Characterization of biofilm formation in natural water subjected to low-frequency electromagnetic fields

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ABSTRACT
Electromagnetic field (EMF) treatment has proven to be effective against mineral scaling in water systems. Therefore, it should be assessed for the treatment of other deposits such as biofilms. In this study, a commercial device producing low-frequency EMF (1–10 kHz) was applied to a reactor fed with natural water for 45 days. The treatment promoted the concentration of microorganisms in suspension and limited the amount of sessile microorganisms in the biofilm, as determined by the measurement of total DNA, qPCR and microscopy. The structure of the bacterial community was assessed by t-RFLP and pyrosequencing analysis. The results showed that EMF treatment affected both planktonic and sessile community composition. EMFs were responsible for a shift in classes of Proteobacteria during development of the biofilm. It may be speculated that the EMF treatment affected particle solubility and/or microorganism hydration. This study indicated that EMFs modulated biofilm formation in natural water.

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Introduction
Biofilms, corrosion and calcium carbonate scale deposits induce significant damage in industrial, domestic and agricultural equipment using natural water. Indeed, a reduction in hydrodynamic performance through pipe blocking and membrane clogging, alteration of surface structure, a decrease in heat transfer efficiency and higher energy consumption are frequently observed (Beech & Sunner 2004). With the potential multiplication of opportunistic pathogens and antibiotic-resistant microorganisms, the control of biofilms is a major concern in water supplies and is commonly achieved in many countries through disinfection methods using biocides (Morgenrooth & Wilderer 2000; Zhang et al. 2012). Currently, however, the use of these methods is limited due to the risk in storing and handling chemicals, changes to the chemical composition of water, water pollution and environmental degradations, disposal of chemical residues, the high cost of chemical products and long-term inefficiency due to the enrichment of disinfectant-resistant microorganisms (Mah & O’Toole 2001; Zhang et al. 2012). To replace chemical treatments, both biological and physical methods can be used to control microbial growth in water (Bott 2001; Huchler 2002). Recently, the use of electromagnetic fields (EMFs) has been described as a promising anti-scale and/or antifouling treatment (Xiaokai 2008; Lipus et al. 2011; Xuefei et al. 2013). It appears that this treatment affects calcium carbonate nucleation and crystallization, preventing the formation of scale at the solid–liquid interface (Lipus et al. 2011). Also EMF treatment has been assessed on bacterial cells, especially targeting extremely low frequencies (in the range 1–300 Hz) in relation to the electrical power supply (Mittenzwey et al. 1996; Del Re et al. 2004; Cellini et al. 2008; Giorgi et al. 2011; Huwiler et al. 2012). The results show that EMFs might affect the growth rate, viability, antibiotic susceptibility and adhesion of microorganisms. As biofilms are made of microorganisms and minerals, it can be hypothesized that EMFs will have an impact on biofilm formation. In the literature, this hypothesis has been tested in only three publications. In the first of these, the EMF treatment decreased cell adhesion in a monospecies biofilm of Helicobacter pylori.
(Di Campli et al. 2010). In the others, the EMF treatment induced partial removal of a mature biofilm adhered to the internal surface of heat exchanger-condenser tubes cooled by seawater and weakened the growing biofilms in initially clean tubes (Trueba et al. 2014, 2015). In addition, EMF treatments in the literature used differing frequencies and methods that could influence their efficiency.

In the present study, the effect of low-frequency EMF treatment on a microbial community in a circuit fed with river water was assessed. The growth of biofilm was followed by DNA quantification, qPCR and microscopic observations in EMF-treated water. Additionally, the structure of the planktonic and sessile microbial communities was analyzed by t-RFLP and pyrosequencing analysis.

Materials and methods

Experimental system

An experimental platform was designed with two circuits fed with natural water sampled in June 2013 from the Vienne river in Bonneuil-Matours (46°68’N, 00°55’E, located 30 km from Poitiers, France). The water was filtered through a 50 μm sieve to remove coarse suspended particles and part of the microplankton. After decanting overnight, the filtrate was homogenized and divided equally into two water tanks, pre-rinsed with sample water. The water characteristics were determined in an accredited laboratory (IANESCO, Poitiers, France) using standardized procedures (SM1, Supplemental material Table S1).

