Article

Pseudomonas aeruginosa in Swimming Pool Water: Evidences and Perspectives for a New Control Strategy

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Abstract: *Pseudomonas aeruginosa* is frequently isolated in swimming pool settings. Nine recreational and rehabilitative swimming pools were monitored according to the local legislation. The presence of *P. aeruginosa* was correlated to chlorine concentration. The ability of the isolates to form a biofilm on plastic materials was also investigated. In 59.5% of the samples, microbial contamination exceeded the threshold values. *P. aeruginosa* was isolated in 50.8% of these samples. The presence of *P. aeruginosa* was not correlated with free or total chlorine amount ($R^2 < 0.1$). All the isolates were moderate- to strong-forming biofilm (Optical Density O.D.$_{570}$ range 0.7–1.2). To control biofilm formation and *P. aeruginosa* colonization, Quantum FreeBioEnergy© (QFBE, FreeBioEnergy, Brisighella, Italy), has been applied with encouraging preliminary results. It is a new, promising control strategy based on the change of an electromagnetic field which is responsible for the proliferation of some microorganisms involved in biofilm formation, such as *P. aeruginosa*.

Keywords: *P. aeruginosa* contamination; biofilm formation; control strategy

1. Introduction

Many people attend swimming pools for sport, recreational, or medical activities. In these environments, the elderly, pregnant women, babies, people with handicaps or movement disabilities, and athletes can be predisposed to contracting infections [1].

Several types of opportunistic or pathogenic micro-organisms can be transmitted in this setting through water, contaminated surfaces, or via direct contact with infected individuals. Although this concept has been known for some time, the systematic study of communicable diseases in pool settings is lacking [2].

Pseudomonas aeruginosa accounts for many episodes of infections associated with attendance at swimming pools. The genus *Pseudomonas* includes free-living bacteria that are highly versatile and able to adapt to different environments and conditions. It is responsible for a series of diseases ranging from skin and eye infections in healthy individuals to serious life-threatening illnesses in burn, surgical, or immunocompromised subjects, often sustained by multi-drug resistant strains [3,4].

Due to its ability to form a biofilm on virtually all surfaces, *Pseudomonas aeruginosa* can survive in treated water with residual chlorine levels < 1 mg/L, in distilled water, and in disinfectant solutions, and it shows high resistance to mechanical cleaning processes. Therefore, contamination of pool, hot tub, and whirlpool water is frequently associated with outbreaks of *P. aeruginosa* [5,6]. Several regulations and guidelines consider this risk and define the measures to control *P. aeruginosa* contamination in recreational waters [7–9].

In Italy, the regulation concerning the hygiene of swimming pools reports threshold values for *P. aeruginosa* concentration, recommended available control measures, and establishes the type and frequency of inspections for swimming facilities [10].

At the present time, many procedures and products are available to manage and reduce the risk of infection in swimming pool settings. Recently, some studies have shown the efficacy of electric or electromagnetic fields on microbial contamination of water due to their effects on biofilm formation [11–13]. Quantum FreeBioEnergy© (QFBE, FreeBioEnergy, Brisighella, Italy) is a new promising technology with water decontaminating effects based on the coherent electrodynamic models developed in the last twenty years. These models might provide a new conception of the physics of water [14,15]. According to these models, water particles can be differentiated into two states: a "coherent state", where the particles move in unison in space and in time, influenced by an electromagnetic field which makes them organized, and an "incoherent state", where their movement is random and disorganized. The electrons of water molecules, under the effects of an electromagnetic field, vary their vibration period, forming the "coherence domain", with a consequent increase in density. Therefore, the water is thermodynamically in a biphasic condition: coherent and incoherent. The coherent state is at a lower energy level than that of the incoherent state. So, in the passage from the incoherent to coherent state (for example from vapor to liquid state), there is an energy profit with a relative decrease in entropy.

The molecules oscillating in phase are said to be within coherence domains (CD). The association between the coherence domains creates a situation thermodynamically more favorable because it implies a lower energetic level. QFBE, using the electromagnetic potentials due to the different energetic levels that electrons can occupy, acts on the water coherence state, putting the coherence domains in resonance and creating a super-coherent state. This information transfer between the different energetic levels is preserved over time, creating a real "memory" of water.

Certain materials such as silver, aluminum, and glass, with a particular shape and arrangement in space are able to yield electromagnetic signals to water particles from a distance of several meters, using natural fields (Earth's magnetic field, cosmic radiations, and electromagnetic motions typical of the ionosphere) as environmental carriers. With this new configuration, the water can interact with immersed biological structures, transforming them from a suitable environment for growing bacteria to one not suitable for bacterial species, and these properties can be maintained for long distances and a long time. Therefore, QFBE is classifiable as an "informational technology" (Figure 1).

Figure 1. Quantum 30 Hotel model.

In this study, we analyzed the presence of *P. aeruginosa* in swimming pool water in relation to the concentration of chlorine and microbiological parameters. Then, we employed the QFBE system (Quantum 30 Hotel model) to reduce water contamination, evaluating its efficacy.

2. Materials and Methods

During the period from September 2013 to December 2014, nine recreational and rehabilitative swimming pools in the Naples area were monitored according to the local legislation to assess the microbiological face of their water [16].

