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Research Paper

Antimicrobial mechanisms of *ortho*-phthalaldehyde action

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Biocides generally have multiple biochemical targets. Such a feature easily entangles the analysis of the mechanisms of antimicrobial action. In this study, the action of the dialdehyde biocide *ortho*-phtalaldehyde (OPA), on bacteria, was investigated using the Gram-negative *Pseudomonas fluorescens*. The targets of the biocide action were studied using different bacterial physiological indices. The respiratory activity, membrane permeabilization, physico-chemical characterization of the bacterial surfaces, outer membrane proteins (OMP) expression, concomitant influence of pH, contact time and presence of bovine serum albumin (BSA) on respiratory activity, morphological changes and OPA-DNA interactions were assessed for different OPA concentrations.

With the process conditions used, the minimum inhibitory concentration was 1500 mg/l, the concentration to promote total loss of bacterial culturability was 65 mg/l and the concentration needed to inactivate respiratory activity was 80 mg/l. These data are evidence that culturability and respiratory activity were markedly affected by the biocide. OPA lead, moreover, to a significant change in cell surface hydrophobicity and induced propidium iodide uptake. Such results suggest cytoplasmic membrane damage, although no release of ATP was detected. At pH 5, the bactericidal action of OPA was stronger, though not influenced by BSA presence. Nevertheless, at pH 9, BSA noticeably ($p < 0.05$) impaired biocide action. A time-dependent effect in OPA action was evident when contemplating respiratory activity variation, mainly for the lower exposure times. Scanning electron microscopy allowed to detect bacterial morphological changes, translated on cellular elongation, for OPA concentrations higher than 100 mg/l. Interferences at DNA level were, however, restricted to extreme biocide concentrations. The overall bactericidal events occurred without detectable OMP expression changes.

In conclusion, the results indicated a sequence of events responsible for the antimicrobial action of OPA: it binds to membrane receptors due to cross-linkage; impairs the membrane functions allowing the biocide to enter through the permeabilized membrane; it interacts with intracellular reactive molecules, such as RNA, compromising the growth cycle of the cells and, at last, with DNA.

Keywords: Antimicrobial action / Antimicrobial resistance / Biocide / Disinfection / Mechanisms of action / O*rtho*-phtalaldehyde

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Introduction

Control of microbial growth is required in many microbiologically sensitive environments, particularly when wet surfaces provide favourable conditions for proliferation of microorganisms (Simões *et al.* 2005a, 2006). An effective and wide spectrum disinfection strategy helps to overcome not only cross-resistance problems and existence of persister populations, but also the formation of recalcitrant and multi-resistant biofilms in disinfection dependent processes (Gilbert and McBain 2003; Simões *et al.* 2003a, 2003b, 2005a, 2006).

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Ortho-phtalaldehyde (OPA), an aromatic compound with two aldehyde groups, has been claimed to have an effective bactericidal character, having therefore been suggested as a replacement for glutaraldehyde, for high-level disinfection (Cabrera-Martinez *et al.* 2002, Mcdonnell and Russell 1999, Walsh *et al.* 1999a). This FDA (Food and Drug Administration) approved biocide has several potential advantages comparing to glutaraldehyde: it is virtually odourless, stable, effective over a wide 3–9 pH range, non-irritant to the eyes and nasal passages, and does not require activation before its use (Cabrera-Martinez *et al.* 2002, Rutala *et al.* 2001, Simões *et al.* 2006, Walsh *et al.* 1999a). Moreover, microorganisms that have acquired resistance to glutaraldehyde have not yet gained cross-resistance to OPA (Cabrera-Martinez *et al.* 2002, Simões *et al.* 2006, Walsh *et al.* 1999a). So far, toxic effects associated with amino acids interaction, and cellular cross-link, have commonly been used to explain the antimicrobial action of OPA (Simons *et al.* 2000, Walsh *et al.* 1999a). However, specific mechanisms in the antimicrobial action of OPA, against Gram-negative bacteria, remain poorly characterized. Some authors (Tomlinson and Palombo 2005, Russell 2003) have stated that there is an urgent need to deeper investigate the nature of the inhibitory and lethal effects of both biocides and disinfectants. This need emerges from the fact that the rise in the resistance to biocides might result in cross-resistance to other antimicrobial agents, especially at low concentrations. A wide range of possible multi-target cell sites would therefore constitute an important aspect of such studies.

Previous reports have recognized OPA as being a multi-target biocide (Cabrera-Martinez *et al.* 2002, Mcdonnell and Russell 1999, Rutala *et al.* 2001), Simons *et al.* 2000, Simões *et al.* 2003a, 2003b, Walsh *et al.* 1999a). Thus, the chance for most bacterial cells to develop resistance to the in-use biocidal concentrations is unlikely. At high concentrations, the toxic agent induces rapid kill of bacterial cells, through the implication of multi-target sites (Champlin *et al.* 2005, Massi *et al.* 2003).

In order to ascertain the antimicrobial action mechanism of a common multi-target biocide, a point-by-point analysis, from non-lethal to lethal concentrations, must be performed. Such a need rises from the fact that the susceptibility of a particular target is likely to vary, being also dependent on the antimicrobial concentration (Russell 2003).

P. fluorescens was used as a representative, well studied, Gram-negative bacteria ubiquitous in nature, medical and industrial environments and has potential to cause serious problems in a wide range of areas in its planktonic and biofilm states (Hsueh *et al.* 1998, Simões *et al.* 2005a, 2005b, 2006, Tuttlebee *et al.* 2002). This bacterium has a strong ability to form disinfectant-resistant biofilms (Simões *et al.* 2003a, 2003b, 2005a, 2006).

The purpose of this study was to investigate, using different physiological indices, the mechanisms of antimicrobial action of OPA against the Gram-negative bacterium *P. fluorescens*.

Materials and methods

Microorganism and culture conditions

Pseudomonas fluorescens ATCC 13525^T was used throughout this study.

