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Highlights

- Low-frequency ultrasound was studied on drinking water biofilms and $CaCO_3$ deposits
- Ultrasounds affect both biofilm and planktonic cell number and activity
- US treatment did not prevent initial bacterial adhesion and $CaCO_3$ deposition
- Low-frequency ultrasounds markedly limit biofilm formation and scaling

Abstract

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

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

1. Introduction

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

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

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

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

In heat exchangers, precipitation fouling, ie the formation of a solid layer of deposits of inverse solubility ($CaCO_3$, $CaSO_4$, $Ca_3(PO_4)_2$, etc.), and viscous hydrated organic deposits on the side of plates in contact with coolants determine heat exchange limitation, pressure drop and eventually complete clogging of the systems [23,24]. Such phenomena are observed with river waters but also when drinking waters are used for cooling the systems, especially if they are oversaturated with respect to $CaCO_3$.

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

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

obtained ~~here~~ that show for the first time that although ultrasound treatment did not prevent initial bacterial adhesion and $CaCO_3$ deposition, it markedly limits biofilm formation and scaling. This highlights the value of continuous application of low-frequency ultrasound waves in preventing biofilm formation and scale deposits in drinking water systems.

2. Materials and methods

2.1. Characteristics of the waters

Drinking waters from two origins were used for cooling the heat exchanger and feeding the Propella^{TM} reactor. The water 'N' was a relatively soft water from the network of the city of Nancy, F and was spiked with $Ca(OH)_2$ (final concentration 100 mg l^{-1}), and pH adjusted to 9.0 with HCl to get oversaturated water (saturation index, SI = 1.6). The water 'M' was a scaling water issued from the network of the city of Metz, F. Table 1 shows some of the physicochemical and microbiological characteristics of these two waters. The calcium carbonate equilibrium of the waters was expressed through the saturation index (SI) such as SI = pH – pH_s. The pH_s is obtained from the equation: $pH_s = pK_2 - pK_s + p[Ca^{2+}] + p[HCO_3^-] + 5pfm$; where K_2 is the carbonic acid dissociation constant; K_s is the $CaCO_3$ solubility constant; $[Ca^{2+}]$ is the concentration of calcium ions (g-moles L^{-1}); $[HCO_3^-]$ is the concentration of hydrogen carbonate ions (g-moles L^{-1}); and f_m is the ionic strength [29]. The two waters were oversaturated with respect to $CaCO_3$ and non-corrosive (SI > 0), which should favour scaling of the materials.

2.2. Heat exchanger

Two lab-scale heat exchangers (Flexiplak GCP-012 equipped with six stainless-steel plates - length 42 cm; width 12 cm; thickness 0.06 cm) (Figure S1 in the Supplemental material) - one for the assay, the other one as control - were connected on one side to deionized warmed water (> 40°C) and on the other side to cooling drinking water 'N' (average temperature 23 ± 1 °C) spiked with $Ca(OH)_2$ (final concentration 100 mg l^{-1}). A schematic view of the pilot setup is shown on Figure S2 in the Supplemental material. At T0, the whole system was filled with 25 L of oversaturated 'N' water. As

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

2.3. Propella reactors

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

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 cm^2 surface area for heat exchanger plate; 2 cm^2 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

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

2.5. Cultivable bacteria counting and their identification

The number of cultivable bacteria in water from heat exchangers and Propella was determined by counting colonies grown on R2A agar (Oxoïd, CM0906, Thermo Fisher Scientific, Dardilly, France) after 2-day incubation at 30 ± 1°C (to be close to the water temperature during the assays). The results were expressed as colony forming unit (CFU-2d) per milliliter. Identification of the bacterial colonies grown on R2A agar was done by MALDI-TOF-MS (Microflex LT®, Bruker, Champs sur Marne, France). MALDI-TOF (Matrix-assisted laser desorption/ionisation-time of-flight) is a technology based on mass spectrometry, analysing the proteins of microorganisms to serve as a landmark for acute identification. Proteins are first co-crystallised in a matrix then ionised by a laser beam (MALDI). The released ions are accelerated and separated in a vacuum tube under the action of an electric field based on their mass/charge ratio, and detected individually over time by a particle detector (TOF). The entire set of molecules is therefore be displayed as a series of peaks forming a spectrum which is characteristic of an organism and is compared to a database of reference spectra, thus enabling identification [30]. ~~(matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry) (Microflex LT®, Bruker, Champs sur Marne, France).~~ For each sample, three colonies of each morphological type were re-isolated on R2A agar and one colony per isolate was identified. Direct spotting of bacteria cells and full protein extraction using formic acid were performed following the manufacturer's recommendations. After drying each spot at room temperature, 1 µL matrix1 HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) was added before analysis. The identification criteria used were those recommended by the manufacturer.

