichia coli* to different cultivation conditions has been studied using two fluorescent dyes for staining prior to analysis by FCM [23]. However, the results were not evaluated with multivariate data analysis. Furthermore, Davey et al. [24] have combined multi-staining with FCM multivariate data analysis for identifying different bacteria in a background of other microorganisms. That report focuses on identity on an event level rather than a sample level. This study presents a new method for characterising microbial communities, based on multiply staining major cell constituents and FCM multivariate data analysis on a sample level. This method is compared with genotypic profiling using RAPD during the degradation of naphthalene in three different types of aromatic-contaminated groundwater and one type of bio-basin water.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli DH5α [25] was grown in Luria–Bertani (LB) medium [26] at 37°C to an optical density (OD) of 0.71 (600 nm) corresponding to 2.41×10^8 (S.D. 3.6×10^7; $n=6$) cfu ml^{-1} on LB agar plates, *Bacillus subtilis*

Table 1
Background contamination profiles of the four types of water included in the mixed population and biomonitoring study

Chemical	AS (μg dm^{-3})	XB (μg dm^{-3})	R18 (μg dm^{-3})	R19 (μg dm^{-3})
HMX[a]	–	–	1 700	23
RDX[b]	–	–	6 900	68
1,3,5-TNB[c]	–	–	680	–
1,3-DNB[d]	–	–	220	–
TNT[e]	–	–	34 000	130
NB[f]	–	–	1 400	–
4A-2,6-DNT[g]	–	–	–	28
2A-4,6-DNT[h]	–	–	2 500	11
2,6-DNT[i]	–	–	5 000	340
2,4-DNT[i]	–	–	7 100	83
2-NT[j]	–	–	17 000	71
4-NT[j]	–	–	14 000	36
3-NT[jf]	–	–	740	2
2-ring PAH[k]	16	–	–	–
3-ring PAH[k]	85	–	–	–
4–6-ring PAH[k]	2000	0.05	–	–
Heterocyclic aromatics	250	–	–	–

[a]Hexa-methylxylene.
[b]Random diatomic xylene.
[c]Trinitrobenzene.
[d]Dinitrobenzene.
[e]Trinitrotoluene.
[f]Nitrobenzene.
[g]4-Amino-2,6-dinitrotoluene.
[h]2-Amino-4,6-dinitrotoluene.
[i]Dinitrotoluene.
[j]Nitrotoluene.
[k]Polyaromatic hydrocarbons.

(ATCC 6633) were grown in LB medium at 30°C to OD 0.79 (1.12×10^8 (S.D. 3.6×10^7; $n=6$) cfu ml^{-1} on LB agar plates), and *Pseudomonas putida* CF600 [27] were grown in LB medium at 30°C to OD 0.73 (2.35×10^8 (S.D. 4.3×10^7; $n=6$) cfu ml^{-1} on LB agar plates). These respective cell densities were referred to as 1× dilution. All three strains were also diluted 10× and 100× with FACSFlow™ (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

2.2. Microbial communities

Four 250-ml Ehrlenmeyer flasks with Teflon-coated screwcaps were prepared, containing 20 ml 1× M9 medium [28], 10 mg naphthalene (approximately twice the saturation level, to ensure a relatively high naphthalene concentration), and 20 ml water originating from (i) polycyclic aromatic hydrocarbon (PAH)-contaminated water (AS), (ii) groundwater from a PAH-contaminated site (XB) with only traces of PAHs in the water and (iii) ground water from two different sampling wells at a site contaminated with nitroaromatics (R18 and R19) (Table 1). Sampling well R19 is located 100 m upstream of R18. These microcosms were shaken at 26°C for 9 days, and quickly opened and closed every day to maintain an aerobic atmosphere. Triplet samples (0.5 ml) were withdrawn from each flask for both FCM and RAPD analysis on four occasions: on days 0, 5, 7 and 9.

The protein concentration was used to estimate the biomass in the microcosms at three points in time. Eighty μl from each microcosm was withdrawn on day 1 and duplicate samples of 300–480 μl were withdrawn on days 5 and 9.

The samples were diluted with water to a total volume of 800 μl, mixed with 200 μl of Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA) and measured spectrophotometrically at 595 nm. Standard curves were prepared from a dilution series of bovine serum albumin solution.

2.3. Mixing design

Three different bacteria, *E. coli*, *B. subtilis* and *P. putida*, were mixed according to Table 2. Samples 1–24 were 1×, 25–48 were 10×, and 49–72 were 100× diluted. Three different undiluted populations (AS, XB and R18) were mixed according to the same design (Table 2).