2.1. Sampling

Water samples were collected as recommended by the norm ISO 5667-3:2012 [17].

In brief, one liter of water was collected from each sampling point (pool and intake tap) with a sterile plastic bottle, and sodium thiosulfate was added to a final concentration of 20 mg/L.

Sampling was undertaken on a monthly basis before the opening of the pools.

2.2. Total Microbial Count Determination

Total Microbial Counts (TMCs) of both mesophilic and psychrophilic bacteria were detected on the basis of UNI EN ISO 6222:2001 [18]. Two milliliters from each sample were inoculated, one each, in a Petri plate containing Water Plate Count Agar (Oxoid, Basingstoke, Hampshire, England) and incubated at $37 \pm 1\,^{\circ}$C for 40–48 h and $22 \pm 1\,^{\circ}$C for 64–72 h, respectively. After incubation, visible colonies were counted and results were expressed as Colony Forming Units per milliliter (CFU/mL).

2.3. Other Microorganisms Detection

Other microbiological parameters were evaluated on the basis of corresponding guidelines (*Escherichia coli*: UNI EN ISO 9308-1:2002; *Enterococcus* spp.: UNI EN ISO 7899-2:2003; *Staphylococcus aureus*: UNI 10678) [19–21]. All the results were expressed in CFU/100 mL.

2.4. Pseudomonas aeruginosa Detection

The detection of *P. aeruginosa* was performed as recommended by the UNI EN ISO 16266:2008 [22]. Briefly, 100 mL of each sample were filtered with a sterile 0.45-μm Ø cellulose membrane, which was incubated on a *Pseudomonas* agar base/CN-agar (Oxoid) at $36 \pm 2\,^{\circ}$C for 44 ± 4 h. Blue-green, fluorescent and reddish brown colonies were counted, confirmed, and expressed in CFU/100 mL.

The threshold values for psychrophilic microbial count are ≤ 100 CFU/mL for intake water and ≤ 200 CFU/mL for pool water. For the mesophilic count, the limits were ≤ 10 CFU/mL for intake water and ≤ 100 CFU/mL for pool water. The guidelines indicate that *E. coli* and enterococci should not be present in water samples, and the limits for *S. aureus* are ≤ 1 CFU/100 mL for water inside the pool and absence in 100 mL for intake water. The recommended threshold values for *P. aeruginosa* are 0 CFU/100 mL in intake water and < 1 CFU/100 mL in pool water [16].

2.5. Chlorine Determination

Free and combined chlorine levels were also measured in water samples using a photometer (226 Multi-parameter Photometers for Swimming Pools, Hanna Instruments, Woonsocket, RI, USA) with the 4500-Cl G standard method. Threshold values for free chlorine are 0.6–1.8 mg/L for intake water and 0.7–1.5 mg/L for pool water while recommended values for combined chlorine are ≤ 0.2 mg/L and ≤ 0.4 mg/L, respectively [16].

The presence of *P. aeruginosa* has been related to chlorine concentration and the presence of other microorganisms, calculating the coefficient of determination R^2.

2.6. Biofilm Production

The ability of *P. aeruginosa* to produce biofilm was investigated through colorimetric assay by Stepanović et al. to the first strain isolated from each facility [23].

For each isolate, 200 μL of a microbial suspension (0.5 Standard McFarland, 0.125 O.D. λ = 550 nm) in Tryptic Soya Broth (Oxoid, Basingstoke, Hampshire, England) were inoculated in a 96-well polyethylene microtiter plate (Beckton-Dickinson, Franklin Lakes, NJ, USA). A negative control made of 200 μL of the sole culture medium was included. The multiwall plates were incubated at $37 \pm 1\,^{\circ}C$. After 24 h, the wells were washed with a Phosphate Buffered Saline (Oxoid, Basingstoke, Hampshire, England) solution, fixed with methanol, and colored with 150 μL of crystal violet at 2%; after rinsing with distilled water, glacial acetic acid 30% was added to each well. Then, the absorbance at 570 nm was read with a spectrophotometer. Cut-off values were calculated as the mean Optical Density (OD) value plus three standard deviations (SD) of the negative controls. The values were used to classify the isolates as non-biofilm-producing or weak, moderate or strong-biofilm-producing, based upon the previously calculated OD values: OD ≤ ODc = no biofilm producer; ODc < OD ≤ 2 × ODc = weak biofilm producer; 2 × ODc < OD ≤ 4 × ODc = moderate biofilm producer; 4 × ODc < OD = strong biofilm producer.

2.7. QFBE Application

The QFBE (Quantum 30 Hotel model) was placed on a swimming pool wall free of obstacles and furniture for at least 1 m to the right and to the left and at a height of about 2.5 m, where there were no objects between the floor and the device. Therefore, we assessed the microbial contamination of intake- and pool-water samples monthly, as described before. The instrument consists of a plastic box (size: $12.4 \times 8.4 \times 4.2$ cm). Inside, there are two 6.6 cm long cylinders, antiparallel to the aluminum (Al), that are fixed on the smaller faces of the instrument.