A continuous pure culture of this bacterium was grown in an aerated (air flow rate = 0.425 min^{-1}) 2 l glass chemostat (Pobel 2000, Portugal), at 27 °C, agitated with a magnetic stirrer (Heidolph Mr 3001, Germany), providing exponential-phase bacteria. The chemostat was continuously fed with 40 ml/h of sterile medium containing 5 g/l glucose, 2.5 g/l peptone and 1.25 g/l yeast extract in 0.02 M phosphate buffer (KH_2PO_4; Na_2HPO_4) pH 7.0.

Biocide

The biocide used was *ortho*-phthalaldehyde (OPA) obtained as powder from Sigma (P-1378), with a purity ≥97%. Before each experiment, biocide solutions were prepared to the required concentration with sterile distilled water.

Growth inhibitory activity – minimum inhibitory concentration

To determine whether the presence of OPA had effect on the bacteria ability to grow in a liquid culture, the minimum inhibitory concentration was assessed, using the macrobroth dilution method (Champlin *et al.* 2005). Several bacterial cultures were prepared in sterile flasks, containing 200 ml of sterile growth medium (5 g/l glucose, 2.5 g/l peptone and 1.25 g/l yeast extract in 0.02 M phosphate buffer) and a suitable volume of bacterial inoculum. The optical density, at 640 nm (OD_{640}), was set to 0.2, corresponding approximately to 5×10^8 cells/ml. In each of these bacterial cultures, a different concentration of OPA was established, followed by incubation in an orbital shaker (120 rpm, 27 °C).

Sterile flasks containing growth medium, at a desired OPA concentration, were used to ascertain the interference between the biocide and the growth media components, on the final absorbance values (results not shown). Bacterial growth was measured at specific time points (0, 0.5, 2, 3, 5, 10, 11, 22, 23, 24, 26, 27, 28, 29,

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34, 35, 46, 47 and 48 h) by aseptically sampling 1 ml from each flask and subsequently measuring the OD_{640} (Spectronic 20 Genesys, Spectronic Instruments). The relevant OD_{640} value was obtained by subtracting, to the absorbance of the bacterial suspensions with OPA, the absorbance of growth media with OPA. These growth assays were performed in triplicate.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of biocide, where no growth was detected (Gilbert and McBain 2003).

At the end of the experiment, 30 μl of bacterial suspension were streaked onto solid (12% v/v agar) growth medium. This step ensured the viability, as assessed by culturability, and the purity of the suspensions.

Bacterial exposure to OPA

A volume of 50 ± 5 ml of bacterial culture was harvested from the 2 l reactor, washed with saline (0.85% NaCl) phosphate buffer (0.02 M) by three consecutive steps of centrifugation (3777 *g*, 5 min), and resuspended in 0.02 M phosphate buffer pH 7, in order to obtain a bacterial suspension with an OD_{640} of 0.2. This bacterial suspension was then divided by several sterilised glass flasks of 100 ml (containing 50 ml of bacterial suspension), and put on an orbital shaker (120 rpm, 27 °C). After a 30 min standard OPA exposure time (Simões *et al.* 2003a, 2003b, 2005a, 2005b), the residual biocide was neutralized, as described below, and bacterial cultures used for further testing. Three replicate experiments, each with triplicate samples, were performed per condition tested.

Biocide neutralization

Sodium bisulphite (Aldrich), at a final concentration of 0.5% (w/v), was added to the bacterial cultures and left to react for 10 min (at a proportion of 8 ml of neutralizer for 1 ml of bacterial suspension) immediately after the 30 min biocide contact time (Cabrera-Martinez *et al.* 2002, Walsh *et al.* 1999a). Control experiments were performed, having been detected no interference ($p > 0.1$) between sodium bisulphite, at the concentration used, and *P. fluorescens* viability and respiratory activity (data not shown).

Culturability method on Plate Count Agar – kill curve

The selection of an adequate medium for heterotrophic microbial growth is an important factor to be taken into account, when using the plate count method. Being so, tests were carried out in order to choose the appropriate medium.

Plate Count Agar (PCA; Merck) was selected as it allowed small colonies to grow, while preventing larger colonies from growing excessively, through medium components diffusion limitation. After biocide neutralization, the bacterial samples were diluted to an adequate cellular concentration (from 10^6 to 10^0) in phosphate buffer. Then, a volume of 30 μl of each suspension was transferred onto PCA plates and incubated at 27 °C. Colony enumeration was carried out after 48 h.

Assessment of the bacterial respiratory activity

The respiratory activity (respirometry) of the bacterial samples was determined by measuring oxygen uptake rates in a Yellow Springs Instruments (Ohio, USA) BOM – biological oxygen monitor (Model 53), as previously described (Simões *et al.* 2005b). The samples were placed in the temperature-controlled BOM vessels ($T = 27\ ^{o}C \pm 1$ °C), each containing a dissolved oxygen (DO) probe connected to a DO meter. Once inside the vessels, the samples were aerated for 30 min to ensure oxygen saturation ($[O_2] = 9.2$ mg/l, 1 atm, 27 °C). The vessels were then closed and the decrease of the oxygen concentration monitored over time. The initial linear decrease corresponded to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, 50 μl of a glucose solution (100 mg/l) was injected into each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponds to the total respiration rate. The difference between the two respiration rates discloses the oxygen uptake rate due to glucose oxidation. This respiratory activity was expressed in mg of oxygen *per* g of dry bacterial mass *per* min. The bacterial dry mass was assessed by the determination of the total volatile solids of the bacterial suspension, according to the 2540 A-D Standard Methods of Analysis (APHA, AWWA, WPCF 1989).

