

Efficacy of copper and silver ions with iodine in the inactivation of *Pseudomonas cepacia*

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3573/01/91: accepted 11 July 1991

B.H. PYLE, S.C. BROADAWAY AND G.A. MCFETERS. 1992. Alternatives to chlorination of water have been sought for reasons which include trihalomethane formation, possible bacterial regrowth, the high concentrations of chlorine required in certain circumstances, and the taste, odour and bodily irritation in chlorine-treated water. Electrolytically generated Cu and Ag ions at low levels, in addition to very low chlorine concentrations, have been suggested as an alternative to routine chlorination. We have examined the combination of Cu and Ag ions with low levels of iodine. *Pseudomonas cepacia* was grown either in rich medium or under nutrient restriction prior to disinfection. Survival of the organism and its ability to regrow after treatment as well as the effects of varying buffers, metal ion and iodine concentrations were determined. Low concentrations of metal ions (100 ppb Cu and 11 ppb Ag) and iodine (200 ppb) were more effective than either metal ions or iodine alone against *Ps. cepacia* grown on rich agar or in low nutrient buffer. After iodination, buffer-grown suspensions recovered to their original cell concentrations within 7 d. When Cu and Ag ions were used with or without iodine, regrowth was prevented. The results show that low concentrations of Cu and Ag in combination with iodine permit effective disinfection of bacteria after cultivation on either rich media or under nutrient restriction. These results, along with published data, suggest that the combination of these metals with halogenation may have applications in the disinfection of both recreational and potable water.

INTRODUCTION

Chlorination has been the predominant means of water disinfection since its antibacterial action was discovered in the 19th century and its introduction for water purification early this century (Trueman 1971; Hugo 1982; Dychdala 1983). Several factors have, in part, suggested the need for alternative water disinfectants. One of these is the production of hazardous levels of trihalomethanes in chlorinated water containing organic materials (Stevens *et al.* 1978). Furthermore, unexplained increases in bacterial populations have been observed in chlorinated water distribution systems (Olivieri *et al.* 1985), and the presence of legionellas in source water has been correlated with its detection in water distribution mains (Colbourne *et al.* 1988). It is sometimes difficult to maintain an adequate concentration of residual chlorine throughout the distribution system (O'Connor *et al.* 1975; Fliermans *et al.* 1982; Goshko *et al.* 1983). In addition, the taste, odour, and bodily irritation caused by chlorine (Clarke & Berman 1983), particularly at

concentrations above 0.6 mg/l (Yahya *et al.* 1989a), are significant drawbacks to its current usage.

The disinfectant activity of chlorine is thought to be related largely to oxidation reactions by which enzymes are inactivated, although it may also react with membrane proteins forming toxic products and possibly disrupting cell membranes (Dychdala 1983). However, chlorine is not effective against some micro-organisms at the concentrations used for routine disinfection of water. For example, legionellas may be particularly resistant to chlorine (Skaliy *et al.* 1980; Lee & West 1991), and these bacteria may develop increased chlorine resistance in systems with high chlorine levels (Kutchka *et al.* 1983). Corrosion of plumbing systems is possible at the extreme levels of chlorine required for disinfection of legionellas in cooling towers (Skaliy *et al.* 1980). Bacterial biofilms may also contribute to the difficulty of achieving adequate disinfection in some situations (LeChevallier *et al.* 1990; Pyle & McFeters 1990a; Lee & West 1991).

Initially used as an antiseptic (Sykes 1965; Trueman 1971; Hugo & Russell 1982; Gottardi 1983) since World War I, iodine has also been used for water treatment in

mainly emergency situations (Black *et al.* 1968; Gottardi 1983) and for disinfection of small individual water supplies and swimming pools (Favero & Drake 1966; Clarke & Berman 1983). Iodine is slower acting than chlorine (Davis 1962), and its mode of action is thought to be by iodination of amino acids and nucleotides, oxidation of S-H groups of cysteine, and reaction with the phenolic group of tyrosine of the C=C bond of unsaturated fatty acids (Gottardi 1983). Since it is less reactive than chlorine, it is inactivated by organic matter to a lesser extent than is chlorine (Sykes 1965; Williams & Russell 1991). It is also less corrosive than chlorine and is considered to be safer because it does not produce explosive gases. For these reasons, anion exchange resins have been developed (Taylor *et al.* 1970; Hatch *et al.* 1980; Lambert & Fina 1980; Marchin *et al.* 1985) for use as demand-type disinfectants on United States spacecraft.