Both water recirculation circuits (Figure 1) consisted of a storage water tank (25 l capacity), a peristaltic pump (L/S 6-600 rpm, Masterflex, Bélignieux, France) equipped with a peristaltic head (Masterflex Easy Load HP LS PPS/stainless steel T15-36), an EMF device and a water aquarium (455 × 255 × 190 mm, 12 l capacity). AISI304 stainless steel (SS) slides (76 × 26 mm) were washed three times with acetone, rinsed with deionized water and with 70% ethanol before being dried and autoclaved twice for 20 min at 121°C. Sterile slides were inserted vertically in SS racks and immersed in the aquaria from both circuits, parallel to the water flow and with no hydraulic obstructions.

Both circuits were equipped with an Aqua-4D system (Planet Horizons Technologies, Sierre, Switzerland). This device consists of two independent solenoid coils wrapped into a PVC-C tube (436 mm long and 50 mm in internal diameter) connected to a pulsed voltage generator, thus producing and transmitting electromagnetic signals into the water passing through the tube. The EMFs covered weak fields with range of 1–5 mT, a voltage of tens of volts, an intensity of one ampere and a frequency of 1–10 kHz (confidential data protected by patent: EP2364954A1 from Thut et al. 2011). Two waves of harmonic frequencies were transmitted simultaneously in the tube. No EMF-water treatment occurred in the control circuit due to the absence of connection between the Aqua-4D® tube and the generator (Figure 1). Both EMF-treated and control circuits were maintained under dynamic conditions with constant flow velocity (25 l h⁻¹) and hydraulic continuity (flow loop) using C-flex L/S tubing (Masterflex). Moreover, both circuits were positioned to avoid any additional electromagnetic interference from the EMF generator or other electronic materials.

Experiments were conducted for 45 days in the dark at room temperature (20–25°C). Conditions within the aquaria were monitored weekly with a HI 9828 multiparameter meter (HANNA Instruments, Lingolsheim, France) measuring temperature, pH, dissolved oxygen, conductivity, and oxido-reduction potential.

Sampling and DNA extraction

Water was sampled in triplicate (50 ml) from the surfaces of both aquaria above the SS slides at days 2, 7, 15, 30 and 45 of the experiment. Water samples were also taken immediately before the start of the experiment in both tanks. A volume of 10 ml from each water sample was filtered onto a polycarbonate membrane (0.2-μm pore size, 25-mm diameter, HealthCare, Mérganac, France) held in a SS vacuum filtration unit (Nalgene, Villebon-sur-Yvette, France). The membrane was transferred to a bead-beater tube (MP Biomedicals, Illkirch, France) containing 0.50 ± 0.05 g of glass beads 106 μm diameter and four glass beads 2 mm in diameter and stored at –20°C before DNA extraction. Similarly, at each time point, three SS slides were taken from both circuits and washed successively by complete immersion steps with moderate movements in three sterile beakers containing sterile water to remove non-adherent cells. The total biofilm from each slide was thoroughly scraped off for 5 min using a sterile cell scraper perpendicular to the surface and collected in 2 ml of sterile water. Suspensions of the biofilm were filtered onto polycarbonate membranes as described above. DNA was recovered from the membranes by adding 1 ml of a solution containing 100 mM Tris–HCl (pH 8.0), 100 mM ethylenediaminetetraacetic acid (EDTA; pH 8.0), 50 mM NaCl, and 2% (w v⁻¹) sodium dodecyl sulfate (SDS). Samples were homogenized for 20 s at speed 5 using a FastPrep instrument (MP Biomedicals, Illkirch, France), incubated twice for 15 min at 70°C and centrifuged (12,000 rpm, 15 min, 20°C). The supernatants were incubated for 10 min on ice with 1/10 volume of 3 M potassium acetate (pH 5.5) and centrifuged (15,000 rpm, 10 min, 20°C). After precipitation with one volume of ice-cold isopropanol, the nucleic acids were washed with 70% ethanol. Aliquots of DNA extracts were quantified