2.4. Staining for FCM

Each sample was centrifuged for 5 min at $13\,000\times g$. The wet pellet was resuspended and stained with (i) 5 μl nile red (NR) dissolved in acetone (200 μg ml^{-1}), (ii) 5 μl fluorescein-5-isothiocyanate (FITC) dissolved in dimethylformamide (200 μg ml^{-1}), (iii) 5 μl rhodamine 123 (Rho123) dissolved in methanol (200 μg ml^{-1}), (iv) 5 μl propidium iodide (PI) dissolved in methanol (200 μg ml^{-1}) and (v) 5 μl ethidium bromide (EtBr) dissolved in H_2O (300 μl ml^{-1}). The dyes have the following emission peaks: NR, 636 nm; FITC, 525 nm; Rho123, 580 nm; PI 623 nm, and EtBr, 595 nm [20]. NR binds lipids, FITC is a protein-specific dye, both PI and EtBr specifically bind nucleic acids, and Rho123 stains metabolically active bacteria since it is accumulated intracellularly when the cytoplasmic membrane is polarised. The multi-stained microorganism-containing pellet was incubated at room temperature, in darkness for 30 min, resuspended and washed with 1 ml CellWASH (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), then centrifuged for 5 min at $13\,000\times g$. The washed pellet was resuspended in 0.5 ml FACSFlow™ prior to analyses by FCM.

Table 2
Permutations used in the mixed bacterial strain and mixed bacterial population studies

Sample No.[a]	Set[b]	A[c,d]	B[e,d]	C[f,d]
1, 25, 49	WS	980	10	10
2, 26, 50	WS	10	980	10
3, 27, 51	WS	10	10	980
4, 28, 52	WS	495	495	10
5, 29, 53	WS	495	10	495
6, 30, 54	WS	10	495	495
7, 31, 55	WS	657	172	172
8, 32, 56	WS	172	657	172
9, 33, 57	WS	172	172	657
10, 34, 58	WS	333	333	333
11, 35, 59	WS	333	333	333
12, 36, 60	WS	333	333	333
13, 37, 61	TS	100	400	500
14, 38, 62	TS	300	100	600
15, 39, 63	TS	300	600	100
16, 40, 64	TS	50	150	800
17, 41, 65	TS	800	150	50
18, 42, 66	TS	100	250	650
19, 43, 67	TS	700	200	100
20, 44, 68	TS	150	800	50
21, 45, 69	TS	400	350	250
22, 46, 70	TS	200	500	300
23, 47, 71	TS	275	325	400
24, 48, 72	TS	600	50	350

[a]Sample No. 1–24: 1× dilution, 25–48: 10× dilution, 49–72: 100× dilution.
[b]WS: work set, TS: test set.
[c]A: *E. coli* for mixed strain and R18 for mixed population experiments.
[d]Volume of each bacterial solution (μl).
[e]B: *B. subtilis* for mixed strain and XB for mixed population experiments.
[f]C: *P. putida* for mixed strain and AS for mixed population experiments.

2.5. FCM protocol

The flow was set to 35 μl min^{-1} for all FCM runs. A total of 30 000 events were collected in all runs.

The instrument used for the FCM analyses was a FACSSort (Becton Dickinson Immunocytometry Systems,

San Jose, CA, USA) equipped with an Argon laser (488 nm), and the data collecting software was SyQuest (SyQuest Technology, Fremont, CA, USA).

2.6. DNA extraction

Samples of DNA were extracted, in triplicate, at four time points from all four microcosms using a modified bead-beater method [29]. Three hundred μl phosphate buffer (100 mM NaH_2PO_4, pH 8), 300 μl sodium dodecyl sulfate (SDS) solution (100 mM NaCl, 500 mM Tris (pH 8), 10% (w/v) SDS), 300 μl chloroform:isoamylalcohol (24:1), and 1 g zirconia/silica beads (0.1 mm) (Biospec Products, Bartlesville, OK, USA) were added to each 500-μl sample. The mix was vigorously shaken in a cell disrupter (FastPrep™ FP120, BIO 101/Savant, Vista, CA, USA) at 4.0 m s^{-1} for 20 s. The aqueous phase was separated from the particles and $CHCl_3$ by centrifugation at $10\,000 \times g$ for 10 s. The nucleic acid-containing aqueous phase was extracted with an equal volume of phenol:$CHCl_3$:isoamylalcohol (25:24:1) followed by extraction with 2-butanol to reduce the volume to 100 μl. The remaining aqueous phase was purified using a Microspin S-400 HR column (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The extracted and purified nucleic acids were stored at −20°C prior to use.

2.7. RAPD procedure and analysis

The DNA extracted from each sample (1 μl of the extract, equivalent to approximately 10 ng) was amplified by PCR, using the RAPD protocol of [15] with the exception of 25 instead of 35 temperature cycles. Thus, there were three RAPD analyses for each sampling occasion for each of the four microcosms, giving a total of 48 RAPD reactions or genetic profiles. The method used to analyse the RAPD products generated has also been described previously [15]. Essentially, the fragments in each sample were separated on a polyacrylamide gel (6%), then scanned with a laser detector and the computerised image was transformed to a data matrix consisting of 48 rows (samples) and 471 columns (variables).