The use of copper and silver ions to control bacteria, viruses, algae and fungi in water is well documented (Sykes 1965; Kushner 1971; Clarke & Berman 1983; Grier 1983; Landeen *et al.* 1989a, b; Yahya *et al.* 1989a, 1990). The antimicrobial activity of copper is related to oxidation of sulphhydryl groups of enzymes (Hugo & Russell 1982) which inhibits enzymatic activity and interferes with cell respiration (Domek *et al.* 1984, 1987). Silver also interferes with enzyme activity by binding to proteins, and both Cu and Ag bind to DNA molecules (Yahya *et al.* 1990). While the bactericidal action of copper and silver may be slower than that of free chlorine (Kutz *et al.* 1988), there appears to be a synergistic effect when these metals are used in combination with chlorine (Yahya *et al.* 1989a, 1990).

The sensitivity of bacteria to disinfection is related in part to the conditions under which the bacteria were grown. Cells grown in a low nutrient medium may be more resistant to iodine than those grown in rich medium, although the opposite may be true for some strains (Pyle & McFeters 1989). With increasing growth rate in a chemostat, N- and C-limited cells of *Escherichia coli* became more sensitive to chlorhexidine, while Mg- and PO₄-limited cells became less sensitive (Wright & Gilbert 1987). These effects could not be correlated with cell envelope composition. Surface attachment of bacteria may also affect their halogen or metal ion sensitivity (Goodson & Rowbury 1986; Hicks & Rowbury 1986, 1988; Pyle & McFeters 1990a). Pseudomonads are usually resistant to disinfection by a number of methods (Brown 1971) and it has been found that iodine-resistant bacteria, particularly pseudomonads, may proliferate in iodinated water (Favero & Drake 1964, 1966; Pyle & McFeters 1990b). It would thus be advantageous if other disinfectants could be included to control bacterial numbers in iodinated systems.

The studies reported here were done to determine whether electrolytically generated silver and copper ions

would be effective in the disinfection of *Pseudomonas cepacia* when combined with low levels of chlorine or iodine. Cells were exposed to different disinfection conditions, including: (1) chlorine and Ag/Cu ions in two buffer systems; (2) varying iodine concentrations; (3) varying Cu/Ag ion concentrations; and (4) iodine plus Cu/Ag-treated water at two metal ion and iodine levels. The effects of iodine and Cu/Ag exposure on cells grown under either rich or low nutrient conditions and their ability to regrow after different disinfection conditions were also determined.

MATERIALS AND METHODS

Experimental conditions and solutions

Containers used for preparation of solutions and disinfection were acid washed and sterilized before use. High density polyethylene (HDPE) bottles were used and each experiment was completed within 1 h of ion generation to minimize adsorption of metals. All experiments were done at room temperature. The pH of buffers was 7.2. The contents of inoculated test bottles were mixed constantly with magnetic stir bars. All disinfections were done with cells suspended in buffer so that the pH and ionic strength were consistent and also to minimize interactions between the metals and medium (Bird *et al.* 1985).

Cultures

The organism used in these experiments was a *Ps. cepacia*, Montana State University strain Pc3, which was isolated by the National Aeronautics and Space Administration (Johnson Space Center, Houston) from a Space Shuttle water system. This strain was shown to be relatively resistant to iodine when grown under nutrient restriction (Pyle & McFeters 1989) and in biofilms (Pyle & McFeters 1990a). It was also capable of growth after iodination (Pyle & McFeters 1990b).

For rich nutrient conditions, the cells were grown on R2A agar (Reasoner & Geldreich 1985) for 24 h, then re-inoculated on R2A agar and allowed to grow for an additional 24 h. R2A agar is a low-nutrient medium designed to enumerate aerobic heterotrophs in water (Anon. 1985a). These cells were harvested, suspended in water, and filtered through a 5 µm nylon screen to eliminate clumps. The suspensions were then diluted and adjusted with a McFarland No. 1 standard to ca 3×10^8 cfu/ml before introduction into test bottles. For nutrient-restricted conditions, Pc3 cells which had previously been adapted for growth in phosphate-buffered water (PBW) (Pyle & McFeters 1989), were inoculated into sterile PBW at about 3×10^2 cfu/ml.

The suspension was then left at room temperature for 7 d. Cell numbers reached about 10^5 cfu/ml by day 7.