2.6. Calcium carbonate analysis on the coupons

For measurements on heat exchanger plate, thanks to an home-made plastic frame, deposits from a 3.80 cm² surface area were directly acidified to pH near 0 with 10 ml of ultra-pure HNO_3 (65 %) and filtered through 0.2 µm polyethersulfone filters~~)~~ (Millex GP, Merck Millipore Ltd, Molsheim, France) ~~to reach a pH near 0. The calcium was analysed by HORIBA, Longjumeau, France).~~ The elemental analysis of calcium, which is a direct indicator of scaling, was performed by inductively coupled plasma optical emission spectrometry, ICP OES (HORIBA, Longjumeau, France) [31]. For Propella™ reactor, the whole coupon (2 cm²) was immerged into 10 ml of ultra-pure HNO_3 and the dosage was performed as for heat exchanger plate.

3. Results

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

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

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

the assays.

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

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

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

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

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

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

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

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

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

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

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

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

Flow-through reactors mimicking water distribution pipes (Propella^{TM} reactors) were equipped with suspended coupons and continuously fed with drinking water ‘M’. In the bulk of water, the number of bacterial cells measured at pseudo-stationary state (between days 18 and 28) was systematically higher

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

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

4. Discussion

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

Vasyliev et al. [35] have recently confirmed the effect of ultrasounds on scale demonstrating that depending on their intensity, ultrasound can cause either a surface clean-up by cavitation [36] or a rise of $CaCO_3$ nucleation site density, building a protective scale layer on the surface. However understanding the mechanisms through which ultrasonic waves affect such carbonate deposition is not straightforward. On the one hand, the complexity of the $CaCO_3$ deposition on a flow surface arises from the numerous multicomponents of the system that include all ionic species, mass transfer and chemical reactions [37], and the synergistic effect of suspended solids [23]. On the other hand, ultrasound can promote carbonate precipitation in solution rather than on the solid surface [38]. In other words, the lower deposition of calcium on the heat exchanger plate could be explained by both cavitation erosion and changes in the calcocarbonic equilibrium of the bulk.

These continuous low-intensity and low frequency ultrasound waves were affecting both the biofilm and planktonic cell number and activity. Indeed, whatever the tested systems (heat exchangers and Propella^{TM} reactor) the number of attached cells was 1 log lower with ultrasound treatment than in the control without ultrasound treatment. The number of attached cells measured throughout the assays subjected to ultrasound was equivalent to the number of bacteria generally measured after a few hours of immersion and in the order of 10^4 cm^{-2} [39, 26]. It means that the initial adhesion of the drinking water bacteria on the surfaces was not significantly affected by the vibrations (as previously reported by [40] with *S. aureus* adhering on polyethylene rods), but that their activity was hindered preventing their growth. As a result, the number of cultivable bacteria exposed to ultrasound was lower. The low bacterial activity could explain the lower attachment strength of the cells to the sonicated materials as the matrix exopolymer synthesis may have also been inhibited, lowered or delayed. Our observation contrasts with that of [40,41] who reported about the growth stimulation of some bacteria and fungy. However our results are in line with that of Joyce et al. [42,43] and Gao et al. [44], who reported a loss of cultivability. Another unexpected finding from our work is that the cytoplasmic membrane permeability was not really compromised as the permeability to specific fluorochrome such as SYTOX Orange was not modified. This is contrary to previous results [45,46,47] who reported a membrane permeability enhancement but for assays done at much higher frequencies (500 kHz and 1.65 MHz,), or higher power, excepted Dong et al. [48] who worked in the

