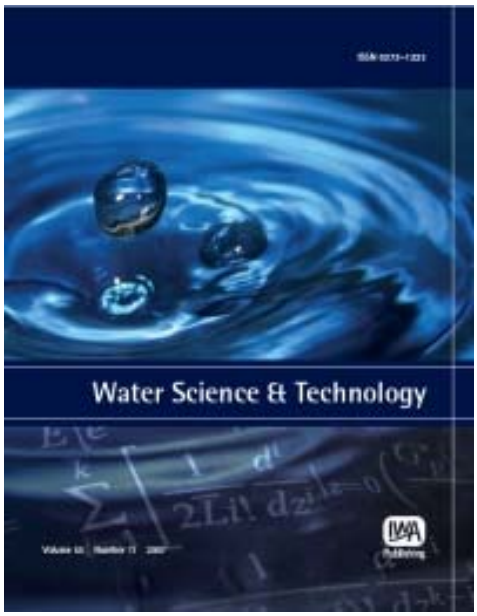

Biofilm control in water by advanced oxidation process (AOP) pre-treatment: effect of natural organic matter (NOM)

Anat Lakretz, Eliora Z. Ron, Tali Harif and Hadas Mamane

ABSTRACT

The main goal of this study was to examine the influence of natural organic matter (NOM) on the efficiency of H_2O_2/UV advanced oxidation process (AOP) as a preventive treatment for biofilm control. *Pseudomonas aeruginosa* PAO1 biofilm-forming bacteria were suspended in water and exposed to various AOP conditions with different NOM concentrations, and compared to natural waters. H_2O_2/UV prevented biofilm formation: (a) up to 24 h post treatment – when residual H_2O_2 was neutralized; (b) completely (days) – when residual H_2O_2 was maintained. At high NOM concentrations (i.e. 25 mg/L NOM or 12.5 mg/L DOC) an additive biofilm control effect was observed for the combined H_2O_2/UV system compared to UV irradiation alone, after short biofilm incubation times (<24 h). This effect was H_2O_2 concentration dependent and can be explained by the high organic content of these water samples, whereby an increase in NOM could enhance $^{\bullet}OH$ production and promote the formation of additional reactive oxygen species. In addition, maintaining an appropriate ratio of *bacterial surviving conc.: residual H_2O_2 conc.* post-treatment could prevent bacterial regrowth and biofilm formation.

Key words | advanced oxidation, biofouling, disinfection, hydrogen peroxide, NOM, ultraviolet

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INTRODUCTION

Biofouling is the unwanted deposition of suspended micro-organisms (i.e. biofilm) on surfaces. Biofouling can induce severe operational problems in many water sectors, including water distribution pipes and membrane filtration systems. An advanced oxidation process (AOP) is suggested herein as a preventive strategy for biofilm control. AOPs are processes where pollutants are chemically oxidized by free hydroxyl radical. The AOP process investigated in this study was medium-pressure (MP) ultra-violet (UV) irradiation with the addition of hydrogen peroxide (H_2O_2/UV). The H_2O_2/UV process includes two direct reactions with the microorganism cells: UV direct photolysis by inducing the formation of DNA photoproducts and H_2O_2 oxidation (or diffusion into cell); and an indirect reaction, oxidation by highly reactive hydroxyl radical ($^{\bullet}OH$) generated from a direct photolysis of H_2O_2. These oxidation processes may result in DNA oxidation and peroxidation of proteins and lipids. Many studies have demonstrated the oxidizing potential of hydroxyl radical for the removal of organic contaminants, including micro-pollutants from drinking water and wastewater. However, only a few papers investigated the disinfecting and oxidizing activity of hydroxyl radical on bacteria and viruses in general, and even fewer still on hydroxyl radical generated by the combined application of H_2O_2/UV (Bianchini *et al.* 2002; Lubello *et al.* 2002; Koivunen & Heinonen-Tanski 2005; Alkan *et al.* 2007; Mamane *et al.* 2007; Labas *et al.* 2009; Metz *et al.* 2011).

A number of constituents in water may influence UV and UV based AOPs such as iron, nitrate and natural organic matter (NOM). NOM is a complex matrix of various organic molecules originating from the natural biological activity in water. NOM impacts AOP process efficiency through several mechanisms: (1) NOM can absorb light in both the UV and the visible ranges, thereby hindering the initiation of H_2O_2 photolysis and the subsequent $^{\bullet}OH$ production; (2) photosensitization reactions from NOM could lead to the formation of long-lived (triplet) excited states,

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and thus to the formation of reactive species (e.g. singlet oxygen $^1O_2$, peroxyl radical $^{\bullet}RO_2$, superoxide anion radical $HO_2^{\bullet}/O_2^{-\bullet}$, hydroxyl radical $^{\bullet}OH$, hydrated electrons e_{aq}^-, carbonate radical $CO_3^{-\bullet}$, etc.) and (3) NOM can act as either a radical inhibitor or a radical promoter, depending on its functional groups and molecular size. As an inhibitor, NOM reacts with $^{\bullet}OH$ forms non-reactive species, and terminates the chain reaction. As a radical promoter, NOM reacts with $^{\bullet}OH$ and forms reactive species such as $HO_2^{\bullet}/O_2^{-\bullet}$ and secondary carbon centered radical (Bianchini *et al.* 2002) and promotes the chain process. (4) The sorption of NOM and particles might also impact UV based AOP disinfection processes.