[288] A. MERCIER ET AL.
using SYBR Green I dye (Invitrogen, Villebon-sur-Yvette, France) on a LightCycler 480 Instrument (Roche Applied Science, Meylan, France). A standard curve of HindIII-digested λ DNA fragments (Promega, Charbonnières-les-Bains, France) was used to estimate the final DNA concentration. All DNA samples were stored at –20°C. Quantitative polymerase chain reaction (qPCR)

Quantitative PCR was performed in a LightCycler 480 Instrument (Roche Applied Science), using the LightCycler FastStart DNA MasterPLUS Sybr Green I mix (Roche Applied Science), according to the manufacturer’s instructions. The 341F (5′-CCTACGGGAGGCAGCAG-3′) and 515R (5′-ATTACCGCGGCTGCTGGCA-3′) primer set (Baker et al. 2003) was used to amplify the bacterial 16S rRNA gene. Thermal cycling conditions were as follows: an initial activation cycle at 95°C for 10 min and 35 cycles at 95°C for 15 s, 60°C for 10 s and 72°C for 15 s. A standard curve was obtained with serial dilutions of a known amount of DNA containing a fragment of the 16S rRNA gene from Pseudomonas aeruginosa PA14 at concentrations ranging from 10² to 10⁶ gene copies μl⁻¹. The DNA samples were previously tested for inhibitory effects of co-extracted substances.

The FF390 (5′-CGATAACGAGACGATC-3′) - FR1 (5′-AKCCATCCATCGTATCT-3′) primer set was used to amplify the fungal 18S rRNA gene according to the amplification protocol described by Chemidlin Prévost-Bouré et al. (2011). A standard curve was generated with serial dilutions of a known amount of DNA containing a fragment of the 18S rRNA gene from Candida albicans ATCC 3153 at concentrations ranging from 10² to 10⁶ gene copies μl⁻¹.

Terminal restriction fragment length polymorphism (t-RFLP)

The diversity of the bacterial communities both in water and adhered to slides was analyzed by capillary electrophoresis–polymerase chain reaction–terminal restriction fragment length polymorphism (CE-PCR-t-RFLP) of the
16S rRNA gene (Liu et al. 1997). The bacterial primers 8F (5’-AGAGAGTTTTGATCMGGCTAG-3’), labeled at the 5’- end with 6-carboxyfluorescein (6-FAM), and 1492R (5’-TACGGHTACCTTGGTATCAG-3’) were used to amplify an approximately 1,500 bp fragment of the 16S rRNA gene (Baker et al. 2003). PCR amplification was performed in a vapo.protect Mastercycler (Eppendorf) in a 50 μl reaction mixture containing 1× PCR buffer, 200 μM of each dNTP, 0.2 μM of each primer, 1 U of DNA polymerase (GoTaq DNA polymerase, Promega) and 5 μl of a 0.05 ng μl⁻¹ DNA-diluted template. Reaction mixtures were held at 95°C for 3 min, followed by 30 cycles: 94°C for 30 s, 56°C for 30 s and 72°C for 45 s and a final extension of 72°C for 10 min. PCR products were purified using the PCR clean-up Kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer’s instructions. One hundred nanograms of each fluorescently labeled PCR product were digested with 2.5 units of HaeIII (Promega) at 37°C for 3 h in a 15 μl reaction. Fluorescently labeled terminal-restriction fragments were resolved by capillary electrophoresis in an ABI 3130 genetic analyzer using an internal size standard (GeneScan 500 ROX, Applied Biosystems, Saint-Aubin, France).

Quantification of total polysaccharides in the biofilm

The polysaccharide part of the biofilm matrix was assayed using the phenol-sulfuric acid method described by Dubois et al. (1956), using D-glucose as a standard. Briefly, three slides from both the control and EMF-treated circuits were scraped as described in the previous section at the different sampling dates. The total biofilm from each slide was collected in 1 ml of sterile water, mixed with 1 ml of 5% phenol in water (w/w) (Amresco) and immediately vortexed after the addition of 5 ml of concentrated sulfuric acid (Fisher). After incubation for 30 min at 37°C in the dark, hexose monosaccharide concentrations were obtained by measuring the absorbance of the yellow-orange color at 490 nm using a spectrophotometer (Biomate3 ThermoSpectronic, Illkirch, France).