Assessment of membrane integrity – propidium iodide uptake

The Live/Dead® *Bac*Light™ kit (Molecular Probes, L-7012, Leiden, Netherlands) assesses membrane integrity by selective stain exclusion (Simões *et al.* 2005b). After biocide treatment (30 min) and neutralization, the various bacterial suspensions were diluted 1:10. Three hundred microliters of each diluted suspension were filtered through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm) and stained with 250 μl diluted component A (SYTO 9) and 250 μl diluted component B (propidium iodide – PI). The dyes were left to react for 15 min in the dark, at 27 ± 1 °C. The membrane was then mounted on *Bac*Light mounting oil, as described in the instructions provided by the manufacturer. Solutions containing the dyes were previously prepared by dissolving 3 μl of each

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component in 1 ml of sterile-filtered (pore size 0.22 μm) water. The observation of stained bacteria was performed using a Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination and a 100 × oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained mounts consisted of a 480 to 500 nm excitation filter in combination with a 485 nm emission filter. Several microphotographs of the stained bacterial samples were obtained using a microscope camera (AxioCam HRC, Carl Zeiss) and a program path (AxioVision, Carl Zeiss Vision) involving image acquisition and image processing. A program path (Sigma Scan Pro 5) involving object measurement and data output was used to obtain the total number of cells (both stains) and the number of PI stained cells. Both the total number of cells and the number of PI stained cells on each membrane was estimated from counts of a minimum of 20 fields of view. The range of total cells per field was between 50−200 cells.

Assessment of membrane integrity – adenosine triphosphate measurement

The adenosine triphosphate (ATP) released from the cells was measured using the luciferase-luciferine System/Sigma FL-AAM. After the required contact time with OPA, 100 μl of each bacterial suspension was added to 100 μl of a 25-fold dilution mixture of luciferine and luciferase. The light transmission was measured in a bioluminometer (Lumac, Biocounter M 25000) and the output values were recorded in Relative Light Units (RLU). In order to investigate possible interference between the biocide and the bioluminescent method, control experiments were conducted using phosphate buffer, in the presence and absence of OPA. The OPA effect on membrane integrity was evaluated in terms of RLU, as an estimate of the intracellular ATP content released. This methodology has already been successfully applied in the assessment of *P. fluorescens* membrane integrity, after a 30 min contact with a cationic surfactant (Simões 2005; Simões *et al.* 2005a). The RLU was calculated according to Dalzell and Christofi (2002), using the following equation:

$$\text{Relative light units} = (RLU_1/RLU_0) \quad (1)$$

with RLU_0 standing for the relative light units of the control assay (bacteria without chemical addition) and RLU_1 for the relative light units of the test sample.

Physicochemical characterization of bacterial surfaces

The physicochemical properties of the bacterial surface (exposed and not exposed to OPA) were determined by the sessile drop contact angle measurement on bacterial lawns, prepared as described by Busscher *et al.* (1984). Determination of contact angles was performed automatically using an OCA 15 Plus (DATAPHYSICS, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. Contact angle measurements (at least 25 per liquid and OPA concentration tested) were carried out according to Simões *et al.* (2007). The liquids surface tension components reference values were obtained from literature (Janczuk *et al*. 1993). Hydrophobicity was evaluated after contact angles measurements, following the van Oss *et al.* approach (1987, 1988, 1989), where the degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (w) – ΔG_{sws} (mJ/m^2). If the interaction between the two entities is stronger than the interaction of each entity with water, $\Delta G_{sws} < 0$, the material is considered hydrophobic. Conversely, if $\Delta G_{sws} > 0$, the material is hydrophilic. ΔG_{sws} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (2)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy, and γ^+ and γ^- are, respectively, the electron acceptor and electron donor parameters, of the Lewis acid-base component (γ^{AB}), being $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$.

The surface tension components, of a solid material, are obtained by measuring the contact angles of the three liquids (l), the apolar α-bromonaphtalene, and the polar formamide and water. All these three pure liquids hold well known surface tension components. Once the values are obtained, three equations of the type below can be solved:

$$(1+\cos\theta)\,\gamma_1^{Tot} = 2\left(\sqrt{\gamma_s^{LW}\gamma_w^{LW}} + \sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+}\right) \quad (3)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

Influence of pH, contact time and proteins in the biocide action

To assess the influence of these parameters in OPA antimicrobial action, *P. fluorescens* suspended cultures were sampled from the 2 l reactor, centrifuged (3777 *g*, 5 min) and washed three times with saline (NaCl 0.85%) phosphate buffer pH 7. Afterwards, the pellets were

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resuspended in different buffers at pH 5 (0.02 M citrate buffer), 7 (0.02 M phosphate buffer) and 9 (0.02 M borate buffer), to a final OD_{640} of 0.2, respecting the different initial pH values. Each of the bacterial cultures was then equally divided between several sterilised glass flasks, and exposed to a 10 mg/l sub-lethal OPA concentration for 5, 30 and 180 min, along with 120 rpm shaking at 27 °C. In order to evaluate the influence of proteins, namely bovine serum albumin (BSA), in the biocide action, 0.72%, 0.36% and 0.18% (w/v) BSA was added to the bacterial suspensions buffered at different pH, prior to OPA exposure (Simões *et al.* 2006). After the pre-determined contact time of 5 min, 30 min and 180 min, the bacterial suspensions were put in contact with the neutralizer. The contact, at a proportion of 1:8 – volume of cells per volume of neutralizer, lasted for 10 min, being followed by a 3777 *g* and 5 min centrifugation. The pellets were resuspended in buffer, to 0.2 (OD_{640}). Samples were then submitted to the determination of bacterial respiratory activity. Three replicate experiments, of triplicate samples, were performed for each condition tested. The influence of pH was also assessed on cellular viability and respiratory activity, having been found no variability ($p > 0.1$) on such parameters for the range of pH values (5–9) used (results not shown).

Outer membrane protein isolation and analysis

The outer membrane proteins (OMP) were isolated according to the method described by Winder *et al.* (2000). The cells, before and after a 30 min 100 mg/l OPA treatment, were harvested by centrifugation (3777 *g*, 5 min, 4 °C), according to the procedure described above. The pellet was resuspended in 25 mM Tris and 1 mM $MgCl_2$ buffer (pH = 7.4) and the resulting suspension sonicated for 2 min (Vibracell, 60 W) in ice, to promote cell lysis. Following sonication, the solution was centrifuged (7000 *g*, 10 min, 4 °C) to remove non-lysed cells. The supernatant was collected and N-lauroylsarcosine (Sigma) was added to achieve a final concentration of 2% (w/v), for inner membrane proteins solubilisation, and left on ice for 30 min. Afterwards, the solution was centrifuged (27000 *g*, 1 h, 4 °C) to retrieve the outer membrane proteins (OMP). The pellet containing the OMP was suspended in deionised water (1 ml) and stored at –20 °C until needed.

