Control of *Legionella* contamination and risk of corrosion following various disinfecting procedures in hospital water networks

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Running title: *Legionella* and corrosion following disinfection

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ABSTRACT

Physical and chemical disinfection methods have been proposed with the aim of controlling *Legionella* water contamination. To date, the most effective procedures for reducing bacterial contamination have not yet been defined. The aim of this study is to assess the long-term effectiveness of various disinfection procedures in order to reduce both culturable and not culturable (NC) legionellae in different hospital water networks treated with heat, chlorine dioxide, monochloramine and hydrogen peroxide. The temperature levels and the biocide concentrations that proved to obtain reliable results were analysed. In order to study the possible effects on the water pipes, we verified the extent of corrosion on experimental coupons after applying each method for six months. The percentage of positive points was at its lowest after treatment with monochloramine, followed by chlorine dioxide,

hydrogen peroxide and hyperthermia. A different selection of *Legionella* spp was observed, as networks treated with chlorine-based disinfectants were mainly contaminated by *L. pneumophila* serogroup 1, hyperthermia was associated with the serogroups 2-14 and hydrogen peroxide mainly with non-*pneumophila* species. NC cells were only detected in heat-treated waters, also when the temperature was approximately 60°C. The corrosion rate of the coupons was within a satisfactory limit for water networks, but the morphology changed. We confirm that chemical disinfection controls *Legionella* colonization more effectively than hyperthermia. Monochloramine was the most effective treatment, while hydrogen peroxide could be a promising alternative to chlorine-based disinfectants due to its ability to select for other less virulent or non-pathogenic species.

INTRODUCTION

The number of cases of Legionnaires' disease has increased steadily over the years especially in Italy and Europe (1, 2). In 2013 in Italy, most cases were community-acquired (83.4%), followed by travel-associated (9.8%) and healthcare-associated (4.6%) (3). Despite the lower percentage of nosocomial cases, the control of *Legionella* spp contamination is essential in healthcare settings where patients, in particular those with compromised immune systems, are at high risk of contracting the disease with possible fatal outcome (4). For this reason, national and international guidelines recommend using preventive measures to control *Legionella* water contamination with particular reference to healthcare structures (3,5). A range of physical and chemical disinfection methods have been proposed, but to date, the most effective procedures for controlling contamination have not yet been defined (6). Moreover, their impact on pipe deterioration/corrosion has not been extensively documented and is mainly studied in models water distribution systems (7-10).

Molecular investigation tools were used together with conventional culture for monitoring corrective actions (11-13). Culture is essential for identifying and typing the bacterial strains, however it is time

consuming and could give false negative results due to the possible presence of cells in a Viable But Not Culturable (VBNC) state (14). In the VBNC state, pathogens are not generally able to initiate disease, but virulence is retained, and infection can follow their resuscitation to the actively metabolizing state (15). Quantitative PCR (qPCR) techniques prove to have several advantages including high sensitivity, accuracy and rapid evaluation of germ contamination. The main disadvantage of these techniques is they that cannot distinguish viable and not viable cells which is an important factor to take into account when evaluating the effectiveness of corrective actions (16, 17). In order to overcome this problem, new PCR-based strategies, collectively called molecular viability analyses, have been developed (18). Among these, methods based on DNA-intercalating dyes such as ethidium monoazide (EMA) have been proposed together with qPCR (19-21). EMA selectively binds the DNA of cells with compromised membranes, while intact cell membrane represents a barrier for the dye. In this bound state, the DNA cannot be amplified by qPCR, while DNA from intact membrane cells can be amplified and quantified (21). The only insurmountable limitation of this method is the inability to detect bacteria inactivated by conditions that do not alter membrane permeability such as shortwave UV and low-temperature pasteurization (18).

The main aim of this study is to assess the long-term effectiveness of monochloramine, chlorine dioxide, hydrogen peroxide and heat in reducing/eliminating both culturable and not culturable (NC) legionellae in various hospital water networks. For this purpose, traditional culture was used with qPCR alone and in combination with EMA. In our study, the EMA-qPCR method proved to be suitable for measuring bacterial viability as oxidative disinfectants and heat rapidly act against bacterial cells causing damage to cellular components including the cytoplasmic membrane (18). In order to verify the possible corrosion action of each disinfection procedure, carbon-steel coupons were inserted along the water distribution systems, and were periodically examined for weight loss and morphology.