Chemical analyses

Copper concentrations were determined with a colorimetric kit (model STC, LaMotte Chemical Products Co., Chestertown, MD). It was assumed that silver concentrations would be proportional to copper concentrations since the electrode used was 90Cu : 10Ag, thus silver ion concentrations are expressed relative to copper levels. Samples were sent to an independent laboratory for verification of metal ion concentrations. Chlorine and iodine concentrations were tested by amperometric titration using phenylarsine oxide titrant (Anon. 1985b).

Effect of buffers

The relative performance of PBW and carbonate buffered water (CBW) was compared for use in subsequent disinfection experiments. PBW was prepared from stock solution (Anon. 1985c) without $MgCl_2$. The final concentration of PO_4 was 42 ppm. CBW was prepared as described by Domek *et al.* (1987).

Generation of copper and silver ions

A Tarn-Pure (TP) Cu/Ag 90 : 10 unit (Tarn-Pure USA, Las Vegas, NV) was closed at one end so that it could be filled with buffer for generation of Cu and Ag ions. It was rinsed with hot sterilized distilled water, allowed to cool then filled with 1.5 l of either PBW or CBW and operated for 40–90 s. The treated buffer was diluted with untreated buffer until the copper concentration was 400 ppb. A suspension of rich grown Pc3 was added to a series of HDPE bottles containing either PBW or CBW with one of the following: (1) 400 ppb Cu/44 ppb Ag ions; (2) 50 ppb chlorine; or (3) a combination of 400 ppb copper/44 ppb silver and 50 ppb chlorine. A control bottle contained only buffer with no chlorine or metal ions added. The concentration of cells was about 3×10^6 cfu/ml before disinfection. Samples were withdrawn at timed intervals and placed in vials containing 14.6% $Na_2S_2O_3 \cdot 5H_2O$ and 10% sodium thioglycollate (10 μ l/ml of sample), to neutralize the metal ions and iodine. Samples were then diluted and plated on R2A agar.

Effect of varying iodine concentrations

Tests were performed to establish a suitable concentration of iodine. Cells were grown on R2A agar as described above, and suspended in PBW at about 10^6 cfu/ml. Different concentrations of iodine (0.5, 0.25, and 0.1 ppm) were added to

the PBW suspensions. Samples were withdrawn at timed intervals, treated with the neutralizing solution, diluted and plated on R2A agar.

Effect of varying metal ion concentrations

Similar experiments were done with Cu/Ag ions over a range of concentrations. The TP unit was run for 90 s and the treated PBW diluted as required. Survival of Pc3 in copper levels of 400, 200 and 100 ppb was compared.

Effect of metal ions plus iodine

For the remainder of the study, PBW was treated in the TP unit for 5 to 7 min, giving a copper concentration of 1.5 to 3.0 ppm. Samples of this treated PBW were added to PBW containing suspensions of cells that had been grown on R2A agar or in PBW. For studies with low levels of metal ions and iodine, the final copper concentration was 100 ppb and the iodine concentration was 200 ppb. Experiments with higher disinfectant levels, in which the copper concentration was 400 ppb and the iodine concentration was 500 ppb, were done with PBW grown cells only. The copper level was checked at the beginning and end of each experiment.

For each experiment, four bottles of PBW with either low nutrient (PBW) or rich grown (R2A agar) Pc3 at 10^5 to 10^6 cfu/ml were prepared. A sample of one of the following was added to this suspension: (1) Cu/Ag-treated PBW plus iodine; (2) Cu/Ag-treated PBW only; (3) untreated PBW plus iodine only; or (4) untreated PBW only. Samples were taken at timed intervals up to 15 min, added to neutralizing solution, diluted as needed, and plated on R2A to enumerate the survivors. Suspensions treated with higher concentrations of disinfectants were held for a week and sampled to determine if regrowth occurred.

Statistical analysis

Except for the initial buffer comparison, experiments were repeated three to five times, and the mean and variance of the replicate results for each treatment and sampling time calculated using the MSUSTAT statistical analysis package, version 4.10 (developed by Richard Lund, Montana State University). Standard errors were calculated manually and indicated on the figures where n was ≥ 3 .