2.8. Processing the FCM data

Histogram data (horizontally aligned) from each run was saved as a text-file in WinMDI 2.8 [30], imported into SIMCA-P 8.0 (Umetrics AB, Umeå, Sweden) and vertically transposed to be suitable for the final compiling of the data into data matrices in Microsoft® Excel 97 SR-2. Each of the five FCM channels describes each sample with 1024 histogram data variables, implying that each sample was described by a total of 5120 variables. The first and last two or three variables from each channel were excluded from further analysis since they described the number of events located outside the recorded channel range. The remaining data from each sample were normalised to restore equivalence in the number of events among the samples.

Dot plot data from samples 1, 2 and 3 in the mixed strain experiment were saved as text-files in WinMDI 2.8 [30], compiled into a data matrix in Microsoft® Excel 97 SR-2 and then imported into SIMCA-P 8.0 (Umetrics AB, Umeå, Sweden). The same procedure was performed for FCM analyses of the AS, XB and R18 microcosms for both days 1 and 9. Each event was described by five variables namely, FSC, SSC, FL1, FL2 and FL3. FSC is most dependent on size, and SSC on granularity, whereas FL1–3 represent data from three windows that detect fluorescent signals in three different wavelength intervals: FL1, 512–545 nm; FL2, 564–606 nm, and FL3, >650 nm. Five hundred events from each sample/FCM run were analysed with respect to similarities and differences at the event level.

2.9. Multivariate data analysis

Principal component analysis (PCA) [31] was used to identify dyes that contributed with unique information. The distance between projected samples in a PCA scoreplot is correlated to the sample similarity. Clustered samples disclose similarity, while samples projected distantly disclose dissimilarity, i.e. containing unique information compared to each other. The microbial communities in the microcosms were described by 5099 and 471 variables at each time point originating from the microorganisms' FCM and RAPD profiles, respectively. All RAPD and FCM variables were mean-centred and remained unscaled prior to analysis. PCA was performed on the FCM and RAPD data separately to monitor variations in the microbial communities over time in the microcosms.

Projection to latent structures by means of partial least squares analysis (PLS) [32] was used to evaluate the quantitative properties of the FCM method in the mixed strain and mixed population experiments. PLS models were calculated for the first 12 samples (the work set; WS) of the mixed strain experiment and further tested with the next 12 samples (the test set; TS). This procedure was repeated for the 10× and 100× dilutions, and for the mixed population experiment. These PLS calculations consisted of FCM data fitted to the actual microbial proportions in the mixtures. Observed (true) proportions were compared with predicted contents. Root mean square error of prediction (RMSEP) [33] values were calculated from this comparison for the 12 samples in the TS.

PLS-discriminant analysis (PLS-DA) was used to investigate which variables differed between FCM runs. In PLS-DA, 500 events from each sample were classified by introducing dummy variables (i.e. 0 or 1) to which the FCM data were fitted. The most significant coefficients for each microcosm were reported.

To evaluate the (dis-)similarities of events originating from the different microcosms compared between days 1 and 9, eight separate PCA models were calculated based on 500 events from each microcosm on days 1 and 9. The average probability of the events from all four microcosms being part of the different modelled sets was calculated.

The FCM variables were mean-centred and scaled to unit variance prior to event analysis. Simca-P 8.0 (Umetrics AB, Umeå, Sweden) was used for all multivariate data analysis.

2.10. Chemical analysis

A screening analysis was performed on the four different types of water as follows: the solid phase in the samples was collected on a glass fibre filter (APFA 09050; Millipore), which was dried at room temperature and extracted twice with dichloromethane in an ultrasonic bath (Ultrasonic cleaner, Fungilab). The extract was dried with anhydrous sodium sulfate, Na_2SO_4 (purified at 550°C) before determining the final concentration of the extract.

The water (AS: 25 ml; XB, R18, R19: 500 ml) was extracted twice with dichloromethane (AS: 5 ml; XB, R18, R19: 25 ml) (Burdick and Jackson, High Purity Solvent), which was dried with Na_2SO_4. These extracts were initially concentrated, to 5 ml, in a rotary evaporator (Büchi R-114) and the final concentration of the extracts was achieved by reducing the volume to 1 ml by evaporating the solvent with dry nitrogen.

PAHs in the organic phase were analysed by a gas chromatograph/mass spectrometer (GC/MS), Hewlett-Packard 6890/Hewlett-Packard 5973, using a 30 m × 0.25 mm × 0.25 μm DB5MS capillary column (J&W Scientific). The sample was injected (HP 7673 GC/SFC injector) in splitless mode, and PAHs were separated using a GC temperature program starting at 40°C for 1 min, then rising 10°C min^{-1} to 270°C, which was held for 10 min. Helium was used as carrier gas with a constant linear velocity of 35 cm s^{-1}. The PAHs were quantified by an external standard technique, using anthracene and phenanthrene as calibrating substances.

