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Effect of oxygen and temperature on the dynamic of the dominant bacterial populations of pig manure and on the persistence of pig-associated genetic markers, assessed in river water microcosms

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Abstract :

Aims : The aim is to evaluate the dynamic of *Bacteroides–Prevotella* and *Bacillus–Streptococcus–Lactobacillus* populations originating from pig manure and the persistence of pig-associated markers belonging to these groups according to temperature and oxygen.

Methods and Results : River water was inoculated with pig manure and incubated under microaerophilic and aerobic conditions, at 4 and 20°C over 43 days. The diversity of bacterial populations was analysed by capillary electrophoresis-single-strand conformation polymorphism. The persistence of the pig-associated markers was measured by real-time PCR and compared with the survival of *Escherichia coli* and enterococci. Decay was characterized by the estimation of the time needed to produce a 1-log reduction (T90). The greatest changes were observed at 20°C under aerobic conditions, leading to a reduction in the diversity of the bacterial populations and in the concentrations of the Pig-1-Bac, Pig-2-Bac and *Lactobacillus amylovorus* markers with a T90 of 10·5, 8·1 and 17·2 days, respectively.

Conclusions : Oxygen and temperature were found to have a combined effect on the persistence of the pig-associated markers in river waters.

Significance and Impact of the Study : The persistence profiles of the Pig-1-Bac, Pig-2-Bac and *Lact. amylovorus* markers in addition to their high specificity and sensitivity support their use as relevant markers to identify pig faecal contamination in river waters.

Keywords : *Lactobacillus amylovorus* ; microbial source tracking ; oxygen ; persistence ; pig-associated *Bacteroidales* markers ; quantitative real-time PCR ; river water ; temperature

1. Introduction

Microbial Source Tracking (MST) methods have been selected to distinguish between human and animal sources of faecal pollution (Marvin et al., 2000; Bernhard and Field 2000b; Glassmeyer et al., 2005; Ufnar et al., 2007). Among them, promising library-independent MST methods based on molecular techniques such as the Polymerase Chain Reaction (PCR) have been developed to target host-associated bacteria (Bernhard and Field 2000a; 2000b). Real-time PCR (qPCR) was developed to quantify human-, pig- and ruminant-associated bacteria (Savill et al., 2001; Seurinck et al., 2005; Kildare et al., 2007; Reischer et al., 2007; Gourmelon et al., 2010; Marti et al., 2010). Previous studies suggested that *Bacteroidales*, represented mainly by the genera *Bacteroides* and *Prevotella*, could be used as alternative faecal indicator organisms for humans and for mammals such as pig and cows, as they are numerically dominant in the intestinal flora, highly abundant in faeces with 10^9 to 10^{11} bacteria per g of faeces, are restricted to warm-blooded animals and cannot multiply in the environment (Allsop and Stickler 1985; Fiksdal et al., 1985; Kreader 1995; Suau et al., 1999; Dick et al., 2005; Furet et al., 2009). To date, only four pig-associated *Bacteroidales* 16S rRNA markers have been developed to identify and quantify pig faecal pollution in environmental waters with qPCR (Okabe et al., 2007; Mieszkin et al., 2009). The two markers developed by Okabe et al. (2007) in Japan were tested on a small number of faecal samples and they showed some cross-reactions with human and cow faeces samples or weak concentrations in target faecal samples whereas the Pig-1-Bac and Pig-2-Bac markers developed by Mieszkin et al. (2009) demonstrated a high sensitivity and specificity on faeces samples The *Bacillus–Streptococcus-Lactobacillus* (BSL) group represents also a dominant bacterial population of the pig manure (Peu et al., 2006; Marti et al., 2009). Within this group, the genus *Lactobacillus* is highly present in the pig intestinal tract with 10^8 to 10^{10} bacteria per g of faeces (Konstantinov et al., 2004; Tzortzis et al., 2005; Yin and Zheng 2005; Konstantinov et al., 2008; Pieper et al., 2008) and one species, *Lactobacillus amylovorus,* appears to be a relevant indicator of pig faecal pollution (Marti et al., 2010).

Although host specificity markers are valuable for discriminating the origin of a faecal contamination, their persistence in environmental surface waters is also an important factor to be considered. Given that their habitat is the digestive tract, *Bacteroidales* and lactobacilli are well adapted to anaerobic and microaerophilic conditions in nutrient-rich environments (Felis and Dellaglio 2007; Wexler 2007). However, in aerobic conditions, their behaviour is not well known and may differ according to the species. For example, *Bacteroides fragilis* has a high oxygen tolerance and can survive several days in aerobic fresh waters whereas most *Bacteroidales* species can only survive for a few hours (Fiksdal et al., 1985; Walters and Field 2006). Since DNA can remain in the environment and be amplified by PCR methods even when a microorganism is not alive (Espinosa et al., 2008), it is important to know how long host-associated bacterial markers persist for and whether their persistence differs from that of culturable faecal indicators (*Escherichia coli* and enterococci) in contaminated environmental waters. To our knowledge, no data have been reported regarding the impact of abiotic factors on the behaviour of *L. amylovorus* in water. A few studies, using molecular methods such as bromodeoxyuridine immunocapture, fluorescent in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probe (LDS-FISH) and conventional and real-time PCR describe the persistence of *Bacteroidales* from pure culture or faeces under environmental factors by using microcosms (Kreader 1995; Savichtcheva et al., 2005; Seurinck et al., 2005; Walters and Field, 2006; Okabe and Shimazu 2007; Balleste and Blanch 2010). Overall, these authors observed a decrease in the persistence of *Bacteroidales* with an increase in temperature and oxygen concentration and with low salinity. The effects of temperature, sunlight, salinity and predation were also studied for host-associated *Bacteroidales* markers using qPCR (Seurinck et al., 2005; Walters and Field, 2006; Okabe and Shimazu 2007; Bell et al., 2009; Walter and Field 2009; Walter et al., 2009;

Dick et al., 2010). However, only one study concerning the persistence of pig-associated *Bacteroidales* has been published (Okabe and Shimazu, 2007).

There are scarcely any reports about the capacity of pig-associated bacterial markers belonging to *Bacteroides-Prevotella* and BSL bacterial groups to persist in natural water bodies, so we designed this study to examine their persistence under controlled conditions.

The aims of this study were: (i) to examine the dynamic of *Bacteroides-Prevotella* and BSL populations according to two temperatures (4 and 20°C) and two conditions of oxygen concentration (aerobic and microaerophilic), using Capillary Electrophoresis-Single Strand Conformation Polymorphism analysis (CE-SSCP; fingerprinting technique based on the electrophoretic behaviour of a single-stranded DNA fragment which is separated according to the conformation of their secondary structure; Hebenbrock et al., 1995), and (ii) to compare the persistence of DNA from *Bacteroidales*, lactobacilli, Pig-1-Bac, Pig-2-Bac and *L. amylovorus* markers with the survival of conventional faecal indicators, *Escherichia coli* (*E. coli)* and enterococci, in river water microcosms inoculated with pig manure.

2. Materials and methods

2.1. River water microcosms

Water samples were collected from the Neven River, Brittany, France, where the concentration of *E. coli* was lower than 1 Colony Forming Unit (CFU) per 100 mL water, and stored 24h at 4°C before experimentation. The samples were filtered through 3-µm nitrocellulose membrane filters (Millipore, Ireland) to eliminate most grazing protozoa (Weinbauer and Hofle 1998). Fresh pig manure (age < 1 month) originating from a farm located in Brittany was sampled from a storage tank after homogenisation with propeller agitator for 20 minutes. The chemical characteristics of the manure were pH 7.6, dry matter 4.6% (wt/wt), total Kjeldahl nitrogen content 4.1 g per L, ammonium 2.6 g per L, total Chemical Oygen Demand (COD) 51 g O_2 per L.

Microcosms, which consisted of 100-fold dilution of pig manure in filtered river water, were prepared in sterile 2-liter or 5-liter flasks. Four treatments were tested combining two levels of temperature with two oxygen conditions: 4°C or 20°C under an aerobic (5-liter flasks with 2 l of river water with pig manure) or a microaerophilic (2-liter flasks with 2 l of river water with pig manure) atmosphere. Microaerophilic flasks were hermetically sealed with silicone caps. Aerobic conditions were established by flushing the river water microcosms with 0.22 µm filter-sterilized air (Millipore, St Quentin-en-Yvelines, France) to obtain dissolved oxygen saturation. Microaerophilic conditions were established by flushing the river water microcosms with filter-sterilized nitrogen (0.22 µm) at the beginning of the experiment and after each sampling. In these microaerophilic conditions, oxygen concentrations ranged from 0.4 to 1.9 mg oxygen per L (corresponding to 1.5 to 16.9 % of oxygen saturation). The four river water microcosms and their respective control river water microcosms (same conditions without manure) were kept in the dark and gently stirred with a magnetic barrel. Fourteen samples were collected in duplicate over a 43-day period from each microcosm.