RESULTS

Effect of buffers

There were fewer survivors with the combination of Cu/Ag-treated water plus chlorine than with either Cu/Ag ions

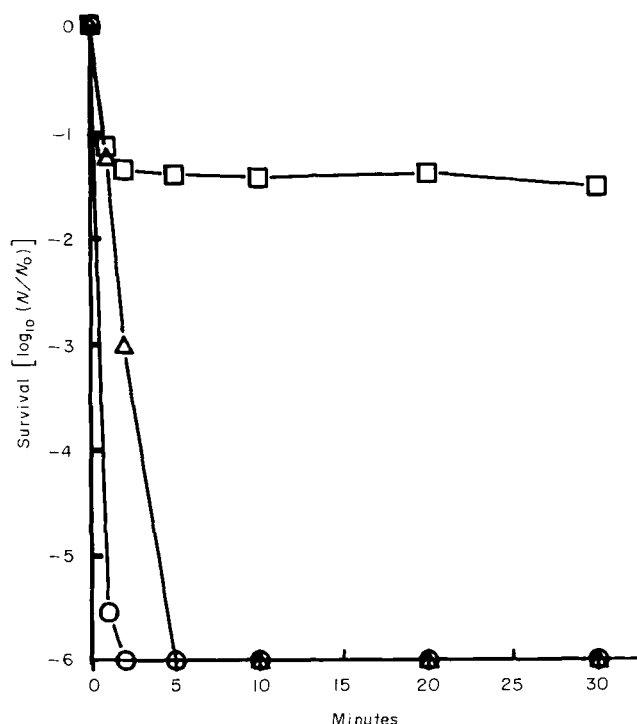


Fig. 1 Survival of *Pseudomonas cepacia* following treatment in phosphate buffered water. Disinfection conditions were 400 ppb Cu/40 ppb Ag ions (Δ), 50 ppb chlorine ($\square$), and Cu/Ag plus chlorine ($\circ$)

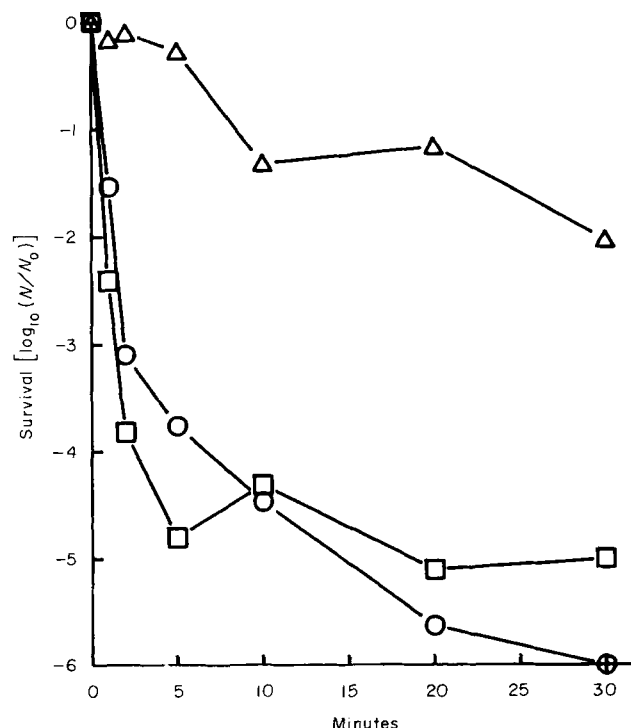


Fig. 2 Survival of *Pseudomonas cepacia* following treatment in carbonate buffered water with Cu/Ag ions (Δ), chlorine ($\square$), or Cu/Ag ions plus chlorine ($\circ$). Disinfection conditions are as for Fig. 1

or chlorine alone in PBW (Fig. 1) or CBW (Fig. 2). However, the survival of Pc3 exposed to either Ag/Cu ions or chlorine depended on the type of buffer used. Disinfection in CBW was slower than in PBW. Since PBW had been used previously for pseudomonad disinfection experiments in this laboratory (Pyle & McFeters 1989), and because pseudomonads can be grown in PBW for low nutrient studies PBW was used in all subsequent experiments.

Effect of varying iodine concentrations

An iodine concentration of 200–300 ppb was chosen for low level disinfection experiments because it gave a reduction in cell numbers of between 1 and 2 logs over 10 min (Fig. 3). A higher iodine concentration of 500 ppb reduced cell numbers nearly 3 logs in 1 min.

Effect of varying metal ion concentrations

Cu/Ag concentrations of 100/11 ppb were selected for subsequent experiments with the metals plus low levels of halogen disinfectants. At these concentrations of Cu/Ag, there was a 4 to 6 log decrease in cell numbers in 10 min,

with about a 1 log loss in 2 min (Fig. 4). Metal concentrations of 400 ppm copper/44 ppm silver used in experiments with higher concentrations of disinfectants reduced cell numbers over 3 logs in 2 min and over 5 logs in 5 min (Fig. 4).

