Comparison of the Effectiveness of Chlorine, Ozone, and Photocatalytic Disinfection in Reducing the Risk of Antibiotic Resistance Pollution

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Abstract:

Effectiveness of conventional chlorine and ozone disinfection on reduction of antibiotic resistance was compared with less commonly applied heterogeneous photocatalytic process. For this purpose plasmid DNA isolated from a multi-resistant *Escherichia coli* (*E. coli*) HB101 was treated in two different concentrations with the three oxidation processes. Oxidative damage on the plasmid DNA was analyzed with gel electrophoresis by comparing the extent of conformational changes in the DNA structure. The effectiveness of the applied oxidant in reducing the risk of resistance transfer was also evaluated by comparing the ability of treated plasmid DNA to transform competent cells. Chlorine did not affect plasmid DNA structure at the studied doses, while ozone and photocatalytic treatment resulted in conformational changes and the damage increased with increasing oxidant doses. Transformation experiments confirmed a similar trend. Chlorine did not affect the transformability and the cell counts of competent cells transformed with chlorine treated plasmid DNA were similar to those transformed by non-treated plasmid DNA in the control experiments.

Keywords: Antibiotic resistance; Plasmid DNA; Chlorine; Ozone; Heterogeneous photocatalysis

Introduction

Currently, biological treatment remains to be the most commonly applied wastewater treatment technology, but has proven to be insufficient for the complete removal of many antibiotics (1, 2). As a result, antibiotics are released into the environment from domestic wastewater treatment plant effluents (3) and animal farming units (4, 5) and lead to development of antibiotic resistance in local bacterial communities (6-8). Microorganisms can bear natural primary resistance, which can be inherited and passed through generations by vertical gene transfer; however secondary resistance transferred by conjugation is also common due to antibiotic exposure. In the latter, genetic elements such as plasmids can be exchanged among members of different classes of bacteria (9). Through exchange of these mobile genetic elements, resistance can be transferred for example from non-pathogenic to pathogenic microorganisms. Even very low concentrations of antibiotics in the environment can lead to resistance development (10). Antibiotic resistant bacteria have been detected in aquatic environments (11-13) and in drinking water (14, 15). While improvement of current water treatment technologies for complete or maximum antibiotic degradation has an urgent importance, improvement of water disinfection for the destruction of bacteria that have already attained antibiotic resistance, as well as the released bacterial elements bears equivalent significance in order to reduce further spread of antibiotic resistance in the environment.

Conventional chlorine continues to be the most commonly applied water disinfection technology, which is especially preferred due to its economical affordability compared to more advanced disinfection technologies and due to the provided disinfectant residual that is left after disinfection. However, studies show that chlorine is not very effective in the removal of antibiotic resistant bacteria (16, 17) and sometimes even leads to selection of these (17-19). Ozone on the other hand is a powerful disinfectant which has been shown to remove pathogens that are relatively resistant to chlorine and chloramine (20, 21). Studies on the mechanism of ozone disinfection showed that ozone can penetrate the bacterial cells, reacting with cell ingredients (22) and causing damage to the DNA (23). The ability of oxidant to cause DNA damage can be considered an important feature when transfer of antibacterial resistance through genetic materials is considered. The damaging effect on DNA has also been shown with heterogeneous photocatalysis by TiO_2-photocatalyst (24-27). Although TiO_2-photocatalysis is a less commonly applied technology due

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to the limited performance of TiO_2 owing to its wide band gap (28), this technology can still be suitable as a post- treatment process in drinking water treatment.

The purpose of this study is to compare the effectiveness of the three oxidation processes, chlorination, ozonation and heterogeneous photocatalysis in reducing the risk of antibiotic resistance pollution by resistance carriers, which inevitably reach drinking water sources and therefore conclude on their suitability as current or potential disinfection technologies for water treatment. Disinfection kinetics with the three oxidation processes has been deeply investigated (21, 29-30). Although separate studies have also shown the effect of the oxidants on the DNA structure, there is no work comparing their effect on eliminating the risk of antibiotic resistance transfer. Here, the approach followed for this purpose involves isolation of plasmid DNA of a multi-resistant *E. coli* HB101 and its treatment by various oxidant doses and/or treatment periods of chlorine, ozone, and heterogeneous photocatalysis followed by transformation of competent cells by the treated plasmid DNA in order to understand the effect of treatment on resistance transfer potential. Effect of plasmid DNA concentration on oxidation performance has also been investigated.