MATERIALS AND METHODS

Hospital setting

The study was carried out in two hospitals situated in Modena, northern Italy: a private hospital built in the 1980s, and an university hospital consisting of a central block indicated as building 1 and four separate blocks built between the 1970s and the 1990s. The same municipality provides the incoming cold groundwater in both hospitals. Hot water is produced *in situ* using heat exchangers and reaches the peripheral points through a water recirculation system. Three different water networks (A, B and C) distribute hot water in parallel in building 1, while the other four buildings of the university hospital have their own hot water network (D, E, F and G), as previously described (22). The private hospital has a unique hot water distribution network (H).

Since 2000, sampling plans have been implemented in order to assess *Legionella* spp contamination in the water distribution systems. In both hospitals, the samples collected prior to intervention were mainly contaminated by *L. pneumophila* serogroup 2-14 at concentrations higher than 10^4 CFU liter^{-1} which led to the implementation of a wide range of control strategies (23). The disinfection strategies still operating in both hospitals are described below.

• University hospital, building 1 (nine-floors, 40 years old):

- a monochloramine device operating since March 2009 on water network C; monochloramine is produced in situ from the chemical reaction between a stabilized chlorine-based precursor and an ammonium salt (Sanipur S.r.l., Brescia, Italy). The monochloramine generator is set to maintain a concentration of biocide in the recirculation loop between 2.0 and 4.0 mg liter $^{-1}$. Residual levels are in line with the guideline value of 3 mg liter $^{-1}$ and the maximum contaminant level of 4 mg liter $^{-1}$, established by WHO and the United States Environmental Protection Agency (EPA), respectively (24, 25).

- two chlorine dioxide devices (Sanipur S.r.l., Brescia, Italy) operating since January 2005 on water network A and since November 2005 on water network B. Chlorine dioxide is produced in situ by injecting hydrochloric acid and sodium chlorite into the recirculating hot water. The system is set up to ensure concentrations of at least 0.30 mg liter $^{-1}$ at distal points as previously reported (22), without exceeding the EPA maximum residual disinfectant level of 0.80 mg liter $^{-1}$ (25).

• University hospital, building D (four-floors, 40 years old): an experimental hydrogen peroxide device operating since January 2012 (O2 S.r.l., Bergamo, Italy). 48% hydrogen peroxide solution is continuously injected into the recirculating hot water by a dosing pump in order to ensure concentrations of 15-20 mg liter $^{-1}$ at distal outlets.

• Private hospital H (four-floors, 35 years old): hyperthermia has been used since April 2012; the hot water is produced at a temperature of at least 60°C and distributed at temperatures constantly >50°C.

Samples collection

Over a three-year period (January 2012 – December 2014), hot water samples (n. 662) were taken from heaters, return loops, and distal outlets (showers and/or taps) of the water networks treated with the disinfection strategies listed above. In both hospitals, the protocols anticipated sampling from at least one remote point every 50 beds, repeating sampling in the same sites every 3 or 4 months. The network that was experimentally treated with hydrogen peroxide was monitored more frequently: every week for the first three months, every month until the end of the second year and every 4 months during the last year. Water was collected in sterile plastic bottles without flaming and after flushing for 1 minute. Sodium thiosulphate (10 mg liter $^{-1}$) was added (1 ml liter $^{-1}$, final concentration) to neutralize residual free chlorine. At sampling, water temperature (digital thermometer), chlorine dioxide (DPD method, Microquant, Merck, Darmstadt, Germany), monochloramine (Indophenol method, Hach Lange, Milan,

119 Italy) and hydrogen peroxide (reflectometer RQflex 2, Merck, Darmstadt, Germany) were measured.
120 The samples were returned to the laboratory immediately after collection and analysed within 24h as
121 described elsewhere (26).

122

123 Laboratory methods

124 Culture and identification of *Legionella* spp were carried out with the ISO 11731:1998 method (27), as
125 previously described (28). The results were expressed as CFU liter $^{-1}$, and the limit of detection (LOD)
126 of the procedure was 25 CFU liter $^{-1}$.