A few studies have demonstrated an increase in radical formation and the anti-microbial effect of H_2O_2/UV processes in the presence of organic matter (Bianchini *et al.* 2002; Lubello *et al.* 2002; Koivunen & Heinonen-Tanski 2005; Alkan *et al.* 2007; Mamane *et al.* 2007).

A synergistic effect between UV and other disinfectants (e.g. Cl_2, ClO_2, NH_2Cl) in mitigating biofilms in water distribution systems has also been reported (Dykstra *et al.* 2007; Rand *et al.* 2007; Murphy *et al.* 2008; Wenjun & Wenjun 2009). Song *et al.* (2004) showed that H_2O_2/UV pre-treatment significantly reduced the permeate flux decline of nano-filtration membranes by transforming NOM fractions and polysaccharides into less sorbable organic acids, subsequently mitigating organic fouling and potentially also biofouling. Sarathy & Mohseni (2007, 2009) showed that UV/H_2O_2 increased the ratio of hydrophilic to hydrophobic compounds, and Metz *et al.* (2011) suggested that polar compounds which tend to be hydrophilic are less adsorbed than non-polar compounds. A question arises as to whether the presence of NOM could impact UV or AOP in a way that would affect biofilm control. In fact, different oxidative disinfectants have been shown to enhance biofilm formation by reducing the molecular weight of organic material and thereby increasing its availability to microorganisms (Momba *et al.* 2000). However, when using UV doses of 480 mJ/cm^2 there was no change in the nature of NOM or its biodegradable fraction in water (Camper *et al.* 2001). Metz *et al.* (2011) found that UV photolysis (MP) at a dose of ~280 mJ/cm^2 did not alter NOM into assimilable organic carbon (AOC), while at a dose of ~800 mJ/cm^2 (low pressure, LP) it produced a 36% increase in AOC. These authors showed that UV/H_2O_2 increased AOC and the formation of oxygenated species such as carboxylic acids, as a function of NOM concentration. Another phenomenon that can impact biofouling is the sorption of NOM to surfaces (e.g. membranes, bacteria). Thus, it is apparent that using AOP treatments in the presence of NOM may impact biofouling.

In summary, there are gaps in the literature with respect to the use of $^{\bullet}OH$ or H_2O_2/UV for the prevention of biofilms in water in the presence of NOM. The main goal of this study was to examine the influence of NOM on the use of H_2O_2/UV as a preventive treatment for biofilm control. The evaluation of the H_2O_2/UV process efficiency was determined by assessing the inactivation of suspended (free swimming) biofilm-forming bacteria (*Pseudomonas aeruginosa* PAO1), and subsequent biofilm formation.

MATERIALS AND METHODS

Preparation and enumeration of model organism

Pseudomonas aeruginosa PAO1 was used as model bacteria. Inocula of pure cultures were grown in minimal M9 media and incubated overnight at 30 °C with shaking. The inocula were diluted and incubated at 30 °C to mid-exponential phase. Working suspensions were prepared with a cell density of ~10^6 CFU/mL. Enumeration was performed on Difco LB agar plates and incubating at 25 °C for 2 days.

Preparation of water samples

Buffered water samples were prepared using 1 mmol/L phosphate buffer saline (PBS). All NOM samples were prepared using Suwannee River NOM obtained from the International Humic Substances Society. Sea water, surface water and secondary effluent samples were obtained from pilot sites in Palmachim Desalination Plant, kibbutz Ginosar-Sea of Galilee, and from Shafdan Waste Water Treatment Plant, (Israel) respectively, and filtered with a 0.45 μm filter to remove suspended solids. Chemical analyses of water samples were obtained by Magnet Consortium, Atlantium Technologies Ltd, and Shafdan Waste Water Treatment Plant.

Experimental set-up

Each water sample (30 mL) had an initial concentration of ~10^6 CFU/mL *P. aeruginosa* and was exposed to different UV doses and H_2O_2 concentrations (up to 50 mg/L). After irradiation, each sample was serially diluted, viable cells were enumerated to determine the reduction in suspended cells, and dose–response curves were developed. Mean

concentration (CFU/mL) of suspended microorganisms without UV exposure (~10^6 CFU/mL) was taken as N_0, while mean bacterial concentration per sample after each treatment was N_d. The dose–response curve was computed as log reduction [$\log_{10}(N_0/N_D)$] as a function of average UV dose (mJ/cm^2) with first-order kinetics. The water samples were further incubated to evaluate biofilm formation potential.