Fluorescence microscopy

To observe the microbial colonization, two SS slides from both circuits were immediately stained with 6.6 μM SYTO 9 fluorescent stain (Invitrogen) in Citifluor AF1 (Biovalley, Nanterre, France) at each sampling time and examined using an Axio Observer A1 fluorescence microscope coupled with an Apotome (Carl Zeiss, Marly-le-Roi, France). The microscope was equipped with a mercury lamp, a filter specific for SYTO 9 (Zeiss Filter Set 44) and a Plan Neofluar 63×/1.25 oil immersion objective (Zeiss) using Immersol 518F oil (Zeiss). Images were taken and processed using the software Axiosvision or its upgrade Zen (Zeiss). At least three mosaic image acquisitions (MosaicX, Carl Zeiss, Marly-le-Roi, France) covering an average area of 1 mm² were performed for each slide, using a z-stack (1 μm interval). The maximum biofilm thickness at day 45 was measured from z-stacks across five 100 μm × 200 μm windows in each of the four images acquired (20 measures per treatment).

Statistics and data analysis

DNA quantification, microbial qPCR, total polysaccharide content data and maximum biofilm thickness were analyzed using non-parametric Mann–Whitney and Kruskal–Wallis tests (α = 0.05). T-RFLP electropherograms were analyzed using the StatFingerprints Version 2 software.
(Michelland et al. 2009). The data obtained were converted into a data frame (bacterial communities as rows and normalized peaks as columns) and subjected to principal component analysis (PCA) providing an ordination of the bacterial communities in a factorial map based on the scores of the first two principal components. Statistical ellipses representing 90% confidence intervals were drawn over the replicates. PCA was performed using ADE-4, an R software program (R Development Core Team 2004; Thioulouse & Dray 2007).

Pyrosequencing data were analyzed using the GNS-PIPE (version 1.1.11) pipeline described by Terrat et al. (2012, 2015). The parameters used for each bioinformatic step can be found in SM2 (Table S1). Briefly, the raw reads were sorted according to MIDs. All reads with mismatches in the primer sequence, ambiguities in sequence, sequence length < 300 bp or without the exact primer sequence at the start of the sequence were discarded. Rigorous dereplication (ie clustering of strictly identical sequences) was performed using a PERL program. Normalization was conducted to obtain the same number of reads for each sample and to avoid biased bacterial community comparisons. The retained dereplicated reads were aligned using INFERNAL alignment (Cole et al. 2014) and clustered into operational taxonomic units (OTU) with a 5% dissimilarity cut-off at the genus level. These parameters were recommended by the Gns-pipe pipeline to obtain a reliable representation of bacterial communities through the taxonomic classification of short 16S rRNA gene sequences, considering the error rate from high-throughput sequencing and also the variability of the 16S rRNA gene in bacterial genomes (Větrovský & Baldrian 2013). Retained high-quality reads were taxonomically assigned according to the Silva r114 reference database (Quast et al. 2013). During the analysis, all singletons corresponding to reads detected only once and not clustered (which might be artifacts such as PCR chimeras or large sequencing errors produced by the PCR and the pyrosequencing) were checked based on the quality of their taxonomic assignments (Terrat et al. 2015). Heatmap and principal coordinate analysis (PCoA) were also performed using the R software. The dataset is available on the EBI database system in the Sequence-Read Archive (SRA) under study accession number PRJEB9525 (http://www.ebi.ac.uk/ena/data/view/ PRJEB9525).

Results

Effects of EMFs on the physico-chemical parameters of the water

The water characteristics determined in an accredited laboratory showed that the river water used in this study was moderately hard, slightly alkaline and conductive (SM1, Table S1). The water temperature was 20–22°C during the first 25 days and then settled at ~25°C until the end of the experiment (Figure 2A). The temperature of the water in the tank subjected to EMFs showed a slight but statistically non-significant 0.43 ± 0.15°C increase compared to the untreated water tank. The oxygen concentration in the water from the control circuit was almost constant at 5.94 ± 0.36 mg O₂ l⁻¹ throughout the experiment (Figure 2B). A slight increase in the oxygen concentration was observed in the water subjected to EMFs, with a mean of 6.37 ± 0.42 mg O₂ l⁻¹ over the experiment (Figure 2B). In addition, pH (Figure 2C), electrical conductivity (measured electrometrically with temperature compensation) and the oxido-reduction potential (data not shown) did not change as a result of EMF-water treatment, although some fluctuations were observed during the experiment. Changes in particle/floc size and settleability were observed between water from the surface of both the control and EMF-treated aquaria (Figure 2D).