The protein content of the samples was determined using the Bicinchoninic Acid Protein Assay Kit (BCA – PIERCE Cat. No. 23225), with bovine serum albumin as standard.

The OMP fractions obtained were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970), with 12% (w/v) acrylamide. Electrophoresis was performed at a constant current of 10 mA. After electrophoresis, the proteins were silver stained and the gels analyzed by Quantity One 4.3.1 software from BioRad (Simões *et al.* 2006).

DNA isolation and analysis

The DNA of *P. fluorescens* cells treated with 100 (a concentration where all the cells were PI stained), 500 and 1500 mg/l of OPA (concentrations higher than needed to lyse all the bacteria), for 30 min, was isolated using the GenElute Bacterial Genomic DNA kit (Sigma Cat. No. NA 2110), according to the manufacturer's procedure. For further comparison, isolation of the DNA of untreated cells was also performed. Ensuing isolation, the four DNA isolated samples were subjected to a 0.7% agarose gel electrophoresis, in 1 × TBE (40 mM Tris-acetate, 1 mM EDTA, pH 8), at 70 V. Following ethidium bromide staining (BioRad), DNA visualization was performed using a UV transilluminator (Gel Doc 2000, BioRad, CA, USA). Quantity One 4.3.1 software (BioRad) was used for result analyzes.

Scanning electron microscopy

During the experiments, both untreated and OPA treated cells were observed by scanning electron microscopy (SEM). Prior to SEM observations, bacterial cells were dehydrated by heat (60 °C, 2 h) and stored in a desiccator for 3 d. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10–15 kV. SEM observations were documented through the acquisition of at least 20 representative microphotographs.

Statistical analysis

The data were analysed using the statistical program SPSS version 14.0 (Statistical Package for the Social Sciences). Both mean and standard deviation, within samples, were calculated for all cases. Paired *t*-test analyses were performed for data assuming a normal distribution. Other data were statistically analyzed by the non-parametric Wilcoxon test. Statistical calculations were based on a confidence level equal or higher than 95% ($p < 0.05$ was considered statistically significant).

Results

Growth inhibition studies – MIC determination

In order to assess which OPA concentration inhibits the planktonic growth of *P. fluorescens* and thus, to establish the MIC, the bacterial growth in the presence of several

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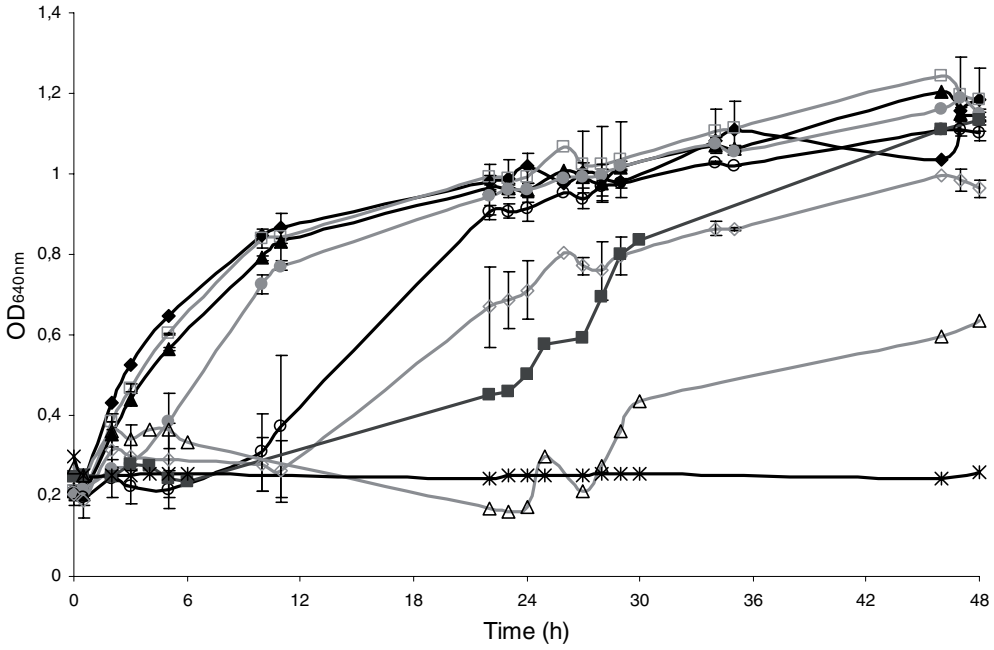

Figure 1. Growth curves of *P. fluorescens* in the absence (control) and presence of several OPA concentrations: 10 mg/l, 20 mg/l, 50 mg/l, 100 mg/l, 200 mg/l, 500 mg/l, 1000 mg/l, 1500 mg/l. The means ± SDs for at least three replicates are illustrated.

OPA concentrations was followed over time (Fig. 1). Some considerations needed to be taken into account in this experiment, as OPA increases the optical density of bacterial cell suspension. Furthermore, it also reacts with the growth medium proteins (Walsh *et al.* 1999b). So, in order to minimize interferences, the final OD_{640} values corresponded to the difference between the values recorded for the bacterial suspension exposed to OPA, and the growth medium in the presence of OPA.

The observation of the *P. fluorescens* growth curves revealed that complete growth inhibition was only detected for an OPA concentration of 1500 mg/l (MIC = 1500 mg/l). OPA concentrations lower than 100 mg/l did not cause any disturbance in bacteria growth, as the resulting growth curves have profiles similar to that of the control test ($p > 0.1$). All these curves presented a very short lag period (lesser than 1 h) followed by a 10 h exponential growth phase. For OPA concentrations equal or higher than 100 mg/l, bacterial growth seems to be affected in a concentration-dependent way. For OPA doses between 100 and 500 mg/l, bacterial growth curves patterns, though similar in between ($p > 0.05$), are significantly different from the control ($p < 0.05$). The growth curves of treated samples display a lag phase of around 10 h. Once the bacteria begin to grow, they reach OD_{640} values equivalent to those of the control. This lag phase, either allowed the bacterial adaptation to the stress conditions imposed by the very high OPA concentrations, or was associated with the population recovery from a decreased number following the lethal activity of OPA.