Naphthalene analysis was performed on the four different types of water, sampled over time as follows. Five hundred μl from each microcosm was taken for naphthalene analysis. To these samples 30 μl dibenzofuran (1.79 μg μl^{-1}) was added as an internal standard, and 0.1 g sodium chloride to increase their ionic strength. The samples were extracted with 3 × 1 ml toluene. The organic phases were combined and dried on a small column of activated sodium sulfate. The samples were analysed using a Fisons GC 8000 Top (30 m × 0.32 mm × 0.25 μm film thickness, SPB-5 capillary column, Supelco, Bellefonte, PA, USA) GC coupled to a Fisons MD800 mass selective detector (MS). The GC was operated in splitless mode, and 1 μl of the extracts was injected using an autosampler. The MS was operated in full-scan mode. The naphthalene in the samples was identified and quantified by reference to a known standard solution containing naphthalene (128 μg) and dibenzofuran (89.6 μg).

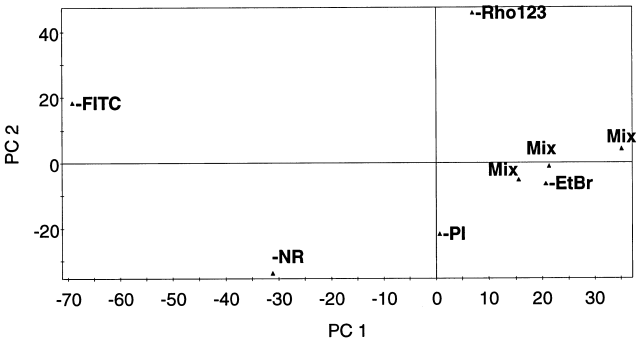

Fig. 1. Scoreplot with three objects representing penta-stained *P. putida* samples (denoted Mix) and five objects representing tetra-stained *P. putida* samples each lacking one of the five fluorescent dyes: nile red (NR), propidium iodide (PI), rhodamine 123 (Rho123), ethidium bromide (EtBr), fluorescein-5-isothiocyanate (FITC). Omissions are denoted with a minus sign and the name of the omitted dye. R^2X for PCs 1 and 2 were 0.30 and 0.15, respectively.

3. Results

3.1. Evaluation of the FCM phenotypic profiling method

3.1.1. Staining evaluation

Samples containing *P. putida* were stained with all combinations of four of the five fluorescent dyes being tested (NR, FITC, Rho123, PI and EtBr). Triplicate samples were also stained with all five dyes. The PCA designed to identify fluorescent dyes that contribute unique information revealed that the sample in which EtBr was omitted had the same phenotypic profile as the penta-stained triplet (Fig. 1). Thus, EtBr did not appear to contribute any unique phenotypic information and it was consequently omitted from further staining experiments. This experiment also indicated that FITC contributed the most unique phenotypic information (Fig. 1).

3.1.2. Resolution of tertiary bacterial mixtures

Three different bacteria, *E. coli*, *B. subtilis* and *P. putida*, were mixed according to Table 2. Fig. 2 shows the relationships between the observed and predicted proportions of these mixtures for both the WS and TS. The RMSEP values, which indicate how precisely the PLS model could predict the ratios in the 12 samples of the TS, are shown in the legend to Fig. 2. The prediction precision ranged from 6.9 to 16.9% and improved as the dilution factor increased for all three strains. Graphical presentation of the observed values versus those predicted by the PLS model (Fig. 2) shows that the method is quantitative and reproducible enough to permit predictions within an acceptable range for individual bacterial strains in the presence of the two other bacteria.