Effects of EMFs on microbial biomass in water

In the control circuit, the microbial biomass was almost constant with a mean of 8.7 ± 1.5 ng DNA ml⁻¹ of water over the 45 days of the experiment (Figure 3A). In contrast, the suspended microbial biomass was significantly higher in the water treated with EMFs (Figure 3A). It was 2.4-fold higher than in the control circuit during the first week, reaching 20.6 ± 2.4 ng DNA ml⁻¹ of water on day 2. The ratio between the microbial biomass in the water from both circuits remained constant from day 15 to the end of the experiment. Based on the qPCR results, the bacterial biomass in the water from the surface of the aquarium decreased over the 45 days of experiment in both circuits, in contrast to the fungal biomass (Table 1). Otherwise, the bacterial and fungal communities were significantly more abundant in suspension in the EMF-treated water, especially during the first week.

Effects of EMFs on the microbial biomass in the biofilm

The colonization of the SS slides was rapid, as indicated by the DNA quantified after contact between the slides and the river water in the control and treated systems for only two days (Figure 3B). At that time, the adhering microbial biomass was similar in the two circuits (2.5 ± 0.3 ng DNA cm⁻² in the control and 1.4 ± 0.1 ng DNA cm⁻² in the EMF-treated circuits). With fluorescence microscopy, single adhering microorganisms and isolated clusters were observed, dispersed on the slides (data not shown). In the control circuit, adhered microbial biomass increased throughout the experiment, up to 12.3 ± 6.5 ng DNA cm⁻².
of biofilm at day 45 (Figure 3B). In contrast, in the water subjected to EMFs, the microbial biomass in the biofilm remained constant, with a mean of $2.2 \pm 0.9$ ng DNA cm$^{-2}$ of biofilm throughout the experiment (non-parametric test, $p = 0.05$). In both circuits, biofilms were heterogeneous, insofar as their thickness and coverage varied across each supporting slide (Figure 4). However, the control biofilms appeared thicker than the EMF-treated biofilms throughout the duration of the experiment. Hence, after 45 days, in the EMF-treated circuit, a significant 39.9% reduction in the maximum biofilm thickness was revealed compared with the biofilm from the control circuit ($11.6 \pm 2.1$ µm vs $19.3 \pm 5.6$ µm, $p < 0.05$, Figure 4). Congruently, from day 7 to day 45, the adhering microbial biomass in the EMF setup was approximately 2.4- to 3.7-fold lower than in the control circuit (Figure 3B). Additionally, the bacterial biomass monitored by qPCR was lower in the EMF-treated circuit than in the control throughout the 45-day experiment (Table 2). The same pattern was observed with the fungal biomass, but it was only significantly lower at day 30 (Table 2).

**Effects of EMFs on bacterial community structure in the water and biofilm using t-RFLP**

The genetic structures of the bacterial community in the water and biofilm from both circuits were first investigated by t-RFLP. In the PCA analyses, the ellipses (representing 90% confidence intervals) from the water tanks sampled in the control and EMF-treated circuits immediately before the beginning of the experiment overlapped (Figure 5A). These independent replicates show that the river water was well homogenized when divided into the water tanks. However, from day 2, the ellipses from the two circuits segregated and were also distinct from the original bacterial community in the water tanks. The latter may result from the start of the circulation pump and the hydraulic continuity (flow loop) conditions, hiding a possible water treatment effect at day 2. The PCA analysis showed that the genetic structure of the bacterial communities in suspension in water from both circuits was discriminated on the first axis (Figure 5A). Additionally, in the control circuit, the genetic structures of the bacterial communities at days 2, 7 and 15 were separated on the second axis.
structures of the bacterial communities sampled in both circuits (Figure 5B). Moreover, in the control circuit, the genetic structure of the bacterial community at day 2 differed from the structure at day 7. Both were separated on the y-axis, which explained 9.5% of the total variability from days 15 and 30 to day 45. This pattern was not observed in the EMF-treated circuit.