The main tested conditions were:

1. H_2O_2 alone – effect of H_2O_2 only.
2. UV exposures alone – effect of UV photons only.
3. H_2O_2/UV – effect of combined UV photons, $^{\bullet}OH$ and residual H_2O_2.
4. H_2O_2/UV/Catalase – addition of catalase (immediately after exposure) to eliminate the residual H_2O_2, leaving the effect of UV photons and $^{\bullet}OH$ only.

UV exposures

UV exposures were carried out with a MP bench-scale collimated beam apparatus using a 0.45 kW polychromatic lamp (Ace-Hanovia Lamp Cat. No. 7830-61). A neutral density (ND) filter (1% transmittance) (type OFR-2.0, POB 82, Caldwell, NJ, USA) was placed in the polychromatic light path to enable appropriate exposure times for an acceptable counting range of microbial inactivation. The integrated average irradiance (E_{avg}) between 220 and 300 nm, to which the microorganisms were exposed when the filter was used, was 0.005–0.010 mW/cm^2 and calculated according to Bolton & Linden (2003) using the incident spectral irradiance obtained from a calibrated spectroradiometer (USB4000, Ocean Optics, FL, USA, measured without filters), multiplied (weighted) by the transmittance at each wavelength, and taking into account the spectral absorbance of the water (with or without addition of H_2O_2), reflection at the sample surface and the measured Petri-factor for the dish. Average UV fluence (dose) was calculated by multiplying the average integrated irradiance with exposure time. Residual H_2O_2 was measured by the method outlined in Klassen *et al.* (1994).

Biofilm formation and quantification

Biofilm formation (%) was evaluated using a standard method using 96 microtitre plate (O'Toole & Kolter 1998) and detailed in Lakretz *et al.* (2010). This method allows for the examination of biofilm formation in many experimental conditions in parallel (batch conditions). All selected waters were treated with 15 min UV exposure with 1% transmittance filter with or without H_2O_2. Samples were diluted in M9 medium and further incubated at 30 °C without shaking for short (<24 h) or long (2 days) incubation times. Confocal laser scanning microscopy (ZEISS, LSM 510) was used to demonstrate qualitatively the effect of UV/AOP on the viability and adherence of *P. aeruginosa*, and on the production of extracellular polymeric substances (EPSs), as detailed in Lakretz *et al.* (2011).

RESULTS AND DISCUSSION

Effect of H_2O_2/UV on suspended PAO1 inactivation

NOM was added to buffered water (termed herein NOM-added water) at concentrations of 2.5, 5 and 25 mg/L (obtaining ~1.25, ~2.5, and ~12.5 mg dissolved organic carbon (DOC)/L) to study the effect of NOM on H_2O_2/UV process efficiency. Buffered water (NOM = 0 mg/L) was used as a controlled water make-up, maintaining constant pH value. Since NOM concentration is difficult to directly quantify, DOC concentration was measured as an indicator of NOM presence. Filtered sea water, surface water and secondary effluents simulated water contained DOC concentrations of ~1, ~2, ~12 mg DOC/L, respectively (Table 1).

Figure 1 illustrates the log survival of suspended (free swimming) *P. aeruginosa* PAO1 in water samples as a function of average UV fluence (dose). Overall, any addition of H_2O_2 (up to 50 mg/L) to buffered water did not significantly improve inactivation of suspended *P. aeruginosa* PAO1 cells compared to UV irradiation alone (Figure 1(a)). These results concur with our previous study whereby addition of 5, 10, and 50 mg/L H_2O_2 to a MP-UV system (<8 mJ/cm^2) did not improve inactivation of suspended *P. aeruginosa* PAO1 cells compared to MP-UV alone (Lakretz *et al.* 2011). In contrast, it was shown in this paper that when the UVC (200–300 nm) wavelengths (germicidal range) were filtered, leaving only wavelengths above >295 nm, additions of 10 and 50 mg/L H_2O_2 with UV showed significantly improved

Table 1 | UVT and DOC values of water samples

	PBS ~ 1 mM	Sea water	Surface water	Secondary effluents	PBS + 25 mg/L NOM
UVT (%)	~95%	~90%	~90%	~50%	~30%
DOC (mg/L)	0	~1	~2	~12	~12.5