127 DNA was extracted using the QIAamp DNAMini kit (Qiagen, Hilden, Germany), according to
128 manufacturer's instructions as reported elsewhere (19). The extracted DNA was stored at -20°C until
129 use.

130 The minimum number of samples, that were to be analysed by molecular methods in order to have
131 statistical power, was determined by carrying out a power analysis based on the results of a previous
132 pilot study (29). For each treatment, the first 22 negative samples with culture (total n. 88) were
133 analysed by the qPCR with and without the EMA pre-treatment. The water samples were treated with
134 EMA (Sigma Chemical Co., St Louis, MO, USA) at a final concentration of 6 μM (2.53 μg ml^{-1}) prior
135 to DNA extraction as reported by Mansi et al. (19).

136 The DNA amplification was carried out with a Rotor-Gene Q 2plex instrument (Qiagen, Hilden,
137 Germany) using the commercial "New *Legionella* spp Quantitative kit" (Diatheva, Fano, Italy),
138 validated in agreement to ISO/TS 12869:2012 (30, 31). The results were expressed as Genome Units
139 per Liter (GU liter $^{-1}$). Under the experimental conditions used in this study, the LOD and the limit of
140 quantification of the qPCR method were estimated to be 100 GU liter $^{-1}$ and 500 GU liter $^{-1}$,
141 respectively.

142

143 Corrosion study

144 Rectangular coupons (area 21.81 cm^2, density 7.87 g cm^{-3}) of carbon steel C1010 foils with frosted
145 surfaces were used for evaluating the type and extent of corrosion according to the standard practices
146 ASTM G1-03:2011 and ASTM G4-01:2014 (32, 33). The non-alloy steel with carbon content up to
147 0.22% is suitable for the conveyance of aqueous liquids, including water for human consumption (34).
148 We selected the C1010 steel with maximum carbon content of 0.13% because it is easily found in the
149 market as coupons proper for our experimental conditions. The coupons were inserted into five
150 separate racks made from polytetrafluoroethylene (see fig.1). The racks were connected to the return
151 loops of the treated networks (A, B, D and H) and an untreated network (F). Before the beginning of
152 the study, four coupons were inserted into each rack. After 2 and 4 months, one coupon from each rack
153 was removed and a new coupon was added. After 6 months all the coupons were removed. In line with
154 this practice, we analysed six coupons for each rack, two for each exposure time (2, 4, 6 months). After
155 collecting the coupons for corrosion analysis they were immediately dried with dimethyl ketone and
156 placed in vials containing silica gel for transportation to the laboratory. The weight loss method was
157 used in order to determine the corrosion rate. The coupons were scraped with a brass brush to remove
158 surface deposits, washed in an ultrasonic bath for 6 minutes and then weighted. The cleaning cycles
159 were suspended as soon as weight value was stabilized. With the aim of determining the mass loss of
160 the base metal when removing the corrosion products, an uncorroded control coupon was cleaned using
161 the same procedure performed on the test coupons. The average corrosion rates were calculated by
162 means of the following formula:

163 $$\text{corrosion rate (mm y}^{-1}) = (K \times W)/(A \times T \times D)$$

164 where K, constant (3.65×10^4), W, mass loss (g), T, time of exposure (days), A, area (cm^2) of carbon
165 steel coupon, D, density (carbon steel)(g cm^{-3}).