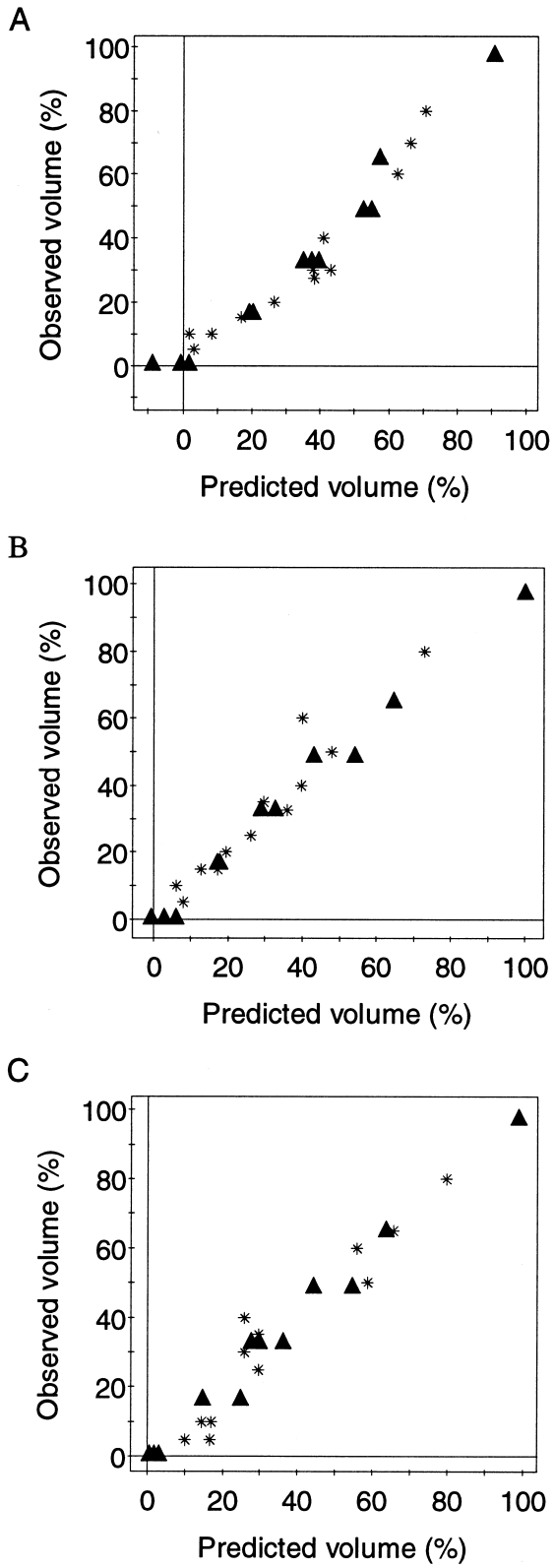

Fig. 2. The observed (true) volume percentage of (A) *E. coli*, (B) *B. subtilis* and (C) *P. putida* in relation to the volume percentage predicted by the PLS model at 100× dilution. The RMSEP values for the TS predictions were 15.9, 15.2 and 6.9% for *E. coli*; 11.1, 7.1 and 6.7% for *B. subtilis* and 16.9, 8.9 and 7.0% for *P. putida* at 1×, 10× and 100× dilutions, respectively. ▲ = WS, * = TS.

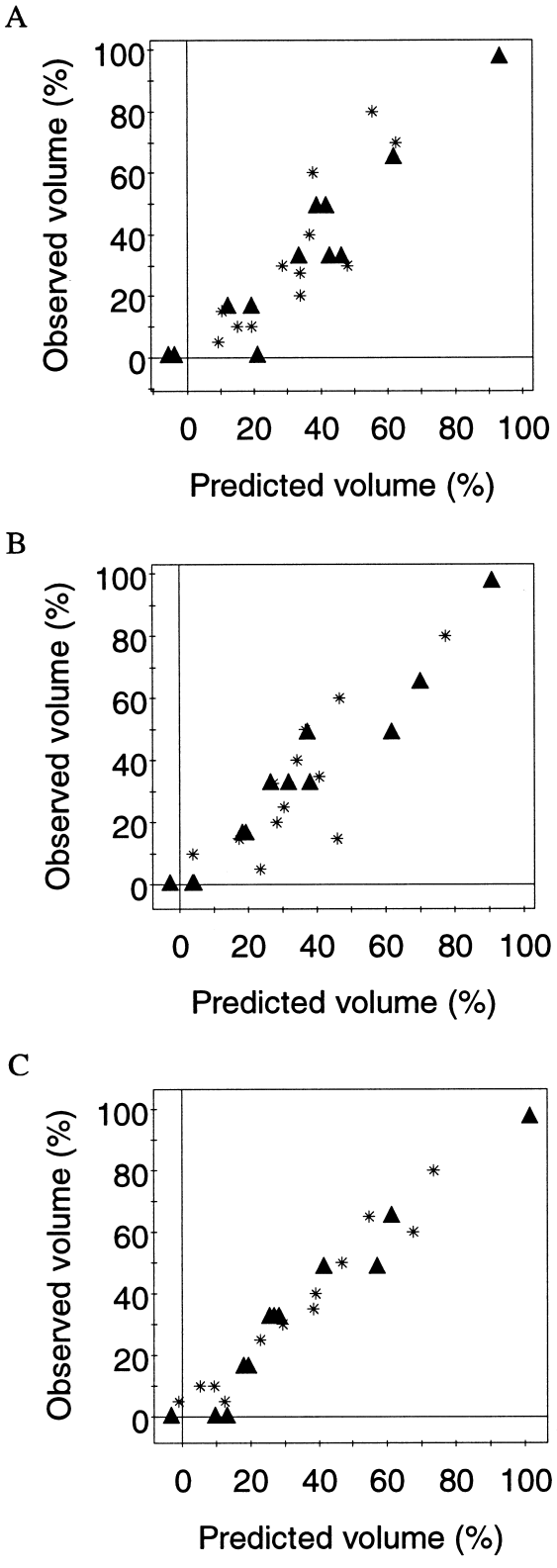

Fig. 3. The observed (true) volume percentage of (A) R18 microcosm, (B) XB microcosm and (C) AS microcosm in relation to the volume percentage predicted by the PLS model in the mixed population experiment. The RMSEP values for the TS predictions were 12.5, 12.6 and 5.4% for R18, XB and AS, respectively. ▲ = WS, * = TS.