2.8. Statistical Analyses

T tests were performed to compare the means of the TMC at 22 $^{\circ}C$ and 37 $^{\circ}C$ and the presence of *Pseudomonas aeruginosa* in the intake- and pool-water before and after the installation of the QFBE. In June 2015 the equipment was removed and the T test was applied to compare the same medium values pre-installation and after uninstallation. The results are expressed as mean ± standard error of mean (SEM). A *p* value of 0.05 was set as the level of significance.

3. Results

On a total of 126 samples, 75 (59.5%) showed a microbial contamination exceeding threshold values, especially in intake water samples (45 samples, 60%). *P. aeruginosa* was isolated from 67 (50.8%) of these samples while *E. coli* was never isolated.

In eight (6.4%) samples, *P. aeruginosa* contamination was associated with exceeding values of *Staphylococcus* spp., *Enterococcus* spp., or TMCs, while eight (6.4%) showed only contamination from *Staphylococcus* spp., *Enterococcus* spp., and TMCs.

The presence of *P. aeruginosa* was correlated with the presence of other microorganisms ($R^2 = 0.9$ for TMC 37 $^{\circ}C$ and $R^2 = 0.6$ for TMC 22 $^{\circ}C$) (Figures 2 and 3), while no correlations were found with free or combined chlorine concentration ($R^2 < 0.1$).

As for the ability to form biofilm, all the isolated strains were moderate- to strong-producers of biofilm (O.D.$_{570}$ range 0.7–1.2) (Figure 4).
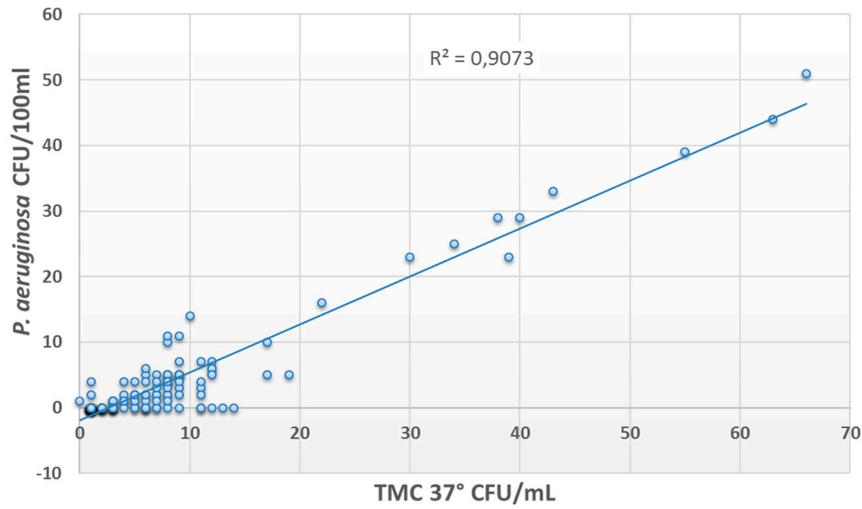
Figure 2. Correlation between *P. aeruginosa* contamination and mesophilic microbial count.
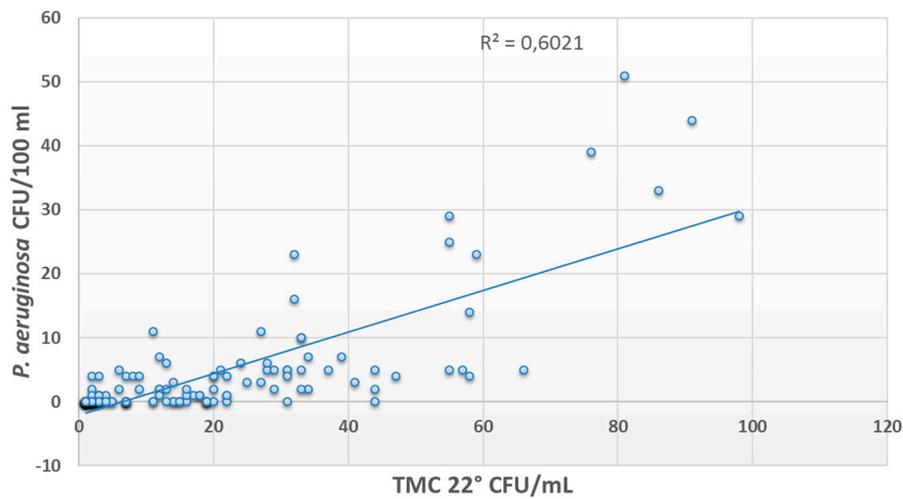