166 An optical microscope (Carl Zeiss, Milan, Italy) equipped with a system of automatic digitization of
167 the images was used in order to characterize the corrosive phenomena.
168
169 Statistical analysis
170 All statistical analyses were performed with PASW statistic version 21.0 (SPSS Inc, Chicago, IL,
171 USA). Logarithmic transformations were used to normalize the bacteriological data and the results are
172 presented as geometric mean values. Chi-square test, paired t test, one-way analysis of variance
173 (ANOVA) with Bonferroni test were applied whenever necessary.
174
175 RESULTS
176 In total, 237 out of 662 samples (35.8%) were contaminated by *Legionella* spp. The disinfection
177 treatments significantly affected both the percentage of positive samples ($\chi^2 = 104.385$, $P < 0.001$) as
178 well as the bacterial load of positives expressed as geometric mean ($F = 26.007$, $P < 0.001$).
179 Monochloramine showed the lowest percentage of positives (9/95, 9.5%), followed by chlorine dioxide
180 (60/201, 29.8%), hydrogen peroxide (80/208, 38.5%) and hyperthermia (36/66, 54.5%). Regarding
181 *Legionella* concentrations, no differences in the geometric mean were observed according to chemical
182 treatments (2.2×10^2 CFU liter^{-1} for monochloramine, 3.0×10^2 CFU liter^{-1} for chlorine dioxide and 1.3
183 $\times 10^2$ CFU liter^{-1} for hydrogen peroxide), while a significantly higher geometric mean (1.7×10^3 CFU
184 liter^{-1}) was observed for the heat-treated positive samples than for the samples treated with all biocides
185 (Bonferroni test, $P < 0.05$).
186 Table 1 shows the number, percentage of positive samples and their geometric mean according to the
187 biocide/temperature levels. No significant difference in the percentage of positives relating to the
188 concentrations of monochloramine was observed and 3 mg liters $^{-1}$ are required in order to obtain
189 legionellae $< 10^2$ CFU liter^{-1}. Levels of chlorine dioxide ≥ 0.50 mg litre $^{-1}$ significantly reduced the

190 percentage of positive points below 30% ($\chi^2 = 3.930$, $P < 0.05$). Hydrogen peroxide between 15 and
191 19.9 mg liter $^{-1}$ was associated with a significant reduction in positive points ($\chi^2 = 3.823$, $P < 0.05$), yet
192 levels ≥ 20 mg liter $^{-1}$ are required to obtain less than 30% of positive distal points. A significant
193 reduction of positives was observed by increasing the temperature to 55 - 59.9°C ($\chi^2 = 7.796$, $P <$
194 0.010) but no positive sample was observed when the temperature reached 60°C. For all treatments, the
195 bacterial load did not differ according to the biocide/temperature levels and only a limited number of
196 samples exceeded 1.0 x 10^4 CFU liter^{-1} (one with monochloramine and hyperthermia, two with chlorine
197 dioxide).
198 Table 2 shows that waters treated with chlorine-based systems were mainly contaminated with *L.*
199 *pneumophila* serogroup 1, hyperthermia was strictly associated with *L. pneumophila* serogroups 2-14
200 and hydrogen peroxide was mainly associated with non-*pneumophila* species (54.5% *L.*
201 *jamestowniensis*, 36.4% *L. anisa* and 9.1% both).
202 Among the 394 water samples whose cultures were negative, 88 were analysed with molecular
203 methods (Table 3). Chemical biocides showed a similar percentage of positives by qPCR, but no
204 positive sample by EMA-qPCR. Over 95% of the heat-treated samples were positive with qPCR and
205 50% with EMA-qPCR. Positive EMA-qPCR analyses were also associated to samples at temperatures
206 over 60°C (6/11, 54.5%).
207 The average loss of thickness of the coupons exposed to treated and untreated waters did not
208 significantly differ according to the type of treatment and time of exposure. As a mean, the corrosion
209 rate was 0.17 ± 0.03 mm/year for the coupons exposed to hydrogen peroxide, 0.15 ± 0.03 mm/year for
210 hyperthermia, 0.14 ± 0.04 mm/year for the untreated network, 0.14 ± 0.03 mm/year for chlorine
211 dioxide and 0.11 ± 0.05 mm/year for monochloramine.
212 The morphology of corrosion did not change over time, but differed according to the treatment. As an
213 example, the morphology of corrosion according to treatment following six months of water exposure

