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ORIGINAL ARTICLE

Culture dependent and independent analyses of bacterial communities involved in copper plumbing corrosion

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Abstract

Aims: This study used culture-dependent and culture-independent approaches to characterize bacterial communities in copper plumbing corrosion and to assess biofilm formation and copper resistance of heterotrophic bacteria isolated from copper pipes.

Methods and Results: Water and copper pipes were collected from a cold-water household distribution system affected by 'blue water' corrosion and presenting biofilm formation. Corrosion-promoting ageing experiments were performed with conditioned unused copper pipes filled with unfiltered and filtered sampled water as nonsterile and sterile treatments, respectively. During 8 weeks, stagnant water within the pipes was replaced with aerated fresh water every 2 or 3 days. Total copper and pH were determined in sampled water, and copper pipe coupons were cut for microscopic analyses. Biofilms were extracted from field and laboratory pipes, and total DNA was isolated. Bacterial communities' composition was analysed by terminal restriction fragment length polymorphism (T-RFLP) and clonal libraries of 16S rRNA genes. Heterotrophic bacterial isolates were obtained from water and biofilm extracts and characterized in terms of biofilm formation capacity and copper minimum inhibitory concentration. The results indicated that copper concentration in stagnant water from nonsterile treatments was much higher than in sterile treatments and corrosion by-products structure in coupon surfaces was different. Multivariate analysis of T-RFLP profiles and clone sequencing showed significant dissimilarity between field and laboratory biofilm communities, and a low richness and the dominant presence of *Gamma-* and *Betaproteobacteria* in both cases. Several bacterial isolates formed biofilm and tolerated high copper concentrations.

Conclusions: The study demonstrates microbially influenced corrosion (MIC) in copper plumbing. *Gamma-* and *Betaproteobacteria* dominated the corroded copper piping bacterial community, whose ability to form biofilms may be important for bacterial corrosion promotion and survival in MIC events.

Significance and Impact of the Study: The characterization of micro-organisms that influence copper plumbing corrosion has significant implications for distribution system management and copper corrosion control.

Introduction

Copper is a preferred plumbing material in domestic water distribution systems because of its relative resistance to corrosion and beneficial properties preventing biofouling (Boulay and Edwards 2001; Keevil 2004). However, copper piping involves the release of copper to water through corrosion processes, deteriorating the metallic

pipe surface and increasing bulk water copper concentration to health-threatening levels. Plumbing infrastructure failures and human health effects have been reported as important consequences of copper corrosion (Dietrich *et al.* 2004). Copper corrosion initiation and development in piping systems is a complex phenomenon determined by physical, chemical, and biological water quality parameters, and hydrodynamic conditions imposed by operation conditions. Several studies have given insights into the influence of water quality parameters (Edwards *et al.* 1994, 1996; Boulay and Edwards 2001; Edwards and Sprague 2001; Pehkonen *et al.* 2002), water stagnation time and flushing (Merkel *et al.* 2002; Calle *et al.* 2007), and pipe age (Edwards *et al.* 2001; Vargas *et al.* 2009) in corrosion by-products release. However, less usual corrosion types including cold-water pitting corrosion and 'blue water' corrosion, reported worldwide, have been related with microbial activity and biofilm accumulation (Bremer *et al.* 2001; Critchley *et al.* 2004; Keevil 2004; Calle *et al.* 2007). Microbially influenced corrosion (MIC) in plumbing systems depends on the establishment of micro-organisms on the inner surface of pipes. Biofilms are considered a prominent mode of microbial colonization and survival in metal-enriched environments (Harrison *et al.* 2007), and biofilms in corroding copper pipes may be composed of different groups of micro-organisms, as a spatial and chemical heterogeneity has been described in their surfaces (Keevil 2004). Microbial studies in copper plumbing corrosion biofilms has been addressed utilizing culture-dependent methods (Dutkiewicz and Fallowfield 1998; Bremer *et al.* 2001; Critchley *et al.* 2004). It is well known that culturability of environmental micro-organisms is very low (Amann *et al.* 1995); hence, it is important to use molecular approaches to gain more information on microbial communities. In addition, bacterial effects and biofilm influence on copper corrosion have been inferred indirectly, or by pure bacterial culture (Davidson *et al.* 1995; Bremer *et al.* 2001; Critchley *et al.* 2003). Few studies have demonstrated direct microbial involvement in corrosion of household copper piping systems (Critchley *et al.* 2004), and to our knowledge a culture-independent characterization of biofilm microbial communities has not been addressed yet. In pure culture biofilms of bacteria isolated from corroded copper pipes, corrosive and protective effects have been described which may depend on water quality (Critchley *et al.* 2003). Thus, it is important to determine the key microbial groups and activities in corrosive biofilms. Our previous work in a cold-water domestic distribution system evidenced copper corrosion by-products together with a biofilm, forming a reactive barrier that accumulates high amounts of copper and controls its release into water (Calle *et al.* 2007).

The identification of the mechanisms that control the reactivity of the barrier and the characterization of microbial communities involved in MIC constitutes a next step in understanding copper pipe corrosion. The objectives of this study were to investigate the effect of bacterial communities associated with MIC in copper plumbing, to characterize the molecular diversity of these communities, and to examine biofilm formation and copper resistance of heterotrophic bacteria isolated from water and biofilms obtained from field and laboratory corroded copper pipes.

Materials and methods

Water and pipe sampling

Water and pipe samples were taken from a household copper plumbing system supplied by well water disinfected by a UV-radiation system, in an area affected by 'blue water' corrosion, located in a rural suburb of the city of Talca (257 km south from Santiago, Chile). For water sampling, an outdoor pipe was completely flushed and then 20 l of running water were collected in sterile glass bottles. From this sample, 10 l were twice filtered *in situ* with a sterile vacuum unit, using 0·22 μm pore size cellulose ester membranes (Millipore, Bedford, MA, USA). Pipe sampling was carried out with a sterilized handsaw to remove 1 m pieces. The sampled pipes were collected after 1 year in use. The pipe samples were taken and filled with sampled water and sealed with parafilm. All samples were stored at 4°C and rapidly taken to the laboratory for analytical and experimental procedures.