Effect of metal ions plus iodine

For cultures grown on R2A agar treated with a low level of iodine alone, cell numbers tended to level off after 5 min following a 2 log decrease (Fig. 5). At low concentrations of Cu/Ag without iodine, there was a 3 log decrease in cell numbers by 15 min, but the initial rate was slower than with iodine alone, giving only one log decrease in 5 min. More complete disinfection occurred when a combination of iodine and Cu/Ag-treated PBW was used. There was a 3 log bacterial loss in 5 min and more than a 5.5 log decrease in 15 min.

For Pc3 grown under low nutrient conditions, cell numbers dropped over 1 log in 5 min, and 3 logs in 15 min when treated with a low concentration of metal ions, i.e. 100 ppb of copper (Fig. 6). PBW cultures of Pc3, when exposed to 200 ppb of iodine, decreased over 2 logs in 5 min and 3 logs in 15, indicating they were somewhat more

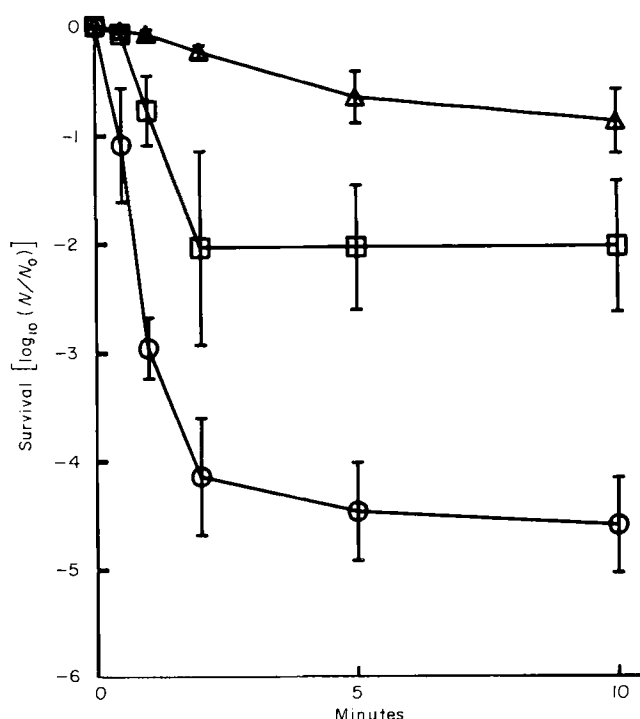


Fig. 3 Survival of R2A grown *Pseudomonas cepacia* following treatment with iodine at concentrations of 100 ppb (Δ), 250 ppb ($\square$) and 500 ppb ($\circ$)

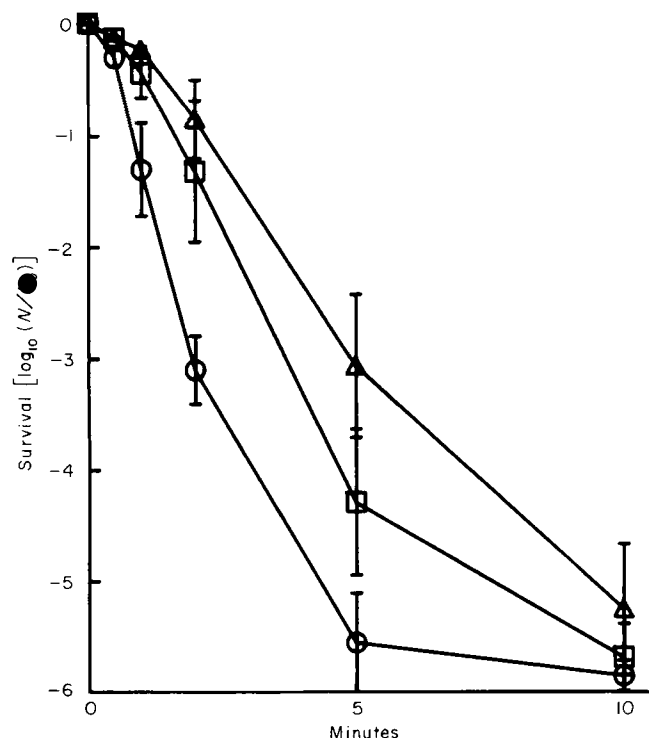


Fig. 4 Survival of *Pseudomonas cepacia* grown on R2A agar following disinfection with Tarn-Pure treated phosphate buffered water. Copper and silver concentrations were 100/11 ppb (Δ), 200/22 ppb ($\square$), and 400/44 ppb ($\circ$)