214 is reported in fig 2. The coupons exposed to chlorine dioxide presented a uniform corrosion with
215 pitting and ulcerations (image A), those exposed to monochloramine showed a uniform corrosion as
216 well as rare pitting (image B), and those exposed to hydrogen peroxide were characterized by pitting,
217 whose size ranged from a few microns up to several millimetres (image C). Finally, coupons of the
218 heat-treated waters (image D) showed a uniform corrosion with some ulcerations similarly to those
219 observed on untreated samples (*image not shown*).
220
221 DISCUSSION
222 In this study, we followed the trend of contamination by *Legionella* spp in hospital hot water networks
223 treated with different disinfection procedures. The effectiveness of these procedures was evaluated
224 using the traditional culture, the qPCR and the EMA-qPCR in order to detect culturable and NC
225 *Legionella* cells. Among the studied disinfection strategies, we included monochloramine, an
226 innovative method which proved to be effective in controlling *Legionella* contamination in a hospital
227 water network (22, 35). We also studied hydrogen peroxide which has not yet been extensively used for
228 controlling *Legionella* in hospital water distribution systems (36, 37). A comparison was carried out
229 between these new procedures and two popular methods such as chlorine dioxide and hyperthermia,
230 which have been widely reported to be effective (38-42).
231 Our study confirms the effectiveness of continuous chemical disinfection, but we emphasize that all
232 systems must be continuously monitored since none of them eradicates legionellae from water
233 distribution systems (6, 36, 43). On comparing the three disinfectants, monochloramine proves to be
234 the most effective approach, as it gave the best results in reducing the percentage of positive points by
235 culture, followed by chlorine dioxide and hydrogen peroxide. Moreover, for all biocides approximately
236 50% of the culture negative samples analysed using molecular methods was found positive by qPCR,
237 but negative by EMA-qPCR. This confirms that the qPCR can give false positives when the biocides

238 are applied in a contaminated system as previously reported (17), and that a continuous injection of
239 chemicals that are capable of killing the circulating microbes, avoids the induction of VBNC forms of
240 legionellae.
241 On the contrary, the network treated with hyperthermia is more contaminated both in terms of
242 percentage of positive sites and bacterial load. The Italian and European guidelines recommend
243 maintaining the water temperature between 55 and 60°C constantly in order to prevent *Legionella*
244 contamination (44, 45), yet in our study this range proved to be ineffective as over 60% of the samples
245 remained positive. Interestingly, the presence of NC legionellae was also observed at temperatures
246 around 60°C, which is considered to be a safe value for preventing *Legionella* contamination (46).
247 Recent studies demonstrate that VBNC legionellae are again culturable upon resuscitation within
248 amoebae and that infection can initiate following their resuscitation (14). In this respect, the NC cells
249 generated following the thermal treatment used in our hospital could constitute a potential public health
250 hazard. For all these reasons, we do not advise using hyperthermia as the only method for controlling
251 *Legionella* contamination in hospital water networks.
252 We stress the importance of finding an adequate level of biocide for controlling *Legionella*
253 contamination as our long-term experience suggests that the effectiveness of chlorine-based chemicals
254 changes over time. After one year of disinfection applications, we proposed a level of chlorine dioxide
255 between 0.30 - 0.40 mg liter $^{-1}$ and 2.0 mg liter $^{-1}$ of monochloramine to lower contamination below 10^2
256 CFU liter^{-1} (35). In the following 3 years, the levels required for obtaining the same reduction were
257 0.50 - 0.70 mg liter $^{-1}$ for chlorine dioxide, and 2 - 3 mg liter $^{-1}$ for monochloramine (22). In this study,
258 chlorine dioxide $\geq$ 0.50 mg liter $^{-1}$ was associated with 10^3 CFU liter^{-1} but the percentage of positives
259 was below 30%, a value reported as being an indicator of low risk for disease transmission (47).
260 Similarly, a monochloramine $\geq$ 3 mg liter $^{-1}$ was required to maintain *Legionella* below 10^2 CFU liter^{-1},
261 in accordance with other authors (48-50), but the percentage of positive sites was less than 30%