OPA effect on culturability and respiratory activity

The influence of OPA concentration in the culturability and respiratory activity (oxygen uptake ability) of *P. fluorescens* cells are presented in Fig. 2.

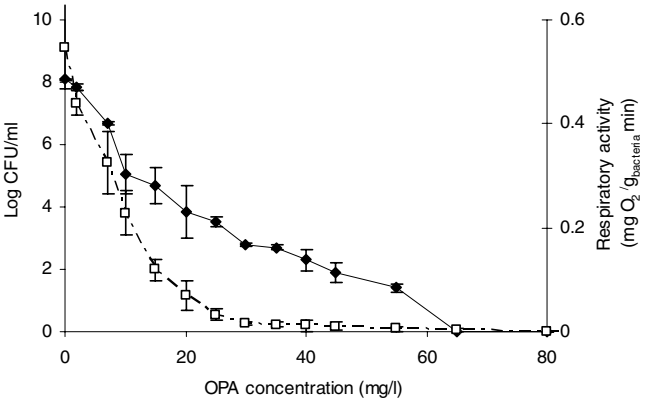

Figure 2. Bacterial culturability (- - - ◆ - - - colony forming units – CFU), and respiratory activity (— □ —) as a function of OPA concentration. The means ± SDs for at least three replicates are illustrated.

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OPA induced a sharp decrease in culturability (CFU counts) where 65 mg/l of OPA promoted total loss of bacterial culturability on PCA. For concentrations of 7 mg/l, the decrease of bacterial culturability corresponded to a log reduction of 1.4, value that rose to 5 for concentrations higher than 25 mg/l. Fig. 2 also showed that the increase of OPA concentration promoted the decrease of the bacterial oxygen uptake rate, therefore reducing the respiratory activity. As an increase in OPA concentration promoted a decrease in the oxygen uptake rate, it can be said that this effect was concentration dependent. For concentrations equal and higher than 80 mg/l, the bacteria were completely inactivated, as evidenced by the null oxygen uptake rate. The linear adjustment between loss of respiratory activity and loss of culturability are only correlated by a factor of 0.549 ($p < 0.02$). Further comparisons between culturability and respiratory activity results (Fig. 2) show that bacteria can retain respiratory activity after an OPA treatment ranging from 65 to 80 mg/l, even though most of them could not form colonies on conventional medium. This loss of culturability led to the assumption that bacterial cells, when exposed to OPA concentrations higher than 65 mg/l, could be in a viable but not culturable (VBNC) state.

Effect of OPA on the membrane integrity, physicochemical surface properties and OMP expression

Propidium iodide (PI) is commonly used as an indicator of cytoplasmic membrane permeability. Once inside the cytoplasm, it binds to single- and double-stranded nucleic acids, yielding fluorescence in the red wavelength region. Data related with PI uptake by OPA-treated cells are shown in Fig. 3.

OPA promoted significant damage (30 min exposure) to the cytoplasmic membrane as suggested by the PI uptake results (Fig. 3). Furthermore, the PI uptake was OPA concentration dependent ($p < 0.05$), with a logarithmic distribution of data ($R^2 = 0.962$). For an OPA concentration of 2 mg/l, about 20% of the cells presented a damaged cytoplamic membrane, value that rose to 100% for 100 mg/l. For concentrations higher than 20 mg/l, more than 80% of cells evidenced already damages in the cytoplasmic membrane.

The ATP bioluminescent assay (Fig. 4), carried out to assess whether OPA could have some effect on the exclusion of intracellular molecules, revealed that no significant amount of ATP was released from the bacterial cells treated with OPA.

Table 1 presents the hydrophobicity, and the apolar and polar components of the surface tension of *P. fluorescens*, without OPA treatment and when exposed to several concentrations of the biocide. *P. fluorescens* cells present hydrophilic properties as suggested by Table 1 ($\Delta G_{sws}^{Tot} > 0$ mJ/m^2). The application of OPA promoted the decrease of their hydrophilic characteristics, this decrease being more evident for the lower concentration tested (20 mg/l). These results appear to suggest that OPA, at low concentrations, promote the complete saturation of the OPA-surface reactive sites of cells, allowing further biocidal events. For the other concentrations tested, the OPA-induced surface properties were not concentration dependent ($p > 0.1$). The values of the surface tension components demonstrated that cells acquired polar properties after OPA treatment, translated by the increase in the γ^{AB} values. The apolar component (γ^{LW}) was almost unaffected by the OPA treatment, except for the treatment with 20 mg/l ($p > 0.05$).

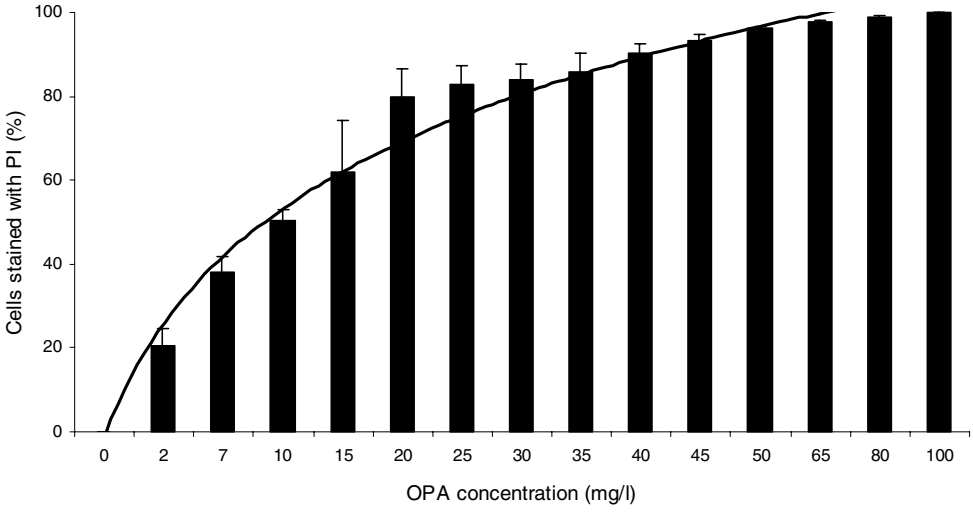

Figure 3. Permeability of OPA treated cells to propidium iodide. The means ± SDs for at least three replicates are illustrated.