Water analysis

Running water was analysed *in situ* for pH with a pH meter (Thermo Orion 420A, West Chester, PA, USA). Total copper concentration was measured using ICP spectrometry (iCAP 6300; Thermo Fisher Scientific, Waltham, MA, USA). Total copper measurements were performed after digestion with *aqua regia* (Method 303OF) (APHA, AWWA and WEF 2005).

Corrosion tests

The experimental set-up for the corrosion tests is presented in Fig. 1. Unused ¾-inch internal diameter copper pipes were cut into 25 -cm length pieces, washed three times with 0·1 mol l^{-1} NaOH, and rinsed with sterile MilliQ water (Millipore). Copper pipes were filled with filter-sterilized sampled water to condition the inner surface for 24 h at room temperature (about 22°C). After conditioning, two triplicate sets of pipes were exposed to

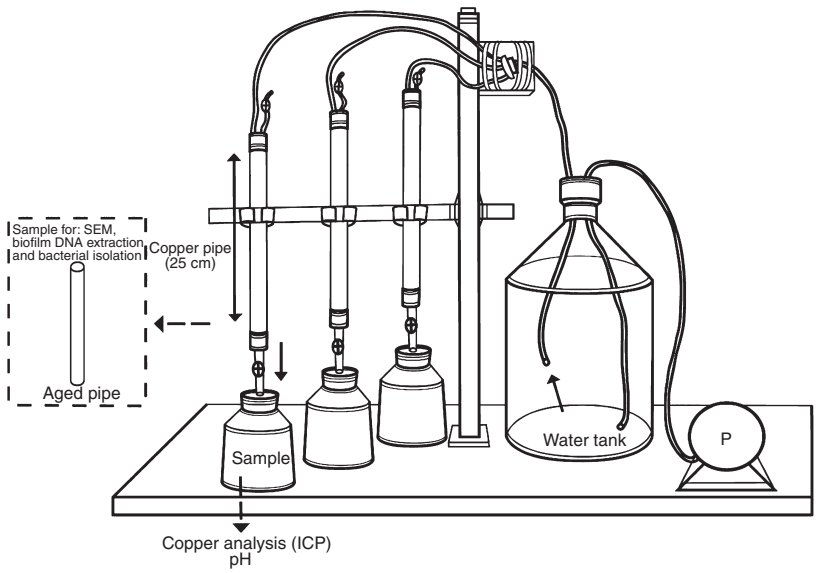

Figure 1 Experimental set-up for laboratory copper pipe corrosion tests.

different ageing treatments with sampled MIC water. One set (S1) was filled with filter-sterilized water representing an abiotic condition, and the other (S2) was filled with unfiltered water to make a biotic test. Each pipe was sealed with autoclaved rubber stoppers. During a test period of 8 weeks, stagnant water within the pipes was replaced every 2 or 3 days after filling the feeding tank with the corresponding treatment water, which was kept in sterile bottles at 4°C. Pipes were refilled after valve draining, by pressurizing water from reservoirs with 0·22 μm-filtered air using a vacuum pump. Air flux was regulated to avoid turbulent pipe filling, and excessive pressure in the pipes was relieved through a cap valve. To maintain dissolved oxygen concentrations, tank water was aerated for 2 min before pipe filling. Stagnant water samples were collected weekly for pH and total copper measurements, discarding the tube outlet volume before collecting the entire stagnant content in sterile polypropylene bottles. For imaging purposes, additional pipes exposed for 3 weeks to the same treatments were used.

Electron microscopy

Several coupons of 1 × 1 cm from field and laboratory copper pipes were aseptically cut for microscopic analyses. Samples were kept hydrated with the corresponding test water before preparation for electron microscopy. Coupons were fixed with 3% (w/v) glutaraldehyde buffered with 0·27 mol l^{-1} sodium cacodylate (pH 7·2) for 2 h. Samples were rinsed with sterile distilled water, postfixed with 1% (w/v) osmium tetroxide for 1 h and dehydrated in serial ethanol (50–100%) and acetone (100%) baths. After dehydration, coupons were dried to critical point and coated with gold. Scanning electron microscopy (SEM) was used to study the morphology and structures formed on the inner surface of copper pipes using a LEO 1420VP microscope (Cambridge, UK).

Biofilm and DNA extraction

Biofilm extraction was performed on field pipe samples and laboratory corrosion test pipes. Field pipes were aseptically cut into 25-cm length pieces. Biofilms were extracted from laboratory corrosion test pipes at the end of the experiments. The inner surface of the pipes was carefully washed and filled with autoclaved saline isotonic solution 0·85% (w/v) NaCl trying to avoid any biofilm loss. Biofilms were released by sonication on ice in a 40 -kHz ultrasonic bath (Branson 2510, Danbury, CT, USA) for 10 min. After the first 5 min, sonication was paused and pipes were manually agitated for 10 s. Biofilm extracts were pelleted in 50 ml sterile polypropylene tubes by centrifugation at 12 452 ***g*** for 10 min. Supernatants were withdrawn leaving 2 ml to resuspend the pellet. The pellet suspension was used for DNA extraction and heterotrophic bacteria isolation. Metagenomic DNA isolation was performed using a phenol–chloroform extraction protocol with some modifications in the first steps (Morán *et al.* 2008). An initial 0·85% (w/v) NaCl pellet suspension of 500 μl was used and 100 μl of 2% (w/v) trimethylammonium bromide solution (0·75 mol l^{-1} NaCl, 50 mmol l^{-1} Tris, 10 mmol l^{-1} ethylenediaminetetraacetate, pH 8) were added. The mixture was incubated at 60°C for 90 min. Extraction was carried out, and the quality and quantity of DNA was verified by 1% (w/v) agarose gel electrophoresis prepared in 1 × SYBR

green DNA binding dye (Invitrogen, Carlsbad, CA, USA). DNA preparations were stored at −20°C prior to analysis.

