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Assessment of a low-frequency ultrasound device on prevention of biofilm formation and carbonate deposition in drinking water systems

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27 **Highlights**

- 28 – Low-frequency ultrasound was studied on drinking water biofilms and CaCO₃ deposits
- 29 – Ultrasounds affect both biofilm and planktonic cell number and activity
- 30 – US treatment did not prevent initial bacterial adhesion and CaCO₃ deposition
- 31 – Low-frequency ultrasounds markedly limit biofilm formation and scaling
- 32

33 **Abstract**

34 A device generating low-frequency and low-intensity ultrasound waves was used for mitigating
35 biofilm accumulation and scaling. Two systems were tested: a lab-scale plate heat exchanger operated
36 with continuously recycled water and a continually fed flow-through drinking water pilot used for
37 mimicking water circulation in pipes. Initial deposition of bacterial cells was not prevented by
38 ultrasound wave treatment. However, whatever the tested system, both further calcium carbonate
39 deposition and biofilm growth were markedly inhibited. Biofilms formed in reactors subjected to low-
40 frequency and low-intensity ultrasound waves were weakly attached to the material. Even though the
41 activity of bacteria was affected as shown by their lower cultivability, membrane permeability did not
42 appear compromised. Ultrasound technology sounds very promising in both the mitigation of drinking
43 water biofilm and carbonate accumulations.

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50 *Keywords: drinking water biofilm, low-frequency ultrasound, carbonate deposition, heat*
51 *exchangers, water pipes.*

52

53 **1. Introduction**

54 Bio-fouling of any materials in contact with water results in a range of adverse issues for industrial
55 engineered water systems such as decrease in water transport efficiency, heat exchange limitation,
56 corrosion, increased maintenance costs, and environmental impact (as traditional cleaning strategies
57 sometimes involve the use of environmentally unfriendly chemicals). Alternative procedures, i.e. less
58 drastic, eco-aware, with a low water footprint and respectful of material integrity are required,
59 particularly to reduce their impact on the environment.

60 In this context, ultrasonic treatments represent a very promising alternative. Indeed, ultrasonic waves
61 have been used in many environmental, industrial and medical sectors: algal bloom control, food and
62 beverage processing, sonochemistry, nanotechnology, mineral processing, welding, surface cleaning,
63 medical scanning and non-destructive testing [1,2,3,4,5]. Likewise, ultrasonic waves (US) are proved
64 to have ‘synergistic curative’ effects on deposits when combined with other disinfection/cleaning
65 processes: eg US+UV [6], US+enzymes [7], US+microbubbles [8], US+turbulence [9], US+antibiotics
66 [10,11,12,13], US+bleach [14], US+ozone [15].

67 More recently, ultrasonic waves have been successfully applied to prevent marine macro-
68 fouling (caused by the attachment of large organisms such as barnacles, seaweed, mussels, and
69 diatoms) on vessel hulls with devices emitting mechanical waves in the ultrasonic (>20 KHz)
70 frequency range [16,17,18]. Moreover a few successful experimental studies have been reported on
71 inhibition of fouling in heat exchangers and pipes by application of a few short pulses of high power
72 ultrasonic waves: pulses of 0.2 s with 100 s inter-pulse intervals per day [19], or pulses of 3×30 s per
73 day [20]. Mechanisms explaining such an effect are generally not reported but they appear to be
74 frequency and power dependent, and they could be related to both mechanical effects and local
75 cavitation phenomena [21]. Additionally, experimental systems used for generating ultrasound are
76 very diverse and difficult to compare, because of differences in the applied piezoelectric transducers,
77 electrical discharge, ultrasound water bath, etc. Although very limited, the literature indicates that low
78 ultrasonic frequencies could be accurate for preventing the fouling of surfaces by bacterial biofilm.
79 However, the demonstration of ultrasound efficiency in inhibiting biofilm formation is not an easy
80 task as ultrasound waves should *de facto* prevent deposition/attachment of soft colloids such as single

81 bacterial cells, multiplication of attached living cells or the formation of viscoelastic clusters described
82 in young water biofilms [22]. Indeed, experimental demonstrations are needed to get a clear proof of
83 concept of ultrasound technology under conditions relevant to engineered water systems.

84 In heat exchangers, precipitation fouling, ie the formation of a solid layer of deposits of
85 inverse solubility (CaCO_3 , CaSO_4 , $\text{Ca}_3(\text{PO}_4)_2$, etc.), and viscous hydrated organic deposits on the side
86 of plates in contact with coolants determine heat exchange limitation, pressure drop and eventually
87 complete clogging of the systems [23,24]. Such phenomena are observed with river waters but also
88 when drinking waters are used for cooling the systems, especially if they are oversaturated with
89 respect to CaCO_3 .

90 In drinking water pipes, relatively thin viscoelastic biofilms are made up of highly diversified
91 bacterial populations embedded in an exopolymer matrix [25,26]. They represent a major reservoir for
92 many undesirable bugs and sometimes pathogens [27] that distribution system managers want to
93 prevent.

94 [Despite the above-mentioned studies documenting the inactivation effect of US waves on biofilms](#)
95 [composed of pure bacterial strains, almost nothing is known on the effect of ultrasound on complex](#)
96 [bacterial consortia from drinking water and scaling.](#) Thus in this work we assessed the preventive
97 effect on drinking water biofouling of continuous power ultrasonic waves of low frequency generated
98 by transducers of low electric power (10 and 25 W) [provided by SOFCHEM; TWIN f@ Ultrasonic](#)
99 [system](#), already described by [28]. [The novelty of the study resides first on the dual experimental](#)
100 [approach used.](#) Two drinking water systems with specific geometry and operating conditions were
101 tested: (i) a lab-scale plate heat exchanger operating in batch with continuously recycled water, i.e.
102 planktonic bacteria were constantly exposed to ultrasonic waves over the 14-day assay period and (ii)
103 a continuously fed flow-through pilot used for mimicking water circulation in pipes (Propella™
104 reactor), i.e. planktonic bacteria were exposed to ultrasonic waves for a time equal to the hydraulic
105 residence time of the reactor, which is 48 h. Both systems were fed with drinking water of different
106 chemical composition ('N' and 'M', respectively). The number of bacterial cells (planktonic and
107 sessile), the saturation index of water and the calcium deposited on the materials were used as
108 indicators of the ultrasound efficiency. [Finally, the originality also belongs to the pioneering results](#)

109 obtained ~~here~~ that show for the first time that although ultrasound treatment did not prevent initial
110 bacterial adhesion and CaCO₃ deposition, it markedly limits biofilm formation and scaling. This
111 highlights the value of continuous application of low-frequency ultrasound waves in preventing
112 biofilm formation and scale deposits in drinking water systems.