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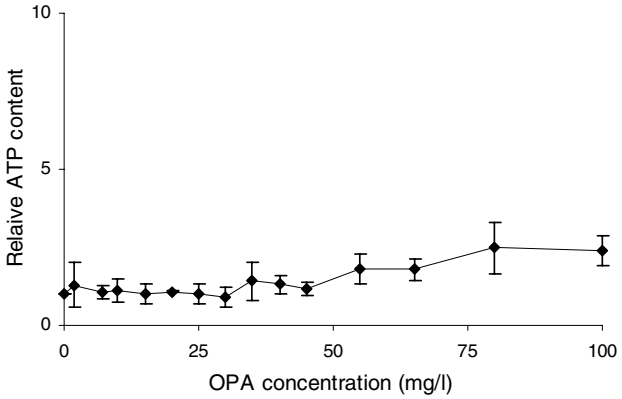

Figure 4. Relative ATP content released from the bacterial cells after treatment with several concentrations of OPA. The means ± SDs for at least three replicates are illustrated.

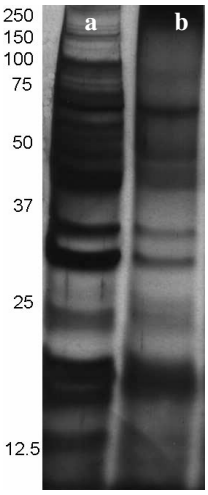

Figure 5. OMP profiles of *P. fluorescens* cells without biocide treatment (a) and after treatment with 100 mg/l of OPA (b). Numbers on the left represent molecular weights in kDa.

The OMP profile, assessed after bacterial treatment with 100 mg/l of OPA (100% of the cells stained with PI), was performed in order to inspect the possible effect of the aldehyde biocide on the expression of outer membrane proteins.

Fig. 5 shows that no significant difference was evident between the OMP profile of the OPA-treated bacteria, and the one of untreated cells. This phenomenon was reinforced when comparing the number and molecular weight of the profiles obtained using Quantity One 4.3.1 software.

Effect of pH, proteins and contact time in the OPA action

The effect of pH, presence of proteins and contact time, on OPA action, was evaluated by measuring the bacterial respiratory activity of suspended cultures. As shown by Table 2, the measurement was conducted before and after exposure to 10 mg/l of OPA, for several contact times, and cultures with different initial pH values and BSA concentrations.

A 10 mg/l application of OPA demonstrated to promote a 30% respiratory inactivation and a 3 log loss in culturability (Fig. 2). The use of sub-lethally injured or stressed cells is a warranty of the existence of a behavioural bacterial response, like respiratory activity variation, when bacteria face other stress conditions.

The analysis of data from Table 2 demonstrated that the variation of respiratory activity, due to OPA application, in the absence of BSA, is pH dependent ($p < 0.05$). In fact, for every OPA contact time and pH value, the application of the biocide caused the decrease of the respiratory activity. However, such a decrease was more pronounced when cells were suspended at pH 5 and 9.

Concerning OPA contact time, its influence is only noticeable when comparing the results 5 and 30 min after exposure ($p < 0.05$). The comparison of the data obtained 30 and 180 min after OPA exposure revealed no significant differences ($p > 0.1$).

The analysis of the respiratory activity variation, due to OPA application, in the presence of BSA, demonstrated that the synergistic association of BSA and pH change has a pronounced effect on respiratory activity. In fact, the application of OPA to the bacterial cells suspended in the pH 9 buffered medium, supplemented with BSA, did not cause any respiratory inactivation. Conversely, the cellular respiratory activity even increases ($p < 0.05$), being an undeniable sign of OPA antimicrobial action quenching. This effect was revealed to be BSA-concentration dependent, as an

Table 1. Hydrophobicity (ΔG_{sws}^{Tot}), and apolar (γ^{LW}) and polar (γ^{AB}) components of the surface tension of untreated and OPA treated cells. Values are means ± SDs

	Without treatment	[OPA] mg/l		
		20	50	100
ΔG_{sws}^{Tot} (mJ/m^2)	65.7 ± 4.8	6.08 ± 1.3	19.8 ± 2.1	23.4 ± 0.79
γ^{LW} (mJ/m^2)	24.1 ± 1.1	11.7 ± 1.2	23.4 ± 1.7	24.4 ± 1.1
γ^{AB} (mJ/m^2)	7.20 ± 0.78	52.3 ± 2.1	33.1 ± 2.4	31.4 ± 3.2

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increase in its concentration augments in 43% the bacterial respiratory activity measured for untreated cells ($p < 0.05$). At pH 5, the variation in the respiratory activity, for every exposure time, did not suffer any significant influence due to BSA ($p > 0.05$). Concerning the experiments at pH 7, the optimal pH for *P. fluorescens* growth, BSA also impaired OPA antimicrobial action. This effect was exposed by the lower respiratory activity resulting from biocide combination with BSA ($p < 0.05$). However, that effect was not so marked as for experiments at pH 9.

Bacterial structure

The influence of OPA on the bacterial structure was assessed by SEM, through the morphological comparison between untreated cells and cells exposed to 100 mg/l of OPA (Fig. 6).

These SEM inspections revealed a morphological alteration induced by the biocide, where the treated cells seem to present a higher length (Fig. 6). The septation of cells seems to have been blocked, thus cells appear to be elongated.