262 independently of the biocide levels, thus confirming the satisfactory results obtained with this
263 disinfectant.
264 Hydrogen peroxide only produced satisfactory results in reducing *Legionella* contamination when
265 biocide was ≥ 20 mg liter $^{-1}$, in agreement with other studies (36, 37). For this procedure, the high
266 percentage of positive points, although at low levels, was due to the difficulty in regulating the
267 disinfectant concentration, probably because the building was under renovation and many of the outlets
268 were seldom used. Hydrogen peroxide appears to be a promising alternative for decreasing *Legionella*
269 colonization; however, further field studies in other healthcare and community settings are required in
270 order to confirm its effectiveness.
271 The chlorine-based biocides caused a shift from *L. pneumophila* serogroups 2-14 to *L. pneumophila*
272 serogroup 1 while hydrogen peroxide favoured the switch from *L. pneumophila* to other species,
273 mainly *L. jamestowniensis*, which has not yet been associated with human disease (51, 52).
274 Contrastingly, no shift was observed with hyperthermia, which is in line with its ineffectiveness in
275 reducing the *Legionella* colonization. The continuous injection of chlorine-based biocides evidently
276 selects the most resistant *Legionella* spp, in our experience the *L. pneumophila* serogroup 1 which is
277 also the most virulent (1). In order to support this hypothesis, other authors reported the persistence of
278 serogroup 1 in hospital water systems despite the adoption of chlorine–based disinfection strategies
279 (53, 54). Duda et al. (50) reported a shift from *L. pneumophila* serogroup 1 to *L. bozemanii*, which are
280 both associated with human pathologies, following 24 months of monochloramine applications.
281 It is well known that disinfection can speed up corrosion and cause plumbing leaks, even if
282 contradictory results are reported in literature concerning the impact of disinfection on corrosion (7-
283 10). To complete the study, we studied the appearance of a favourable environment for corrosion within
284 the water networks according to the disinfection methods applied. The loss of thickness of the carbon
285 steel foils which were used to evaluate the extent of corrosion over a six-month period, does not exceed

the average values of 0.50 mm/year that are considered satisfactory for water networks (55), and no significant differences were observed between treated and untreated networks. On the other hand differences were observed regarding the morphology of corrosion. Hydrogen peroxide and chlorine dioxide caused pitting, which is a type of corrosion that can create holes in tubes (9). Monochloramine and hyperthermia appeared to be less aggressive, since monochloramine caused a uniform corrosion with rare formation of pitting, and hyperthermia showed a morphology of corrosion similar to that observed in the untreated coupons. The results encouraged us to continue the corrosion study therefore we are now analysing the long-term corrosive effect of these four disinfection procedures on commonly used plumbing materials such as copper, stainless steel, galvanized steel and polyvinyl chloride, the last two also used in our hospitals.

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442

443 Table 1. Number, percentage of positive samples and their geometric mean by culture according to the

444 biocide/temperature levels.

	positive n. (%)	geom. mean of positives (range) CFU liter^{-1}
Monochloramine		
<2.0 mg liter $^{-1}$	2/8 (25.0)	2.0 x 10^3 (1.2 x 10^2 – 3.1 x 10^4)
2.0 – 2.9 mg liter $^{-1}$	4/29 (13.8)	3.7 x 10^2 (25 – 5.7 x 10^3)
≥3.0 mg liter $^{-1}$	3/58 (5.2)	25 (25)
	χ^2=4.131, ns	F=1.963, ns
Chlorine dioxide		
<0.30 mg liter $^{-1}$	31/84 (36.9)	4.0 x 10^2 (25 – 2.5 x 10^4)
0.30-0.49 mg liter $^{-1}$	18/54 (33.3)	3.5 x 10^2 (25 – 4.1 x 10^4)
≥0.50 mg liter $^{-1}$	11/63 (17.5)	1.0 x 10^2 (25 – 5.5 x 10^3)
	χ^2=6.928, $P < 0.05$	F=2.058, ns
Hydrogen peroxide		
<15.0 mg liter $^{-1}$	46/91 (50.5)	1.1 x 10^2 (25 – 1.4 x 10^3)
15-19.9 mg liter $^{-1}$	14/43 (32.6)	2.6 x 10^2 (25 – 5.0 x 10^3)
≥20.0 mg liter $^{-1}$	20/74 (27.0)	1.1 x 10^2 (25 – 2.3 x 10^3)
	χ^2=10.339, $P < 0.001$	F=2.468, ns
Hyperthermia		
50-54.9°C	19/21 (90.5)	2.5 x 10^3 (25 – 1.2 x 10^4)
55-59.9°C	17/27 (63.0)	1.2 x 10^3 (25 – 7.9 x 10^3)
≥60°C	0/18 (0)	-
	χ^2=33.31, $P < 0.001$	F=0.481, ns

445

446

447 Table 2. Number and percentage of species and serogroups of *Legionella* according to the treatments

448 (χ^2=270.042, $P < 0.001$).