2.2. Sample preparation and DNA extraction

Sampling was carried out daily for the first five days, then every two days until day 11 (D11) and then every four days until D26. Additional samples were collected on D34 and D43. At each sampling point, 55 mL of water were collected in duplicate either with a sterile pipette, for aerobic conditions, or with a 20-mL sterile syringe, for microaerophilic conditions, and

transferred to sterile vials for molecular and cultural analyses. After sampling, microaerophilic microcosms were flushed with filter-sterilized nitrogen for at least 15 min.

For each duplicate sample collected per river microcosm, 5 mL were kept for enumeration of faecal indicators and 50 mL were filtered through 0.22 µm Nuclepore membrane filters (Whatman, Brumath, Germany). The filters were then placed in 1.6 mL of Stool Lysis (ASL, QIAGEN, Courtaboeuf, France) buffer and frozen at -20°C until DNA extraction. DNA was extracted using the QIAamp DNA stool kit (QIAGEN, Courtaboeuf, France) following the manufacturer's instructions. The final elution volume was 50 µL.

2.3. Enumeration of *E. coli* and enterococci

For each sample, a volume of 5 mL of water was used for the enumeration (in duplicate) of faecal indicators. *Escherichia coli* were counted using 3M™ Petrifilm *E. coli* (3M, Cergy-Pontoise, France). The gel of the Petrifilm was rehydrated with 1 mL of sample and incubated for 24 h at 44°C. Blue colonies (Glucuronidase positive) were counted to determine the concentration of *E. coli* (Vail et al., 2003). The detection limit was 1×10^2 CFU per 100 mL of water. Enterococci were detected according to the Standard Procedures for Water Analysis (ISO 7899-2:2000). Filtration was replaced by the plating of duplicate 0.1 mL volumes of sample onto Slanetz and Bartley agar (Biokar Diagnostics, Beauvais, France). After incubation at 37°C for 48 h, typical colonies (pink, red or maroon) were transferred onto Bile-Esculin-Azide agar (BEA) (Biokar Diagnostics, Beauvais, France) and incubated for 2 h at 44°C. Black colonies on BEA were counted as enterococci. The detection limit was 1×10^3 CFU per 100 mL water.

2.4. PCR for CE-SSCP analysis

Conventional PCRs were performed for the *Bacteroides-Prevotella* and BSL groups. The reaction mix was 200 nM dNTP, 350 nM of each primer (Table 1), 1× AccuPrime *Taq* DNA polymerase buffer II, 2.5 U AccuPrime *Taq* DNA polymerase (Invitrogen, Illkirch, France) and 1 µL DNA extracts (1/10 dilutions) from each river water microcosm and control river water microcosm at D0 and D43 of the experiment. The final reaction volume was 20 µL. The annealing temperature was 55°C for the *Bacteroides-Prevotella* group and 61°C for the BSL group. After a denaturation step at 94°C for 2 min, the reactions consisted of 30 cycles at 94°C for 30 s, at the annealing temperature for 90 s, and at 68°C for 90 s. As recommended by the supplier (Invitrogen), no final elongation was performed. The size of the PCR products was confirmed by agarose gel electrophoresis (TBE 1X 1.5 % agarose (wt/vol)). The PCR products were visualised under UV light after gel staining with GelRed 1X. A volume of 1 µL of each PCR product was used as template for the CE-SSCP analyses.

The CE-SSCP-PCRs were performed with primers W34 and W49 targeting the 16S rDNA V3 region (200 base pairs) (Table 1). The reaction mix was 200 nM dNTP, 390 nM of each primer, 1× *Pfu* turbo buffer, 0.625 U *Pfu* turbo (Stratagene, La Jolla, CA) and 1 µL of the previous PCR products. The final reaction volume was 20 µL. The amplification conditions were 2 min at 94°C followed by 25 cycles of 30 s at 94°C, 30 s at 61°C then 30 s at 72°C, and a final elongation step of 10 min at 72°C. The resulting PCR products were separated by SSCP capillary electrophoresis using an ABI 310 genetic analyser (Applied Biosystems, Villebon sur Yvette, France) as described by Delbes et al. (2001) but using a CAP 5.58% - Glycerol 10% polymer (Applied Biosystems).

2.5. Cloning and sequencing

Cloning was performed on a mixture of two PCR products selected according to the CE-SSCP profiles (with the most numerous and highest peaks) of the *Bacteroides-Prevotella* and BSL groups. The mixed PCR products were cloned and transformed into competent *E. coli* cells with the StrataClone PCR cloning kit (Stratagene, La Jolla, CA) following the manufacturer's instructions except for the ligation time, which was increased from 5 to 15 min.

A total of 208 clones were further analyzed: 104 from each bacterial group. The clones were randomly picked, and their inserts were screened by nested PCR followed by CE-SSCP analysis. In the first step, plasmid inserts were amplified by PCR with plasmid-targeted primers T7 (5'-TAATACGACTCACTATAGGG-3') and P13 (5'-GACCATGATTACGCCA-3') (Stratagene, La Jolla, CA). The reaction mixture was 0.2 mM dNTPs, 700 nM each primer, 1× Red*Taq* buffer, 2.5 U Red*Taq* polymerase, 1 µl of plasmid inserts and deionized water to bring the volume to 25 µl. The amplification conditions were 10 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C and a final elongation step of 10 min at 72°C for. One microliter of these PCR products was used to perform a CE-SSCP PCR as described above. Inserts yielding a peak that co-migrated with distinguishable peaks from the manure CE-SSCP profiles were sequenced for peak identification.

A total of 44 clones (28 for *Bacteroides-Prevotella* and 16 for BSL) were sequenced. Sequence reactions were performed at the Ouest Genopole Sequencing Facility (CNRS, Roscoff, France) with primer T7. DNA sequences were identified by comparison with their closest relatives available in databases using Basic Local Alignment Search Tool *(*BLAST*)* from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) and Ribosomal Database Project II (RDPII; http://rdp.cme.msu.edu/). The sequences were deposited in EMBL under accession numbers HQ442268 to HQ442285.

2.6. QPCR for general and pig-associated markers

The general All-*Bacteria*, All-*Bacteroidales* and total lactobacilli markers and the three pig-associated bacterial markers were quantified in the samples of river water, river water microcosms and pig manure.

2.6.1. Oligonucleotide primers and probes

The primers and probe All-*Bacteria* (Bact2, modified from Suzuki et al., (2000) and All-*Bacteroidales* (AllBac) from Layton et al. (2006) were used to amplify total bacterial and total *Bacteroidales* 16S rRNA genes. The primers lactobacilli LAC1 (Walter et al., 2000), and Lab0677 (Heilig et al., 2002) were used to amplify total lactobacilli 16S rRNA genes (Table 1). The two pig-associated *Bacteroidales* 16S rRNA gene markers (Pig-1-Bac and Pig-2-Bac) and the Representational Difference Analysis (RDA) fragment A of the pig-associated *L. amylovorus* marker were quantified with the primers and probe described by Mieszkin et al. (2009) and Konstantinov et al. (2005), respectively (Table 1).

2.6.2. QPCR assays

All amplifications were performed using the Chromo4 real-time detection system associated with Bio-Rad Opticon Manager software version 3.1 (Bio-Rad, Hercules, CA). For the All-*Bacteria*, All-*Bacteroidales*, Pig-1-Bac and Pig-2-Bac markers, qPCR assays were performed

using the TaqMan® Brilliant II QPCR Master Mix kit (Stratagene) whereas the IQ SYBR-Green Supermix (Bio-Rad) was used for the total lactobacilli and *L. amylovorus* markers.

The cycle conditions for the TaqMan® assays were 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. The cycle conditions for the SYBR-Green® assay were 1 cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, 60°C for 45 s. A dissociation step was added at 95°C for 1 min, 55°C for 30 s and 95°C for 30 s to control amplification specificity.

Reactions were carried out in a final volume of 25 µL with appropriate final concentrations of primers and probe (Table 1). The presence/absence of PCR inhibitors was verified using an Internal Positive Control (IPC; Applied Biosystems, France). Each sample was analysed in duplicate for each marker and was diluted to verify if inhibitors were present. Negative controls (no template DNA) were performed in triplicate for each run.

2.6.3. DNA standard curves

Linear plasmids used to generate standard curves for the quantification of *Bacteroidales* markers were extracted with the QIAquick Miniprep Extraction Kit (Qiagen), following the manufacturer's instructions. The linear forms of plasmids were obtained by digestion, with *Not*I enzyme (Roche Diagnostics, Meylan, France), in a final volume of 50 µL for 3 h at 37°C (Mieszkin et al., 2009). For the quantification of *Bacteroidales* markers, standard curves were generated from 10-fold dilutions of a known concentration of plasmid DNA. Plasmid concentrations ranged from 1.6×10^7 to 1.6 copies per PCR well.