3.1.3. *Resolution of tertiary mixtures of different microbial communities*

Two different aromatic-contaminated ground water samples (XB and R18) and a sample of water originating from a bio-basin for treating aromatic-contaminated industrial process water (AS) were mixed according to the experimental design shown in Table 2.

The RMSEP values from the mixed population PLS models, which ranged from 5.4% (AS) to 12.6% (XB), are shown in the legend to Fig. 3. Graphical presentation of the observed values versus those predicted by the PLS model shows that the method could quantitatively and reproducibly resolve each of the individual microbial communities against the background of the two other microbial communities.

3.2. *Phenotypic and genotypic profile characterisation of different microbial communities during biodegradation of naphthalene*

Four microcosms containing aromatic-contaminated water (AS, XB, R18 and R19, Table 1) from different locations were augmented with excess amounts of naphthalene. The biomass increased in all microcosms, but most markedly in XB and R18 and least in the AS microcosm (Fig. 4). These data correlated well with the extent of the naphthalene biodegradation in the different microcosms. Complete biodegradation occurred in both the XB and R18 microcosms (Fig. 5), whereas the naphthalene concentration remained stable over time in the AS microcosm. An intermediate level of biodegradation occurred in the R19 microcosm (Fig. 5), which correlated well with its moderate increase in biomass (Fig. 4).

The change in phenotypic and genotypic profiles of the microbial communities over time is shown in Fig. 6. The microbial communities in the R18 and R19 ground water samples were relatively similar phenotypically, at first. However, R18 changed significantly phenotypically between days 7 and 9, probably as a consequence of the growth-linked biodegradation of naphthalene (Fig. 5). In contrast, R19 showed little phenotypic change. Genotypically, both R18 and R19 exhibited little change over time. The AS community remained phenotypically clustered throughout the 9-day period. However, genotypically, a major change occurred between days 1 and 5. For the XB microcosm, a major shift in the microbial community, both phenotypically and genotypically, occurred between days 1 and 5.

The phenotypic results were confirmed by event analysis for the microcosms on days 1 and 9 (Table 3). R18 and R19 were similar on day 1 but differed quite substantially

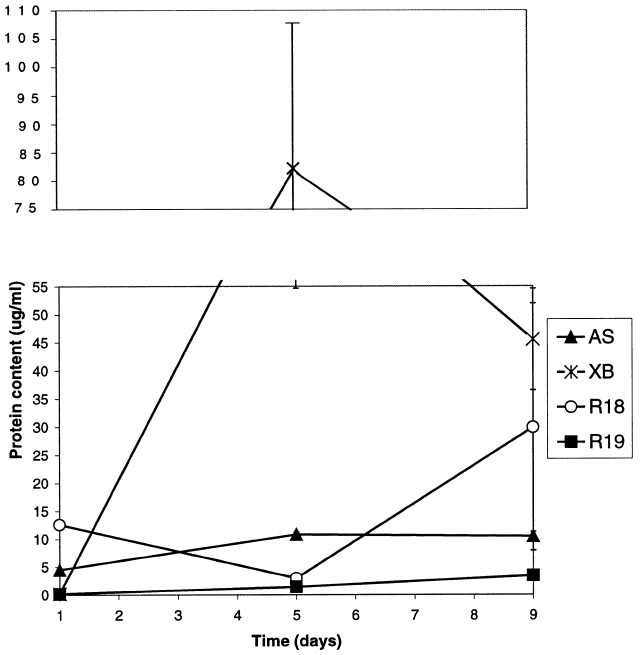

Fig. 4. Variation of biomass over time in the biomonitoring experiment, expressed as protein content (μg ml^{-1}). The error bars show the standard deviation ($n = 1$ for day 1, and $n = 3$ for days 5 and 9).