Figure 3. Correlation between *P. aeruginosa* contamination and psychrophilic microbial count.
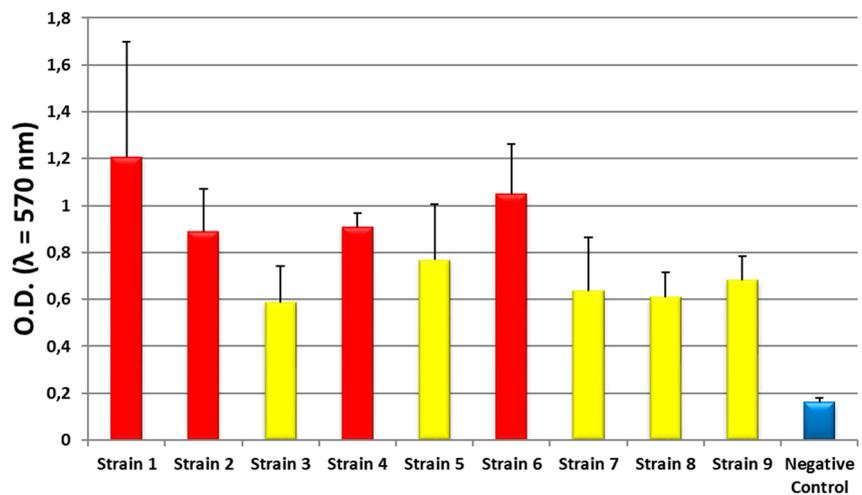

Figure 4.　Ability to produce biofilm in first isolation of *P. aeruginosa* first (black bars: Strong-biofilm-producing strains; gray bars: Moderate-biofilm-producing strains; white bar: Negative control).

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Considering these results, different control measures were adopted in the contaminated facilities. QFBE technology was applied to control biofilm production and *P. aeruginosa* colonization in the swimming pool where the strongest biofilm-producer strain (strain 1) of *P. aeruginosa* was isolated. In this pool data were collected up to December 2015.

Figures 5 and 6 show the results of water monitoring in this facility before and after the installation of QFBE. In the first month after equipment installation, it was possible to observe an increase in *P. aeruginosa* concentration and other microbial parameters, probably due to the release of microorganisms from the detached biofilm. Later, the contamination values decreased progressively until reaching the recommended values. (Figures 7 and 8).
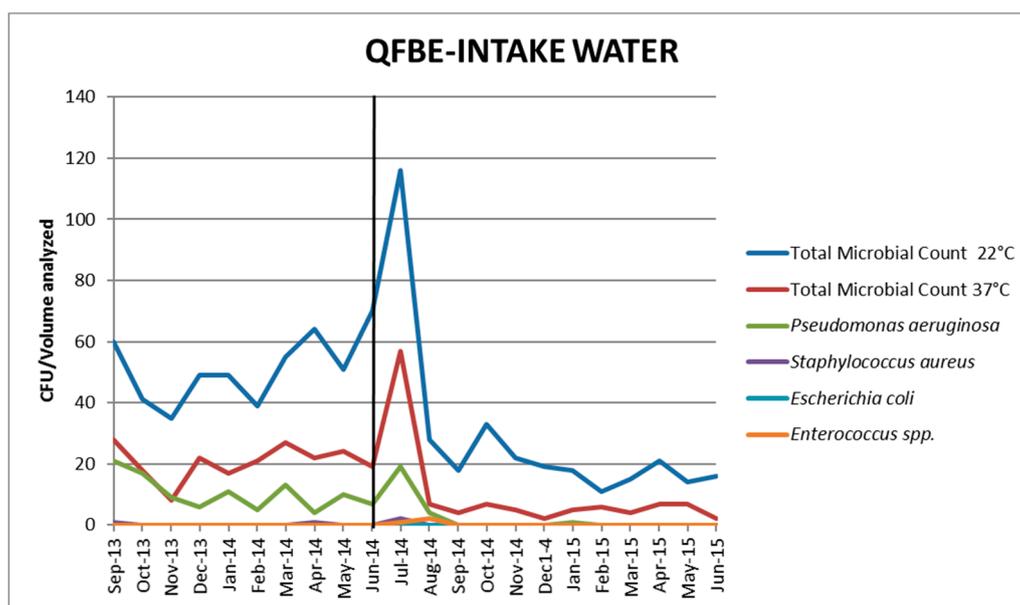

Figure 5. Trends of microbial parameters in intake water before and after Quantum FreeBioEnergy© (QFBE) installation (June 2014 marked with the black line).
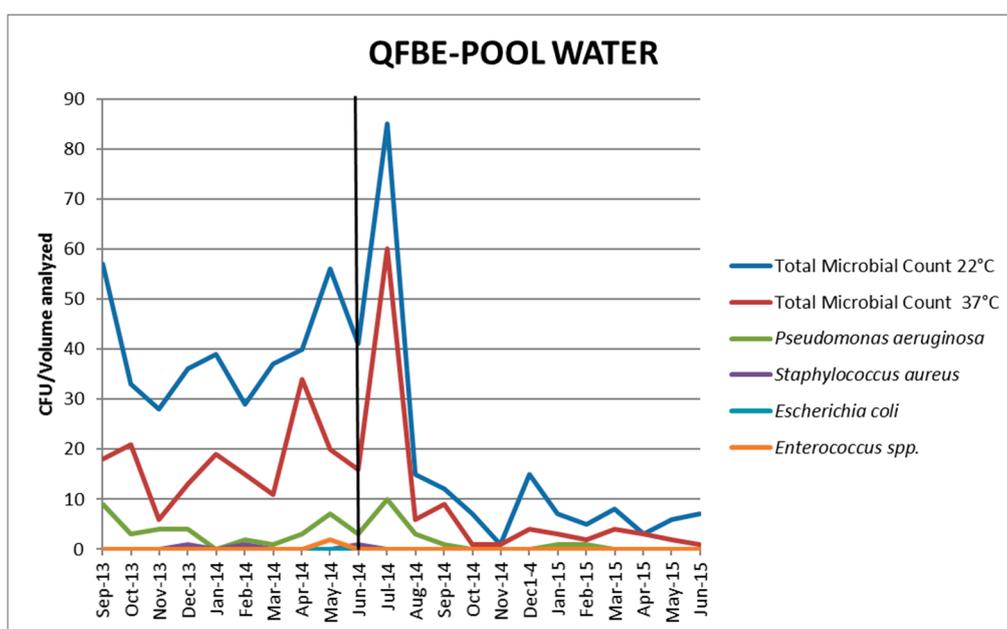