113

114 2. Materials and methods

115 2.1. Characteristics of the waters

116 Drinking waters from two origins were used for cooling the heat exchanger and feeding the Propella™
117 reactor. The water 'N' was a relatively soft water from the network of the city of Nancy, F and was
118 spiked with Ca(OH)₂ (final concentration 100 mg l⁻¹), and pH adjusted to 9.0 with HCl to get
119 oversaturated water (saturation index, SI = 1.6). The water 'M' was a scaling water issued from the
120 network of the city of Metz, F. Table 1 shows some of the physicochemical and microbiological
121 characteristics of these two waters. The calcium carbonate equilibrium of the waters was expressed
122 through the saturation index (SI) such as $SI = pH - pH_s$. The pH_s is obtained from the equation: $pH_s =$
123 $pK_2 - pK_s + p[Ca^{2+}] + p[HCO_3^-] + 5pfm$; where K_2 is the carbonic acid dissociation constant; K_s is
124 the CaCO₃ solubility constant; $[Ca^{2+}]$ is the concentration of calcium ions (g-moles L⁻¹); $[HCO_3^-]$ is the
125 concentration of hydrogen carbonate ions (g-moles L⁻¹); and f_m is the ionic strength [29]. The two
126 waters were oversaturated with respect to CaCO₃ and non-corrosive (SI > 0), which should favour
127 scaling of the materials.

128

129 2.2. Heat exchanger

130 Two lab-scale heat exchangers (Flexiplak GCP-012 equipped with six stainless-steel plates - length 42
131 cm; width 12 cm; thickness 0.06 cm) (Figure S1 in the Supplemental material) - one for the assay, the
132 other one as control - were connected on one side to deionized warmed water (> 40°C) and on the
133 other side to cooling drinking water 'N' (average temperature 23 ± 1 °C) spiked with Ca(OH)₂ (final
134 concentration 100 mg l⁻¹). A schematic view of the pilot setup is shown on Figure S2 in the
135 Supplemental material. At T0, the whole system was filled with 25 L of oversaturated 'N' water. As

136 water was continuously recycled throughout the assays (batch), the hydraulic residence time (HRT)
137 was equivalent to the duration of the assays (14 days in most of the assays). The transducer generating
138 ultrasonic waves provided by the Sofchem company (Rueil Malmaisons, France) (Figure S1) consisted
139 of a single-frequency device connected to a computer-driven generator. The transducer was tightly
140 fastened to a metal bar in contact with the six heat exchanger plates. The ultrasound frequency
141 delivered by the transducer was selected from previous works [18,28] and two electric powers were
142 tested: 10 and 25 W corresponding to a theoretical intensity of 0.5 and 1.25 W cm⁻² of the transducer.
143 The good transmission of the ultrasonic waves to all plates and the control of the frequency were
144 checked thanks to a piezometric sensor (Piezotech, Arkema, Lyon, France).

145

146 2.3. Propella reactors

147 Two Propella™ reactors (one assay, one control) were made up of 100 mm diameter by 500 mm long
148 stainless-steel pipes, (Figure S3 in the Supplemental material). A schematic view of the pilot system is
149 shown in Figure S4 in the Supplemental material. The water velocity in the reactor was controlled
150 with a marine propeller (300 rpm), which pushed the water through an inner stainless-steel cylinder,
151 generating a flow parallel to the pipe wall ($\sim 0.1 \text{ m s}^{-1}$). These flow-through fed reactors were
152 continuously fed with drinking water 'M' (37.5 ml h⁻¹) providing a hydraulic residence time of 48
153 hours. Four lines of four 2 cm² coupons made of unpolished stainless steel (SS 316L) were immersed
154 in the Propella™ reactors [in the annular space between the pipe wall and the inner cylinder \(Figure](#)
155 [S3\)](#) in order to analyze the deposits (bacteria + scale). The ultrasound transducer provided by the
156 Sofchem Company was tightly secured on the side of the Propella™ reactor at ~ 10 cm from the
157 bottom (Figure S3). The supplied electrical power to the transducer was fixed at 10 W. Proper
158 transmission of the ultrasonic waves into the pipe wall was checked thanks to a piezometric sensor
159 (Piezotech, Arkema, Lyon, France), and the distribution of ultrasound waves inside the bulk water was
160 measured thanks to a 0.5 mm needle hydrophone (Precision acoustics Ltd) connected to an
161 oscilloscope (HDO4022 - 200 MHz 154 High Definition 2.5 GS/s), via a booster amplifier of a
162 minimum gain of 25 dB.

163

164 2.4. Bacterial cell counting in water and in the biofilm

165 The total number of cells was determined after bacterial staining with SYBR™ Green I Nucleic Acid
166 Gel Stain (S7563, Invitrogen, France) at a final concentration 1X for 15 min in the dark. For some
167 water analysis, quantification of the membrane-altered bacterial populations was determined using
168 SYTOX™ Orange Nucleic Acid Stain (S11368, Invitrogen, France) at a final concentration of 0.25
169 µM and an incubation for 15 min in the dark). Biofilms analysis were assessed after dispersion of the
170 attached bacteria cells from the surfaces (20 cm² surface area for heat exchanger plate; 2 cm² for the
171 coupons immersed in Propella reactor) by gentle sonication (20 kHz; power output 10 W) for 2 × 30 s,
172 using an immersion ultrasonic probe (Ø 9 mm; Labsonic B. Braun) placed in 10 ml distilled-bacteria-
173 free water covering the analyzed surface. Then staining of the dispersed biofilm and counting were
174 done as for water samples.

175 For the heat exchanger experimentations, both the water and dispersed-biofilm samples were
176 analysed by flow cytometry (FCM) performed with the BD Accuri™ C6 flow cytometer (BD
177 Biosciences, USA), equipped with two lasers (a blue one at 488 nm and a green one at 552 nm).
178 Commercial ultrapure water (Aqua B. Braun, Braun, France) was used as sheath fluid. Bacteria DNA-
179 stained with SYBR-I were detected using the FL2 detector (510 ± 15 nm) and bacteria DNA-stained
180 with SYTOX-Orange by the FL1 detector (610 ± 20 nm). Events were triggered on the forward scatter
181 parameter with a threshold 5000 and on FL1 or FL2 with a threshold 2000 according to the detector
182 used. The data were analyzed using BD Accuri™ C6 software (BD Biosciences).