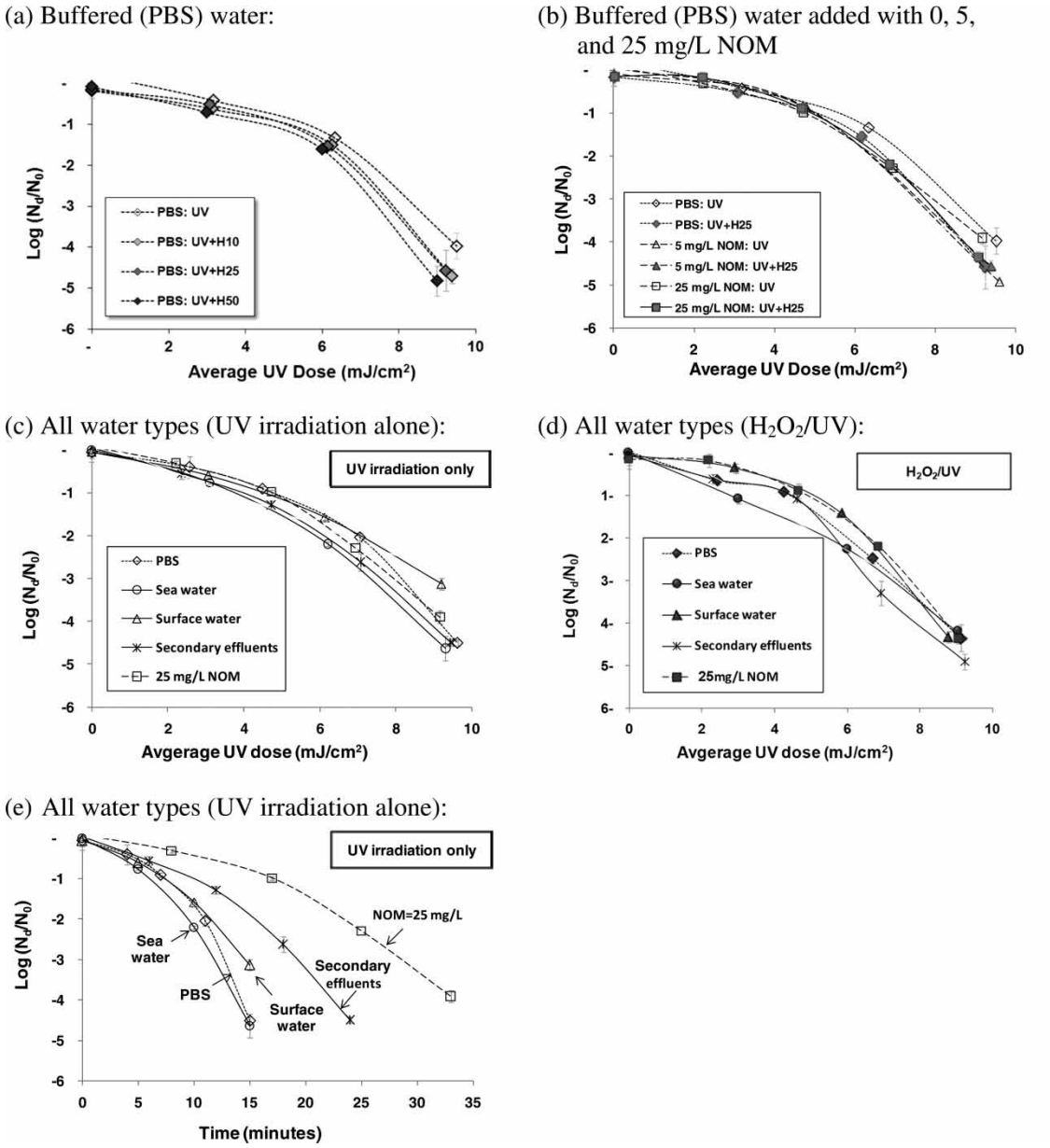

Figure 1 | Dose–response curves of *P. aeruginosa* PAO1 in: (a) buffered water (PBS) irradiated with UV and addition of ◇ 0, ◈ 10; ◆ 25; ◆ 50 mg/L H_2O_2; (b) NOM-added water containing ◇ 0; Δ 5; and □ 25 mg/L NOM, irradiated with UV and addition of 0 (blank); and 25 (bold) mg/L H_2O_2; (c), (d) and (e) ◇ buffered water; ○ sea water; Δ surface water; ✳ secondary effluents; □ 25 mg/L NOM-added water, irradiated with UV and addition of 0 (blank) and 25 (bold) mg/L H_2O_2.

inactivation results compared to UV-filtered irradiation alone. However, the slight overlap between the UV emission spectra using the 295 nm filter and the molar absorption coefficient of H_2O_2 could result in general low production of $^{\bullet}OH$. The additive-inactivation effect of H_2O_2/UV-filtered was explained by the relative contribution of the $^{\bullet}OH$ formed. When H_2O_2 is added to the non-filtered UV system, the germicidal effect gained from the UVC photons appears to mask the effect of the $OH^{\bullet}$.

When 2.5, 5, or 25 mg/L NOM-added water was treated with H_2O_2/UV, there was no significant improvement in the inactivation of suspended *P. aeruginosa* PAO1 cells compared to UV irradiation alone (partly shown in Figure 1(b)). It is possible that inactivation differences between UV and H_2O_2/UV can be affected by the water matrix (e.g. organic content). Alkan *et al.* (2007) report a hampering effect of fulvic acid, even at low concentrations, on UV/H_2O_2 disinfection efficiency, and concluded that a sufficient H_2O_2

concentration is required to overcome the counteracting effect.