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

An abundant literature has been produced on the effect of ultrasonic waves on planktonic and sessile algae (see the review of [~~3,17,4,5~~21,17,49,5]). According to it, there is no universal optimal frequency as alteration of algal cells was obtained in the range of 20 to 1,320 KHz depending on the ultrasound generator and the algal species. Despite evidences supporting the efficiency of low-power ultrasonic waves on algae and invertebrates such as barnacles, few tests have been conducted on bacteria and carbonate deposition under drinking water conditions. *A priori*, the use of low-frequency ultrasound waves for disturbing/inactivating bacterial cells, which behave as 0.2 µm soft colloids, is very challenging. Indeed, in water the ultrasound velocity is of 1,500 m s^{-1}, and the wavelength of the ultrasound applied in this work is close to 3×10^{-2} meter. It means that the small planktonic cells should not be directly affected by such a wavelength/frequency. The same can be said for 100 µm viscoelastic young biofilm clusters, which should absorb ultrasound wave energy with reduced impact on the structure. Indeed, the absorption of sound in water-like materials at 46 kHz is in the order of 0.8 dB/km [50] and capsular material has been identified as a protective structure [51].

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

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

5. Conclusion

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

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

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

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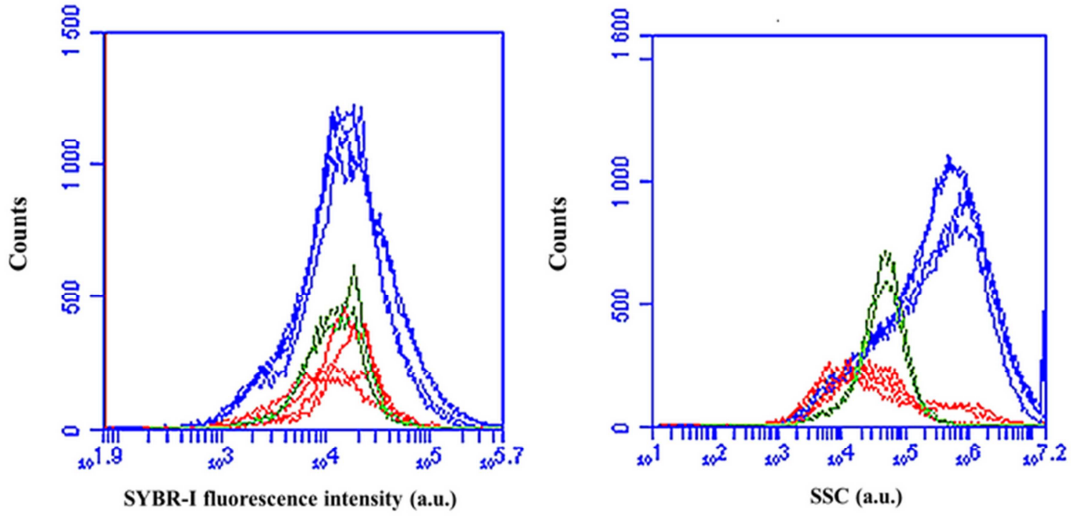

Figure 1: Distribution of the number of SYBR-I stained planktonic bacteria (FCM measurement) in water 'N' at day 14 of experiment in the heat exchanger. (a) Fluorescence intensity (FL2 detector) and (b) granulosity (SCC parameter). Blue curves correspond to control without treatment (n=4), red curves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