183 For Propella™ reactor assays, total bacteria cell counting was done by epifluorescence
184 microscopy. Water and dispersed-biofilm stained samples were filtered through 25 mm diameter, 0.2
185 µm pore-size black polycarbonate membranes (Nucleopore, Whatman, VWR, France). After mounting
186 the membrane in anti-fading mounting oil (Citifluor, Ltd., London), counting was performed with an
187 epifluorescence microscope (BX40, Olympus, Japan) equipped with a ×100 immersion objective lens,
188 a 470–490 nm excitation filter and a 520 nm barrier filter. Between 30 and 100 randomly chosen
189 microscopic fields were counted for each sample. Moreover, direct *in situ* staining with SYBR™
190 Green I was also done on coupons after the biofilm dispersion procedure in order to quantify the
191 residual biofilm cells on the surface. This constituted an indirect estimation of the adhesion strength of

192 the biofilm onto the surface, which permitted to appreciate the role of ultrasound waves on the biofilm
193 cohesion. Bacteria cell counting was done after staining and direct observation of the coupons under
194 epifluorescence microscope as for counting on membrane. As residual biofilm could represent in some
195 conditions a significant fraction of the biofilm, we expressed the total number of biofilm cells as the
196 number of biofilm-dispersed bacteria plus the residual bacteria cells on coupon. Altogether, the
197 number of bacteria was expressed as cells ml⁻¹ and cells cm⁻² for water and biofilm, respectively.

198

199 2.5. Cultivable bacteria counting and their identification

200 The number of cultivable bacteria in water from heat exchangers and Propella was determined by
201 counting colonies grown on R2A agar (Oxoid, CM0906, Thermo Fisher Scientific, Dardilly, France)
202 after 2-day incubation at 30 ± 1°C (to be close to the water temperature during the assays). The results
203 were expressed as colony forming unit (CFU-2d) per milliliter. Identification of the bacterial colonies
204 grown on R2A agar was done by MALDI-TOF-MS (Microflex LT[®], Bruker, Champs sur Marne,
205 France). MALDI-TOF (Matrix-assisted laser desorption/ionisation-time of-flight) is a technology
206 based on mass spectrometry, analysing the proteins of microorganisms to serve as a landmark for
207 acute identification. Proteins are first co-crystallised in a matrix then ionised by a laser beam
208 (MALDI). The released ions are accelerated and separated in a vacuum tube under the action of an
209 electric field based on their mass/charge ratio, and detected individually over time by a particle
210 detector (TOF). The entire set of molecules is therefore displayed as a series of peaks forming a
211 spectrum which is characteristic of an organism and is compared to a database of reference spectra,
212 thus enabling identification [30].-~~(matrix-assisted laser desorption/ionization time-of-flight mass~~
213 ~~spectrometry) (Microflex LT[®], Bruker, Champs sur Marne, France).~~ For each sample, three colonies
214 of each morphological type were re-isolated on R2A agar and one colony per isolate was identified.
215 Direct spotting of bacteria cells and full protein extraction using formic acid were performed following
216 the manufacturer's recommendations. After drying each spot at room temperature, 1 µL matrix
217 HCCA (α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) was added
218 before analysis. The identification criteria used were those recommended by the manufacturer.

219

220 2.6. Calcium carbonate analysis on the coupons

221 For measurements on heat exchanger plate, ~~thanks to an home-made plastic frame,~~ deposits from a
222 3.80 cm² surface area were directly acidified ~~to pH near 0~~ with 10 ml of ultra-pure HNO₃ (65 %) and
223 filtered through 0.2 μm polyethersulfone filters) (Millex GP, Merck Millipore Ltd, Molsheim, France)
224 ~~to reach a pH near 0. The calcium was analysed by HORIBA, Longjumeau, France).~~ The elemental
225 analysis of calcium, which is a direct indicator of scaling, was performed by inductively coupled
226 plasma optical emission spectrometry, ICP OES (HORIBA, Longjumeau, France) [31]. For Propella™
227 reactor, the whole coupon (2 cm²) was immersed into 10 ml of ultra-pure HNO₃ and the dosage was
228 performed as for heat exchanger plate.

229

230 3. Results

231 3.1. Assessment of ultrasound wave transmission in the heat exchangers and the Propella™
232 reactors
233

234 When a transducer is used to generate ultrasound, it is important not only that it is properly coupled to
235 the medium into which it is supposed to emit, but also that transmission is not hampered by severe
236 acoustic mismatches or other issues. If that medium is a solid structure and the aim is to further
237 transmit the ultrasonic waves into the bulk of water present inside the structure, then additional
238 concerns must be raised and checked as to how effectively sound is actually emitted into the liquid. It
239 is with these issues in mind that measurements were taken on the side of the plates of the exchanger
240 (not in the bulk as it was not possible to measure ultrasound distribution in the bulk water in such
241 closed systems) and at different locations in the bulk of the liquid of the Propella™ reactor to verify
242 that indeed sound was effectively transmitted from the generating transducer, through the structure,
243 into the liquid.

244 For the heat exchanger, the electric tension (mV) measured with the piezometric sensor on the side of
245 the plates was five times lower than that measured directly at the surface of the transducer, due to
246 some loss of energy between the transducer and the metal bar, and the metal bar and the plates. As a
247 consequence, the plates have been subjected to a low acoustic intensity close to 2 and 5 W/cm² during

248 the assays.
249 For the Propella™ reactor, due to the geometrical complexity and the inherent diffraction and
250 scattering of acoustic waves, the sound field in the liquid was expected to be complicated and
251 absolutely not homogeneous. The distribution of ultrasound waves inside the bulk water was measured
252 with an hydrophone connected to an oscilloscope (Figure S5). To investigate the local spectra, a
253 discrete Fourier transform was performed using a fast Fourier transform algorithm (FFT). The signal
254 showed a major resonance frequency at 46 KHz and its harmonic at 92 KHz. The occurrence of
255 additional lobes in between the secondary lobes was caused by windowing of the temporal signal.
256 More importantly however, the intensity of the signal measured was highly dependent on the position
257 of the hydrophone, which indicates, as may be expected, an acoustic field as the result of the
258 complicated interaction of sound with the structure under examination, determined by scattering
259 effects in and behind the internal cylinder.

260

261 3.2. Effect of ultrasound waves on the saturation index and planktonic biomass of waters
262 circulating in the heat exchanger
263

264 The saturation index (SI) and the number of bacterial cells were measured in the bulk water ‘N’
265 circulating in the cooling circuit of the heat exchangers after 14 days of work (Table 2). SI was lower
266 in the control (no ultrasound) comparatively to the pilot treated with ultrasonic waves, especially when
267 a high power (25 W) was applied. It means that the scaling potential of the water was decreased in the
268 control after 14 days, and that the accumulation of carbonate on the surface was limited, unlike in the
269 ultrasound assays.