Materials and Methods

Plasmid Isolation

Plasmid DNA extraction was carried out by Plasmid Plus Maxi Kit (Qiagen) following the procedures described by the manufacturer. Briefly, a bacteria starter growth solution was initiated by inoculating a single *E. coli* HB101 colony from a previously prepared plate in 100 mL growth medium which was grown for 8 h at 37 °C, 200 rpm. Then a portion of this was transferred into a higher volume of growth medium (1/1000 dilution) and growth was continued for another 16-18 h till the OD reached approximately 4 (measured by Shimadzu UV-1208 model spectrophotometer with 1 cm optical path at 600 nm). The bacteria were pelleted by centrifugation at 4000 g for 15 min and plasmid DNA was extracted from the pellets utilizing the Plasmid Plus Maxi Kit. The plasmid DNA concentration was determined by Nanodrop Spectrophotometer (ND-1000, NanoDrop) and was stored at -20 °C until further use. Plasmid DNA was prepared in two concentrations by dilution with autoclaved Milli-Q water.

Oxidation of Plasmid DNA

Chlorine stock solution was prepared from sodium hypochlorite solution (purum, ~ 10% RT, Sigma-Aldrich) (Free Cl^- = 20,000 $mg.L^{-1}$, as determined by Free DPD Kit purchased from Hach) which was diluted to different chlorine concentrations (0.5 – 5 $mg\ L^{-1}$). The treatment was carried out by addition of 350 µL chlorine solution to eppendorf tubes containing 350 µL sample solution with 80 µL of 200 $ng.\mu L^{-1}$ or 100 $ng.\mu L^{-1}$ plasmid DNA and 270 µL Milli-Q water (to make two plasmid DNA concentrations of 12.8 $\mu g.mL^{-1}$ and 6.4 $\mu g.mL^{-1}$) and treatment period was 1 min. Chlorine from the treated samples was quenched by addition of 550 µL, 0.4% (w/w) sodium thiosulphate (Merck, > 97%).

Ozonation experiments were carried out with a batch system and ozone stock solution in oxidant demand free (ODF) water which was prepared according to the standard methods (31). Ozone stock solution was prepared by using a laboratory scale ozone generator unit (Fisher OZ 500) operated with dry and pure oxygen. In order to prevent ozone decomposition, the temperature of the stock ozone solution was maintained at 4 °C ± 2 by ice bath. The ozone stock solution concentration was determined by the indigo trisulphonate (Acros Organics) spectroscopic method (31). The ozonation experiments were carried out in eppendorf tubes containing 0.5 mL sample solution (80 µL of plasmid DNA with concentration of 200 $ng.\mu L^{-1}$ or 100 $ng.\mu L^{-1}$ and 420 µL Milli-Q water). 0.5 mL ozone solution with known concentration was slowly pipetted into the samples to obtain desired ozone doses (1 – 4.42 $mg.L^{-1}$) and ozonation was carried out for 1 min. Remaining ozone from the treated samples was quenched by addition of 250 µL, 0.4% (w/w) sodium thiosulphate (to make a total of 1.25 mL of sample as in the experiments with chlorine and TiO_2).

Photocatalytic treatment was carried out in 4 mL borosilicate glass tubes designed specifically for the process. 0.2 mg photocatalyst (TiO_2 P25, Evonik Degussa Corporation) was suspended in 1.17 mL Milli-Q by applying sonication for 1 min. Then 80 µL of plasmid DNA with concentration of 200 $ng.\mu L^{-1}$ or 100 $ng.\mu L^{-1}$ was added and the sample was immediately covered with aluminum foil and sonicated for another 1 min. The suspension was illuminated by a 125W BLB Lamp, the quantum yield of which was measured by ferrioxalate chemical actinometry as $\phi = 6.5 \times 10^{-6}$ $quanta.s^{-1}$. During treatment, the samples were placed in a water bath with temperature control and were slightly mixed at 40 rpm on a mini orbital shaker (Stuart, SSM1). Treatment periods ranged between 5-75 min. Dark control experiments and control experiments under black-light irradiation were also carried out. Method of plasmid DNA recovery from TiO_2 suspension was determined by applying a