**Pyrosequencing analyses of the bacterial diversity in the water and biofilm**

As shifts were observed by t-RFLP, the water and biofilm samples were analyzed by 454 pyrosequencing to better characterize the bacterial communities (Glenn 2011). Considering that the narrow confidence ellipses on the PCA analysis from t-RFLP profiles indicated reproducible DNA extractions (Figure 5), the three replicates were pooled for each sample and at each time for 454 pyrosequencing.

A total of 93,386 reads that passed 454 quality filtering were obtained from the water and biofilm samples. After bioinformatic filtering, 91,771 reads with an average length of 384 bp were retained for community analyses.

The water and biofilm bacterial communities had a similar bacterial composition at the phylum level in the two circuits (Figure 6A), with a preponderance of Proteobacteria across all samples. Members of the Actinobacteria, Bacteroidetes, Firmicutes and other phyla with a lower relative abundance were also found. At the class level, the water and biofilm bacterial communities were dominated by Alpha-, Beta- and Gammaproteobacteria (Figure 6A). A focus on biofilm formation in the control circuit showed a decrease in Actinobacteria (from 23.5% to 0.4%) and Betaproteobacteria, (from 28.8% to 5.7%) and an increase in Gammaproteobacteria (from 18.2% to 64.5%), whereas the relative abundance of classes of Alphaproteobacteria remained almost constant (from 10.8% to 17.9%) during the course of the experiment.

Disregarding the plot at day 30, shifts were also observed from day 30 and day 45. This pattern was not observed in the EMF-treated circuit, with the genetic structures of the bacterial community influenced instead by the x-axis (PC1). Differences were observed between the bacterial communities in suspension in water from both circuits at days 7 and 15, in particular.

Additionally, regarding biofilms, the first axis of the PCA analysis of t-RFLP profiles, accounting for 29.1% of the total variability, discriminated between the genetic

![Figure 3. Amounts of DNA measured after direct extraction from water (A) sampled from the surface and from biofilms (B) adhered to the SS slides in both the control and EMF-treated aquaria. Each value represents the mean of three replicates ± SD, except for the initial analysis. * Biomass significantly different from the EMF-treated circuit compared with the control at each time considered (non-parametric Mann–Whitney test, p < 0.05). # Sampled in the water tanks from the two circuits immediately before the start of the experiment. nd: Not detected.](image)

Table 1. Bacterial and fungal biomass in suspension in water, estimated using 16S rRNA and 18S rRNA gene quantification respectively, from aquaria in the control and EMF-treated circuits.

| Days | Bacterial biomass | Fungal biomass |
|------|-------------------|----------------|
|      | Control | EMF-treated | Control | EMF-treated |
| 0    | 2.87 ± 1.75× |  | 0.77 ± 0.58× |  |
| 2    | 5.21 ± 1.10 | 18.15 ± 4.28* | 0.49 ± 0.16 | 1.43 ± 0.14* |
| 7    | 0.49 ± 0.39 | 8.04 ± 0.32* | 0.19 ± 0.24 | 4.53 ± 1.46* |
| 15   | 1.39 ± 0.32 | 4.16 ± 1.30* | 3.01 ± 0.85 | 3.07 ± 0.24 |
| 30   | 1.34 ± 0.16 | 4.22 ± 0.28* | 4.26 ± 0.90 | 3.71 ± 0.05 |
| 45   | 2.87 ± 0.75 | 4.92 ± 0.98 | 4.75 ± 1.31 | 8.04 ± 2.99 |

Each value represents the mean of three replicates ± SD.

*Sampled in the water tanks from the two circuits just before the start of the experiment (means ± SDs from the two circuits).

*Biomass significantly different in water from the EMF-treated circuit compared with the control circuit at each time considered (non-parametric Mann–Whitney test, p < 0.05).
observed. Except for the biofilm plot at day 30, the bacterial communities were closely related on each sampling date (Figure 6B).