DNA analysis

From Fig. 7 it can be concluded that OPA interacted with *P. fluorescens* DNA, when cells were exposed to biocidal concentrations equal or higher than 500 mg/l. This result suggests that other events, before OPA-DNA interactions, may play a determinant role in the OPA biocidal properties, as for 100 mg/l (all bacteria are PI stained) the DNA profile is similar to the one observed for untreated cells.

Discussion

OPA is a relatively new aromatic dialdehyde antimicrobial agent. Assumptions regarding its action mechanism, little studied so far, have been based on its chemical nature and its amino acids cross-linking properties (Cabrera-Martinez *et al.* 2002, Simões *et al.* 2003a, Walsh *et al.* 1999). The present study gives additional enlightenment on OPA mechanisms of action, against Gram-negative bacteria, going beyond the single OPA-amino acids interactions.

In this work, through growth inhibition studies, the MIC was determined to be 1500 mg/l (Fig. 1). This value

Table 2. Variation of the bacterial respiratory activity of *P. fluorescens*, in different environmental conditions, after treatment with 10 mg/l of OPA for several contact times. Values are means ± SDs.

Time	pH	Variation of respiratory activity (% of control)			
		OPA	OPA + 0.18 % BSA	OPA + 0.36 % BSA	OPA + 0.72 %BSA
5 min	5	−74.3	−71.7	−69.1	−67.1
	7	−26.2	− 4.0	+ 6.8	−15.7
	9	−60.4	+20.8	+28.3	+31.1
30 min	5	−86.8	−78.9	−82.5	−77.2
	7	−31.8	+ 4.7	− 7.2	−11.3
	9	−79.5	+22.7	+25.0	+30.7
180 min	5	−85.6	−80.8	−86.5	−76.0
	7	−38.3	−12.8	−22.5	−24.5
	9	−79.2	+19.5	+35.1	+42.9

(+) Increase in the respiratory activity (–) Decrease in the respiratory activity

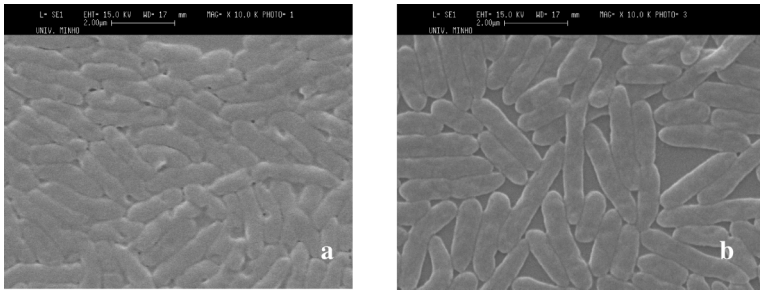

Figure 6. SEM photomicrographs of *P. fluorescens* cells before (a) and after (b) treatment with 100 mg/l of OPA. ×10000 magnification, bar = 2 μm.

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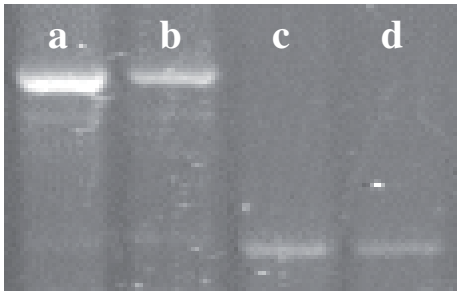

Figure 7. DNA profile of *P. fluorescens* without OPA treatment (a) and after treatment with 100 mg/l (b), 500 mg/l (c) and 1500 mg/l (d) of OPA, for 30 min.

is about four times smaller than the in-use concentration (5.5 g/l) for high-level disinfection (Rutala *et al.* 2001, Walsh *et al.* 1999a). OPA doses between 100 and 500 mg/l seemed to induce a physiological stress adaptation, as indicated by the prolonged lag phase in growth patterns. This phenomenon is probably related with bacterial recovery from a decrease in number of viable cells, following the lethal activity of OPA. At concentrations higher than 500 mg/l, and below the MIC, it is evident a stress adaptation emphasized by the depressed growth rate. Reductions in the growth rate, acknowledged after OPA-induced bacteriostasis, most likely reflect irreversible cell damage.

The total loss of bacterial culturability was detected for an OPA concentration of 65 mg/l, while respiratory inactivation was detected for 80 mg/l. The discrepancy between the MIC and the OPA concentration needed to promote total loss of culturability is probably related with the methodologies used as the MIC was assessed using cells in growth medium while total bacterial counts (kill curve) was performed using bacterial suspensions in phosphate buffer. If the kill curve assays (Fig. 2) would have been carried out under the same conditions as the MIC (Fig. 1), the OPA concentration would clearly have been much higher. Furthermore, a MIC of 1500 mg/l is only an indicative value, influenced by the fact that protein coagulation, the microbial surface or other changes in the opacity of the bacterial constituents will increase the optical density of bacterial suspensions (Walsh *et al.* 1999b).

Increased uptake of the PI, a nucleic acid stain to which cell membrane is normally impermeable, was observed (Fig. 3), revealing cytoplasmic membrane permeabilization. This result was strongly correlated with the inhibition of respiration, following a similar trend (linear correlation $-R^2 = 0.982$; $p > 0.1$). Several authors (Glover *et al.* 1999, Massi *et al.* 2003) have demonstrated that cytoplasmic membrane disturbance may not be immediately responsible for biocidal efficacy of chemicals. According to Maillard (2002), one of the noticeable effects of biocidal interaction with bacterial cells is a change in hydrophobicity. In fact, in this study, the OPA action resulted in a change of cellular hydrophobic interactions (Table 1), suggesting the possible existence of OPA-membrane binding receptors. Such results might indeed be the cause of membrane function impairment, arguably related with cross-linking events. The physico-chemical alterations at cell surface level, and subsequent PI uptake, probably precede OPA uptake. As a result of the uptake and interaction with reactive sites, a respiratory activity inhibition and consequent loss of ability to grow on PCA were recorded (Fig. 2). The indication that OPA might directly inhibit a specific respiration enzyme, involved in glucose consumption, or in the overall bacterial metabolic event, can not be eliminated (Fig. 2). This bactericidal action occurred without any evident OMP break up, as ascertained by comparative OMP analysis of untreated cells and cells exposed to 100 mg/l of OPA (Fig. 5). At this concentration, all cells were PI stained (Fig. 3). In fact, functional membrane proteins are generally supposed to be one of the potential targets of aldehyde-based biocide compounds (Trombetta *et al.* 2002, Walsh *et al.* 1999a). However, the major OMP expressed by untreated cells are similar to those expressed by OPA-treated cells. Furthermore, it was seen that neither the number of low molecular weight bands decreased, nor the number of high molecular weight bands increased, as might be expected from cross-linking events. Such an observation can be an indication of an apparent lack of strong cross-linking interaction between OPA and the OMP.