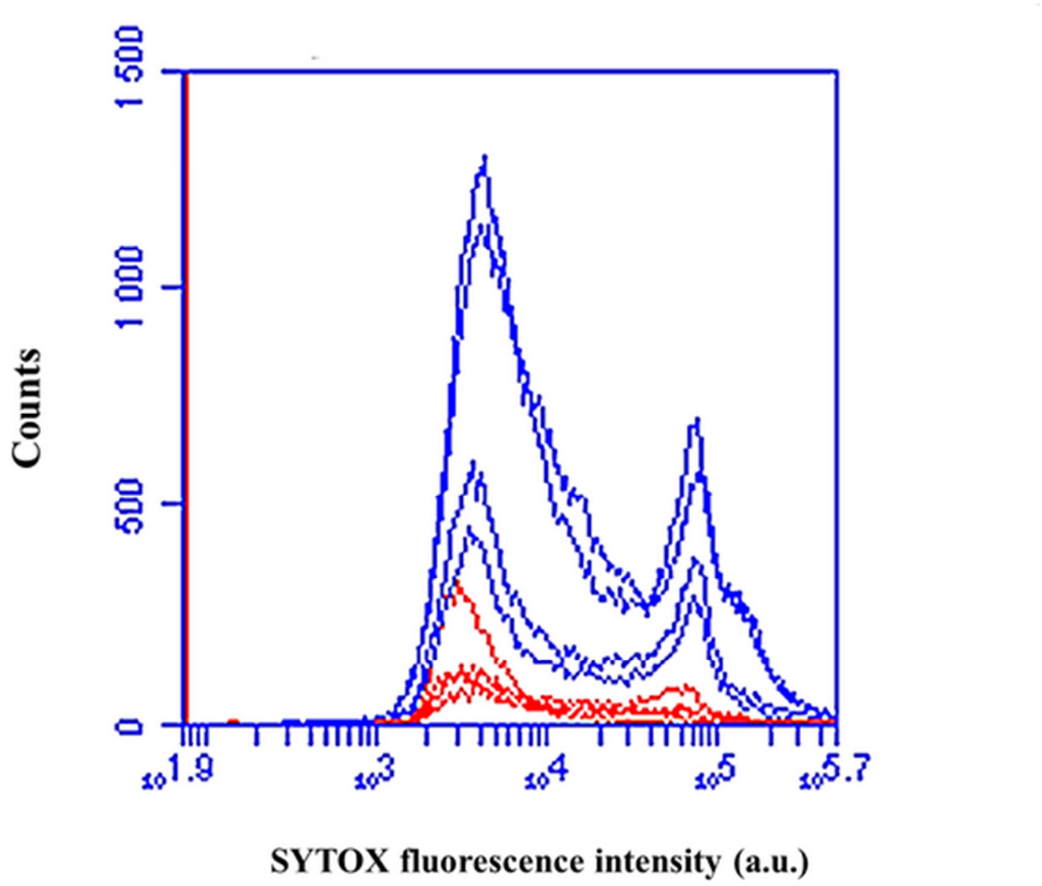

Figure 2: Distribution of the number of planktonic bacteria stained with SYTOX Orange in water 'N' at day 14 of experiment in the heat exchanger. Blue curves represent the control without ultrasounds and red curves correspond to the assays with ultrasounds at 10 W. Four independent assays were performed.

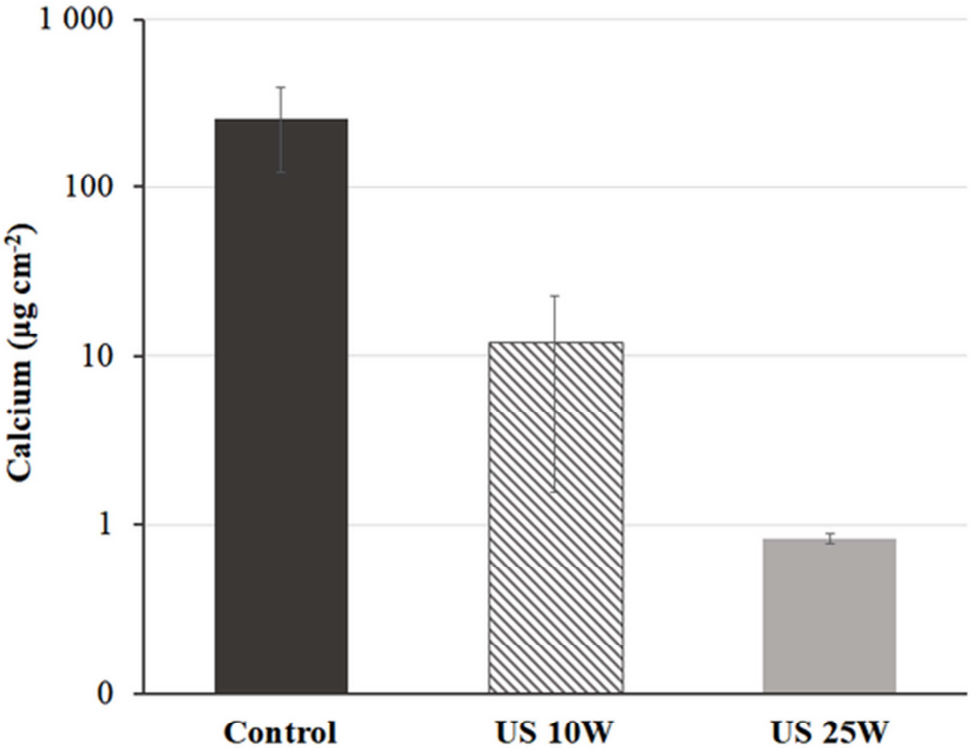

Figure 3: Calcium (µg cm^{-2}) accumulated in 14 days on the surface of the heat exchanger stainless steel plates fed with water 'N' (n= 3 to 4).