procedure that was developed by Suzuki et al. (32). The procedure involved the following steps: A sample solution of 1.25 mL, containing 0.2 mg TiO_2 and 80 µL of 200 ng.µL^{-1} plasmid DNA in Milli-Q water was sonicated in ultrasonic bath for 1 min. The sample was immediately covered and slightly mixed on the mini orbital shaker for 75 min in dark. Then it was centrifuged at 12,000 g for 10 min. The first supernatant was separated and preserved for analysis (S-1). The pellet was resuspended in 1.25 mL Milli-Q water, sonicated for 1 min and again centrifuged at 12,000 g for 10 min. The secondary supernatant was separated (S-2). 100 µL, 0.25 N standard NaOH solution (pH 13) was added to the pellet followed by 1 min sonication and then the sample was inserted into water bath at 98 ºC for 10 min. Following a final sonication for 1 min and centrifugation for 10 min at 12,000 g the final supernatant was separated (S-3). The treatment involving addition of NaOH was applied in order to remove remaining plasmid DNA that might have adsorbed onto TiO_2. S-1, S-2, and S-3 were separately evaluated and recovery of plasmid DNA during the centrifugation and separation steps and the alkaline treatment steps were compared. Samples prepared in these experiments were also utilized as dark control experiments.

Analysis of Oxidation Process Performance

DNA samples treated by the three oxidation processes were analyzed on a 1% (wt/vol) agarose gel (Basica Le Agarose, Prona and 0.5X TBE prepared from 50X TBE, FERMENTAS) and run on gel electrophoresis system (Gel XL Ultra V-2, Colepalmer, Labnet International, Inc.). Plasmid DNA (20 µL + 4 µL 6X loading dye) was loaded to the gel and 90V-100V was applied for 45 min - 1h. The gel was stained with ethidium bromide (5 µg.mL^{-1}). MassRuler DNA Ready-to-use Ladder Mix 103 ng.µL^{-1} (FERMENTAS) (3.5 µL) was utilized as the ladder.

Effect of treatment on the ability of plasmid DNA to transform competent cells was investigated. Frozen XL blue competent cells were utilized as transformants. The transformation process briefly included the following steps: 100 µL of competent cell suspension was placed in pre-chilled plastic micro-centrifuge tubes containing 0.5 µL plasmid DNA. One sample containing the competent cell suspension and PUC19 (a control plasmid that carries ampicillin resistance) were prepared as a positive control. The tubes containing the cell and DNA mixtures were chilled for 20-30 min in ice and then placed in a 42 °C heating block for 1-1.5 min. SOC (Super Optimal broth with Catabolite repression) media was added into the cell samples to make a total of 1mL and these were incubated at 37 °C, 240 rpm for 1 h (post-incubation). The cells were pelleted at 12,000 g for 2 min. The pellet was re-suspended in 100 µL sterile Milli-Q water. Then 100 µL of the samples were plated on selective agar containing ampicillin and were incubated at 37 °C overnight. A negative control plate containing only XL-blue cells and no plasmid DNA was also plated. Plasmid DNA activity was measured by the number of cells transformed (number of cells counted on agar plates).

Results and Discussion

Treatment of Plasmid DNA with Chlorine, Ozone and TiO_2-Photocatalyst

No effect of chlorine on plasmid DNA structure was observed in this study, where gel electrophoresis was utilized in order to analyze oxidant effect on plasmid DNA structural integrity. The gel images of chlorine treated plasmid DNA having concentration of 12.8 µg.mL^{-1} and 6.4 µg.mL^{-1} are displayed in Figure 1a and 1b, respectively. The band that belongs to the supercoiled (sc) or closed-circular plasmid DNA structure has been marked on the gel images. Disruption of plasmid DNA integrity and formation of other plasmid DNA conformations is expected to result in decrease in the band intensity of supercoiled form with subsequent increase in band intensities of other forms.