	***L. pneumophila* sg 1 alone or with others**	***L. pneumophila* 2-14**	***L.* spp**
monochloramine	7 (77.8)	0 (0.0)	2 (22.2)
chlorine dioxide	51 (85.0)	4 (6.7)	5 (8.3)
hydrogen peroxide	21 (26.3)	5 (6.2)	54 (67.5)
hyperthermia	0 (0.0)	36 (100.0)	0 (0.0)

449

450

451 Table 3. Molecular analysis of culture negative samples.

	n (%) positive	
	qPCR	**EMA-qPCR**
monochloramine	11/22 (50.0)	0/22 (0.0)
chlorine dioxide	9/22 (40.9)	0/22 (0.0)
hydrogen peroxide	10/22 (45.4)	0/22 (0.0)
hyperthermia	21/22 (95.4)	11/22 (50.0)
	χ^2=15.246, $P < 0.005$	χ^2=27.957, $P < 0.001$

452

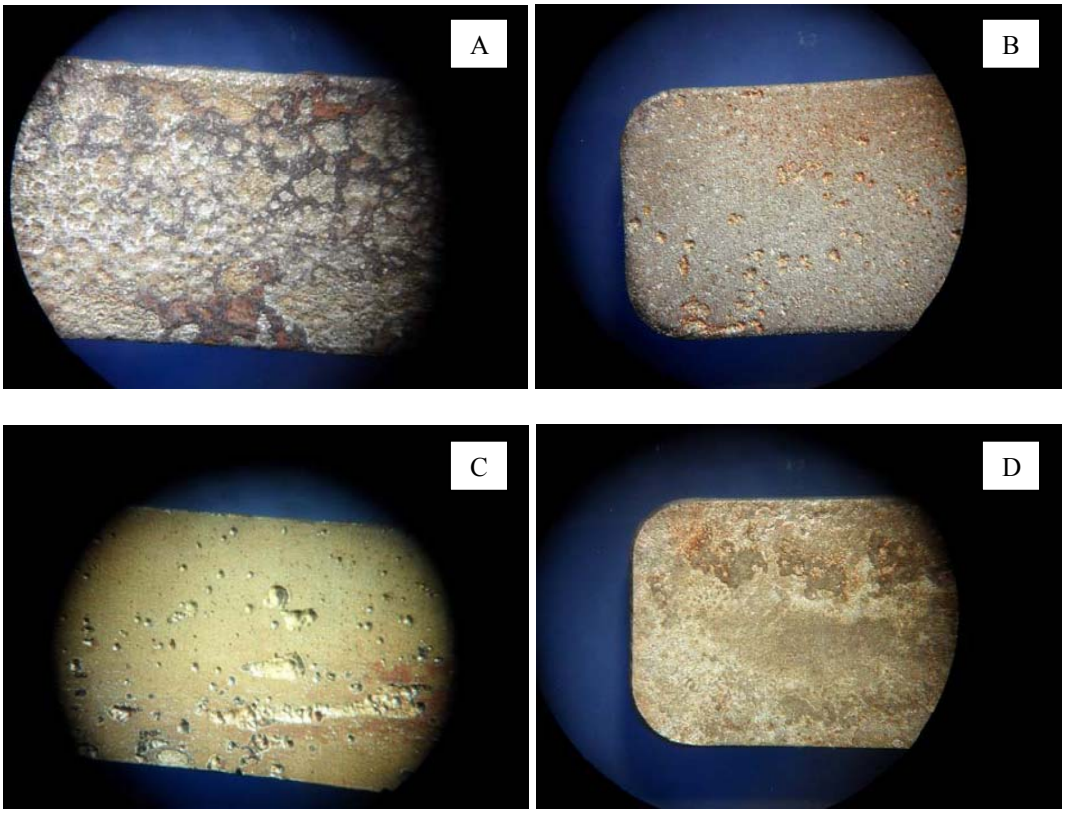
453

Fig. 1. Picture of rack used in order to support coupons for the test of corrosion.

457 Fig. 2. Digitalized images of one of the two coupons removed after 6 months of water exposure for

458 each treatment (3x magnification).

459

460 

461

462 Legend

463 A = coupons treated with chlorine dioxide

464 B = coupons treated with monochloramine

465 C = coupons treated with hydrogen peroxide

466 D = coupons treated with hyperthermia (similar to untreated carbon steel coupons, not shown)

467