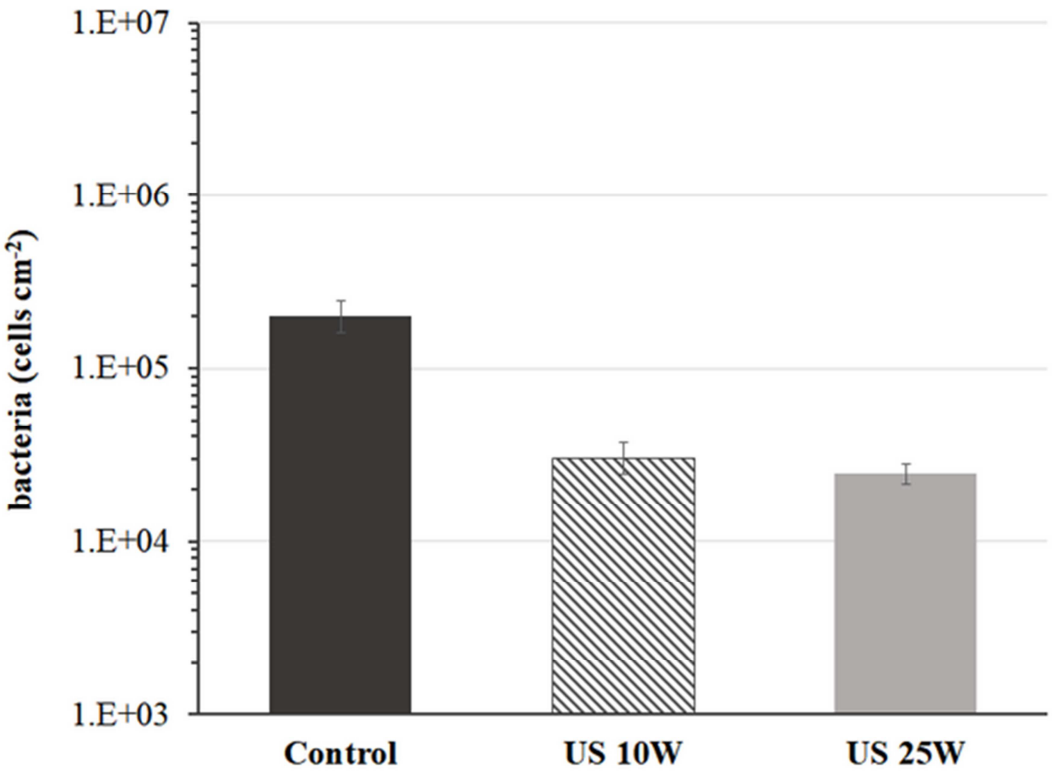

Figure 4: Number of SYBR-I stained bacterial cells measured by FCM in the biofilm accumulated in 14 days on the surface of the heat exchanger plates fed with water 'N'. (n=4).