It can be viewed from the images that band intensities of supercoiled plasmid DNA treated at different concentrations of chlorine (0.5 mg.L^{-1} - 5 mg.L^{-1}), are similar to those of the untreated control sample (Figure 1a). A two-fold decrease in plasmid DNA concentration did not change this observation and again plasmid DNA integrity was not effected (Figure 1b). The proposed most dominant mechanism during chlorine disinfection is the reaction of chlorine with the cell membrane lipids which results in change in cell membrane permeability (33) among various disinfection mechanisms (34). Considering the possible leakage of resistance carrier elements such as plasmid DNA and their presence in free forms and in the light of the findings in this work and those reported in the literature, the relative ineffectiveness of chlorine in antibiotic resistance management can not be ignored. Nonetheless, it should also be noted that in some previous studies in which the effect of generated hypohalites at inflammation sites on the cell components was investigated, oxidative damage and base modifications of DNA by endogenous hypochlorite have been reported (35-37). The contradictory finding in the present work can be attributed to the higher plasmid DNA concentration. Quantities of environmental extracellular DNA detected in marine and freshwater have

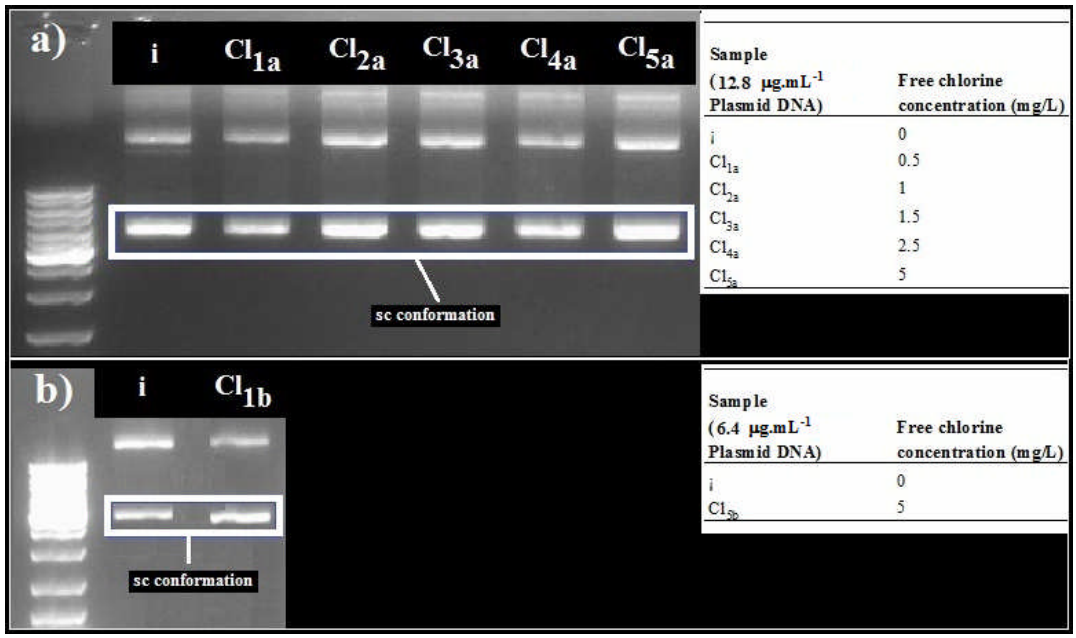

Figure 1. Agarose gel electrophoresis of chlorine treated plasmid DNA (a) high concentration – 12.8 μg.mL^{-1} (b) low concentration – 6.4 μg.mL^{-1} (*sc-conformation:* supercoiled plasmid DNA conformation).

been reported in the range of 0.0002 – 0.044 μg.mL^{-1} (38). However, higher concentrations may be released by leakage after cell lysis as in the case of disinfection of waste water. Also, it is possible that released intact plasmid DNA and extracellular DNA may be protected by binding to particulate or suspended matter in water (39), and then become available under appropriate conditions. The reason why higher concentrations of plasmid DNA were selected in this work was to facilitate the analyses regarding the oxidant effect on plasmid DNA structure.