270 The number of total bacterial cells in the bulk water ‘N’ was relatively high in the control (no
271 ultrasound) (approximately $1.4 \times 10^5 \text{ ml}^{-1}$) (Table 2), as a result of bacterial growth within the
272 experimental system during the 14-day assay period (Figure S6 in Supplemental material). In the
273 assays with ultrasound, the number of total bacterial cells was decreased by a factor 7 ($2 \times 10^4 \text{ ml}^{-1}$)
274 and equal to the number found in the feeding water (Table 1). This lower bacteria density in treated
275 water indicated a bacterial growth inhibition by ultrasonic waves, evidenced after 10 days of work

276 (Figure S6 in Supplemental material). However, at the single cell level, the mean fluorescence
277 intensity of the stained cells was not decreased by the ultrasounds (Figure 1a), suggesting no nucleic
278 acid alteration as SYBR-I still complex to DNA. Only the distribution of the granulosity of cells (SCC
279 parameter) was changed from a multimodal distribution in the control without US waves to a lower
280 and more homogeneous granulosity in the treated samples (Figure 1b).

281 The inhibition of bacteria growth in water was confirmed by the reduction of the number of cultivable
282 bacteria from 18 % of the total number of cells in the control to 2 % in the assays at 10 W (Table 2).
283 Difference in the phenotype of cultivable bacteria was also explored as two types of colony appeared
284 according to the treatment: white- and orange-coloured colonies were largely represented in the
285 control, whereas in the assays only orange-coloured colonies were observed in the assays with
286 ultrasound. Identification with ~~Maldi~~MALDI TOF-MS indicated that *Pseudomonas stutzeri* (high
287 probability at the species level) was representative of half of the white colonies, and *Blastomonas*
288 *ursicola* (high probability at the genus level) was representative of most of the orange colonies.

289 Complementary measurements done with SYTOX® Orange dye used for staining bacterial cells with
290 compromised cytoplasmic membranes showed also two populations, one brighter than the other one
291 (Figure 2). Only the former is representative of damaged membrane of bacteria. This total SYTOX-
292 fluorescent population (ie highly fluorescent bacteria plus slightly fluorescent bacteria) represented
293 approximately 50 % of the total population measured by SYBR-I staining both in the control and in
294 the assay at 10 W (Table 3). In the control, 15 % of the total bacteria were highly-fluorescent with
295 SYTOX meaning that the cells had damaged-membranes, when 34 % of the bacteria were only
296 slightly stained with SYTOX (low permeability to SYTOX). A relatively similar distribution was
297 observed in the assays with US (transducer at 10 W), with 8.6 % of highly-fluorescent cells with
298 SYTOX (damaged membranes), while 35.6 % of slightly fluorescent cells (low permeability to
299 SYTOX). Such a similar distribution in the assay and the control (especially no increase in the highly-
300 fluorescent population) suggested ultrasonic waves did not affect significantly the permeability of the
301 bacterial envelopes.

302

303

304 3.3. Effect of ultrasound waves on the fouling (calcium deposit and biofilm) of heat exchanger
305 plates
306

307 The calcium concentrations deposited at the surface of the heat exchangers plates were low, on
308 average $258 \mu\text{g}/\text{cm}^2$ in the control, and $12 \mu\text{g cm}^{-2}$ and $0.8 \mu\text{g cm}^{-2}$ for the two exchanger subjected to
309 ultrasound (transducers at 10 W and 25 W, respectively) (Figure 3). These results completed and were
310 consistent with our previous SI-related observations, ie the higher the SI of the waters was, the lower
311 the calcium deposit on the plate surface was. It also evidenced that deposits (mineral and biological)
312 occurred even during the US treatment, suggesting that the generated vibrations within the stainless
313 steel plates did not prevent their attachment in the time course of the assay.

314 As reported for water, the number of bacterial cells of the biofilm accumulated on the stainless-steel
315 surfaces of the control plates (2 to 9×10^5 cells cm^{-2}) was decreased by a factor of 7 in the reactors
316 treated by ultrasounds (Figure 4). This is coherent with the lower number of bacteria in 'N' water
317 treated with US waves (transducer 10 W). Flow cytometry (FCM) was applied in order to quantify
318 SYBR-I fluorescence in single cells to discriminate between low and high fluorescent bacteria,
319 revealing an extinction of the fluorescence of the stressed bacteria that could be explained by
320 alteration of the nucleic acids (ie target of SYBR-I). Whereas the bacterial number was lowered by
321 ultrasound, the mean fluorescent intensity of biofilm bacteria subjected to ultrasound was comparable
322 to the control (Figure 5), indicating no detectable alteration of nucleic acids took place in bacteria
323 subjected to ultrasound.

324 All together these results suggest that the ultrasound irradiations mostly inactivate the bacteria
325 activity, which results in growth reduction and cultivability decrease but did not compromised cell
326 membrane permeability (at least to SYTOX Orange).

327

328 3.4. Effect of ultrasound waves on planktonic bacteria and biofilm accumulation in pipe reactors
329

330 Flow-through reactors mimicking water distribution pipes (PropellaTM reactors) were equipped with
331 suspended coupons and continuously fed with drinking water 'M'. In the bulk of water, the number of
332 bacterial cells measured at pseudo-stationary state (between days 18 and 28) was systematically higher

333 in the control than in the reactor subjected to ultrasound waves (power 10 W) (Figure 6 and Figure
334 S7). The dynamic of the bacteria within the reactors was also interesting as differences between
335 control and assays appeared after 7 HRT. Indeed in the Propella™ submitted to ultrasound waves the
336 bacterial number increased in the liquid phase until day 13 and then tended to decrease to the initial
337 values of the feeding water, whereas bacterial density continuously increased in the control reactor
338 (Figure S7). This again suggests that a limitation of the bacterial population activity within the reactor.
339 On the stainless-steel coupons immersed in the Propella™ reactors, the difference in the number of
340 biofilm cells in the assay and the control was approximately of 1 log (Figure 6 and Figure S8). It
341 means that bacteria cells attached to the material in spite of the ‘vibration’ of the surface, but their
342 accumulation or growth was reduced in the time-course of the experiment (28 days). In the reactor
343 subjected to ultrasound waves the lower density of attached bacteria in the reactor submitted to
344 ultrasound waves matched with the lower number of cells in the water bulk. The good reproducibility
345 of the results was interesting although the distribution of the US wave frequency inside the Propella™
346 was not homogeneous (Figure S5).

347 Additionally, the bacterial adhesion strength onto the stainless-steel surfaces was indirectly estimated
348 by comparing the number of bacteria remaining on the coupon after application of the lab-procedure
349 for biofilm detachment (Table 4). The remaining biofilm was equal to 2.5 % of the total biofilm for
350 coupons subjected to ultrasound waves compared to 10 % of the total biofilm for coupons in the
351 Propella™ control.