PCR amplification and T-RFLP analysis

For polymerase chain reaction (PCR) amplification of 16S rRNA genes, universal oligonucleotide primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1392R (5′-ACG GGC GGT GTG TAC-3′) were used (Amann *et al.* 1995). PCR reactions were prepared using similar amounts of template DNA (10–25 ng) as described elsewhere (Morán *et al.* 2008). Reactions were carried out in a thermal cycler (Perkin Elmer 2400, Waltham, MA, USA), and amplified products (*c.* 1·5 kb) were visualized by agarose gel electrophoresis. For T-RFLP analysis, primer 8F for 16S rRNA PCR was labelled at 5′ with NED fluorochrome, and labelled products were generated from each duplicate DNA samples, digested separately with 20 U of *Hha*I and *Msp*I endonucleases and precipitated, as described (Morán *et al.* 2008). DNA fragments were separated and sequenced by capillary electrophoresis (Perkin Elmer ABI Prism 310), and fragment size was estimated using the internal standard ROX 1000 (Applied Biosystems, Foster City, CA, USA). T-RFLP data were plotted as the area (standardized as relative abundance using the sum of all peak areas) of each restriction fragment fluorescence peak against fragment sizes. Terminal restriction fragments (T-RF) with length sizes <50 and >500 bp, and those representing <0·5% of the total area were not included in the analyses (Schütte *et al.* 2008). Richness (S) was calculated as the total number of T-RFs in each profile. A putative assignment was made for T-RFs from an *in silico* restriction analysis of 16S rRNA clone library sequences considering literature recommendations to minimize differences in the estimated fragment sizes (Schütte *et al.* 2008).

Clonal libraries analysis

Unlabeled amplified 16S rRNA gene products derived from DNA of biofilm communities from field and laboratory pipes were cloned in the PCR-TOPO 2.1 TA cloning vector (Invitrogen). About 50 *Escherichia coli* recombinant clones were randomly chosen per library, and the proper plasmid insert length was analysed by PCR and gel electrophoresis. After plasmid detection, 36 and 35 clones were used for the field and biotic tests biofilm libraries, and initial cloning diversity analysis was performed by restriction fragment length polymorphism (RFLP). RFLP endonuclease digestions were performed using *Hha*I and *Msp*I. Restriction fragments were resolved by electrophoresis in 2% (w/v) agarose gel electrophoresis containing 1 × SYBR green, and clones were classified in groups with similar restriction patterns. One to two representative clones for each selected ribotype group were sequenced (Macrogen Inc., Seoul, Korea). Percent coverage value (*C*) was calculated for each cloning library with the equation $C = [1 - (n/N)] \times 100$, where n was the number of clone sequences that occurred once (i.e. unique phylotype) and N was the total number of clone sequences examined (Singleton *et al.* 2001). Sequences were assembled with Vector NTI 9.0 software and chimaeras were screened with Pintail (Ashelford *et al.*, 2005). For sequence identification, a nucleotide Blast similarity search was made in the GenBank database (http://www.ncbi.nlm.nih.gov).

Bacterial isolation

Bacteria were isolated from field sampled water and biofilm suspensions. Isolation was performed on R2A agar plates at 30°C for 5 days. DNA from isolates was extracted using Wizard DNA extraction kit (Promega, Madison, WI, USA) and identification was performed by partial sequencing of the 16S rRNA gene.

Minimum inhibitory copper concentration

For minimum inhibitory copper concentration analysis, a protocol designed to minimize metal precipitation was utilized based on the growth on minimum salts vitamin pyruvate medium (Teitzel and Parsek 2003). Total copper concentrations used were 0·5, 1, 2, 4 and 5 mmol l^{-1}, equivalent to 31·8, 63·6, 127·1, 254·2 and 317·7 mg l^{-1}. The assay was performed in sterile 96-well microtiter plates, and growth was monitored by optical density at 600 nm (OD_{600}) in a microplate reader spectrophotometer (Metertech 960, Taipei, Taiwan), after 72 h at 30°C. For each isolate the determinations were carried out in triplicate. Minimum inhibitory concentration was the lowest concentration at which there was no growth observed, represented by three standard deviations (SD) below the initial mean OD_{600}.

Biofilm formation

The ability to form biofilm was determined in a microtiter plate assay (Djordjevic *et al.* 2002). The liquid from the microtiter plates used in minimum inhibitory copper concentration determinations was removed, and each well was washed five times with sterile MilliQ water. Crystal violet staining, water washing, and ethanol destaining was performed as described (Djordjevic *et al.* 2002). To quantify biofilm production, crystal violet present in the destaining solution was measured at OD_{600} in a microplate reader spectrophotometer.

Nucleotide sequence accession numbers

Sequences from the clone libraries were deposited in GenBank under the accession numbers GU368356 to GU368379.

Statistical analysis

Differences in total copper levels in sampled water from corrosion tests were compared with independent *t*-tests using SIGMAPLOT ver. 10 (Systat Software Inc., Chicago, IL). Multivariate statistical analyses were made with PRIMER ver. 5 (Primer-E Ltd, Plymouth, UK) for T-RFLP data. Similarity matrix was calculated using the Bray–Curtis coefficient, and nonmetric multidimensional scaling analyses were used to group data according to their similarity (Rees *et al.* 2004). One-way crossed analysis of similarity was carried out to examine the statistical significance of grouping (Rees *et al.* 2004). The pair-wise comparisons output statistic R evaluates the level of separation of the group of profiles representing the structure of communities; a value of 0 indicates no separation of the structures because of the factor analysed, and 1·0 indicates total separation (Morán *et al.* 2008). Values of $R \geq 0{\cdot}5$ account for high differences, whereas between 0·25 and 0·5 the differences are considered moderate (Morán *et al.* 2008). Statistical significance was accepted at $P < 0{\cdot}05$.