Gel electrophoresis images of ozone treated plasmid DNA at the high and low concentrations are displayed in Figure 2a and 2b, respectively. Relaxed, linear, and supercoiled plasmid DNA conformations can be viewed in the gel images in the order of slowest migrating to the fastest migrating bands, respectively. As opposed to the results obtained by chlorine, increasing oxidant doses resulted in gradual increase in the intensity of bands belonging to other DNA conformations (i.e. relaxed and linear forms) accompanied with decrease in the supercoiled DNA bands. Progress in this effect as the dose of ozone increases can be viewed in the gel image of the plasmid DNA having higher concentration and at the highest ozone dose (4.2 mg.L^{-1}), the band intensity of supercoiled DNA is minimal (Figure 2a). When plasmid DNA with lower concentration (Figure 2b) was treated with ozone, an ozone dose of 0.90 mg.L^{-1} resulted in complete disappearance of the supercoiled DNA band. As ozone dose increased, intensity of other DNA bands increased initially showing conversion of supercoiled DNA structure, but decreased at higher doses. Complete disappearance of plasmid DNA bands was observed at the highest ozone dose (4 mg.L^{-1}). Ozone has been reported to damage DNA by causing strand breaks as well as base damage and protein cross-linking (40, 41). Sawadaishi et al. (41) explained strand-break formation by the conversion of supercoiled plasmid DNA to open circular plasmid DNA in ozone treated samples initially containing relatively high concentration of plasmid DNA (50 μg). Also, Ishizaki et al. have shown immediate strand scission of chromosomal DNA of *E. coli* upon treatment by ozone (23). Similarly here, ozone treatment resulted in strand scission converting the supercoiled plasmid DNA to relaxed form with subsequent linearization. Increase of intermediate bands in the gel image showed degradation of plasmid DNA and formation of smaller fragments.

The light control experiments showed that in the absence of a photocatalyst, no major effect of illumination occurred on the plasmid DNA having high concentration; while for plasmid DNA of low concentration minimal conformational change took place at the 75th min (Figure 3a). Extended treatment under illumination for 120 min did not lead to any further change in DNA band intensities. Plasmid DNA was effectively recovered by the initial centrifugation step carried out during the dark control experiments. Intensities of plasmid DNA bands from S1 were similar to those of the untreated control sample and no bands were detected in S2 and S3. The plasmid DNA

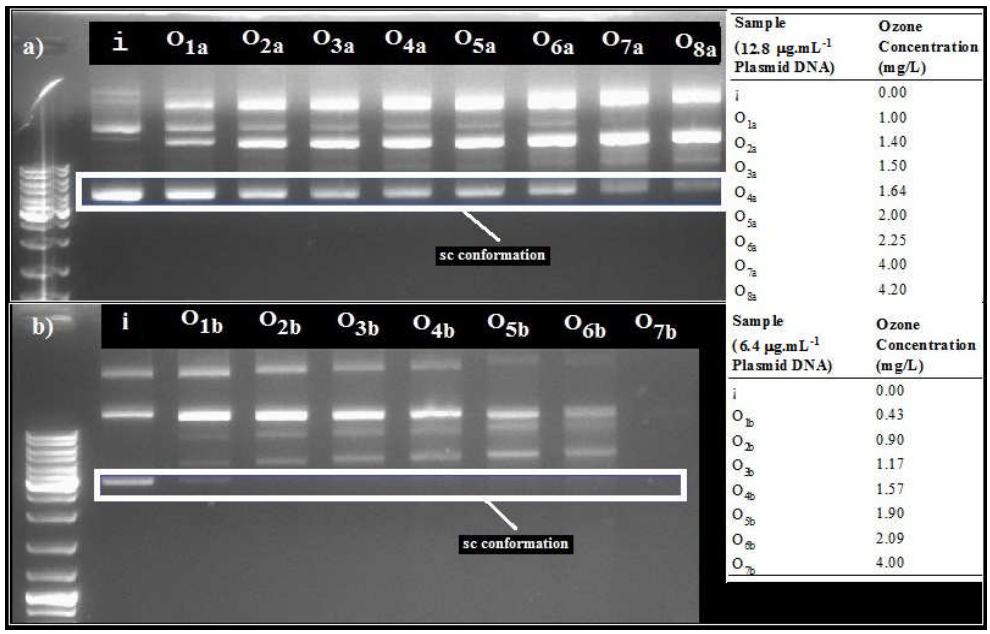

Figure 2. Agarose gel electrophoresis of ozone treated plasmid DNA (a) high concentration – 12.8 μg.mL^{-1} (b) low concentration– 6.4 μg.mL^{-1}.

bands of S1 are displayed in Figure 3b, along with the untreated control sample. Therefore, dark control experiments carried out for 75 min showed that plasmid DNA could be effectively recovered from TiO_2 by a simple centrifugation step.