Similar inactivation results were obtained with 25 mg/L H_2O_2/UV compared to UV irradiation alone for sea water (Figure 1(c), (d)). A small improvement in the H_2O_2/UV inactivation effectiveness of PAO1 was obtained at higher UV doses (>8 mJ/cm^2), compared to UV irradiation alone for surface water and secondary effluents only (Figure 1(c), (d)). These small differences could be due to increases in $^{\bullet}$OH production and other reactive species in these waters, due to the presence of organic (i.e. humic, fulvic) and inorganic (i.e. iron in secondary effluents) materials. For instance, Bianchini *et al.* (2002), found the presence of carbon centered radical as well as $^{\bullet}$OH in H_2O_2/UV treated wastewater. Lubello *et al.* (2002) found synergism between H_2O_2 (at high >20 mg/L doses) and UV irradiation on bacterial inactivation in secondary sewage effluents. Mamane *et al.* (2007) observed higher inactivation using the H_2O_2/UV process for MS2 phage in surface water compared to buffered water, due to the plausible formation of reactive oxygen species (ROS) other than $^{\bullet}$OH.

When plotting the log inactivation results as a function of exposure time (without taking into account the spectral absorbance of the water), differences in inactivation rates between water types were observed in order: buffered water ≈ sea water > surface water > secondary effluents > 25 mg/L NOM-added water (Figure 1(e)). Notably, water with similar DOC values, such as secondary effluents and 25 mg/L NOM-added water, did not have similar UV transmittance (UVT) values (Table 1). This could be explained by differences in specific organic content of these waters or the presence of absorbing inorganic ions in the secondary effluents (i.e. iron). In general, lower UV transmitting water (secondary effluents and 25 mg/L NOM-added water) requires longer exposure times to achieve similar inactivation results compared to higher UV transmitting water (buffer, sea, and surface water, Figure 1(e)). Thus, UVT seems to be the predominant factor affecting both UV and H_2O_2/UV inactivation efficiencies, however the effect of UVT on UV based AOP may be difficult to conclude as no difference was observed in inactivation between UV and H_2O_2/UV. With UV irradiation alone, UV absorbing molecules will compete with the target bacteria thereby reducing UV bacterial inactivation. Whereas for the H_2O_2/UV process, $^{\bullet}$OH formation depends on UV dose and spectrum, H_2O_2 concentration and water matrix. Table 2 presents the primary chemical properties of water that can influence UV or AOP efficiencies. Additionally, inorganic chlorides, alkalinity, and high concentrations of H_2O_2, may act as scavengers and terminate $^{\bullet}$OH chain reactions. Clearly, the efficiency of H_2O_2/UV bacterial inactivation in different water types depends on many factors, some additive or synergistic, and others counteracting.

In addition, due to the high UVC-sensitivity of the model biofilm forming bacteria (i.e. *P. aeruginosa*), low UV doses (<10 mJ/cm^2), produced only slight differences in inactivation between UV and H_2O_2/UV. When examining other microorganisms, which may be more UVC-resistant, the addition of $^{\bullet}$OH in the combined AOP system may be more significant. This should be further investigated.

Effect of H_2O_2/UV on biofilm control

The second goal of this research was to determine whether the inactivation of suspended bacteria by the H_2O_2/UV process correlates with biofilm control in the presence of NOM. To address this issue, biofilm formation potential of the

Table 2 | Chemical properties which may affect UV or AOP efficiency in various water types (chemical property and its conc. from US Army Corps of Engineers 1996)

	Chemical property	Concentration of concern	Sea water (Palmachim)	Surface water (Genosar)	Secondary effluents (Shafdan)
UV interferences	Chloride	>10,000 mg/L	**22,320**	280	273
	Nitrate	>10 mg/L	ND	0.4	0.6
	Nitrite	>10 mg/L	<0.01	0.009	0.77
	Phosphate	>10,000 mg/L	<0.01	<0.01	<0.05
	Ferrous	>50 mg/L Fe^{2+}	<0.05	<0.02	0.07
	Total dissolved solids	>400 mg/L	**42,086**	**850**	**780**
$^{\bullet}$OH Scavengers	Chloride	>1,000 mg/L	22,320	280	273
	Nitrate	>10 mg/L	ND	0.4	0.6
	Alkalinity	>300 mg/L HCO_3^-/CO_3^{2-}	133	85	256

Parameters which obtained measurements higher than those recommended were marked in bold.

surviving bacteria after UV or H_2O_2/UV was evaluated. The water types examined were 25 mg/L NOM-added water, buffered water and secondary effluents. Figure 2 illustrates biofilm formation results. It is important to emphasize that the average UV doses obtained after 15 min irradiation varied between the different water types and correlated to ~10, ~4, and ~6 mJ/cm^2 in buffered water, 25 mg/L NOM-added water and secondary effluents, respectively.