The standard curves for the All-*Bacteria* and for the total lactobacilli and *L. amylovorus* markers were prepared by 10-fold dilutions of bacterial genomic DNA extracted from a pure culture of *E. coli* and a pure culture of *L. amylovorus* DSM16698, respectively, using the Wizard genomic DNA purification Kit (Promega, Charbonnières-les-bains, France) according to the manufacturer's instructions. Dilutions ranged from 7×10^6 to 7×10^1 copies per PCR for *E. coli* and from 7×10^5 to 7 cells per PCR for *L. amylovorus* DSM16698. The number of copies of 16S rRNA genes per genome was considered to be 7 copies for the *E. coli* strain (Klappenbach et al., 2001).

Standard curves were generated by plotting threshold cycles (Ct) against 16S rRNA genes or cells, depending on the marker.

2.7. Limit of quantification of qPCR assays

Limits of quantification were assessed for All-*Bacteroidales*, total lactobacilli and the three pig-associated markers using suspended pig manure in filtered river water (corresponding to a 100-fold dilution suspension of pig manure in water). Series of 10-fold dilutions of this suspension were made in duplicate from 10^{-2} to 10^{-9} in the same filtered river water. Fifty mL of each dilution were filtered through 0.22 µm Nuclepore membrane filters and DNA was extracted using the QIAamp DNA stool kit as described above. QPCR assays were performed for the All-*Bacteroidales*, total lactobacilli, pig-associated *Bacteroidales* and *L. amylovorus* markers. The limit of quantification was defined as the lowest concentration of the bacterial genetic marker still within the linear range of quantification of the qPCR assay in river water.

2.8. Data normalization and T90 calculation

Concentrations of all markers and faecal indicators (number of copies, cells or CFU per 100 mL water), recovered from each sampling day were normalized and a natural log (ln) transformation of the ratio [ln(C/C0); C = concentration at time t, C0 = concentration at day 0] was performed. The values were plotted against the sampling day for each markers and faecal indicators.

According to the results, decay rates calculations were either based on a first order decay model (Chick model) or on the biphasic model of Cerf reported by Lee et al. (2001)

$C(t) = C0 \times e^{-kt}$ or $\ln(C(t)/C0) = -kt$ (Chick Model)

$C(t) = C0 \times (f \times e^{-k1t} + (1-f) \times e^{-k2t})$ or $\ln(C(t)/C0) = \ln(f \times e^{-k1t} + (1-f) \times e^{-k2t})$ (Cerf Model)

where C0 is the average initial concentration of the target, C(t) is the target average concentration at time t, t is the time in days, k is the decay constant in $days^{-1}$, f is the proportion of C0 that declined during the first phase, k_1 is the decay constant of the first phase and k_2 the decay constant of the second phase. The model and associated parameters were obtained with the aid of XLSTAT 2010.4 using the linear or the nonlinear regression modelling.

Decay rates were calculated until the day concentrations were below quantification limit or until day 43 when the detection limit was not reached.

Time (expressed in days) to obtain 90% reductions in initial concentration of markers or faecal indicators was calculated as follows: $T_{90} = -\ln(0.1)/k$ (Chick model) or $T_{90} = -\ln(0.1)/k_1$ (Cerf Model).

3. Results

3.1. CE-SSCP profiles

The effect of temperature and oxygen concentration on *Bacteroides-Prevotella* and BSL groups in river water microcosms was evaluated by 16S rRNA gene-targeted PCR-SSCP analysis.

At the beginning of the experiment (day 0), the *Bacteroides-Prevotella* CE-SSCP profile of the control river water microcosms, presented eleven distinguishable peaks (Fig. 1a). After 43 days, the *Bacteroides-Prevotella* profile had only six peaks, four of which were common with those from the profile at day 0 (Fig. 1a). The BSL CE-SCCP profile of the control river water microcosms had one peak at day 0, which persisted throughout the experiment (Fig. 1b). A second peak was present after 43 days in all the conditions tested (Fig. 1b). None of the peaks present on the profile of the control river water microcosms at day 0 co-migrated with any of the peaks of the river water microcosm after addition of pig manure (Fig. 1).

The CE-SSCP profiles of *Bacteroides-Prevotella* and BSL groups obtained at day 0 and at day 43 for the river water microcosms with pig manure are aligned in Figure 2. At day 0, these profiles were identical in the four microcosms. *Bacteroides-Prevotella* and BSL profiles had eleven and ten distinguishable peaks, respectively that emerged from the background of subdominant bacterial community. At 4°C, in microaerophilic conditions, all peaks observed at day 0 were still present at day 43 (Tab. 2). The two small peaks, that appeared on the BSL

group profiles (indicated by an asterisk in Fig. 2) co-migrated with the two peaks observed at day 43 in the control river water microcosms. At 20°C, in microaerophilic condition, the CE-SSCP profile of the BSL group remained very similar to that observed at 4°C, except for one peak that disappeared (Fig. 2b). The *Bacteroides-Prevotella* group was quite dynamic at 20°C, five peaks were still present but five new peaks appeared, one of them has co-migrated with a peak observed in the control river water microcosms at day 0 and 43 (Fig. 2a).

The CE-SSCP profiles revealed changes in the bacterial community of the two groups of bacteria when exposed to oxygen, especially at 20°C (Tab. 2). At 4°C, eight peaks appeared in the CE-SSCP profile of the BSL group (Figure 2b). The profile had 13 small peaks and two dominant peaks, one of which co-migrated with one peak of the bacterial river water control community. At 20°C, there was a clear reduction in the diversity compared to the CE-SSCP profile obtained at 4°C. Five small peaks remained in the profile suggesting that they persisted in the presence of oxygen. However, only one peak that comigrated with one of the river water peaks became clearly dominant (BSL1). In the presence of oxygen at 4°C, eight initial peaks persisted on the *Bacteroides-Prevotella* profile whereas at 20°C only one peak remained. As observed for the BSL group, one peak became highly dominant (BP10), which was certainly due to relative decrease of the other populations, and two peaks co-migrated with the river water peaks.

The dominant peaks of both bacterial groups (*Bacteroides-Prevotella* and BSL) were identified by cloning and sequencing of the corresponding 16S rRNA gene fragments. A total of 208 clones were screened by CE-SSCP and 44 were sequenced. The phylogenetic affiliation of the clones corresponding to the 19 major peaks of the CE-SSCP profiles is presented in Table 3.

Twelve of the 19 peaks were matched to the closest related known sequences deposited in NCBI and RDP at an identity level higher than 97%. Only three peaks could be identified and link to a species (BSL1, BSL2 and BSL8). The two dominant peaks of the BSL control river water profiles at day 43 (BSL1 and BSL2) were assigned to two lactic acid bacteria (Fig. 1b). The sequence of the peak BSL1, which was present at days 0 and 43, was 99% identical to its closest relative, *Leuconostoc citreum* and the sequence of peak BSL2, which appeared at day 43, was 100% identical to *Weissella cibaria* (Tab. 3). The two peaks BSL1 and BSL2 were also detected in the river water microcosms with pig manure at day 43 for all conditions tested (Fig. 2). Five dominant peaks of the BSL profiles at day 0 could be identified in the river water microcosms with pig manure. The sequences of the four peaks that migrated in the middle of the profile (BSL3 to BSL6) were closely related to the *Erysipelotrichales* order (91 to 99% identity) whereas the sequence of the last peak (BSL8) was related to *Lactobacillus* genus, and was 99% identical to a sequence found in pig manure by Marti et al. (2009). It corresponded to the pig-associated marker *Lactobacillus amylovorus*.

All the sequences of *Bacteroides-Prevotella* corresponded to uncultured bacteria belonging to the family *Cytophagaceae* or the order *Bacteroidales*, but none of them could be assigned at the genus level. Three peaks present in control river water microcosms were identified for the *Bacteroides-Prevotella* group. BP1 and BP11 were 99% and 98% identical, respectively, to their closest relative *Cytophagaceae* and BP2 had 95% identity with an unclassified *Bacteroidetes* which was found in lake water or in suboxic freshwater pond sediment. In the river water microcosms with pig manure, seven of the *Bacteroides-Prevotella* peaks present at the beginning of the experiment and the dominant peak (BP10) observed under aerobic treatment at 20°C (at day 43) were identified. The sequences of three of them, BP3, BP8 and BP10 were identical to sequences of eubacteria found in pig manure (Liu et al., 2009; Marti et al., 2009). The other sequences were identical to a sequence found in soil, water and an anaerobic solid waste digester (Table 3).