Treatment of plasmid DNA with TiO_2-Photocatalyst caused conformational change in plasmid DNA bands as in the ozone treatment experiment (Figure 4a and 4b). For the higher concentration plasmid DNA (Figure 4a) initially, change in the intensity of the band belonging to the supercoiled structure was not apparent as for the ozone experiment, however conversion to other conformations was evident by the increase in their band intensities. Decrease in the supercoiled band intensity occurred at the 60 min treatment period and disappeared completely at the 75 min treatment period. For the sample with lower plasmid DNA concentration, the supercoiled DNA band disappeared within 15 min of treatment. Similar to the effect observed with ozone treated sample having lower plasmid DNA concentration, with shorter treatment periods, intensities of other plasmid DNA bands increased. After 45 min treatment, bands intensities of other conformations decreased as well. All plasmid DNA bands were completely removed from the gel image with 75 min treatment. Similar to ozone, TiO_2-photocatalysis causes strand breaks resulting in conformational change. Moreover bands belonging to smaller fragments show DNA degradation as observed during ozone treatment. Dunford et al. (42) have shown

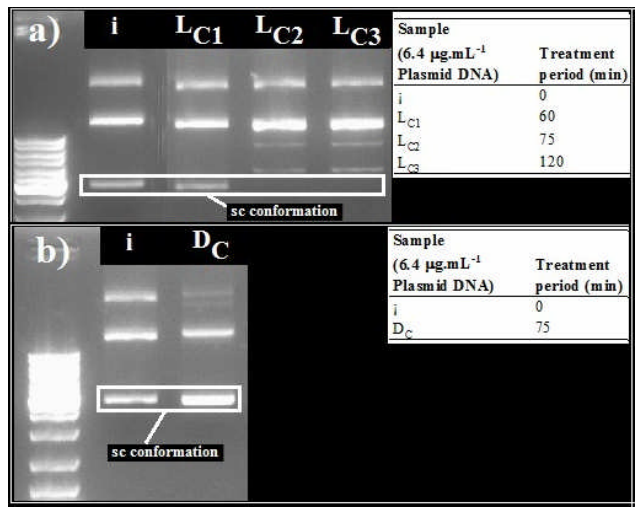

Figure 3. Light, L_C **(**a) and dark control, D_c experiments (b) for low concentration plasmid DNA – 6.4 μg.mL^{-1}.

that strand breakage in plasmid DNA during TiO_2 photocatalysis is by direct oxidation via hydroxyl radicals and Shen et al (27) have confirmed conformational changes in plasmid DNA upon treatment with photosensitized nano-TiO_2 material structure by atomic force microscopy (AFM).

Ability of Plasmid DNA to Transform XL-Blue Competent Cells

Transformability of competent cells with treated plasmid DNA was investigated in order to correlate