Results

Microbial effects on copper piping corrosion

The measured pH in filtered and unfiltered water was 6·4 and mean total copper concentration was 0·05 mg l^{-1}. General water quality physicochemical parameters (i.e. alkalinity, hardness, dissolved oxygen, among others) were assumed to be consistent with our previous work and field campaigns carried out in the same site and area (Calle *et al.* 2007). Running water is slightly acid, slightly hard (160 mg $CaCO_3$ l^{-1}), with a low content of dissolved organic carbon (1·5 mg l^{-1}), chloride (15·6 mg Cl^- l^{-1}) and nutrients (3·2 mg NO_3^--N l^{-1}, 1·66 mg PO_4^{3-} l^{-1}, 30·2 mg SO_4^{2-} l^{-1}), and highly oxygenated (8·11 mg l^{-1}). Microbial counts were estimated in sampled water and, as a sterilization control, in filtered water, by heterotrophic bacteria plate counting in R2A agar plates (Merck, Darmstadt, Germany), at 30°C for 5 days, using triplicate 10-fold serial dilutions. The mean number of heterotrophic bacterial colony forming units (CFU) found in sampled water was $1{\cdot}4 \pm 0{\cdot}1 \times 10^5$ CFU ml^{-1}. No CFU were detected in filtered water, as expected.

To characterize the microbial component of the copper corrosion process laboratory copper pipe tests were carried out. During the first 3 weeks of treatment (ageing), there were statistically significant higher total copper concentrations in laboratory copper pipe tests exposed to biotic than abiotic conditions (Fig. 2a). After this period, there were no significant differences in water copper levels in both corrosion treatments. After 2 weeks of aging, the maximal mean concentration of total copper in biotic tests was $2{\cdot}4 \pm 0{\cdot}4$ mg l^{-1} (mean ± 1 SD). This value is higher than the drinking water standard of 2·0 mg l^{-1} (*c.* 0·03 mmol l^{-1}) (WHO 2004). Total copper released was estimated by integrating the experimental values of copper concentration versus time (Fig. 2b). The ratio of total copper released between biotic and abiotic corrosion tests reached 5·0 after 2 weeks of treatment and decreased to 2·0 at the end of the experiments (8 weeks). There was a pH increment in the outlet water of all pipes, in both treatments. The pH increase in biotic pipes was 6·4–7·8, after 2 weeks, and 6·4–7·5 in abiotic tests, after 3 weeks, coinciding with the corresponding maximal total copper concentrations. The pH differences between inlet and outlet water were very low at the end of corrosion tests (data not shown).

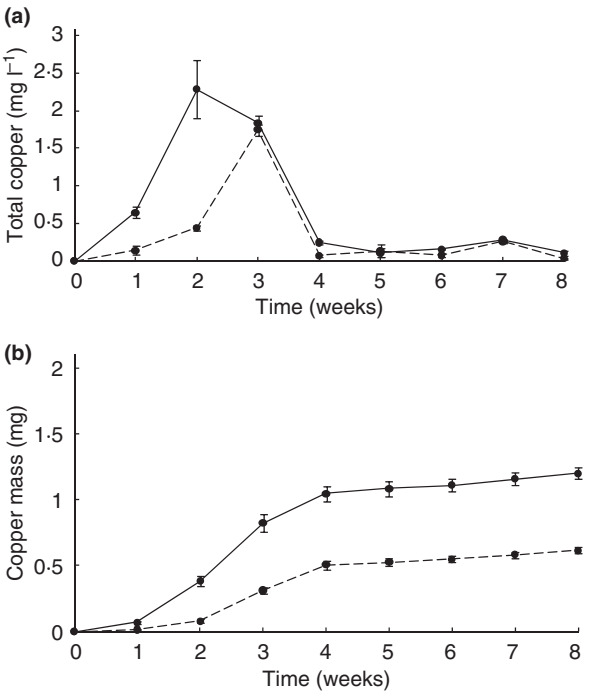

Figure 2 Copper release from pipes in laboratory corrosion tests. (a) Total copper concentration. (b) Accumulated copper mass (solid line, biotic; dashed line, abiotic; mean ± 1 SD).

SEM micrographs of the inner pipe surfaces from initial stages of biotic tests showed a progressive development of copper corrosion and microbial colonization (Fig. 3a–c). The presence of rod- and cocci-shaped micro-organisms was noticed (Fig. 3b,c). Formation of