In buffered water, all treatments prevented biofilm formation up to 24 h post-treatment (~0% biofilm formation,

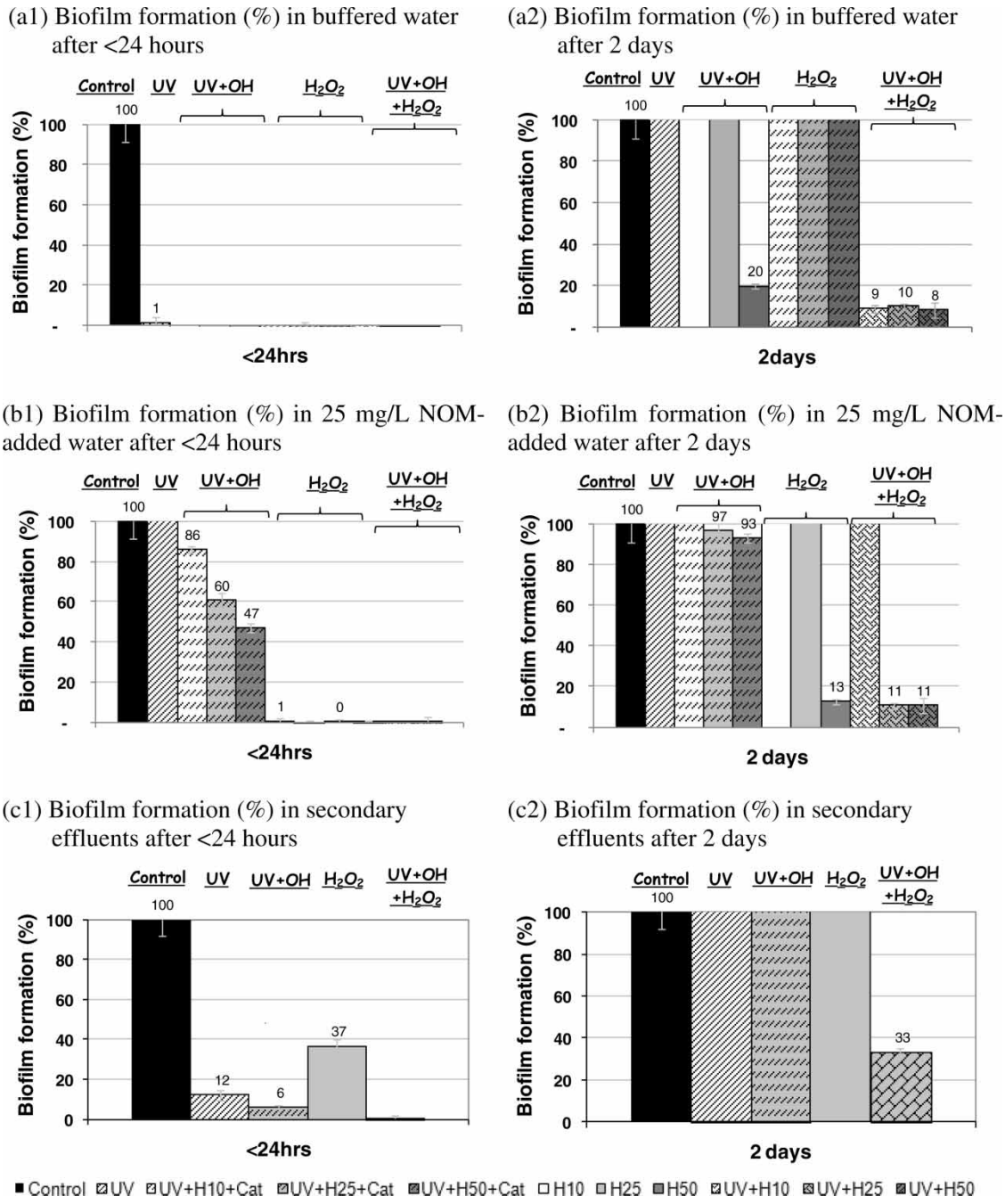

Figure 2 | Biofilm formation (%) of *P. aeruginosa* PAO1 at (a) buffered water; (b) 25 mg/L NOM-added water; (c) secondary effluents; after short (<24 h, left) and long (2 days, right) incubation times post different treatments (left to right): Non-treated control sample (100%); UV irradiation alone; UV + H_2O_2 (10, 25, 50 mg/L) + catalase (eliminated with residual H_2O_2) → effect of UV + $^{\bullet}$OH only; 10, 25, 50 mg/L H_2O_2 alone; UV + H_2O_2 (10, 25, 50 mg/L) including H_2O_2 residual → effect of UV + $^{\bullet}$OH + H_2O_2. All treatments had an exposure time of 15 min UV irradiation.