352

353 **4. Discussion**

354 Our investigation was carried out with ‘power ultrasonic waves’ at low frequency (46 KHz and 92
355 KHz) and low intensity according to the classification of [32,21]. A low intensity is interesting as it
356 could be below the “acoustic cavitation threshold” [33], which results in material damage being
357 avoided. Ultrasound waves applied continuously improved the ‘chemical stability’ of the water and
358 limited the quantity of calcium carbonate deposition. It is in good agreement with the observations of
359 Nakagawa et al. [34] who reported an effective hard deposit exfoliation at the antinodes of vibration.

360 Vasyliiev et al. [35] have recently confirmed the effect of ultrasounds on scale demonstrating that
361 depending on their intensity, ultrasound can cause either a surface clean-up by cavitation [36] or a rise
362 of CaCO₃ nucleation site density, building a protective scale layer on the surface. However
363 understanding the mechanisms through which ultrasonic waves affect such carbonate deposition is not
364 straightforward. On the one hand, the complexity of the CaCO₃ deposition on a flow surface arises
365 from the numerous multicomponents of the system that include all ionic species, mass transfer and
366 chemical reactions [37], and the synergistic effect of suspended solids [23]. On the other hand,
367 ultrasound can promote carbonate precipitation in solution rather than on the solid surface [38]. In
368 other words, the lower deposition of calcium on the heat exchanger plate could be explained by both
369 cavitation erosion and changes in the calcocarbonic equilibrium of the bulk.

370 These continuous low-intensity and low frequency ultrasound waves were affecting both the
371 biofilm and planktonic cell number and activity. Indeed, whatever the tested systems (heat exchangers
372 and Propella™ reactor) the number of attached cells was 1 log lower with ultrasound treatment than in
373 the control without ultrasound treatment. The number of attached cells measured throughout the
374 assays subjected to ultrasound was equivalent to the number of bacteria generally measured after a few
375 hours of immersion and in the order of 10⁴ cm⁻² [39, 26]. It means that the initial adhesion of the
376 drinking water bacteria on the surfaces was not significantly affected by the vibrations (as previously
377 reported by [40] with *S. aureus* adhering on polyethylene rods), but that their activity was hindered
378 preventing their growth. As a result, the number of cultivable bacteria exposed to ultrasound was
379 lower. The low bacterial activity could explain the lower attachment strength of the cells to the
380 sonicated materials as the matrix exopolymer synthesis may have also been inhibited, lowered or
381 delayed. Our observation contrasts with that of [40,41] who reported about the growth stimulation of
382 some bacteria and fungus. However our results are in line with that of Joyce et al. [42,43] and Gao et al.
383 [44], who reported a loss of cultivability. Another unexpected finding from our work is that the
384 cytoplasmic membrane permeability was not really compromised as the permeability to specific
385 fluorochrome such as SYTOX Orange was not modified. This is contrary to previous results
386 [45,46,47] who reported a membrane permeability enhancement but for assays done at much higher
387 frequencies (500 kHz and 1.65 MHz), or higher power, excepted Dong et al. [48] who worked in the

388 same rank of frequency (42 KHz) and intensity ($<1 \text{ W/cm}^2$). One may also suppose that low frequency
389 and low intensity ultrasound perturbations of the envelopes, if any, would be easily and rapidly
390 reversible after the ultrasound irradiation stop. In this study we measured a 46 KHz ultrasound wave in
391 the water and one harmonic of lower intensity at 92 KHz. Then, it is difficult to link the observed
392 effect of ultrasound to one or to the other of these two wavelengths, or their combination. No other
393 frequency, which could have been generated by the vibrations of the plates and pipe walls, was
394 detected in the water.

395 An abundant literature has been produced on the effect of ultrasonic waves on planktonic and
396 sessile algae (see the review of [3,17,4,521,17,49,5]). According to it, there is no universal optimal
397 frequency as alteration of algal cells was obtained in the range of 20 to 1,320 KHz depending on the
398 ultrasound generator and the algal species. Despite evidences supporting the efficiency of low-power
399 ultrasonic waves on algae and invertebrates such as barnacles, few tests have been conducted on
400 bacteria and carbonate deposition under drinking water conditions. *A priori*, the use of low-frequency
401 ultrasound waves for disturbing/inactivating bacterial cells, which behave as $0.2 \mu\text{m}$ soft colloids, is
402 very challenging. Indeed, in water the ultrasound velocity is of $1,500 \text{ m s}^{-1}$, and the wavelength of the
403 ultrasound applied in this work is close to 3×10^{-2} meter. It means that the small planktonic cells should
404 not be directly affected by such a wavelength/frequency. The same can be said for $100 \mu\text{m}$ viscoelastic
405 young biofilm clusters, which should absorb ultrasound wave energy with reduced impact on the
406 structure. Indeed, the absorption of sound in water-like materials at 46 kHz is in the order of 0.8
407 dB/km [50] and capsular material has been identified as a protective structure [51].

408 Ultrasound-induced inhibition of bacterial growth could be explained by some other
409 mechanisms such as cavitation and pressure variations. First, microbubble cavitation (especially
410 ‘asymmetric collapse’ close to the surfaces) generating locally reactive oxidative species (ROS), and
411 the fast flow rate of water (up to $100 \mu\text{m/s}$) generated by microstreaming [9,52,4,53] have a major
412 effect on bacteria and biofilms [54,55]. Cavitation even in the case of low-power, low-frequency
413 treatment represents a possibility that cannot be excluded as such a phenomenon is dependent on many
414 parameters such as temperature, quantity of dissolved gases and physicochemical heterogeneities on
415 surfaces or in the bulk (particles). Second, rapid pressure variations from positive to negative values (=

416 46,000 times per second) may also damage some cell structures such as the membrane and prevent
417 adaptation or repair. Indeed, many bacteria do not adapt so easily to rapid hydrostatic pressure changes
418 [56,57].