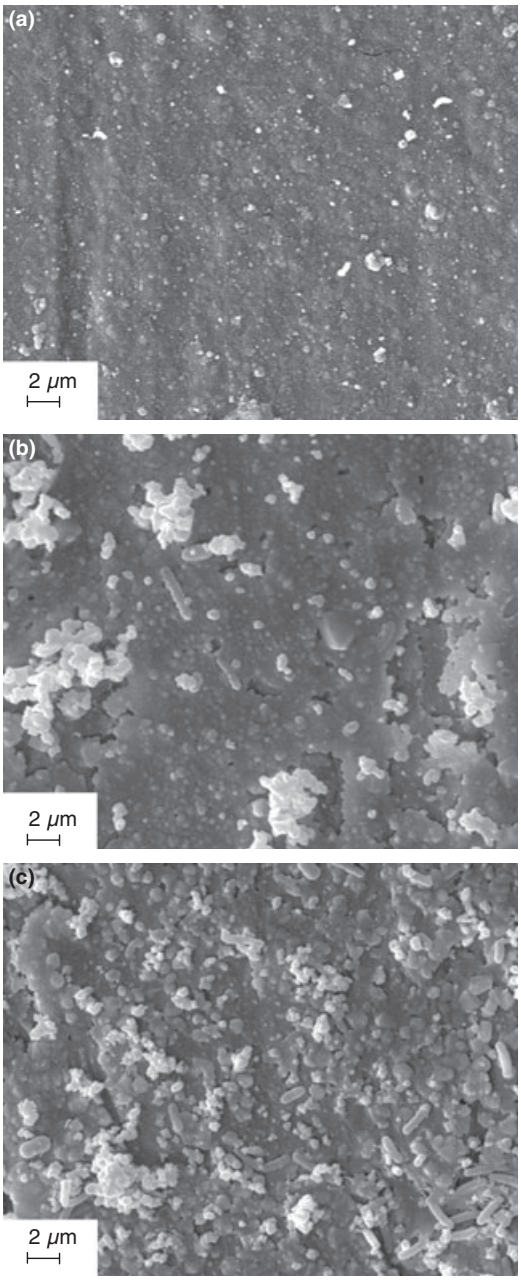

Figure 3 SEM of the inner surface of copper pipes from biotic tests. Representative images of copper pipes after 0 (a), 1 (b), and 2 weeks (c) of exposure to nonsterile water.

primary biofilm and extracellular polymeric substance (EPS) associated with corrosion by-products were observed, after 2 weeks of aging (Fig. 3c). At the end of the corrosion experiments, clear differences were observed between abiotic and biotic tests (Fig. 4). In the biotic condition, the observed corrosion scale had a heterogeneous distribution resembling a patchwork of developing biofilm, EPS, and corrosion by-products (Fig. 4a). The observed morphology of corrosion by-products in this matrix was irregular and diverse (Fig. 4b). In contrast, abiotic corrosion scale looked more compact and less developed (Fig. 4c,d). The inner surface of field-sampled pipe, with 1 year of operation in the plumbing system, showed 'pepper pot' corrosion features (Fig. 4e), typically associated with MIC (Oliphant 2003). Abundant existence of rod- and cocci-shaped micro-organisms and the establishment of a numerous and mature biofilm were also evident in field samples (Fig. 4f).

Structure of biofilm bacterial communities in corroded copper pipes

To compare the structure of the biofilm bacterial communities from the field copper pipes and laboratory biotic condition pipes, the culture independent molecular technique, T-RFLP, using 16S rRNA gene sequences, was applied. Nondetectable DNA was extracted and nonPCR amplification products were obtained from abiotic laboratory pipes; thus, these samples were not included in the analyses. Low richness was observed in T-RFLP profiles of biofilm communities from both kinds of pipes (Fig. 5). A higher richness was detected in the biofilm from the field system (in data obtained with endonuclease *Msp*I: $S = 7 \pm 1$, and *Hha*I: $S = 8 \pm 1$) than in the biotic corrosion test systems (*Msp*I: $S = 4 \pm 1$, *Hha*I: $S = 4 \pm 1$). A main terminal restriction fragment (T-RF) was observed in these T-RFLP profiles (*Msp*I: 490 bp, *Hha*I: 62 bp) (Fig. 5a,b), in contrast to those from laboratory biofilms, where 2–3 abundant fragments were found, in addition to the main T-RF detected also in the field pipe biofilm (Fig. 5c,d). To determine differences in the structure of these bacterial communities, analyses of similarity and nonmetric multidimensional scaling were carried out. The biofilm bacterial community from 1-year-operated sampled pipe was significantly different from those of laboratory tests (*Msp*I T-RFLP profiles: $R = 0{\cdot}74$, *Hha*I T-RFLP profiles: $R = 0{\cdot}52$; $P < 0{\cdot}05$).

Further study on molecular diversity of biofilm bacterial communities was made by analysis of 16S rRNA clone sequences. Field and laboratory pipe clonal libraries showed the presence of bacteria from several phyla (Table 1). The libraries had a high coverage level ($C \geq 95\%$) indicating that representatives of the less-abundant phylogenetic groups might be sampled (Singleton *et al.* 2001). Dominant members in these libraries were closely related to *Gamma-* and *Betaproteobacteria*, such as *Acinetobacter* species in the field copper pipe, and *Pseudomonas* species in the laboratory biofilm (Table 1). Other less abundant groups included *Alpha-* and *Deltaproteobacteria*, *Flavobacteria*, *Sphingobacteria*, and uncultured bacteria. Not a single common sequence was