3.2. qPCR

3.2.1. Quantification limit of the general and pig-associated markers

The linear decrease in the number of copies of All-Bacteroidales, Pig-1-Bac and Pig-2-Bac markers as a function of 10-fold serial dilution of suspended pig manure in filtered river water resulted in a quantification limit of 1.6×10^4, 6.3×10^2 and 2×10^2 16S rRNA gene copies per 100 mL water corresponding to 3.2×10^2, eight and four copies per reaction, respectively. The limits of quantification of total lactobacilli and *L. amylovorus* markers were 4×10^4 and 1.3×10^2 cells per 100 mL water, corresponding to 8.6×10^2 and two cells per reaction, respectively.

Concentrations of bacterial markers and conventional faecal indicators in river water and in river water microcosms after addition of pig manure.

Concentrations of All-*Bacteria*, All-*Bacteroidales*, Pig-1-Bac, Pig-2-Bac, total lactobacilli and *L. amylovorus* markers were evaluated by qPCR and *E. coli* and enterococci were enumerated simultaneously by culture.

In river water samples, geometric mean concentrations of All-*Bacteria,* All-*Bacteroidales* and total lactobacilli were 2.7×10^7, 1.5×10^4 16S rRNA gene copies per 100 mL and 6.3×10^4 cells per 100 mL water, respectively, whereas the three pig-associated markers and the faecal indicators *E. coli*, enterococci were not quantifiable.

At day 0, after addition of pig manure, the geometric mean concentrations of All-*Bacteria,* All-*Bacteroidales* and total lactobacilli were 1.1×10^{11}, 6.0×10^8 16S rRNA gene copies per 100 ml and 1.0×10^6 cells per 100 mL, respectively. For the three pig-associated markers, Pig-1-Bac, Pig-2-Bac and *L. amylovorus*, concentrations were 2.7×10^5, 1.7×10^5 $\log_{10}$ copies and 6.5×10^5 cells per 100 mL, respectively. The geometric mean concentrations of *E. coli* and enterococci were 1.9×10^3 CFU and 1.4×10^4 CFU per 100 mL, respectively.

In the four control river water microcosms, none of the three pig-associated markers or the conventional faecal indicators were detected during the 43 days of the experiment (data not shown).

3.2.2. Effects of oxygen and temperature on the persistence of genetic markers

Figure 3 shows the mean persistence profiles for the AllBacteria, total lactobacilli and *L. amylovorus* and figure 4 shows the persistence profiles for All-*Bacteroidales* and the two pig-associated *Bacteroidales* markers. The behaviour of each group of bacteria depended on the incubation conditions. The level of total bacteria remained stable for the 43 days of the experiment regardless of the temperature and the oxygen concentration (Fig. 3). In most cases, regardless of the microcosm conditions, persistence profiles of the two pig-associated *Bacteroidales* markers were similar to those of All-*Bacteroidales* or presented lower decreases in concentrations whereas persistence profiles of *L. amylovorus* were similar to those of total lactobacilli or presented greater decreases in concentrations (Fig. 3 and 4). Microaerophilic conditions at 4°C did not have any effect on the persistence of the markers, whose concentrations did not change significantly over the course of the experiment (Fig. 3a and Fig. 4a). At 20°C, under microaerophilic conditions, a slight decrease in concentrations was observed (less than one logarithmic unit) for the All-*Bacteroidales* and for the two pig-associated *Bacteroidales* markers whereas concentrations of total lactobacilli and *L. amylovorus* markers remained stable (Fig. 3b and Fig. 4b).

As observed for the CE-SSCP profiles, aerobic conditions led to the greatest changes. Under aerobic conditions at 4°C, concentrations of All-*Bacteroidales* and total lactobacilli markers decreased by 2.6 and 0.4 $\log_{10}$ units after 43 days, respectively (Fig. 3c). Persistence of the Pig-1-Bac and Pig-2-Bac markers was more influenced by oxygen than the one of *L. amylovorus* marker (Figure 3c and 4c). The largest decline was observed at 20°C for the All-*Bacteroidales* marker whose concentration was reduced by about 4 $\log_{10}$ units within 43 days, while the concentration of total lactobacilli only decreased slightly (less than 0.5 $\log_{10}$ unit) (Fig. 3d and 4d). Concentrations of the Pig-1-Bac and Pig-2-Bac markers decreased considerably under these conditions (by more than 3.5 $\log_{10}$ unit) and reached the limit of quantification after 21 and 16 days, respectively. The decrease in the concentration of the *L. amylovorus* marker was less marked but reached 2.9 $\log_{10}$ unit after 43 days.

3.2.3. Effects of oxygen and temperature on the survival of faecal indicators

The survival of *E. coli* and enterococci was also compared. Apart from microcosms under aerobic conditions at 20°C, the decreases in enterococci concentrations were lower than those of *E. coli* (Table 4). The highest decreases in faecal indicator concentrations were observed at 20°C, under microaerophilic conditions for *E. coli* and under aerobic conditions for enterococci.

Although at 4°C the counts of enterococci remained stable throughout the experiment, they decreased at 20°C. The decrease, which started after 27 days under microaerophilic conditions, did not exceed 5 CFU per 100 mL at day 43 whereas the number of enterococci declined after 7 days and reached the quantification limit after 16 days under aerobic conditions (Fig. 5). The concentrations of *E. coli* decreased in all four types of microcosms, suggesting that *E. coli* was less persistent than enterococci in our experimental conditions.

3.2.4. Evaluation of the necessary time to obtain a 1-log reduction in concentrations for bacterial markers and conventional faecal indicators

For the four conditions tested, 1-log reduction in concentrations for the All-Bacteria and total lactobacilli marker was never reached, whereas for All-Bacteroidales marker, 1-log reduction in concentrations was obtained under aerobic conditions at 4°C and at 20°C in 14.4 and 11.7 days, respectively (Table 4). Pig-associated *Bacteroidales* markers reached a 1-log reduction in concentrations earlier than *L. amylovorus* under aerobic conditions at 4°C and at 20°C The weakest T90 values for the Pig-2-Bac and Pig-1-Bac markers and *L. amylovorus* marker were obtained at 20°C (i.e. 1.9, 10.3 and 17.1 days, respectively; Table 4). At 4°C, higher T90 (i.e. 19.3 and 22.0 days, respectively) were obtained for Pig-2-Bac and Pig-1-Bac whereas 1-log reduction in concentrations for *L. amylovorus* marker was never reached over the 43 days of the experiment. In the most unfavorable conditions (aerobic and 20°C), the T90 of the pig-associated *Bacteroidales* markers were lower than those of conventional faecal indicators i.e. *E. coli* and enterococci whereas the T90 of the *L. amylovorus* marker was higher than the T90 obtained for the enterococci.

4. Discussion

In intensive pig farming regions, such as Brittany, runoff of spreading pig manure could contribute to the degradation of water and shellfish quality. It is thus important to have tools that can be used to identify this source of faecal contamination. While the specificity and the sensitivity of the recently developed pig-associated markers, Pig-1-Bac, Pig-2-Bac and

L. amylovorus, had already been successfully tested (Marti et al., 2009; Mieszkin et al., 2009), their persistence in environmental waters were unknown. Furthermore, little information is available on the behaviour of the dominant bacterial population to which they belong, after their discharge into river water. Previous studies focused on the behaviour of the dominant bacterial population during storage (Peu et al., 2006) or after biological treatment of manure (Leung and Topp 2001; Marti et al., 2009) using molecular typing methods but their temporal dynamics have not been described in river water. In this present study, CE-SSCP and qPCR techniques were used to evaluate the dynamics of *Bacteroides-Prevotella* and *Bacillus-Streptococcus-Lactobacillus* taxonomic groups in river water and the persistence of the three pig-associated markers. The CE-SSCP profiles were employed to determine the dominant community structure of the pig manure and to analyse its structural variation after treatment (Peu et al., 2006; Marti et al., 2009). Each peak on a CE-SSCP profile represents a bacterial species when its population represents more than 1% of the total community (Loisel et al., 2006). In this study, where pig manure was diluted in river water microcosms, the analysis of the CE-SSCP profiles indicated differences in the *Bacteroides-Prevotella* and BSL group bacterial composition, depending on temperature and dissolved oxygen concentration. At 4°C, under microaerophilic conditions after 43 days of experiment, the CE-SSCP profiles remained unchanged whereas there was a shift in the composition of both groups of bacteria at 20°C under aerobic conditions, especially for the *Bacteroides-Prevotella*. These results suggest that the dominant species of this group of bacteria are not well adapted to persist in river water with a high level of dissolved oxygen at a temperature of 20°C, i.e. under conditions usually observed in the Brittany rivers during summer. Despite dilution of the pig manure in the river water, under microaerophilic conditions, at least 45% and 90% of the initial peaks of *Bacteroides-Prevotella* and BSL group, respectively are present. The effect of dissolved oxygen at 20°C on the two groups of bacteria is also consistent with the study of Leung and Topp (2001), who observed large changes in bacterial populations during aeration of manure. The disappearance of ten of the 11 peaks present at day 0 and the apparition of one major peak (BP10) in aerobic condition closely related to a sequence of *Bacteroidales* previously found in pig manure highlight that there are variations in oxygen tolerance among the dominant bacteria of *Bacteroides-Prevotella* from pig manure. The BSL group appeared to be less sensitive to oxygen as previously observed by Marti et al. (2009) during the aerobic treatment of manure. Indeed, half of the dominant peaks were still detected in the presence of oxygen at 20°C although a major peak originating from the river water was highly dominant.