Effects of EMFs on total polysaccharides in biofilm

The total polysaccharide content was higher in biofilm sampled in the EMF-treated circuit than in the control during the first week of treatment, with values at day 7 reaching 218.2 ± 53.1 and 135.8 ± 14.9 ng of D-glucose equivalent cm⁻² of biofilm, respectively (Figure 7). A shift was observed between day 7 and day 15 in both circuits, with a decrease in the total polysaccharide content. Then, the content increased in biofilms from the control circuit throughout the duration of experiment. In contrast, after water treatment with EMFs, the total polysaccharide content in the biofilms showed a distinct evolutionary pattern,

Table 2. Bacterial and fungal biomass adhered to SS slides in the form of a biofilm, estimated using 16S rRNA and 18S rRNA gene quantification by qPCR, respectively, from the control and the EMF-treated circuits.

| Days | Bacterial biomass | Fungal biomass |
|------|-------------------|----------------|
|      | × 10⁵ 16S rRNA gene copies cm⁻² of slide | × 10⁴ 18S rRNA gene copies cm⁻² of slide |
|      | Control | EMF-treated | Control | EMF-treated |
| 2    | 1.05 ± 0.46 | 0.22 ± 1.29 | 0.20 ± 0.08 | 0.22 ± 0.67 |
| 7    | 1.97 ± 0.67 | 0.29 ± 1.82 | 0.20 ± 0.04 | 0.24 ± 0.75 |
| 15   | 0.99 ± 0.72 | 0.42 ± 0.90 | 1.56 ± 0.77 | 0.93 ± 0.25 |
| 30   | 2.32 ± 0.54 | 0.23 ± 0.28 | 6.19 ± 0.70 | 0.55 ± 0.17 |
| 45   | 3.23 ± 1.21 | 1.07 ± 0.91 | 7.92 ± 3.23 | 4.72 ± 2.20 |

Each value represents the mean of three slides ± SD.

*Slide biomass significantly different between the EMF-treated circuit and the control at each time considered (non-parametric Mann–Whitney test, p < 0.05).
Figure 5. Principal component (PC1 × PC2) factorial map generated from t-RFLP fingerprints of the 16S rDNA for the control (Cont.) and EMF-treated (EMF) circuits: (A) water and (B) biofilm adhered to SS slides. Statistical ellipses drawn over the plot of three replicates represent 90% confidence. The percentages of the explained variance for the first two principal components are indicated in each ordination.
Figure 6. Analysis of the 16S rRNA gene pyrosequences from bacterial communities in water and in biofilm from the control and EMF-treated circuits over the 45 days of the experiment: (A) heatmap showing the relative abundance (> 0.1%) of each OTU at the phyla and class levels and (B) principal coordinate analysis (PCoA) generated from the OTU tables at the genus level. The first two principal axes are shown.
Globally, under the EMF treatment, the biomass increased in the planktonic microbial community and was limited in the biofilm in the EMF-treated water over the experiment. While slide surface colonization was not delayed, a lower biofilm biomass and a 39.9% reduction in maximum biofilm thickness were observed in EMF-treated water after the 45-day experiment. This result suggests that EMF treatment (1) might prevent or impair microbial adhesion to surfaces, and/or (2) might decrease particle aggregation and thereby promote the quantity of microorganisms found in suspension.

The planktonic bacterial community structure was also modified by the application of EMFs, as revealed by t-RFLP and pyrosequencing analyses. This result suggests that different environmental and growth conditions may be generated during the EMF treatment. A change in the physical interaction forces could be hypothesized. Weakening the physical forces involved in bacterial cluster formation and the attachment of bacteria to organic or inorganic particles would tend to increase the occurrence of suspended bacteria in water. Moreover, a close relationship between the bacterial communities developed on the slides and the bacterial communities in suspension in the water at each sampling time was revealed by the PCoA analysis. Thus, the susceptibility of polarized molecules and ions adsorbed on particle surfaces to low-frequency electromagnetic waves might result in a disturbance of the particle but could also modify microbial membrane organization and structure, with changes in membrane hydration, surface physico-chemical properties/permeability and cell activity. The results from the present study appear to be in agreement with previous studies showing an increase in bacterial susceptibility when treated with a combination of EMF and antibiotics or biocides, which might be explained by closer interactions between bacteria and biocide molecules (McLeod et al. 1999; Matl et al. 2011).