Figure 2(a1)). For the longer incubation periods, i.e. 2 days post-treatment, UV or 10 and 25 mg/L H_2O_2 alone were not sufficient to prevent biofilm formation. Only the highest H_2O_2 concentration (i.e. 50 mg/L) significantly inhibited biofilm formation (~20%, Figure 2(a2)). The combined H_2O_2/UV treatment ($UV + OH + H_2O_2$) did prevent biofilm formation more effectively than either UV or H_2O_2 alone at all periods, and required lower H_2O_2 concentration than H_2O_2 treatment alone (10 mg/L compared to 50 mg/L, Figure 2(a2)). These results are corroborated by previous studies where UV irradiation and 10 mg/L H_2O_2 treatments alone did not prevent biofilm formation for longer periods of time post-treatment, while combined H_2O_2/UV proved effective (Lakretz *et al.* 2011).

Unlike buffered water, UV alone was not sufficient to prevent biofilm formation up to 24 h post-treatment in 25 mg/L NOM-added water (~100% biofilm formation, Figure 2(b1)). The 70% reduction in UVT of the NOM-added water (Table 1) can explain the lowered log inactivation, and the increase in surviving bacteria post-treatment. Also, the presence of NOM may be used as an additional nutrient carbon source for the surviving bacteria and enhance bacterial regrowth (Camper *et al.* 2001; Metz *et al.* 2011). In comparison to the UV treatment alone, the H_2O_2/UV process, with catalase ($UV + OH$, without H_2O_2 residual), at 24 h post-treatments had an additive biofilm control effect (~86, ~60, and ~47% biofilm formation at 10, 25, and 50 mg/L H_2O_2 additions, respectively, Figure 2(b1)). This effect was shown to be H_2O_2 concentration dependent, and may be attributed to enhanced $^{\bullet}OH$ or other ROS formation when high NOM concentrations are present (i.e. 25 mg/L). Once more, UV or H_2O_2 alone (at 10 and 25 mg/L) were not sufficient in preventing biofilm formation after long incubation, and only high H_2O_2 concentrations (i.e. 50 mg/L) succeeded in controlling biofilm (similar to buffered water, Figure 2(b2)). H_2O_2/UV again prevented biofilm formation better than both UV and H_2O_2 alone, and required lower H_2O_2 additions than H_2O_2 alone (i.e. 25 mg/L compared to 50 mg/L, Figure 2(b2)).

In filtered secondary effluents, which contain similar DOC concentrations but have a higher UVT compared to 25 mg/L NOM-added water (~50% compared to ~30%, respectively, Table 1), biofilm was controlled by all treatments up to 24 h post-treatment (Figure 2(c1)). The higher UVT, allows for increased bacterial inactivation and less surviving bacteria. Secondary effluent also showed an additive biofilm control effect with 25 mg/L H_2O_2/UV and catalase ($UV + OH$, without H_2O_2 residual) over UV irradiation alone (~6% compared to ~12% biofilm formation, respectively, Figure 2(c1)). This could be indicative of radical mediated damage to the bacteria in the combined treatment. After 2 day incubation time, only H_2O_2/UV succeeded in controlling biofilm formation (~33% biofilm formation, Figure 2(c2)).

It has been suggested that biofilm control can be maintained by a synergistic effect between UV irradiation and other disinfectants. Dykstra *et al.* (2007) proposed that UV irradiation could increase bacterial vulnerability when used before residual disinfectant in a water distribution system. Lund & Hongve (1994) reported a biofilm inhibitory effect that remained for at least one week in humic surface water that was UV irradiated and attributed this effect to oxidizing species (e.g. $^{\bullet}OH$). Alam & Ohgaki (2002) found that UV irradiation had a persistent residual effect in water, depending on water quality and its organic matter content. This effect was extended in the presence of hydrogen peroxide, and was suggested to involve the formation of reactive species. Kornmueller (2007) suggested that AOPs could substitute conventional biocides for biofouling control in different marine water applications and by combining disinfection strategies it could target different microbial protective mechanisms.

Ratio of bacterial concentration to residual H_2O_2

In order to understand the role of residual H_2O_2 after-treatment on biofilm control, the interaction between H_2O_2 and different bacterial concentrations was investigated. It is important to note that during H_2O_2/UV exposure experiments, which occured over minutes, the change in H_2O_2 concentration was not significant (up to 5% decrease), as only a small fraction of H_2O_2 is consumed for $^{\bullet}OH$ production. Figure 3 illustrates the decrease in 25 mg/L H_2O_2 in buffered water added with: 0, 5, and 25 mg/L NOM and with 10^6 and 10^4 CFU/mL bacteria (achieved by dilution). Immediately after preparing the bacterial cell suspension with H_2O_2 and NOM, the residual H_2O_2 was measured, denoted as time zero.