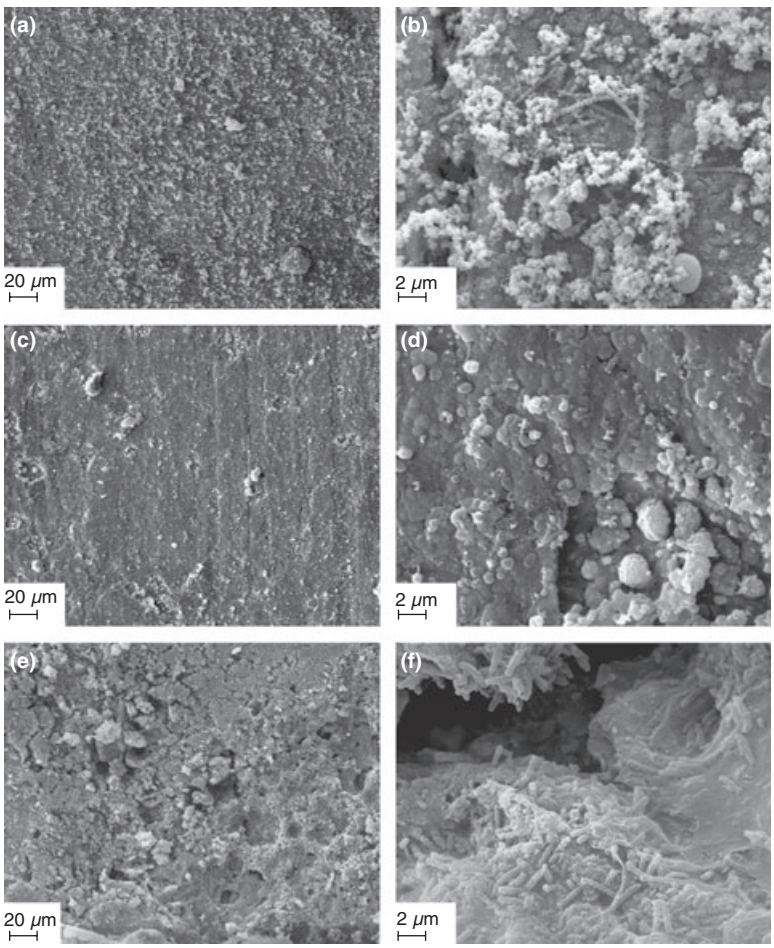

Figure 4 SEM of the inner surface of copper pipes from the end point of corrosion tests and field samples. Representative images of copper pipes from biotic tests (a, b), abiotic tests (c, d), and field samples (e, f).

detected in both libraries, but sequences belonging to the same genus (*Sphingomonas* sp. and *Sphingomonas melonis*) were found (Table 1). Most of the cloned sequences were also detected as the corresponding T-RFs in the T-RFLP profiles (Table 1). *Acinetobacter*-related sequences could be assigned to abundant T-RFs in the T-RFLP profiles from the field biofilm (Table 1, Fig. 5), and *Pseudomonas* sp., *S. melonis*, and *Variovorax* sp. sequences were abundant in the T-RFLP profiles from laboratory biofilms (Table 1, Fig. 5).

Minimum inhibitory copper concentration and biofilm formation ability in bacteria isolated from corroded copper pipes

Several heterotrophic bacterial isolates were obtained from the field samples and the laboratory biotic tests (Table 2). Most of these isolates were *Gamma*-, *Beta*- and *Alphaproteobacteria*. No common isolates were found in these two kinds of copper pipes. Although all isolates showed copper resistance levels of at least 63·6 mg l^{-1}, field system isolates were found to be more resistant to copper than laboratory biofilm isolates (Table 2). *Stenotrophomonas maltophilia* and *Variovorax* sp. found in both water and biofilm from field samples showed higher copper resistance levels when isolated from the latter source.

Interestingly, isolates from the field samples with higher copper resistance levels were also able to form biofilms (Table 2). In general, copper resistance correlated positively with the level of biofilm formation (Table 2). A couple of isolates from laboratory pipe biofilm showed biofilm formation ability but a relatively low level of copper resistance (Table 2).

Discussion

A characterization of bacterial communities from corroded copper plumbing was carried out in a household distribution system affected by 'blue water' corrosion and in laboratory scale experiments. Copper release measurements and SEM analysis of pipes exposed to these communities confirmed their influence in corrosion of copper surfaces. To our knowledge, this study is the first to address the composition of bacterial communities

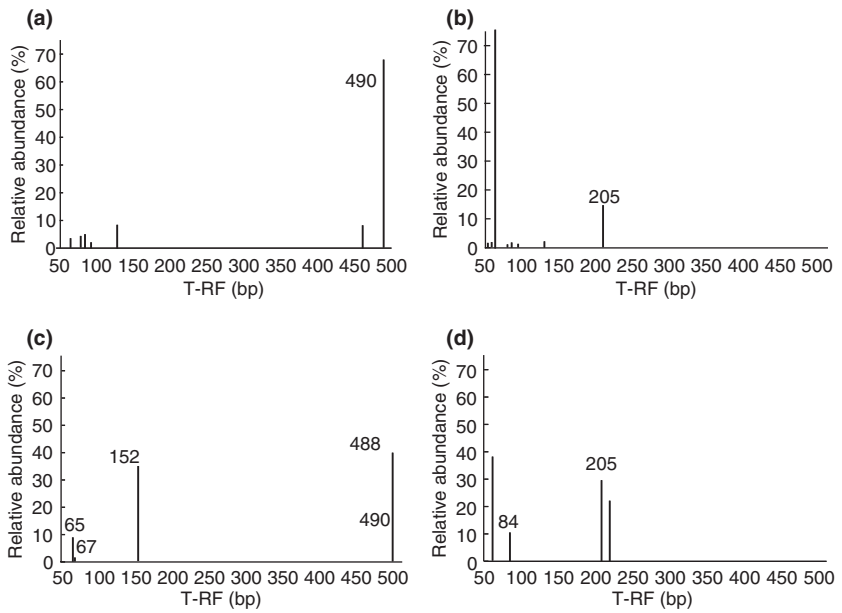

Figure 5 Representative T-RFLP profiles of 16S rRNA gene sequences from copper pipe biofilm communities. Field samples T-RFLP profiles obtained with the *Msp*I (a) or *Hha*I (b) endonuclease. Biotic tests T-RFLP profiles obtained with the *Msp*I (c) or *Hha*I (d) endonuclease. The T-RF lengths that coincide with those determined for clone sequences are indicated.

influencing copper piping corrosion by combining culture-dependent and culture-independent techniques.