419

420 **5. Conclusion**

421 Mitigating (bio)-fouling with a low-frequency and low-intensity ultrasound device was tested
422 under conditions relevant to engineered drinking water systems. The ultrasound devices were tightly
423 fastened outside the systems allowing an ultrasound application without stopping the water system
424 work. Continuous power ultrasonic waves of low frequency (major resonance frequency at 46 KHz
425 and its harmonic at 92 KHz) generated by transducers of low electric power (10 and 25 W) did limit
426 drastically drinking water biofilm accumulation in the two systems tested ~~in this study~~ (a heat
427 exchanger with recycling water loop, and a continuously fed flow-through reactor equivalent to a
428 distribution system pipes). In the two reactors, ~~biofilm bacterial density was limited to approximately~~
429 ~~10^4 to 10^5 cells cm^{-2} equivalent to the initial attachment step without any bacterial attachment to the~~
430 walls was not prevented (approximately 10^4 to 10^5 cells cm^{-2} cells stick in a few hours onto the
431 material surfaces) but the activity of the cells was limited such as no further multiplication of the
432 attached cells and no biofilm growth were observed. Moreover the attachment strength of the ~~biofilm~~
433 bacterial cells in the assay with ~~subjected to~~ ultrasound waves was weaker than in the control,
434 promising an easier further cleaning of the heat exchanger and pipes if needed. Additionally,
435 waterborne planktonic bacteria submitted to the ultrasound waves transmitted throughout the walls
436 behave the same way as the attached cells (no growth, low cultivability on nutritive medium). At such
437 a low frequency and low intensity, cell membrane damaging did not appear as the first cause of ~~no~~
438 growth arrest. Lastly, calcium concentrations deposited at the surface of the heat exchanger were
439 lower thanks to the ultrasound treatment, which should participate to a lower fouling and clogging of
440 heat exchangers.

441 Low-frequency and low-intensity ultrasound technology sounds very promising in drinking
442 water biofilm mitigation. Such an effect was not predictable as preventing attachment of bacteria,
443 which behave as soft colloids, was expected to be very difficult and the thin viscoelastic drinking

444 water biofilms were expected to be insensitive to such waves. Future works should contribute to a
445 better understanding of ultrasound mechanistic effects on bacterial cells and optimizing industrial
446 application (different modes of application e.g. continuous *versus* discontinuous).~~and both different~~
447 ~~modes of application (e.g. continuous *versus* discontinuous) and cellular mechanisms should be~~
448 ~~further explored.~~

449

450

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455

456

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459

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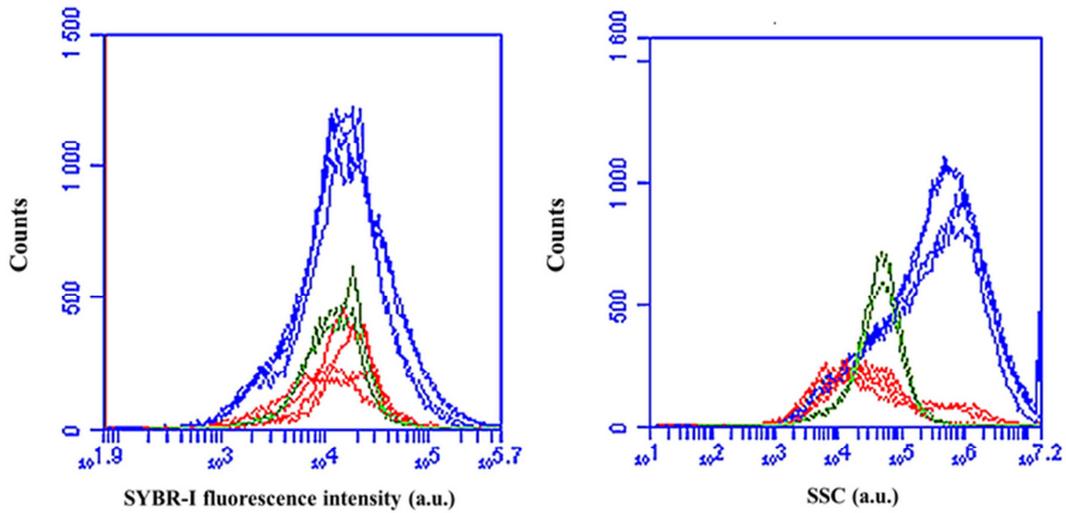
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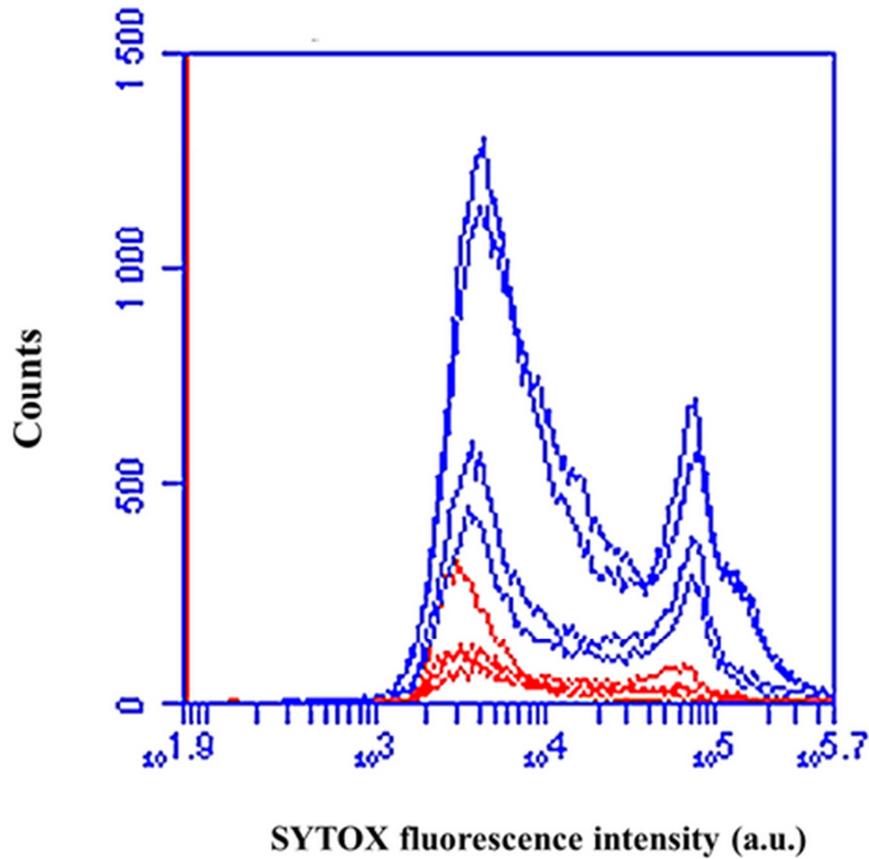
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Figure 1: Distribution of the number of SYBR-I stained planktonic bacteria (FCM measurement) in water 'N' at day 14 of experiment in the heat exchanger. (a) Fluorescence intensity (FL2 detector) and (b) granulosity (SSC parameter). Blue curves correspond to control without treatment (n=4), red curves correspond to the assay with ultrasound (transducer at 10 W) (n=4), green curves correspond to assay with ultrasound (transducer at 25 W) (n=2).