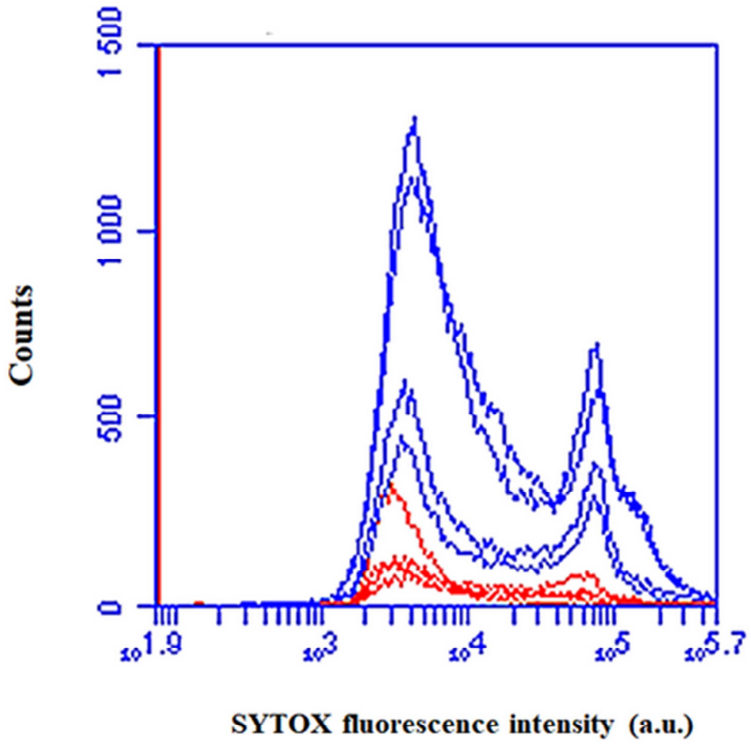

Figure 5: Distribution of Fluorescence intensity (FL2 detector) of the SYBR-I stained biofilm bacteria (FCM measurement) at day 14 of experiment in the heat exchanger fed with water 'N'. Blue curves correspond to control without treatment (n=4), red curves correspond to the assay with ultrasound (transducer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

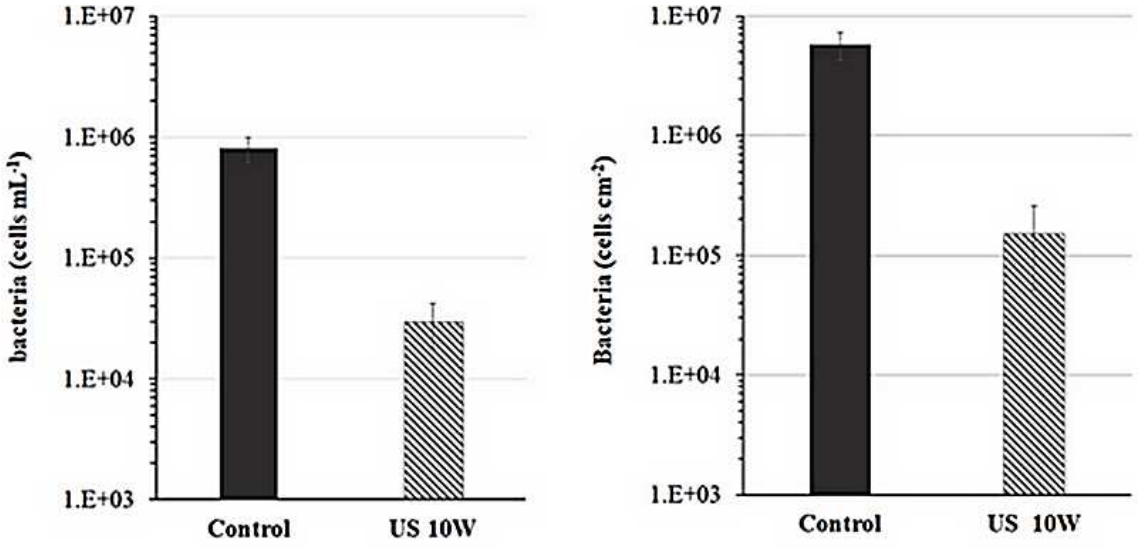

Figure 6: Number of bacterial cells after SYBR-I staining at pseudo-steady state (between days 18 to 28 of kinetics) in the water phase (planktonic cells) (A) and within the biofilms (attached cells) formed on stainless-steel coupon immersed in the flow-through Propella reactors fed with water 'M' (control = 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 ($\mu S\ cm^{-1}$)	1,190	1,031
Chlorine (mg $Cl_2\ L^{-1}$)	< 0.05	< 0.05
Calcium (mg L^{-1})	54	97
Complete alkalimetric title (CAT) (°f)	29	24
Total hardness (TH) (°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} ($\pm 6.9 \times 10^{4}$)	3.6×10^{4}
Assay (10 W)	0.23 (± 0.03)	2.0×10^{4} ($\pm 2.6 \times 10^{3}$)	3.8×10^{2}
Assay (25 W)	0.47 (± 0.01)	2.7×10^{4} ($\pm 3.5 \times 10^{3}$)	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 (± 9.4)	34.7 (±7.4)	15.1 (± 2.2)
Assay (10 W)	100%	56.1 (± 16.7)	35.6 (± 15.9)	8.6 (± 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	4.3×10^6 ($\pm$ 2.9 10^6) (100 %)	1.6×10^5 ($\pm$ 1.0 10^5) (100 %)
Remaining biofilm bacteria after lab-detachment procedure	5.8×10^5 ($\pm$ 4.6 10^5) (13.5 %)	5.9×10^3 ($\pm$ 5.0 10^3) (3.7 %)