Corrosion of distribution systems is an important issue for the management of water utilities (Oliphant 2003). Public health and infrastructure problems arising from copper plumbing deterioration may be enhanced by the presence of a biofilm, as it was shown to increase copper release in this study. The role of biofilms in metallic corrosion, as a reactive barrier controlling the chemistry in the liquid–metal interface, has been attributed to simultaneous mechanisms including formation of microenvironments with different aeration and oxidation–reduction conditions, altered transport of chemical species, changes in structure of inorganic passive layers, and facilitation of removal of protective films (Beech and Sunner 2004; Keevil 2004; Videla and Herrera 2005). Similar microbial effects to those observed in the present study have been previously reported in the corrosion of cold-water household copper distribution systems, but with longer lag phases to induce corrosion (Critchley *et al.* 2004). Our results indicate that the early increase of copper release observed in biotic aged pipes, higher than in abiotic tests, may be because of the rapid colonization of bacteria related to corrosion initiation and the establishment of a biofilm on the pipe inner surface. Severe localized corrosion because of bacterial establishment after short incubation times have been also found in carbon steel gas pipelines (Jan-Roblero *et al.* 2004). Subsequent water copper concentration stabilization in the copper corrosion tests may be explained as a consequence of surface passivation that prevents cuprosolvency (Merkel and Pehkonen 2006). The absence of flux and the growing of an immature biofilm may contribute to the stability of passive layers and to minimize particle detachment, as it has been shown that copper release is augmented in 'blue water' corrosion by the presence of a copious biofilm under flushing conditions (Calle *et al.* 2007). Corrosion scale development differences observed between abiotic and biotic aged pipes suggest that the presence of microorganisms alter the morphology and distribution of corrosion by-products. The existence of a heterogeneous and less-compact scale in biotic conditions suggests also that micro-organisms, biofilm, and inorganic products constitute a complex and mixed assemblage.

Bacterial diversity has been determined in biofilms from copper pipes affected by MIC, but mostly using culture-dependent approaches (Dutkiewicz and Fallowfield 1998; Critchley *et al.* 2003, 2004), which are less informative because of the low levels of culturability of micro-organisms that thrive in these biofilms. Although culture-independent, molecular, PCR-based analyses like T-RFLP and cloning present some biases (Acinas *et al.* 2005; Schütte *et al.* 2008), they are quite useful to assess changes in microbial community structure (Schütte *et al.* 2008; Talbot *et al.* 2008) and to determine its composition (Amann *et al.* 1995; Talbot *et al.* 2008).

The decreased bacterial diversity found in bacterial communities from laboratory and field copper pipe biofilm is probably explained by toxic and bactericidal effects of copper (Turpeinen *et al.* 2004). Particularly in the distribution plumbing system, the most aggressive conditions occur close to the pipe wall between stagnation and flow events where high levels of labile copper are found (Calle *et al.* 2007).

Table 1 16S rRNA cloning libraries and expected T-RF lengths of gene sequences from field and laboratory biotic test biofilms

Clone identification	Best similarity match	16S rRNA identity	Phylum/Class	Frequency* (*N* = 36)	Expected T-RF (bp)†	Detected T-RF (bp)†
Field sample library						
C6 (GU368358)	*Acinetobacter johnsonii* (AB099655)	99%	*Gammaproteobacteria*	39%	492 (*Msp*I), 207 (*Hha*I)	490 (*Msp*I), 205 (*Hha*I)
C2 (GU368356)	*Oxalobacteraceae bacterium* (DQ388765)	99%	*Betaproteobacteria*	25%	491 (*Msp*I), 30 (*Hha*I)	490 (*Msp*I)
C22 (GU368363)	*Acinetobacter junii* (AB101444)	99%	*Gammaproteobacteria*	14%	460 (*Msp*I), 208 (*Hha*I)	458 (*Msp*I), 205 (*Hha*I)
C5 (GU368357)	*Sphingomonas* sp. (EF061133)	99%	*Alphaproteobacteria*	8%	151 (*Msp*I), 83 (*Hha*I)	84 (*Hha*I)
C19 (GU368362)	*Chryseobacterium* sp. (DQ521273)	99%	*Flavobacteria*	8%	486 (*Msp*I), 226 (*Hha*I)	490 (*Msp*I)
C15 (GU368360)	*Anaeromyxobacter* sp. (CP001131)	95%	*Deltaproteobacteria*	6%	87 (*Msp*I), 375 (*Hha*I)	None
Laboratory biotic tests library				(*N* = 35)		
C40 (GU368366)	*Pseudomonas* sp. (AM076674)	99%	*Gammaproteobacteria*	40%	491 (*Msp*I), 208 (*Hha*I)	488–490 (*Msp*I), 205 (*Hha*I)
C28 (GU368364)	*Sphingomonas melonis* (AB334774)	99%	*Alphaproteobacteria*	20%	151 (*Msp*I), 83 (*Hha*I)	152 (*Msp*I), 84 (*Hha*I)
C16 (GU368361)	*Variovorax* sp. (EF471221)	97%	*Betaproteobacteria*	17%	67 (*Msp*I), 207 (*Hha*I)	65–67 (*Msp*I), 205 (*Hha*I)
C14 (GU368359)	*Methylotenera mobila* (CP001672)	99%	*Betaproteobacteria*	11%	491 (*Msp*I), 569 (*Hha*I)	490 (*Msp*I)
C69 (GU368367)	Uncultured bacterium (AM162488)	93%	*Sphingobacteria*	9%	273 (*Msp*I), 354 (*Hha*I)	None
C35 (GU368365)	Uncultured bacterium (AY887012)	96%	None	3%	28 (*Msp*I), 838 (*Hha*I)	None

*Frequency: percentage of clones in the total library.
†*Hha*I, *Msp*I: restriction enzymes used in T-RFLP analysis.

Microbial colonization of the pipe surface has been proposed to follow a sort of ecological succession (Keevil 2004). In this context, the relatively higher bacterial richness and different structure in the biofim from the distribution system with respect to the short-term laboratory tests may reflect a more complex community dominated by adapted micro-organisms. Our results on biofilm bacterial diversity, determined by clonal library analyses, supported the observed differences, as no similar sequences were identified. However, species belonging to *Gamma*- and *Betaproteobacteria* were similarly abundant in each type of biofilm, suggesting that these bacterial groups are important to biofilm formation and development. The differences between both biofilms may be also explained because the experimental set-up may not strictly represent field conditions.