In previous studies, Walsh *et al.* (1999a, 1999b) stated that the glutaraldehyde treatment resulted in a strengthening of the outer envelope, thereby protecting the cell from lysis. Thus, it is not surprising that ATP release was not observed, as its release requires outer membrane destabilization (Simões *et al.* 2005a). In this study, OPA seems to act as a structure stabilizer (Figs. 4 and 6), as reinforced by SEM inspections. The ATP release data, together with the OMP expression results, suggest that the cell structure was not markedly damaged, and that a gradient of intracellular uptake arguably predominated, by a pore-forming system where the membrane stills there, although leaky (Butko 2003). This result is in agreement with a previous OPA chemical study by Zhu *et al.* (2005), where OPA cell penetration was mediated by the medium-induced molecular switching between OPA and 1,3-phthalandiol, and cell-wall penetration *via* this mechanism. This suggests the existence of different biocidal mechanism operating

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versus that of glutaraldehyde (mainly related with cross-linking effects).

In Gram-negative bacteria, the passage across the outer membrane depends on the chemical nature of the antimicrobial agent, with hydrophilic agents utilizing the porin channels – hydrophilic route – and hydrophobic agents entering *via* the hydrophobic route. This occurs probably due to the disturbance of the lipidic fraction of the outer membrane, as the lipophilic nature of OPA will play a key role in the diffusion through the outer membrane (Jarlier and Nikaido 1990). In previous studies (Carson *et al.* 2002), where the mechanisms of action of lipophilic biocides have been examined, effects on the cytoplasmic membrane, and/or on enzymes embedded in it, have been demonstrated.

The results obtained show that OPA could react with BSA and thus, decrease the biocide concentration available to promote respiratory activity inhibition. This phenomenon was strongly dependent on the BSA concentration, as a function of time and pH (Table 2). The pH of a solution determines the charge of a molecule. Consequently, in the presence of BSA, the respiratory activity variation seems to be controlled by the charge associated with the amino acids, due to the number of protonated and unprotonated species. This phenomenon is mainly noticeable at pH 9. According to several authors (Or *et al.* 1998, Simões *et al.* 2006, Singh *et al.* 1998, Walsh *et al.* 1999a), OPA promotes protein cross-linking by reacting with thiols, primary amines and nucleophilic centers of amino acids. In fact, it is known that BSA contains glycine, which can react with OPA, neutralizing it (Rutala *et al.* 2001). This biocide's cross-link with proteins is unstable under acidic conditions (Or *et al.* 1998, Singh *et al.* 1998), leading to the supposition that antimicrobial action, at pH 5, is related with OPA-induced intracellular events. Several authors (Simons *et al.* 2000, Walsh *et al.* 1999) also proposed that OPA interacts strongly with amino acids, mainly at alkaline pH. This fact suggests that protein related interactions are not the key event of biocidal action, as indicated by respiratory activity results variation (Table 2).

Regarding SEM microphotographs of *P. fluorescens* cells, it can be seen that, for OPA concentrations higher than 100 mg/l, cell division is compromised as the cellular septation seems not to occur. A cellular elongation takes then place (Fig. 6). Therefore, it can be said that an interference on bacterial cell cycle occurs at high OPA concentrations. It is already documented an *E. coli* elongation phenomenon after treatment with antibiotics (Garcia and Servais 2004, Goodell *et al.* 1976), with consequent interference with the synthesis of cell wall peptidoglycan. This results in an incapacity of cells to divide without, nevertheless, provoking death. Also, *E. coli* cells exposed to UV, or other SOS-inducing treatments, continue to elongate but fail to septate, thus growing as filaments. It can be argued that nucleic acid damage occurred, probably impairing the synthesis of a specific protein (Böddeker *et al.* 2002). This lack of protein synthesis may be related with RNA damage which, as a single stranded molecule, may be more prone to chemical attack than DNA. Prütz (1998) reported that RNA degradation by hypochlorite is 10 times faster than that of the double stranded DNA. However, if OPA concentration increases, reaction with DNA can also occur. In fact, the analysis of DNA after cell exposure to OPA revealed that, for OPA concentrations higher than 500 mg/l, DNA damage seemed to occur (Fig. 7). This phenomenon is concentration dependent and reinforces the existence of a sequence of events, probably culminating in DNA interference. Moreover, the DNA interactions hinder the protection from further recovery and resistance events, reflecting the strong antibacterial efficacy of OPA.

On the basis of the results presented, it can be said that the antimicrobial action of OPA, on *P. fluorescens,* is a result of a cascade of events, time and concentration dependent. In addition to pH-dependent cross-linking effects, as already stated by several authors (Simons *et al.* 2000, Walsh *et al.* 1999a), the antimicrobial effect of OPA may be due to chemical interactions with membrane molecules, promoting a structural cellular stabilization and alteration of surface hydrophobicity and membrane permeability. Additionally, for the higher concentrations tested, the biocide might cross the cell membranes and interact with intracellular sites, critical for antibacterial activity. For higher OPA concentrations, the growth cycle is compromised provoking cell elongation due to the lack of septation. Bacterial reproduction disability was only detected for concentrations higher than 500 mg/l.

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