Although 64% of the identified peaks showed 97% or greater identity to sequences deposited in NCBI or RDP databases, none of the 11 peaks assigned to the *Bacteroides-Prevotella* CE-SSCP profiles could be identified at the “genus” level, probably because the isolation of members of this group of bacteria is rarely reported. Among the eight peaks assigned to the BSL groups, 62% of them have shown 97% or greater identity to sequences deposited in the databases. Three of them were identified at the "species" level. The presence of *Leuconostoc citreum* (BSL1) and *Weissella cibaria* (BSL2) in the river water is not surprising as the two genera are widely distributed in a variety of sources including vegetables, sewage and soil (Magnusson et al., 2002; Liu et al., 2009). Their dominance in the microcosms especially under aerobic conditions could be explained by their relative insensitivity to oxygen (Björkroth and Holzapfel 2006). Furthermore, the organic matter of the manure likely improved their persistence in the microcosm by acting as a source of nutrients for bacteria, promoting growth or at least extended survival (Pedley et al., 2005). It is interesting to note that the peak BSL8, which was identified to the pig-associated marker *L. amylovorus* was a dominant species of the BSL group regardless the conditions applied to the river water microcosms, indicating its ability to persist in microaerophilic or aerobic environment. The other peaks were phylogenetically close to sequences of bacteria found in faeces, manure or anaerobic digesters, belonging to the *Erysipelotrichaceae* which are facultative anaerobes (Stackebrandt et al., 2006).

In accordance with the CE-SSCP profiles, the concentrations of all target bacteria were affected by the environmental conditions applied to the microcosms. There was a significant decrease in the concentrations of bacteria under aerobic conditions at 20°C, except for *E. coli*, which was most affected by micro-aerophilic conditions. Nevertheless, the initial concentration of *E. coli* at day 0 and the threshold of the method could explain why the detection limit was rapidly reached. The longer survival of enterococci under microaerophilic conditions is consistent with the results of the experiment conducted by Roslev et al. (2009). These authors showed that in drinking water microcosms contaminated with raw sewage, the survival rate of enterococci was higher under anaerobic conditions than under aerobic conditions. Under aerobic conditions at 20°C, during the 16 first days, the concentrations of All-*Bacteroidales*, Pig-1-Bac and Pig-2-Bac markers decreased more rapidly than those of total lactobacilli, *L. amylovorus* and enterococci, as expected due to the lower tolerance to oxygen. Consequently, the Pig-1-Bac and Pig-2-Bac markers were detected only during 21 and 16 days, respectively, showing their capacity to identify recent fecal contamination from porcine origin. It is noteworthy that the pig-associated *Bacteroidales* markers used in this study belong to the genus *Prevotella* (Mieszkin et al., 2009) which contains strictly anaerobic species (Takeuchi et al., 1999; dos Santos et al, 2007, Ueki et al, 2007). Lactobacilli contain a large number of aerotolerant species (Amanatidou et al., 2001; De Angelis and Gobbetti 2004; Brioukhanov and Netrusov 2007; Felis and Dellaglio 2007) and it has been reported that strains of *L. amylovorus* could adapt to the presence of oxygen (Neysens and De Vuyst 2005).

In this study, oxygen and temperature seem to have a combined effect on the persistence of the two pig-associated *Bacteroidales* markers, as their concentrations decreased markedly under aerobic conditions at 20°C. Thus, the T90 values for Pig-2-Bac and Pig-1-Bac markers at 20°C were found to be 8.1 and 10.5 days, respectively whereas they were higher at 4 °C (19.3 and 22.1 days, respectively) or in aerobic conditions (> 43 days). The present results, which showed that lower temperatures increased the persistence of *Bacteroidales,* are in good *agreement with data from the literature (Kreader 1998; Savichtcheva et al., 2005; Seurinck et al., 2005; Okabe and Shimazu 2007)*. Thus, the pig-associated *Bacteroidales* marker – Pig-Bac2 - described by Okabe *et al.* (2007) also had higher T90 values at 4°C (i.e. > 7 days) than at 20°C (5.5 days) in river water with pig faeces in aerobic conditions in the dark. Our results are also consistent with those of Savichtcheva et al. (2005) who observed a decrease in the viability of pure cultures of *B. fragilis* cells and faecal *Bacteroides* spp from human faeces in presence of oxygen and higher temperature.

The temperature and oxygen conditions tested in this study were similar to those measured in river waters where pig-associated *Bacteroidales* markers were previously quantified (Mieszkin et al., 2009). Indeed, for river waters collected on the catchment of the Daoulas estuary (Brittany, France), temperatures ranged between 5 to 7°C and between 14 to 20°C in winter and summer, respectively. Moreover, dissolved oxygen concentrations ranged between 9 to 12 mg/L implying that dissolved oxygen concentrations are relatively high regardless of the season. In this study, oxygen concentrations ranged from 9.3 to 11.8 and from 7.5 to 9.2 mg/L for aerobic microcosms at 4 and 20°C, respectively. Thus, results of the three pig-associated markers obtained in aerobic microcosms may contribute to our understanding of their fate in river water. However, it is important to note that other factors such as predation by protozoans, salinity, sunlight or presence of toxic agents that were not taken into account in this study could also have influenced the behaviour of these markers, and thus, dependently or independently from temperature and oxygen. For example, an increase of temperature was found to decrease the persistence of *Bacteroides* cells or DNA by increasing the level of predation activity (Kreader et al., 1998; Balleste and Blanch, 2010). The two conventional faecal indicators were detected at 20°C until day 16 and they were still present after 43 days at 4°C regardless of the concentration of dissolved oxygen. The changes in the concentrations of the three pig-associated markers were comparable to those

of conventional faecal indicators, suggesting that they may reflect the persistence of these bacteria after transfer to surface waters.

5. Conclusion

The results of this study allow to a better understanding of the dynamic of *Bacteroides-Prevotella* and BSL groups from pig manure in river water as well as of the persistence of three promising pig-associated markers belonging to these groups. They underline the fact that seasonal variations in temperature and the levels of dissolved oxygen that were tested may influence bacterial population of pig manure and the persistence of pig-associated markers. Interestingly, the pig-associated *Bacteroidales* markers and the *L. amylovorus* marker had different persistence in aerobic microcosms. As a consequence, tracing the bacterial source of such faecal contamination would be made easier by the use of a combination of multiple genetic markers. The simultaneous detection of DNA from both types of markers and faecal indicators during the first 15 days of the experiment confirms the relevance of their detection in surface waters to highlight a recent contamination from pig manure origin.

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Tables

Table 1 Oligonucleotide sequences for qPCR assays with the annealing temperature, the final concentration and the expected size for each amplified product.