Figure 6. Trends of microbial parameters in pool water before and after QFBE installation (June 2014 marked with the black line).
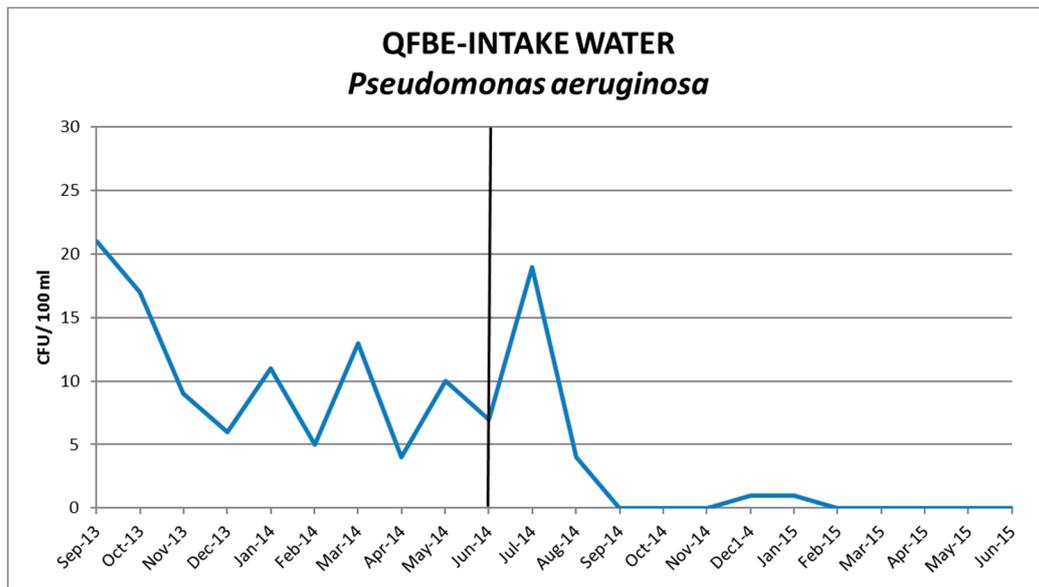
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Figure 7. Trends of *P. aeruginosa* in intake water before and after QFBE installation (June 2014 marked with the black line).
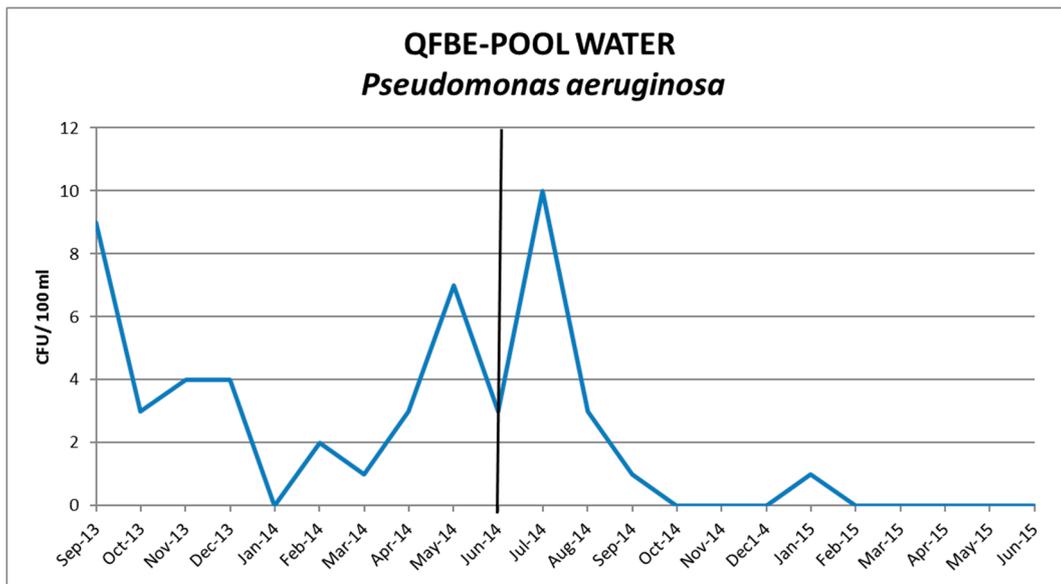

Figure 8. Trends of *P. aeruginosa* in pool water before and after QFBE installation (June 2014 marked with the black line).