Additionally, changes were observed in the composition of the sessile bacterial community in the biofilm throughout the 45 days of the experiment. Pyrosequencing analyses showed a clear shift in the Alpha- and Gammaproteobacteria. The class Gammaproteobacteria, which includes most of the pathogens and opportunistic pathogens carried by water, was notably favored during the initial stages of biofilm formation in water subjected to EMFs, but the occurrence of this class declined with the duration of the water treatment. The changes observed in the microbial biomass and bacterial composition suggest that the application of low-frequency EMFs could affect the stratification and cohesion of the biofilm (Rochex et al. 2009). Nevertheless, the variations observed in the polysaccharide content that represents a part of the biofilm matrix do not allow the estimation of biofilm cohesion.

Discussion

The prevention and control of mineral scaling and biofilm development are required for the long-term performance of industrial, domestic and agricultural equipment using natural water. The detachment of clusters from mature biofilms by abrasion, erosion and/or sloughing allows the continuous propagation of a variety of microorganisms including pathogens, antibiotic-resistant and anaerobic sulfate-reducing bacteria and their efficient re-colonization elsewhere in the equipment (Morgenroth & Wilderer 2000). In this study, a commercial device producing and transmitting two waves of harmonic low-frequency EMF signals was applied to natural water for 45 days to investigate the influence of the treatment on the microbial community of the water and its ability to form biofilms.

Physico-chemical parameters such as temperature and oxygen concentration underwent statistically non-significant variations in the water subjected to EMF treatment over the 45 days. The power of the apparatus (5 W) seems to have been too low to increase the temperature in the water tank. However, the EMF treatment had a clear effect on particle aggregation in the water (Figure 2D). Changes in suspended particle/floc size and settleability suggest that the effects of the treatment could be linked to the properties of the water and/or the surface chemistry of the particles. In particular, physical interaction forces, such as the Zeta potential (Hunter 1981) could be influenced by EMFs.

Figure 7. Total polysaccharides obtained in biofilms adhered to SS slides from the control and the EMF-treated circuits, based on the phenol-sulfuric method. Each value represents the mean of three slides ± SD. *Amount significantly different from the EMF-treated circuit compared with the control circuit at each time considered (non-parametric Mann–Whitney test, p < 0.05). nd: Not detected.

with no significant variation from 30 to 45 days. At day 45, it was half the value in the control, with 87.2 ± 33.0 vs 176.3 ± 16.1 ng of D-glucose equivalent cm⁻² of biofilm.
(Ras et al. 2013). However, changes in cohesiveness should not be excluded as a result of the EMF water treatment, as previously reported under chlorination and hydrodynamic shearing (Mathieu et al. 2014).

Altogether, the results of the present study are in agreement with recent works showing that treatment using low-frequency EMFs, primary targeting water dipole molecules and ions in the bulk solution, affects the microbial community of biofilms (Trueba et al. 2014, 2015). Merlin et al. (2015) reported limited biofilm formation on a glass slide surface in EMF-treated waters and suggested that quantum field theory might provide an explanation for the observed effects. Due to their structure and properties, the water molecules can oscillate between two electronic configurations in phase with an electromagnetic field. This could enable energy transfer to take place in living organisms. This hypothesis also seems consistent with results from the present study.

Conclusions

In conclusion, EMF treatment of natural river water promoted the concentration of planktonic microorganisms and as a consequence limited the amount of sessile microorganisms in the biofilm. The EMF treatment also had a qualitative effect, as it affected the structure of both the planktonic and sessile populations. The main modification was a shift in the classes of Proteobacteria during the biofilm development. Altogether, this study indicates that low-frequency EMFs can modulate biofilm formation. The results reinforce the current interest in studying the application of low-frequency electromagnetic fields to water, and the next step would be to investigate the mechanisms of action.

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Disclosure statement

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