Primers and Probes	Primer and probe sequence (5'→ 3') †	Size of amplicon (bp)	Annealing Temp.(°C)	Final concentration (nmol)	Target	References
Bact2*						
BACT1369F	CGGTGAATACGTTCCCGG	142	60	200	All-*Bacteria*	Suzuki et al. (2000)
PROK1492R	TACGGCTACCTTGTTACGACTT			200		
TM1389F	(FAM)CTTGTACACACCGCCCGTC(NFQ-MGB)			250		
AllBac						
AllBac296F	GAGAGGAAGGTCCCCCAC	106	60	200	All- *Bacteroidales*	Layton et al. (2006)
AllBac467R	CGCTACTTGGCTGGTTCAG			200		
AllBac375Bhqr	(FAM)CCATTGACCAATATTCCTCACTGCTGCT(BHQ-1)			100		
Pig-1-Bac						
Pig-1-Bac32Fm	AACGCTAGCTACAGGCTTAAC	129	60	200	Pig-associated	Mieszkin et al. (2009)
Pig-1-Bac108R	CGGGCTATTCCTGACTATGGG			200	*Bacteroidales*	
Pig-1-Bac44P	(FAM)ATCGAAGCTTGCTTTGATAGATGGCG(BHQ-1)			200		
Pig-2-Bac						
Pig-2-Bac41F	GCATGAATTTAGCTTGCTAAATTTGAT	116	60	300	Pig-associated	Mieszkin et al. (2009)
Pig-2-Bac163Rm	ACCTCATACGGTATTAATCCGC			300	*Bacteroidales*	
Pig-2Bac113MGB	(VIC)TCCACGGGATAGCC(NFQ-MGB)			200		
Lactobacilli						
LAC1	AGCAGTAGGGAATCTTCCA	320	60	200	Lactobacilli	Walter et al. (2000); Heilig et al. (2002)
Lab0677	CACCGCTACACATGGAG			200		
L. amylovorus						
OTU171_RDA_F	TTCTGCCTTTTTGGGATCAA	175	60	200	*L. amylovorus*	Konstantinov et al. (2004)
OTU171_RDA_R	CCTTGTTTATTCAAGTGGGTGA			200		
BSL First SSCP-PCR						
W18	GAGTTTGATC(A/C)TGGCTCAG	670	61	350	*Bacillus* *Streptococcus*	Godon et al. (1997); Heilig et al. (2002)
GE08	ATT(C/T)CACCGCTACACATG			350	*Lactobacillus*	
Bacteroides-Prevotella First SSCP-PCR						
W18	GAGTTTGATC(A/C)TGGCTCAG	880	55	350	*Bacteroides*	Godon et al. (1997);
rBacPre	TCACCGTTGCCGGCGTACTC			350	*Prevotella*	Wood et al. (1998)
BSL and *Bacteroides-Prevotella* Second SSCP-PCR						
W49	ACGGTCCAGACTCCTACGGG	200	61	390	16S rRNA V3 region	Delbes et al. (2001)
W34	TTACCGCGGCTGCTGGCAC(FAM)			390		

*Modified from reference (Suzuki *et al.,* 2000); †FAM, 6-carboxyfluorescein; NFQ-MGB, nonfluorescent quencher group-minor groove binder; BHQ-1, black hole quencher 1

Table 2 Number of peaks of CE-SSCP profiles at day 43 in the four river water microcosms with pig manure that were common to those obtained at day 0 or specific to a profile at day 43.

Conditions at day 43	**Number of peaks common at day 0 and at day 43**		**Number of peaks specific to a profile**	
	BP* (n=11)‡	BSL† (n=10)	BP	BSL
4°C, microaerophilic	11	10	0	0
20°C, microaerophilic	5	9	5	1
4°C, aerobic	8	7	1	5
20°C, aerobic	1	5	7	1

* *Bacteroides-Prevotella* ; † *Bacillus Streptococcus-Lactobacillus*; ‡ number of peaks at day 0

Table 3 Phylogenetic affiliation of 16S rDNA sequences of *Bacteroides-Prevotella* and BSL groups from water and microcosms compared with public databases (NCBI and RDP).

		Closest relative				
Peak designation*	Sequence length (bp)	Name (accession no.) for closest match	Affiliation group	% similarity	Source	Reference
BP1	570	Granotes_9-D11-27F (FN297574)	*Cytophagaceae*	99	Lake water column	Hervas, A. and Casamayor, E.O. (Unpublished, 2009)
BP2	569	Clone Llo_063 (FR667361)	*Bacteroidetes*	95	lake epilithic biofilms	Bartrons, M. (Unpublished, 2010)
BP3	569	clone BS69 (EU358744)	*Bacteroidetes*	99	Pig manure	Liu et al. (2009)
BP4	620	Bacteroidetes bacterium clone MEf05b11F4 (FJ828140)	*Bacteroidetes*	100	Eutrophic lake	Newton, R.I. and McMahon, K.D. (Unpublished 2009)
BP5	619	Clone Hmd24B83 (EF197061)	*Cytophagaceae*	91	Soil samples	Wang et al. (2008)
BP6	570	Clone RSC-II-76 (AJ252694)	*Cytophagaceae*	96	Rhizosphere soil	Lukow (Unpublished, 2000)
BP7	570	Clone M35_D20_H_B_G01 (EF586030)	*Bacteroidales*	99	Anaerobic solid waste digester	Li, T. et al. (Unpublished, 2007)
BP8	569	Clone TraBa 01 (AM991316)	*Bacteroidales*	99	Pig manure	Marti et al. (2009)
BP9	619	Clone 965001H10.y1 (DQ065218)	*Cytophagaceae*	92	Fresh water	Horner-Devine et al. (2003)
BP10	569	Clone TraBa 01 (AM991316)	*Bacteroidales*	99	Pig manure	Marti et al. (2009)
BP11	570	Clone BXHB50(DQ676366)	*Cytophagacea*	98	suboxic freshwater-pond sediment	Briee et al. (2007)
BSL1	360	*Leuconostoc citreum* strain A1 16S (FJ476123)	*Leuconostoc citreum*	99	Beer	Wang, X. et al. (Unpublished, 2008)
BSL2	360	*Weissella cibaria* strain MGD4-4 (HM058481)	*Weissella cibaria*	100	Dairy product	Yu, J. et al. (Unpublished, 2010)
BSL3	360	Clone 24_BS1_19 (FJ825514)	*Erysipelotrichaceae*	95	Biogas plant	Podmirseg, S.M. et al. (Unpublished, 2009)
BSL4	359	Clone E92 (FJ205854)	*Erysipelotrichaceae*	91	Mesophilic biogas plant	Krober et al. (2009)
BSL5	359	Clone orang1_aai53h07 (EU777571)	*Erysipelotrichaceae*	100	Sumatran orangutan feces	Ley et al. (2008)
BSL6	359	TraBSL 03 (AM991312)	*Erysipelotrichaceae*	99	Pig raw manure"	Marti et al. (2009)
BSL7	359	clone QEDR2BC06	*Erysipelotrichaceae*	95	Anaerobic municipal wastewater sludge digester	Rivière et al. (2009)
BSL8	359	*Lactobacillus amylovorus* strain LAB52 (EF120375)	*Lactobacillus amylovorus*	100	Porcine intestine	Jakava-Viljanen et al. (2007)

Table 4 Decay rates and T90 of conventional faecal indicators and pig-associated markers in water microcosms seeded with liquid pig manure (1:100 dilution).

Markers and conventional faecal indicators	**Conditions**											
	Aerobic, 4°C				Aerobic, 20°C				Microaerophilic 4°C	Microaerophilic, 20°C		
	k1(d-1)	k2(d-1)	T90 (d)	R2	k1(d-1)	k2(d-1)	T90 (d)	R2	T90 (d)	k(d-1)	T90 (d)	R2
Bact2			>43				>43		>43		>43	
Lactobacilli			>43				>43		>43		>43	
AllBac	0.140	0.149	16.5	0.89	0.902	0.094	2.5	0.95	>43		>43	
Pig-1-Bac	0.104*		22.0	0.83	0.224	1.058	10.3	0.83	>43		>43	
Pig-2-Bac	0.119*		19.3	0.89	1.215	0.129	1.9	0.91	>43		>43	
L. amylovorus			>43		0.135	1.247	17.1	0.95	>43		>43	
E. coli			>43		0.160	0.184	14.4	0.92	>43		> 43	
enterococci			>43				>43		>43	0.1*	23.0	0.69

* Monophasic model

Figures

Figure 1 CE-SSCP profiles of *Bacteroides-Prevotella* (a) and *Bacillus-Streptococcus-Lactobacillus* (BSL) group (b) at day 0 (upper graphs) and at day 43 (lower graphs) in the control river water microcosms. As the profiles were similar regardless of the temperature and oxygen concentration, only one profile (aerobic conditions at 20°C) is presented. Black arrow heads indicate common peaks at day 0 and day 43. White arrow heads indicate peaks present only at day 43. Peaks that could be identified are designated BP1, BP2, BP11, BSL1 and BSL2.