Figures 9 and 10 show results of the T tests performed to compare the means of the TMC at 22 °C and 37 °C and the presence of *Pseudomonas aeruginosa* in the intake- and pool-water, before and after QFBE installation. In intake water, there are significant differences ($p < 0.05$) of considered parameters, while in pool water there is only a significant difference for TMC at 22 °C.
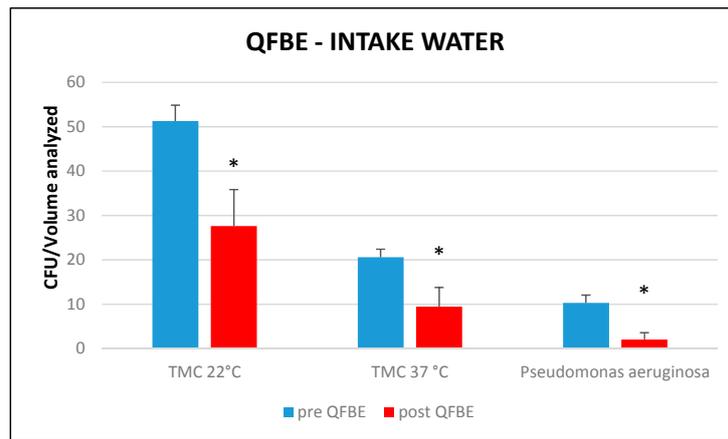
Figure 9. Mean total molecular count (TMC) at 22 °C, mean TMC at 37 °C, and presence of *P. aeruginosa* before and after QFBE installation in intake water. *: significant differences ($p < 0.05$).
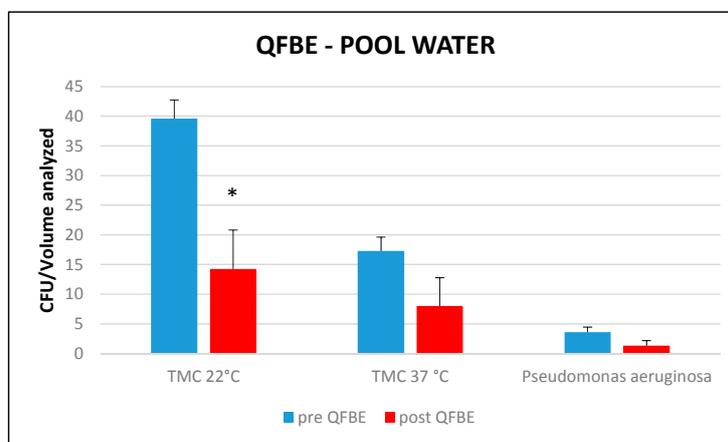

Figure 10. Mean TMC at 22 °C, mean TMC at 37 °C, and presence of *P. aeruginosa* before and after QFBE installation in pool water. *: significant differences ($p < 0.05$).

Comparing mean TMC at 22 °C, mean TMC at 37 °C, and presence of *P. aeruginosa* pre-installation QFBE and after uninstallation QFBE in intake- and in pool-water there are no significant differences ($p < 0.05$) (Figures 11 and 12).
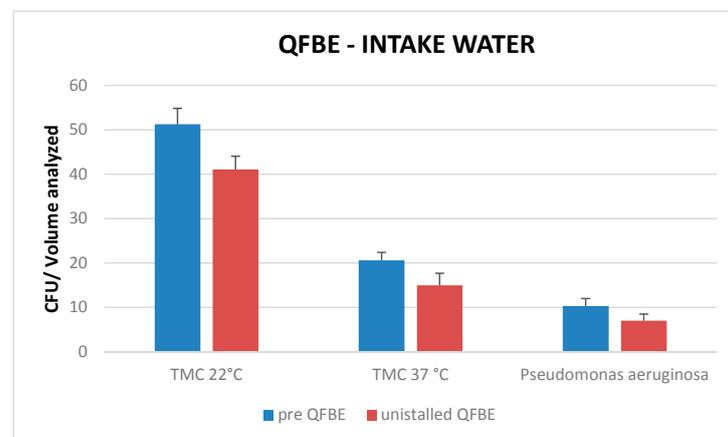

Figure 11. Mean TMC at 22 °C, mean TMC at 37 °C, and presence of *P. aeruginosa* pre-installation QFBE and after uninstallation QFBE in intake water.
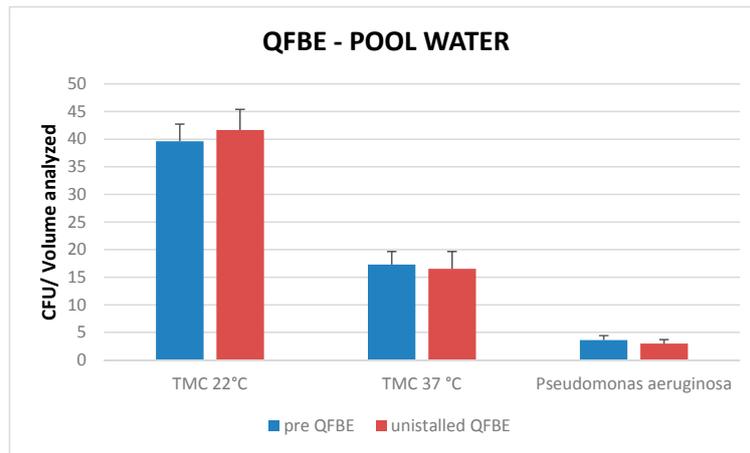
Figure 12. Mean TMC at 22 °C, mean TMC at 37 °C, and presence of *P. aeruginosa* pre-installation QFBE and after uninstallation QFBE in pool water.