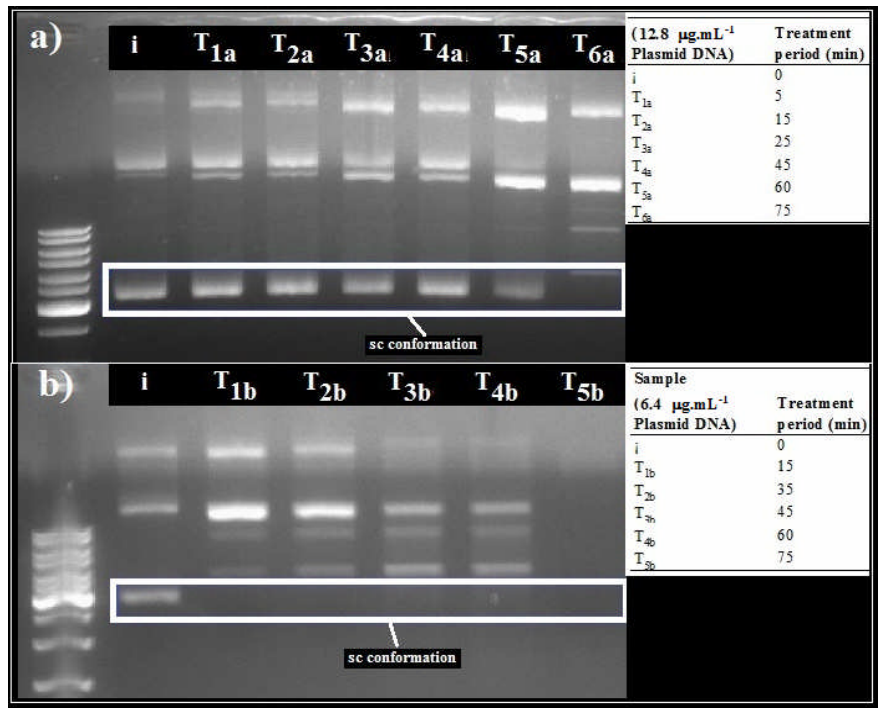

Figure 4. Agarose gel electrophoresis of plasmid DNA treated with TiO_2-photocatalyst (a) high concentration – 12.8 µg.mL^{-1} (b) low concentration plasmid DNA – 6.4 µg.mL^{-1}.

the damaging effect of oxidants with their ability to reduce the potential of the plasmid DNA to transfer antibiotic resistance. For this purpose samples of plasmid DNA treated at several doses of oxidants (typically the lowest and the highest oxidant doses) were selected. Decrease in transformation ability of treated plasmid DNA was calculated relative to transformation ability of non-treated plasmid DNA as $\log(N/N_o)$, where N and N_o are numbers of colony forming units counted on plates containing competent cells combined with treated and non-treated samples, respectively. Transformation ability of plasmid DNA at both concentrations was not affected even with the highest chlorine dose (5 mg.L^{-1}) and thus data was not plot. A similar finding for the effects of chlorine dioxide on transformability was reported in a previous study (43) where both plasmid DNA extracted from the treated microorganisms and treated naked plasmid DNA were investigated. Treated naked plasmid DNA was only minimally affected only at a very high chlorine dose of 20 mg.L^{-1} and with longer treatment periods. Naked plasmid DNA concentration was not reported by the authors; however it is expected to be comparable with the concentration of plasmid DNA obtained from the treated microorganisms, the initial concentration of which was 2×10^8 cells.mL^{-1}. This concentration is much lower than that utilized to obtain the relatively higher plasmid DNA concentrations studied in this study.

Figure 5a and b displays results as $\log(N/N_o)$ plot against ozone dose and treatment period for TiO_2 photocatalysis, respectively.

Samples, the electrophoresis images of which showed minimal change in the plasmid DNA conformation in the gels, also showed minimal effect on the ability to transform competent cells; high concentration plasmid DNA treated with ozone dose of 1 mg.L^{-1} or with TiO_2 for 60 min for instance. Oxidation both with ozone and by TiO_2 photocatalysis resulted in much higher transformability decrease in the lower concentration plasmid DNA samples and no colony forming units were found on the plates of samples treated with 4 mg.L^{-1} ozone dose or by TiO_2 for 75 min. In the gel images of these samples, no plasmid DNA bands were detected. Although natural transformation mechanisms and required conditions for transformation may show variations depending on the nature of the microorganism (9), it has been shown that disruption of plasmid integrity leading to linear plasmid DNA conformations during in vitro ligation reactions can result in up to 2-3 fold decrease in transformability efficiencies in *E. coli* (44). Therefore linearization of plasmid DNA is expected to result in decrease in transformability. However, in transformation experiments with *E. coli* and plasmid DNA of sizes varying from 2 kb to 66 kb, Hanahan et al. (45) have shown that the transformability efficiencies of relaxed plasmid DNA forms were approximately 75%