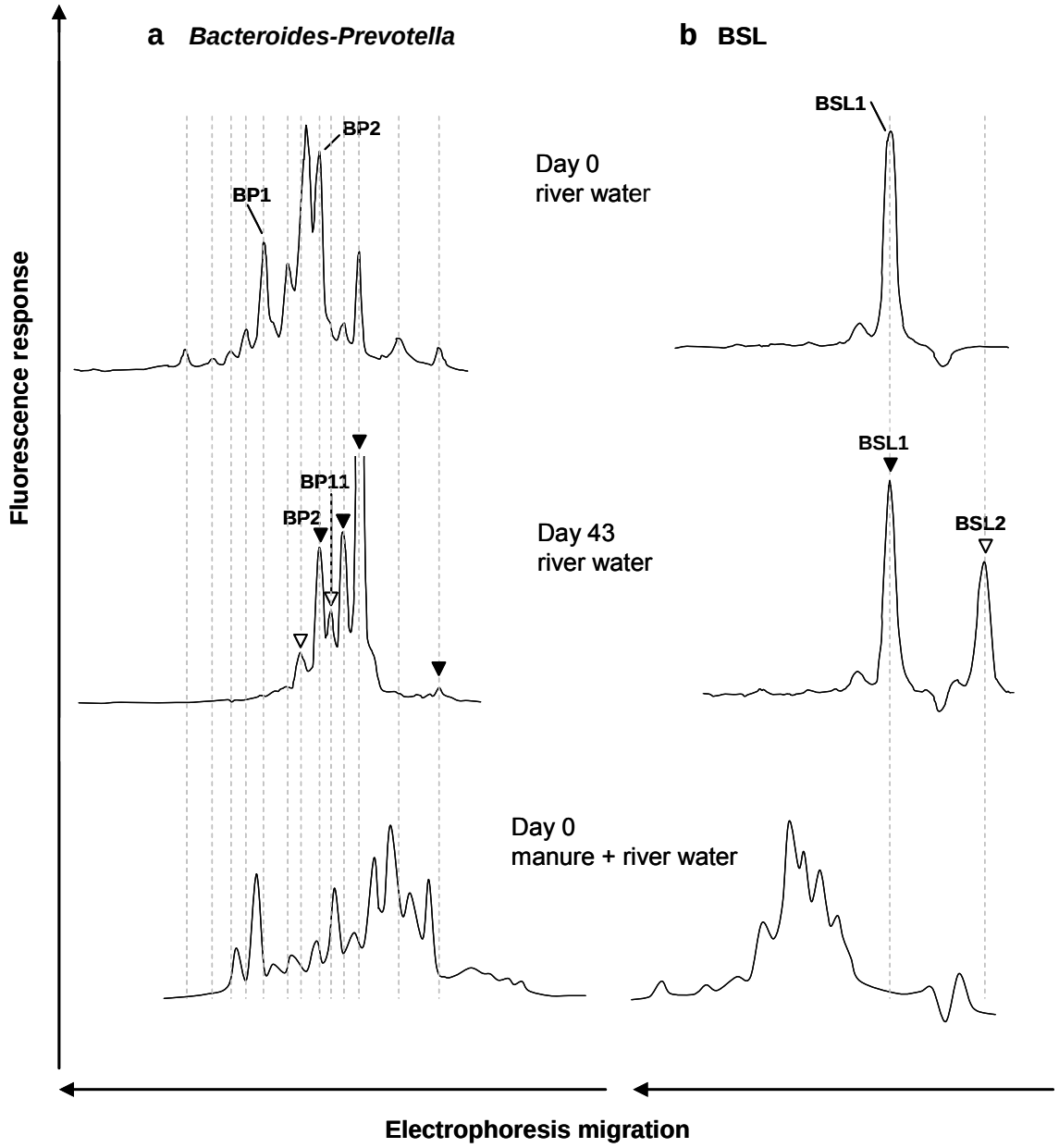


Figure 2 CE-SSCP profiles of *Bacteroides-Prevotella* (a) and *Bacillus-Streptococcus-Lactobacillus* (BSL) group (b) at day 0 (upper graphs) and at day 43 (lower graphs) in microaerophilic conditions at 4°C and 20°C, and in aerobic conditions at 4°C and 20°C in the river water microcosms with pig manure. Black arrow heads indicate common peaks at day 0 and day 43. Grey arrow heads indicate peaks present only on day 43 and common to at least two profiles. White arrow heads indicate peaks present only at day 43 and specific to the profile. Black stars indicate peaks present in the control river water microcosms. Peaks that could be identified are designated BP2 to BP11 and BSL1 to BSL8

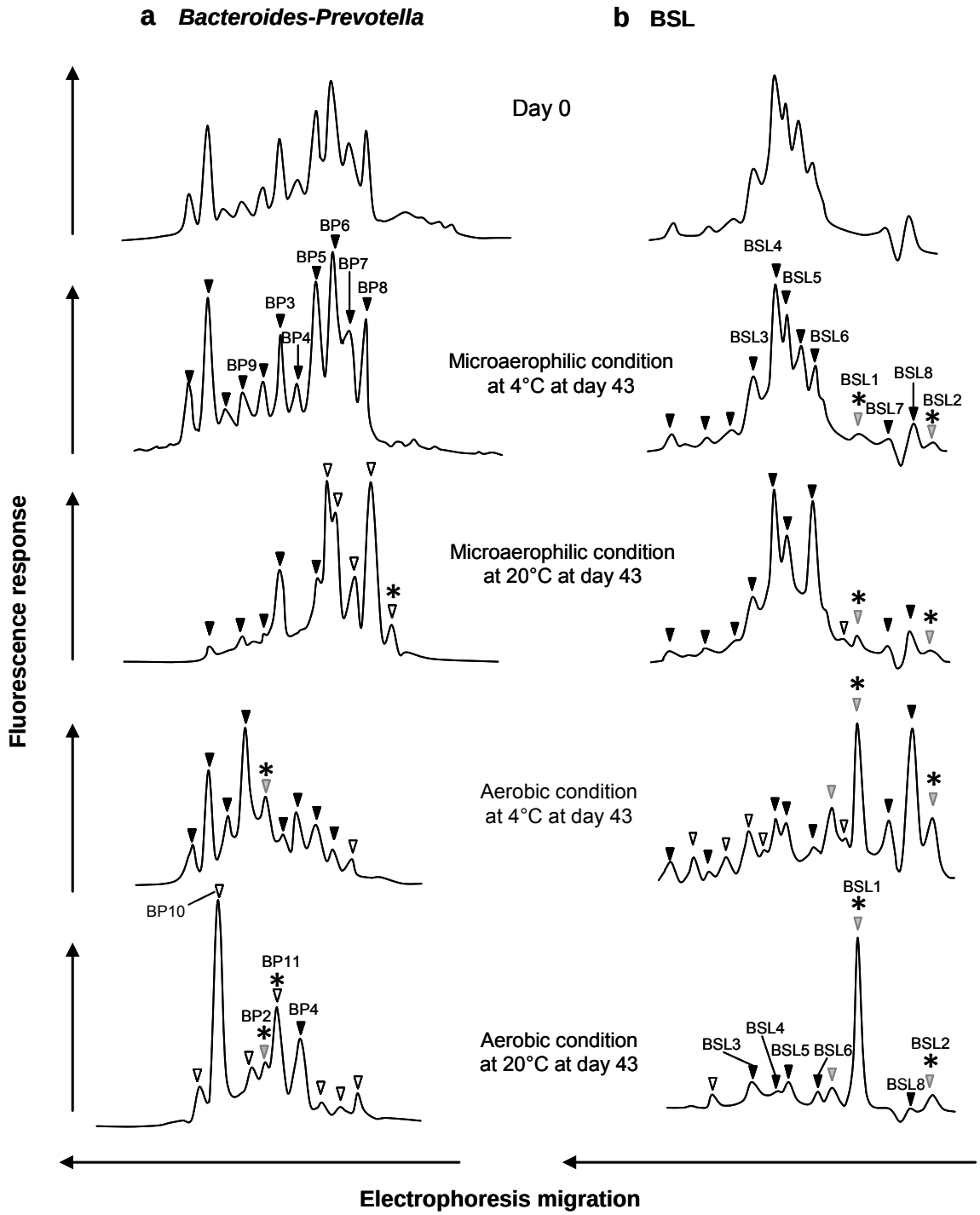


Figure 3 Persistence of All-*Bacteria* (♦), Total lactobacilli (▲) and *L. amylovorus* (○) markers, measured as changes in concentrations [ln(C/C0)] of 16S rDNA marker, in river water microcosms incubated either under microaerophilic conditions at 4°C (a) or at 20°C (b), or under aerobic conditions at 4°C (c) or at 20°C (d) after addition of pig manure. Error bars indicate the standard deviation of reaction (*n=4*). In some cases, the error bars were too small to be illustrated.