In contrast, in the other facilities, where traditional sanitization methods were applied, the mean results of water monitoring did not show similar decreases, both for *P. aeruginosa* concentration and for other microbial parameters (Figures 13 and 14).
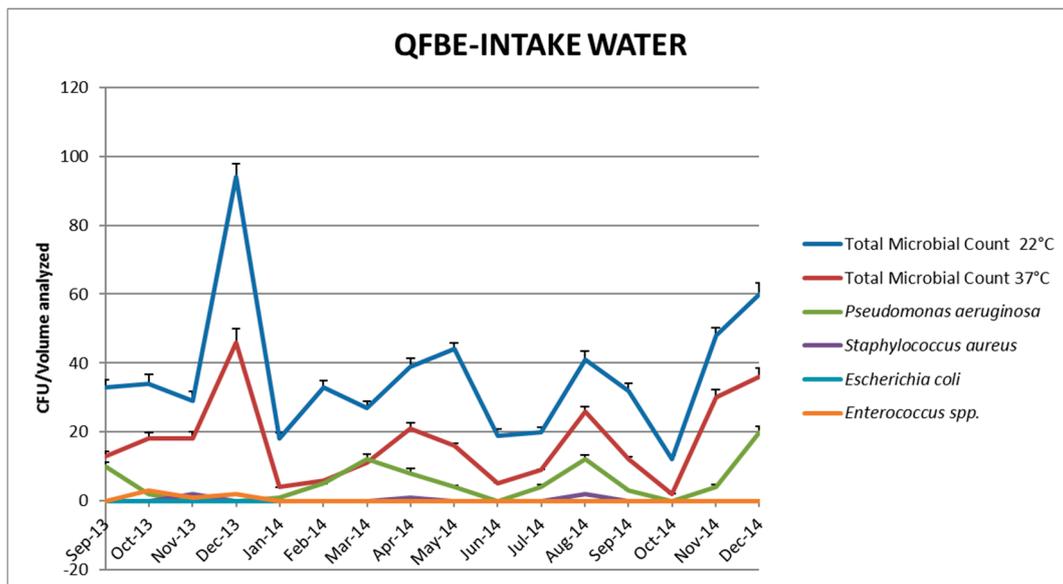

Figure 13. Trends of microbial parameters in intake water without QFBE equipment installation (mean values from eight swimming pools). Data are expressed as means ± standard error of mean (SEM).
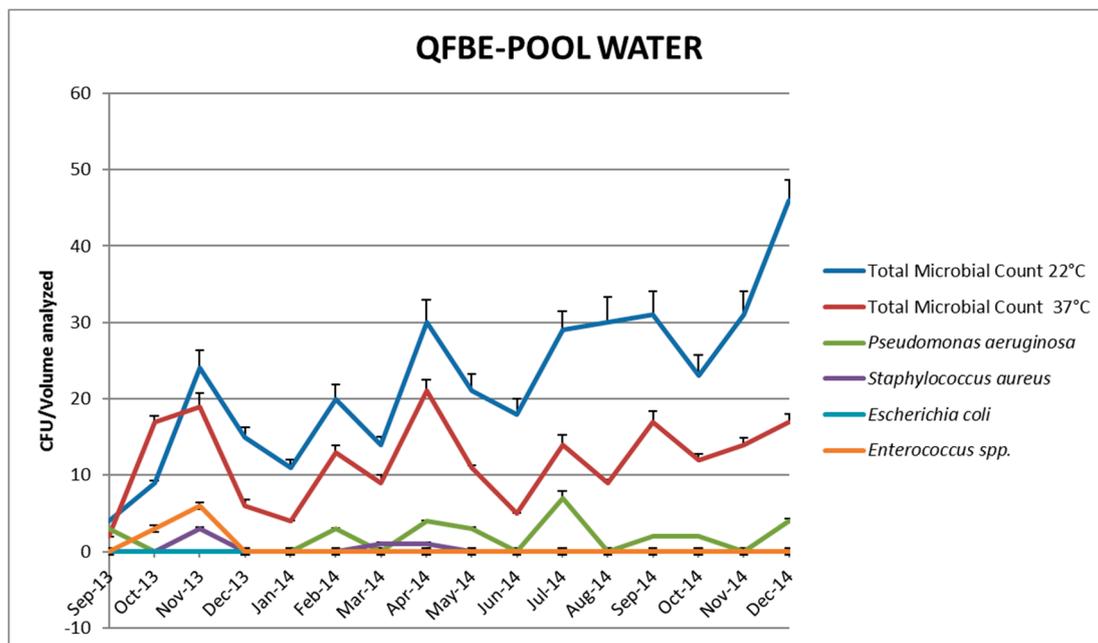
Figure 14. Trends of microbial parameters in pool water without QFBE equipment installation (mean values from eight swimming pools). Data are expressed as means ± standard error of mean (SEM).