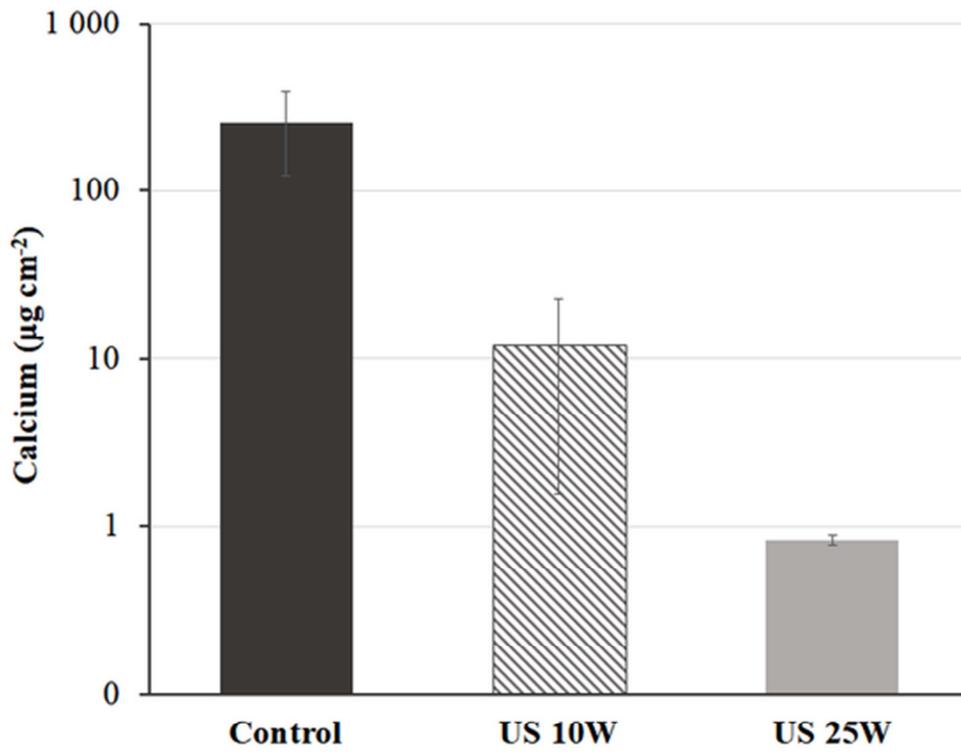
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Figure 2: Distribution of the number of planktonic bacteria stained with SYTOX Orange in water ‘N’ at day 14 of experiment in the heat exchanger. Blue curves represent the control without ultrasounds and red curves correspond to the assays with ultrasounds at 10 W. Four independent assays were performed.

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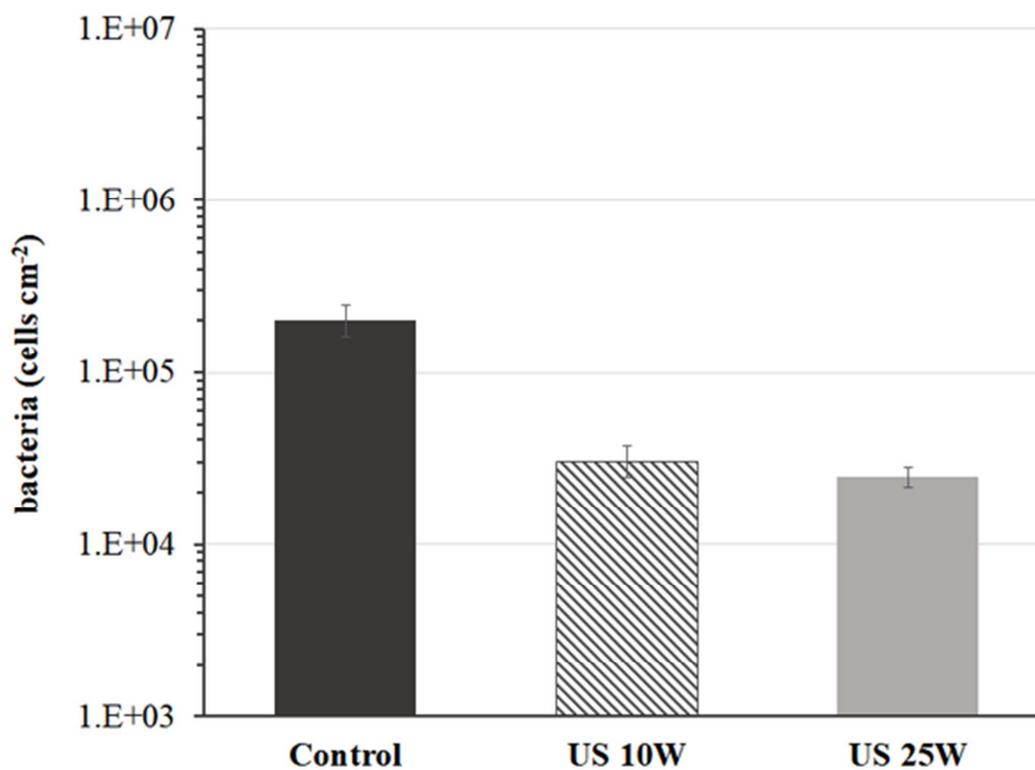


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Figure 3: Calcium ($\mu\text{g cm}^{-2}$) accumulated in 14 days on the surface of the heat exchanger stainless steel plates fed with water 'N' (n= 3 to 4).

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675 **Figure 4:** Number of SYBR-I stained bacterial cells measured by FCM in the biofilm accumulated in
676 14 days on the surface of the heat exchanger plates fed with water 'N'. (n=4).

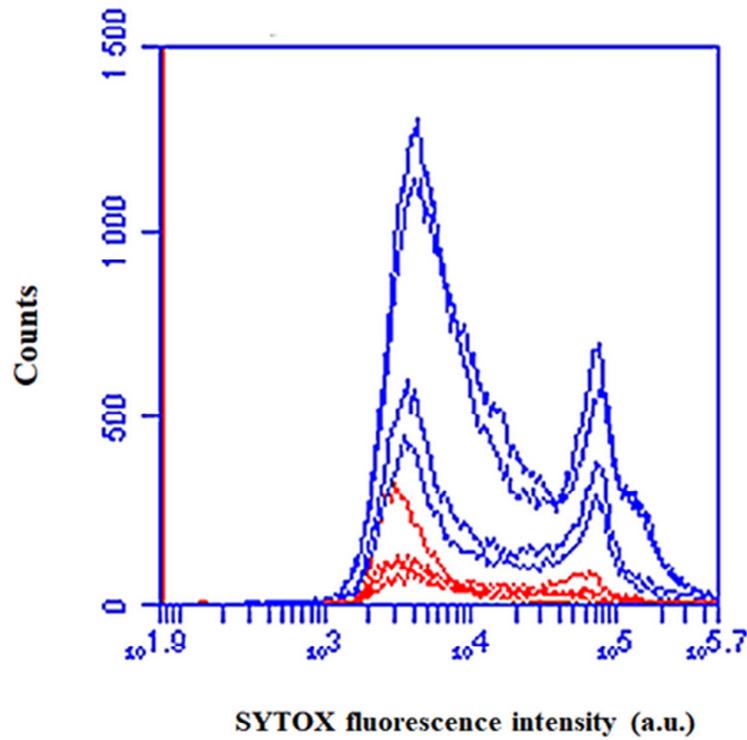
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684 **Figure 5:** Distribution of Fluorescence intensity (FL2 detector) of the SYBR-I stained biofilm bacteria
685 (FCM measurement) at day 14 of experiment in the heat exchanger fed with water 'N'. Blue curves
686 correspond to control without treatment (n=4), red curves correspond to the assay with ultrasound
687 (transducer at 10 W) (n=4), green curves correspond to assay with ultrasound (transducer at 25 W)
688 (n=2).