The T-RFLP assignment and clone frequency analyses suggest that *Acinetobacter*-related clones represent the predominant group in the biofilm from the distribution system. Analysing the heterotrophic culturable fraction, *Acinetobacter* sp. has been shown to be a pioneer bacterium of biofilms in potable water stainless steel pipes (Percival 1999). *Acinetobacter*-related species were also found using molecular characterization in corrosive biofilms from gas steel pipelines (Zhu *et al.* 2003). In turn, *Pseudomonas* sp., *Variovorax* sp., and *S. melonis*-closely related clones were abundant in laboratory biofilm. *Pseudomonas* strains have been shown to increase cuprosolvency, particularly with rising water alkalinity, and have been associated with copper plumbing corrosion in drinking water (Critchley *et al.* 2003). Strains related to *Variovorax* sp. were isolated in the same studied area from pitting corroded pipes biofilms (Reyes *et al.* 2008). Copper-tolerant *Sphingomonas* sp. has been isolated from biofilms in 'blue water' corrosion affected copper tubes (Critchley *et al.* 2004), and its presence has been positively correlated with copper release in cold-water corroding copper plumbing (Arens *et al.* 1995). Moreover, *Sphingomonas*-related bacteria were found to be dominant in a copper-exposed biofilm community

Table 2 Phylogenetic identification, copper resistance levels and biofilm formation ability of bacterial isolates from field samples and biotic corrosion tests

Isolate identification	Best similarity match	16S rRNA identity	Phylum/Class	Copper resistance level (mg l^{-1})	Biofilm formation†
Field sample water					
A1 (GU368368)	*Pseudomonas* sp. (EU013945)	99%	*Gammaproteobacteria*	317·7	3×
A12 (GU368369)	*Stenotrophomonas maltophilia* (DQ991144)	99%	*Gammaproteobacteria*	127·1	3×
A9 (GU368370)	*Sphingomonas* sp. (EF061133)	97%	*Alphaproteobacteria*	127·1	2×
A3 (GU368371)	*Methylobacterium* sp. (AY741717)	98%	*Alphaproteobacteria*	127·1	None
A8 (GU368372)	*Variovorax* sp. (DQ205307)	98%	*Betaproteobacteria*	63·6	1×
A5 (GU368373)	*Arcicella aquatica* (AJ535729)	97%	*Sphingobacteria*	31·8	None
Field sample biofilm					
B13 (GU368369)	*Stenotrophomonas maltophilia* (DQ813325)	99%	*Gammaproteobacteria*	317·7	4×
B5 (GU368374)	*Brevundimonas* sp. (EF177674)	98%	*Alphaproteobacteria*	127·1	3×
B14 (GU368361)*	*Variovorax* sp. (DQ205307)	96%	*Betaproteobacteria*	127·1	1×
Laboratory biotic tests biofilm					
B29 (GU368375)	*Bacillus* sp. (EF690432)	98%	*Bacilli*	127·1	None
B33 (GU368376)	Uncultured bacterium (AY345557)	97%	*Betaproteobacteria*	31·8	2×
B25 (GU368377)	*Pseudomonas* sp. (AM745260)	96%	*Gammaproteobacteria*	31·8	1×
B32 (GU368378)	Uncultured bacterium (AY212678)	97%	*Betaproteobacteria*	31·8	None
B27 (GU368379)	*Pseudomonas lanceolata* (AB021390)	96%	*Gammaproteobacteria*	31·8	None

*Same sequence as C16 in Table 1.

†Biofilm formation is given as a relative factor based on the isolate with the least capacity (1×: least, 2×: 2 times 1×, 3×: three times 1×, 4×: four times 1×).

able to retain large quantities of copper in groundwater treatment (Vílchez *et al.* 2007). Bacterial heterotrophic isolates obtained from biofilms in the present study also included *Variovorax* sp. and, interestingly, *Sten. maltophilia*, a bacterium previously isolated from drinking water biofilms and associated with copper corrosion (Critchley *et al.* 2004). The culturable fraction from the water of the field distribution system samples showed also bacteria closely related with species suggested by molecular analyses to be important in the studied biofilms, like *Pseudomonas* sp., *Sphingomonas* sp., and *Variovorax* sp. Other field water isolate detected was *Methylobacterium* sp. that has been also reported to be present in 'pepper pot' pitting corroded copper pipes (Keevil 2004). The presence of these micro-organisms in the bulk water may be related with the detachment from the biofilm caused by the shear stress associated with flushing conditions.

Biofilm communities are protected from metal toxicity through several physical and biochemical mechanisms (Harrison *et al.* 2007). Because of the metallic binding capacity of the EPS, the increase of solvated copper as a consequence of MIC generates accumulation of copper in biofilms during stagnation periods (Flemming and Wingender 2001). Some EPS components promote also metal ionization because of their acidic nature (Keevil 2004). However, biofilm spatial relations and corrosion scale heterogeneity may protect micro-organisms in these copper-enriched environments, although individual metal tolerance may be important. The resistance levels detected for the isolated strains suggested that bacteria colonizing corroded pipes have no problem to survive within them because of the lower copper concentrations accumulating in the system when compared to the inhibitory copper concentrations. However, copper concentrations within the biofilm matrix may vary and be higher than in the bulk liquid (Calle *et al.* 2007) and minimal inhibitory concentration tests may also overestimate the susceptibility levels in real conditions. Nevertheless, under the same limitations bacterial isolates from the distribution system water and biofilm showed higher copper resistance values than those isolated from laboratory tests. The mature biofilm present in the field pipe may have a community more adapted to the copper threats. Interestingly, the higher copper resistance levels were found in bacterial isolates that also produce high levels of biofilm, including bacteria closely related to members that are dominant in the community.

In conclusion, biofilm formation may be a key element for both the establishment and promotion of corrosion and the survival of microbial communities in copper corroded plumbing. To understand the mechanisms and specific effects on corrosion, further research is required

using, for example, copper-tolerant bacterial isolates belonging to major biofilm producing groups, as those reported here. Incorporation of variable water flux and variable physicochemical water conditions in the experimental set-up would also provide relevant findings.

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