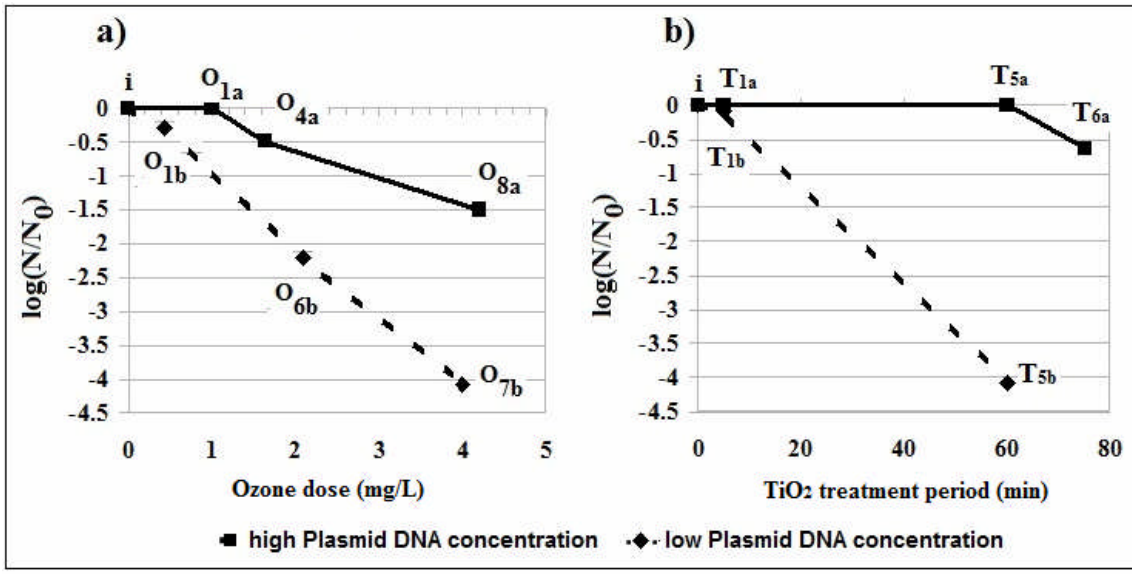

Figure 5. Effect of (a) ozone and (b) TiO_2-photocatalysis on the ability of plasmid to transform competent XL-blue cells. Selected ozone doses are: O_{1a} – 0.43 mg.L^{-1}, O_{4a} – 1.64 mg.L^{-1}, O_{8a} – 4.20 mg.L^{-1} for high concentration plasmid DNA – 12.8 µg.mL^{-1}; O_{1b} – 0.43 mg.L^{-1}, O_{6b} – 2.09 mg.L^{-1}, O_{7b} – 4 mg.L^{-1} for low concentration plasmid DNA – 6.4 µg.mL^{-1}. Selected TiO_2 treatment periods are: T_{1a} – 5 min, T_{5a} – 60 min, T_{6a} – 75 min for high concentration plasmid DNA – 12.8 µg.mL^{-1}; T_{1b} – 15 min, T_{5b} – 75 min for low concentration plasmid DNA – 6.4 µg.mL^{-1}.

of that of their supercoiled forms, showing that relaxed plasmid DNA can still transform competent cells effectively. The results obtained here similarly have shown that although disruption of plasmid DNA integrity did result in decrease in transformability efficiencies, complete loss of the transforming activity was only achieved with complete plasmid DNA destruction by the oxidants. Therefore oxidant damage on plasmid DNA reported in previous studies alone is not sufficient to conclude that the risk of resistance transfer is eliminated. As noted previously, protection of exogenous DNA by suspended material in water matrices further underlies the significance of this finding. Therefore careful monitoring of water and application of effective oxidant doses is important. It can be proposed that ozone and TiO_2-photocatalysis can decrease and prevent ability of plasmid DNA to transform competent cells and thus are both expected to help reducing the risk of antibiotic transfer.

Conclusion

Effect of chlorine, ozone and TiO_2 mediated photocatalytic processes on plasmid DNA was comparatively investigated for the elimination of antibiotic resistance pollution in water. The results revealed that removal of all plasmid DNA bands from the gel image necessitated a high ozone dose and a longer photocatalytic treatment period. However, it is possible to remove lower plasmid DNA concentration with lower ozone doses and shorter photocatalysis treatment durations; but in this case mass transfer limitations should also be considered. While ozone and TiO_2 induced damage in the plasmid DNA structure and therefore leaded to decrease in competent cell transformability, no effect of chlorine was observed during the experiments.

Acknowledgments

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