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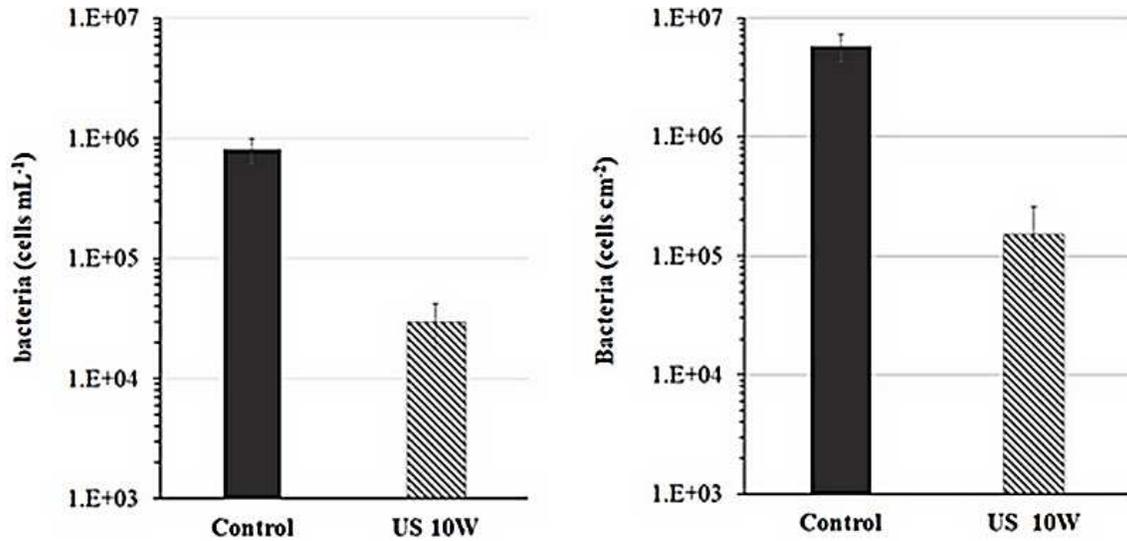
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Figure 6: Number of bacterial cells after SYBR-I staining at pseudo-steady state (between days 18 to 28 of kinetics) in the water phase (planktonic cells) (A) and within the biofilms (attached cells) formed on stainless-steel coupon immersed in the flow-through Propella reactors fed with water ‘M’ (control = no ultrasound; assay = ultrasound – power 10 W). Histograms correspond to averages of 4 values and the error bar represents the SD values.

Table 1: Physicochemical and microbiological characteristics of water ‘N’ (after spiking with Ca(OH)_2 and pH adjustment) and water ‘M’ (n = 2 to 4).

Parameters	Drinking water ‘N’	Drinking water ‘M’
Bacteria (cells mL^{-1})	2.2×10^4	2.6×10^4
pH	9.0	8.3
Conductivity ($\mu\text{S cm}^{-1}$)	1,190	1,031
Chlorine ($\text{mg Cl}_2 \text{L}^{-1}$)	< 0.05	< 0.05
Calcium (mg L^{-1})	54	97
Complete alkalimetric title (CAT) ($^\circ\text{f}$)	29	24
Total hardness (TH) ($^\circ\text{F}$)	9	29
Saturation index (SI)	1.6	1.4

Table 2: Saturation index and number of bacterial cells after SYBR-I staining and FCM quantification at day 14 in the cooling water ‘N’ circulating in the heat exchangers (n=2 to 4). The values between brackets represent the standard deviations.

	Saturation index	Bacteria (cells mL⁻¹)	Bacteria (CFU mL⁻¹)
Control	0.11 (± 0.02)	1.4×10 ⁵ (± 6.9 × 10 ⁴)	3.6×10 ⁴
Assay (10 W)	0.23 (± 0.03)	2.0×10 ⁴ (± 2.6 × 10 ³)	3.8×10 ²
Assay (25 W)	0.47 (± 0.01)	2.7×10 ⁴ (± 3.5 × 10 ³)	not measured

Table 3: Mean proportions (%) of non-fluorescent, slightly fluorescent, and highly fluorescent cells after staining with SYTOX Orange in the 'N' water sampled at day 14 in the heat exchangers (control and assay). Data are average values on 4 independent assays for control and the assays with ultrasound.

	Total cells (a) (See table 2)	Non-fluorescent cells (b)	Slightly fluorescent cells (c)	Highly fluorescent cells (d)
Control	100%	50.6 (\pm 9.4)	34.7 (\pm 7.4)	15.1 (\pm 2.2)
Assay (10 W)	100%	56.1 (\pm 16.7)	35.6 (\pm 15.9)	8.6 (\pm 7.0)

(a) = SYBR-I staining of the cells measured by FCM

(b) = fraction of SYBR-I stained cells not stained by SYTOX Orange on the same sample.

(c) = fraction of the bacteria cells with low fluorescence stained by SYTOX Orange within the total SYBR-I stained cells

(d) = fraction of SYTOX Orange stained cells with high fluorescence compared to total cells stained by SYBR-I

Table 4: Number of total bacterial cells cm^{-2} (SYBR-I staining) on the coupons immersed for 14 days in the Propella reactor (water 'M') and the fraction remaining after the lab-procedure detachment for biofilm analysis (n=3).

	Control (no ultrasound)	Assay (with ultrasound)
Biofilm bacteria	$4.3 \times 10^6 (\pm 2.9 \cdot 10^6)$ (100 %)	$1.6 \times 10^5 (\pm 1.0 \cdot 10^5)$ (100 %)
Remaining biofilm bacteria after lab- detachment procedure	$5.8 \times 10^3 (\pm 4.6 \cdot 10^3)$ (13.5 %)	$5.9 \times 10^3 (\pm 5.0 \cdot 10^3)$ (3.7 %)