Table 3
Average probability of 500 events from each microcosm being components of the four different PCA models on days 1 and 9

	AS model	XB model	R18 model	R19 model
Day 1:				
AS	**0.546**	0.27	0.205	0.312
XB	0.396	**0.51**	*0.212*	0.374
R18	0.182	*0.303*	**0.484**	*0.425*
R19	0.309	0.44	*0.444*	**0.503**
Day 9:				
AS	**0.535**	0.173	0.085	0.249
XB	0.315	**0.601**	*0.361*	0.057
R18	0.414	*0.578*	**0.602**	*0.119*
R19	0.257	0.315	*0.098*	**0.496**

The average probability of events belonging to the corresponding PCA model is shown in bold and should be the largest figure in each column. The most noteworthy changes are the cross similarity on day 1, and difference on day 9, between R18 and R19, and the opposite relationship between XB and R18 on days 1 and 9, all in italics.

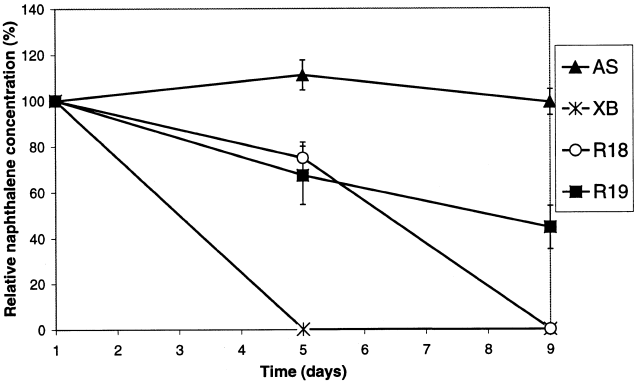

Fig. 5. Variation in the relative naphthalene concentration over time in the biomonitoring experiment (percent of the initial value). The error bars show the standard deviations ($n = 1$ for day 1, and $n = 3$ for days 5 and 9).

on day 9, whereas events from XB and R18 were different on day 1, but they had converged by day 9.

The PLS-DA analysis undertaken to identify FCM variables related to differences between the events from the four microcosms on days 1 and 9, revealed that events from the two best naphthalene-degrading microbial communities, XB and R18, had high FL2 and FL3 values on day 9.

4. Discussion

This study was designed to evaluate the feasibility of using multi-stained microorganisms to monitor phenotypic development and changes in complex microbial communities. To this end, a FCM phenotypic profiling method was explored. Initially, five different dyes were evaluated to assess the value of the information they could contribute to phenotypic profile analysis. EtBr was found to contribute little or no unique information and was consequently omitted. No further optimisation of the staining protocol with respect to dye solution concentrations, fixation, incubation time/temperature, etc. was performed.

Next, to evaluate the quantitative properties of the FCM method, mixtures of different bacterial strains and microbial populations were prepared, according to a design in which 12 of the 24 samples constituted a WS for PLS calculations. The remaining 12 samples were used to test the PLS model, to enable subsequent critical evaluation. The same procedure was repeated with 10× and 100× dilutions of the same bacteria, and with three microbial populations differing in origin.

The RMSEP levels (54–169 μl) were compared to the total volume in each sample (1000 μl) and the results imply that bacteria with over ~5% abundance can be monitored in a diverse microbial background. The fact that the highest dilution (100×) of the strain mixtures (approximately 10^6 bacteria per ml, data not shown) resulted in the best prediction precision was encouraging, and implies that the method would be of value even for more dilute samples.

The biomonitoring experiment in which phenotypic and genotypic changes in four different microcosms over time were analysed by both FCM and RAPD gave several interesting results. Firstly, the four microcosms differed significantly at the start of the experiment, but R18 and R19 were the most similar both phenotypically and genotypically (Fig. 6). Secondly, the biggest phenotypic changes occurred between days 1 and 5 in XB and between days 7 and 9 in the R18 microbial community (Fig. 6). Thirdly, the phenotypic profiles from the AS microbial community changed least over the period studied (Fig. 6). Fourthly, the phenotypic profiles of R18 and XB converged between days 1 and 9, whereas R18 and R19 diverged. These results all correlated well with the patterns of naphthalene degradation and biomass content. A previous study [34]