4. Discussion

P. aeruginosa colonization is a phenomenon of frequent occurrence in facilities such as swimming pools or spas. Moreover, the ability of this bacterium to interact with other microorganisms and to produce biofilm makes it difficult to control its growth. In order to identify the appropriate control strategies for the prevention or the reduction of *P. aeruginosa* contamination, a global risk analysis of pool water is fundamental [5]. Guidelines recommend as paramount: frequent monitoring and adjustment of pH and disinfectant levels, performing microbiological tests, cleaning of surfaces and materials where the microorganism may grow and persist, showering of users, and controlling the number of pool users [7,24].

In this study, the evaluation of the microbial features of pool waters showed a high number of samples contaminated by *P. aeruginosa* and other species. *P. aeruginosa* contamination exceeded threshold values in the majority of the samples, and all the isolates showed the ability to form a biofilm on plastic surfaces. These results, together with the correlation found between *P. aeruginosa* and other microorganisms, allowed hypothesizing that the presence of a microbial community within a biofilm could be responsible for water contamination in examined facilities. Furthermore, the lack of a correlation between *P. aeruginosa* contamination and chlorine concentration suggested the need for an alternative control method.

Subsequently, an innovative sanitization methodology (Quantum FreeBioEnergy©, QFBE, FreeBioEnergy, Brisighella, Italy), based on the change of the electromagnetic field responsible for the proliferation of microorganisms involved in biofilm formation, has been successfully applied in a contaminated facility and it resulted the effective reduction of microbial and *P. aeruginosa* contamination, although not always significantly.

The findings of this study confirm the possible effectiveness of QFBE in a real, critical setting such as a swimming pool, also in comparison with traditional methods for contamination control. Furthermore, it has to be noted that this procedure may be used as an alternative to thermal shock or hyperchlorination, which exposes water systems to mechanical or corrosive stress.

After the installation of this system in an anthropic environment, along a wall without furniture, next to the room where sand filters are present and a good part of the water pipes pass through, we

could observe a continuous "spontaneous" lowering of contaminating flora without the intervention of thermal shock or hyperchlorination, with economic and environmental advantages.

The device does not produce electromagnetic fields, potentially harmful to human health, but exploits already existing fields (earth's magnetic field, cosmic radiation, and electromagnetic motion of ionosphere) by using them as an environmental carriers. The aluminum, for example, when present in the environment and in particular conformation (cylinders with antiparallel orientation, normal to the floor) assume the status of "coherence" (particles that compose it would move in space and time in unison, phase thermodynamically favored). This state would be transferred to the water through the above-mentioned environmental carrier.

Some bacterial and viral DNA sequences have been found to induce low frequency electromagnetic waves in high aqueous dilutions. This phenomenon appears to be triggered by the ambient electromagnetic background of very low frequency. This phenomenon could allow for the development of highly sensitive detection systems for chronic bacterial and viral infections [25].

However, further studies are required to examine, in depth, the new procedure and to compare its long-term effects with those of traditional control strategies.

5. Conclusions

Since the chemical-physical characteristics of water can affect the degree of attachment of microorganisms to a substrate, the first step for the formation of biofilms, and the water can constitute up to 97% of the biofilm, it can be assumed that technologies capable of altering the physical state of water can change a suitable setting for microbial growth into an unsuitable one; in addition, it may also modify the matrix of the biofilm by acting on its most representative constituent or by changing the interaction that this matrix has with the surrounding aqueous environment [26,27].

Due to the lack of in vitro and in vivo research about the effects of QFBE on bacteria growth and persistence, future studies should evaluate them.

In vitro encrustation models represent reliable, cost-effective approaches to the preliminary study and design of materials that resist biofilm formation and encrustation. In future studies, it would be interesting to simulate encrustation formation on different materials with a novel continuous flow encrustation model based on the commercially available CDC (Centers for disease control) biofilm reactor (CBR) (Biosurface Technologies, Bozeman, MT, USA), validated in other studies [28].

The CDC Biofilm Reactor consists of eight polypropylene coupon holders suspended from an ultra high molecular weight (UHMW)-polyethylene ported lid. The coupon holders can accommodate three 1/2 inch (12.7 mm) diameter coupons each. The lid with coupon holders and coupons is mounted in a one liter glass vessel with side-arm discharge port. A liquid growth media/biocide/etc. is circulated through the vessel while mixing, and shear is generated by a magnetic stir bar/vane rotated by a magnetic stir plate.

Sampling of the coupons is conducted by aseptically removing individual coupon holders with accompanying coupons. The coupon holder or a blank is replaced in the lid after sampling to allow the time course experiment to continue. The coupon removed from the reactor vessel is then used for treatment evaluations, scraped to collect the biofilm sample for further study or imaging using microscopy and image analysis. The CDC Biofilm Reactor is autoclavable and re-useable. The total liquid volume is approximately 350 ml. A variety of coupon materials are available, including plastics, metals, and ceramics.

Author Contributions: Marco Guida and Giorgio Liguori conceived and designed the study; Renato Gesuele and Renato Liguori collected and quality-checked the data; Federica Valeriani and Vincenzo Romano Spica interpreted the findings; Valeria Di Onofrio and Francesca Gallè interpreted the findings and wrote the paper.

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