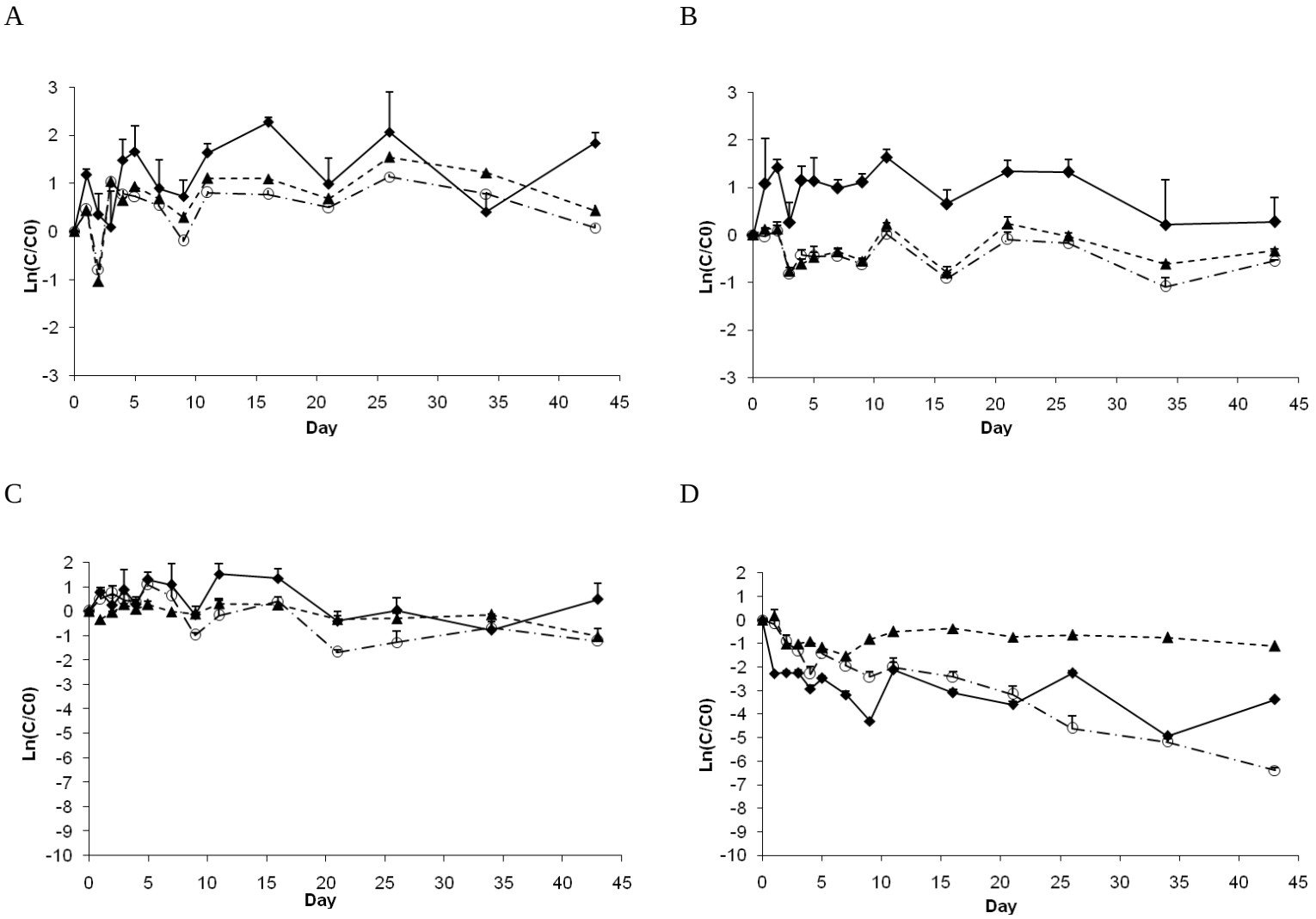


Figure 4 Persistence of All-*Bacteroidales* (●),Pig-1-Bac (□) and Pig-2-Bac (■)markers, measured as changes in concentrations [ln(C/C0)] of marker 16S rDNA, in river water microcosms incubated either under microaerophilic conditions at 4°C (a) or at 20°C (b), or under aerobic conditions at 4°C (c) or at 20°C (d) after addition of pig manure. Error bars indicate the standard deviation of reaction (*n=4*). In some cases, the error bars were too small to be illustrated. Under aerobic conditions at 20°C, the limit of quantification of Pig-2-Bac and Pig-1-Bac was reached after 16 and 21 days, respectively.

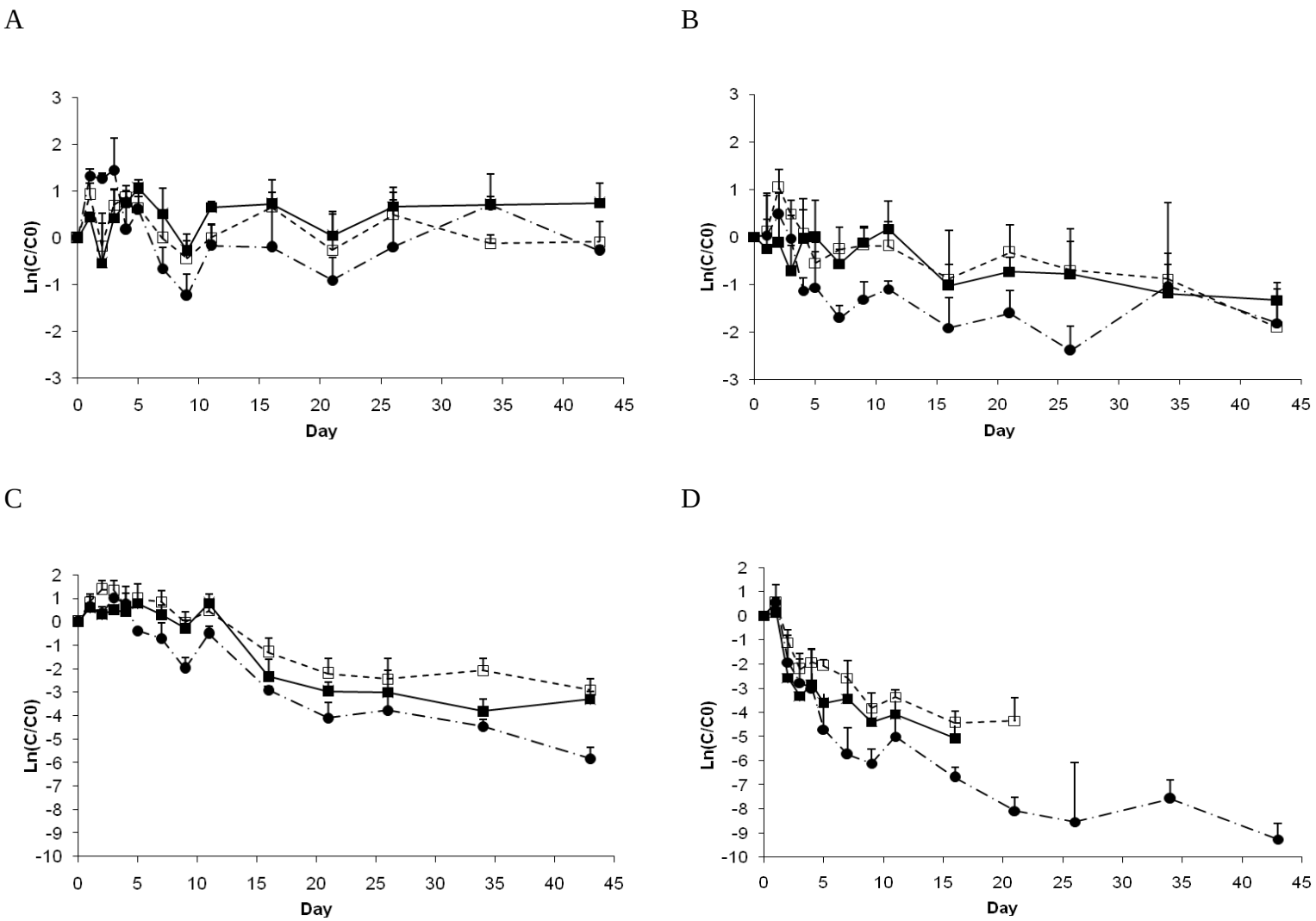
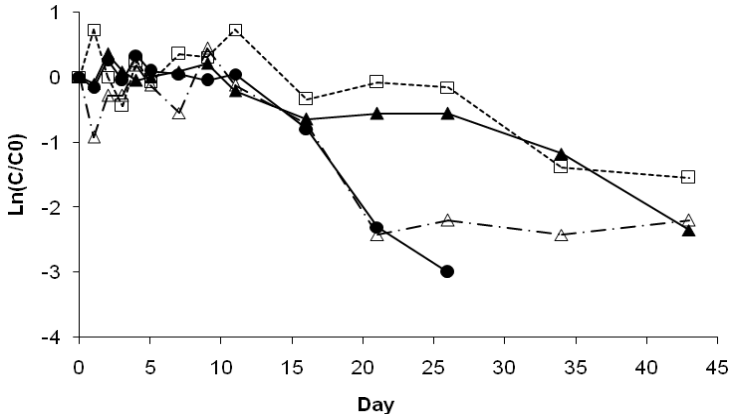


Figure 5 Survival of the faecal indicators *E. coli* (a) and enterococci (b), measured as changes in concentrations [ln(C/C0)] of faecal indicator CFU, in river water microcosms after addition of pig manure and incubation under microaerophilic conditions at 4°C (▲), or at 20°C (●), or under aerobic conditions at 4°C (Δ) or at 20°C (□).Under aerobic conditions at 20°C and microaerophilic conditions at 20°C, the limit of quantification of enterococci and *E. coli* was reached after 16 and 26 days, respectively.

A



B