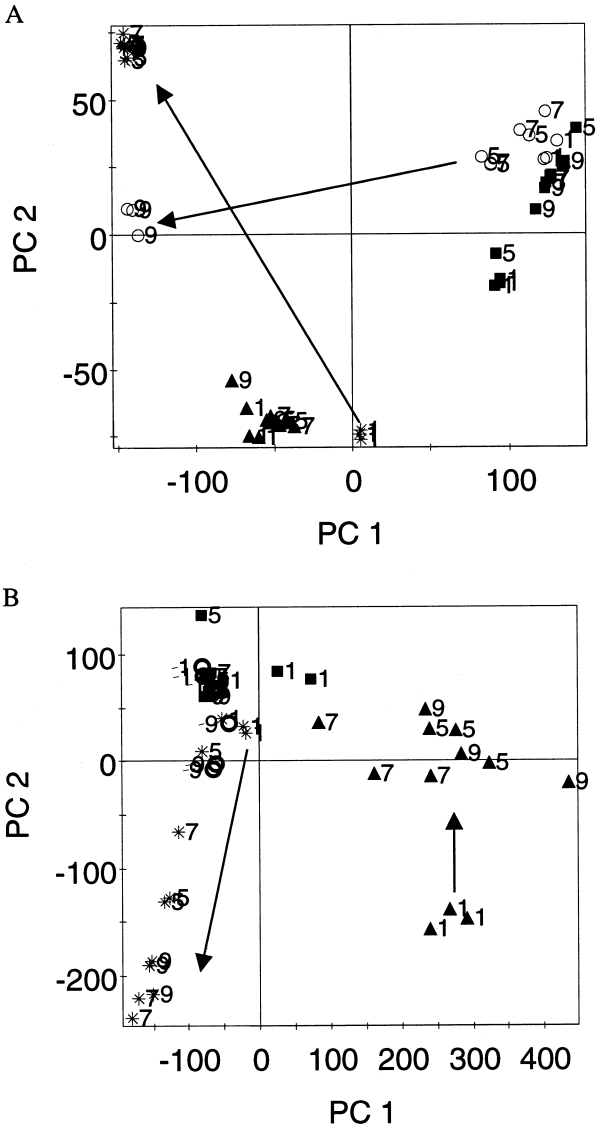

Fig. 6. PCA scoreplots of (A) FCM profiles originating from microbial communities in the four different microcosms over time; ▲ = AS, * = XB, ○ = R18, ■ = R19. The numbers indicate the time (days). The first (PC1) and second (PC2) principal components explained 66 and 15%, respectively, of the total variation in the data. (B) Variations in RAPD profiles of the microbial communities in the four different microcosms over time; ▲ = AS, * = XB, ○ = R18, ■ = R19. The numbers indicate time (days). The first (PC1) and second (PC2) principal components explained 42 and 16%, respectively, of the total variation in the data.

has shown that the site from which the XB water originated was heavily PAH-contaminated. The water, however, contained very low amounts of aromatics. This suggested that despite a lack of dissolved aromatics XB water contained microorganisms that were able to degrade naphthalene quite rapidly. In contrast, the AS water was PAH-contaminated but did not contain microorganisms capable of degrading naphthalene to the same extent as XB. This difference was reflected in the phenotypic profiles (Fig. 6A). The microbial community in the XB microcosm changed dramatically during naphthalene degradation, whereas the AS microbial community remained relatively

constant phenotypically. However, the AS microbial community exhibited a clear genotypic change in relation to the other communities (Fig. 6B). This could be due either to an ongoing succession of microorganisms favourable for naphthalene degradation, or to a toxic effect of the high naphthalene concentration.

In contrast, R18 seemed to be relatively genotypically constant, despite the large phenotypic change, whereas XB was found to change quite dramatically both phenotypically and genotypically. It could be concluded from this observation that the phenotypic change was due to functional change, rather than to changes in the microbial community composition, which remained unchanged.

The genotypic profiles of the microbial communities only described the microbial composition and not the functional change of the microorganisms, whereas the phenotypic profiles seemed to contain information on both microbial composition and functional change. The fact that the microbial communities in none of the microcosms had identical phenotypic profiles at any time confirms this hypothesis (Fig. 6). The first principal component in the PLS model shown in Fig. 6 describes the difference between the AS microbial community over time and the other communities. The second principal component primarily describes the changes that occurred in microbial composition in the XB microcosm over time. Thus, it was evident that the phenotypic and genotypic profiles described different properties and courses of events.

Furthermore, the event analysis shows that high FL2 and FL3 values were most strongly associated with high rates of naphthalene degradation. Since all four dyes used for staining the microorganisms emitted in part in the FL2 window, and both PI and NR emitted in the FL3 window, it was difficult to pinpoint which cell constituent(s) had caused specific shifts in this phenotypic parameter. Further studies are needed to elucidate details of this effect. In this context, a principal advantage, common to all PCR-based microbial community analysis methodologies including RAPD, is that specific DNA fragments correlating with different events may be identified and further analysed after purification by sequencing and linked to a function. However, in this study only overall change in microbial community structure was measured by the RAPD method. It can be envisaged that these measured changes were quantitative since earlier studies have shown that the RAPD method could quantitatively resolve initial proportions of tertiary bacterial mixtures [18]. Moreover, the sensitivity level of RAPD has been estimated to be approximately 2% [18], implying that only dominant parts of the microbial community structure changes should be assumed to be monitored with the RAPD method as well.

In summary, a rapid method for phenotypically profiling microorganisms was developed. This tool, combined with other methods, has the potential to help understand the complex interplays within microbial communities.

An optimised FCM procedure together with data processing including multivariate data analysis has great potential as an on-line method for monitoring microorganisms in aerosols, industrial bioprocesses, wastewater plants and contaminated ground waters.

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