After 3 h incubation with an initial H_2O_2 concentration of 25 mg/L, there was no H_2O_2 decrease with time in samples containing 10^4 CFU/mL, while samples with higher bacteria concentration of 10^6 CFU/mL showed a decrease in H_2O_2 to ~11–12 mg/L H_2O_2 (Figure 3). These trends were consistent up to 24 h of incubation, where samples with 10^6 CFU/mL showed total H_2O_2 decrease up to ~0 mg/L H_2O_2 (data not shown). The results did not change regardless of organic matter (i.e. NOM)

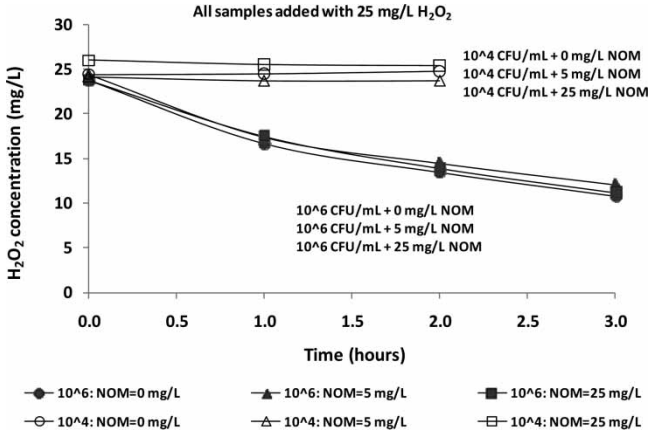

Figure 3 | H_2O_2 concentration (mg/L) as a function of contact time (hours) for buffered water added with ○ 0; 5; and □ 25 mg NOM/L and 10^4 (blank) or 10^6 (bold) CFU/mL *P. aeruginosa* PAO1. All samples were added initially with 25 mg/L H_2O_2.

concentration. These results are in agreement with those obtained for biofilm prevention with H_2O_2/UV that were not quenched with catalase (i.e. UV + OH + H_2O_2) with an initial concentration of 25 mg/L H_2O_2 for all water types (Figure 2(a2), 2(b2), 2(c2)). Possibly, these water samples, which contained less than 10^6 CFU/mL bacteria post-treatment, were maintained with a sufficient residual H_2O_2 concentration and therefore controlled bacterial regrowth. It appears that water that contained 10^6 CFU/mL bacteria and treated with 10 and 25 mg/L H_2O_2 alone did not maintain biofilm prevention due to the lack of residual H_2O_2 post-treatment. The results reinforce the hypothesis suggested in a previous study (Lakretz *et al*. 2011), that the ratio of *bacterial surviving conc.: residual H_2O_2 conc.* post-treatment plays an important role in regrowth prevention when applying AOP processes to control biofilm.

The ability of *P. aeruginosa* PAO1's to form biofilm after H_2O_2/UV treatment in the presence of NOM was assessed using CLSM. Figure 4 is a qualitative illustration of CLSM biolfim images grown in the presence of 2.5 mg/L NOM-added water, after 2 days incubation post-H_2O_2 treatments with or without UV irradiation. H_2O_2 treatment alone allowed the formation of a biofilm layer (~30 μm) of mostly live (green) cells, while with the combined H_2O_2/UV treatment, only a single layer of discrete dead (red) and live (green) cells were observed and no biofilm was present (Figure 4(a) and (b), respectively). This indeed supports the assumption of an additive or synergistic effect between H_2O_2/UV in biofilm prevention, compared to H_2O_2 treatment alone, even in the presence of NOM.

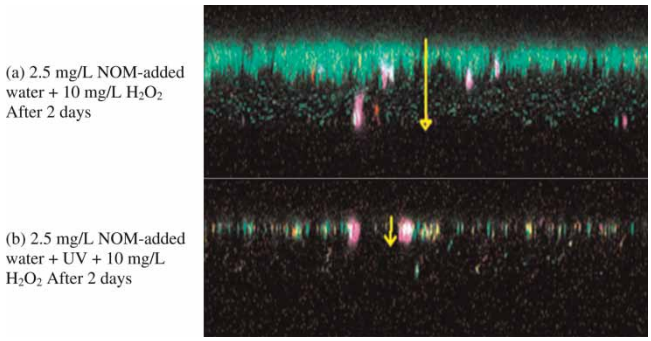

Figure 4 | Biofilm formation, cell viability and EPS presence in 2.5 mg NOM/L water, after 2 days of incubation time post: (a) H_2O_2 (10 mg/L); (b) H_2O_2/UV (maintained with residual H_2O_2).

SUMMARY AND CONCLUSIONS

- H_2O_2/UV showed clear superiority in preventing biofilm formation compared to both UV and H_2O_2 alone in all water types (when H_2O_2 residual was present).
- In water containing organic constituents – H_2O_2/UV (without H_2O_2 residual) showed improved biofilm formation control compared to UV alone. This effect was H_2O_2 concentration dependent, and may be attributed to enhanced radical or other ROS formation.
- For biofilm control, H_2O_2/UV required lower H_2O_2 concentrations than H_2O_2 treatment alone in both buffered and NOM-added water.
- H_2O_2 residual concentrations should be determined relatively to the amount of viable bacteria post-treatment.
- In summary, H_2O_2/UV can serve in certain conditions as a suitable preventive strategy for biofilm control in natural waters, even with high organic content.

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