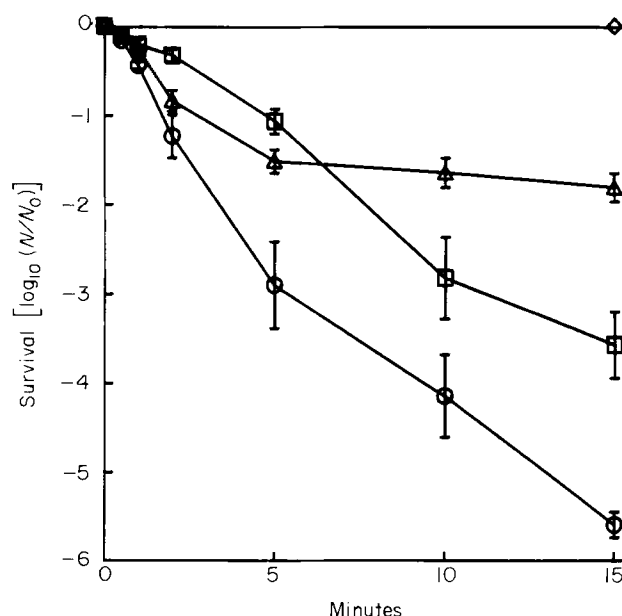


Fig. 5 Survival of R2A grown *Pseudomonas cepacia* following treatment with 200 ppb iodine (Δ), 100 ppb Cu/11 ppb Ag ions ($\square$), and iodine plus Cu/Ag ions ($\circ$) or suspended in PBW with no treatment ($\diamond$)

sensitive to this treatment than R2A grown Pc3. The combination of Cu/Ag-treated water and iodine reduced cell numbers more than either alone, with Pc3 dropping 3 logs in 5 min and nearly 5 logs in 15 min.

Higher concentrations of Cu/Ag and iodine gave a much faster reduction in cell numbers than the lower levels (Fig. 7). The survival of cells when exposed to Cu/Ag plus iodine was almost identical to that of bacteria exposed to iodine alone. Both gave reductions of over 2 logs in 30 s. The Cu/Ag-treated PBW alone took 5 min to give a comparable 2 log reduction.

These treatments, which contained 400 ppb copper and 500 ppb iodine, were held for one week and sampled to determine if regrowth occurred. The results (Fig. 8) indicate growth of Pc3 in samples containing iodine alone by day 3. By day 7, the cell numbers in those samples increased to approximately the same level as the control, which had no disinfectant added. The samples containing Cu/Ag-treated PBW showed no regrowth by day 7.

DISCUSSION

It has previously been shown that electrolytically generated copper and silver ions significantly enhance the effectiveness of low-level chlorination with various laboratory cultures of bacteria suspended in filtered well water (Kutz

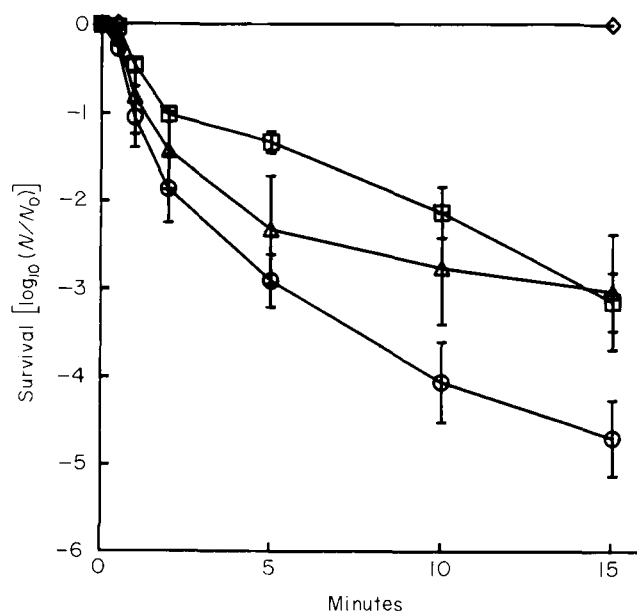


Fig. 6 Survival of PBW grown *Pseudomonas cepacia* following treatment with iodine (Δ), Cu/Ag ions ($\square$), Cu/Ag ions plus iodine ($\circ$) or without treatment ($\diamond$). Disinfection conditions and symbols are as for Fig. 5

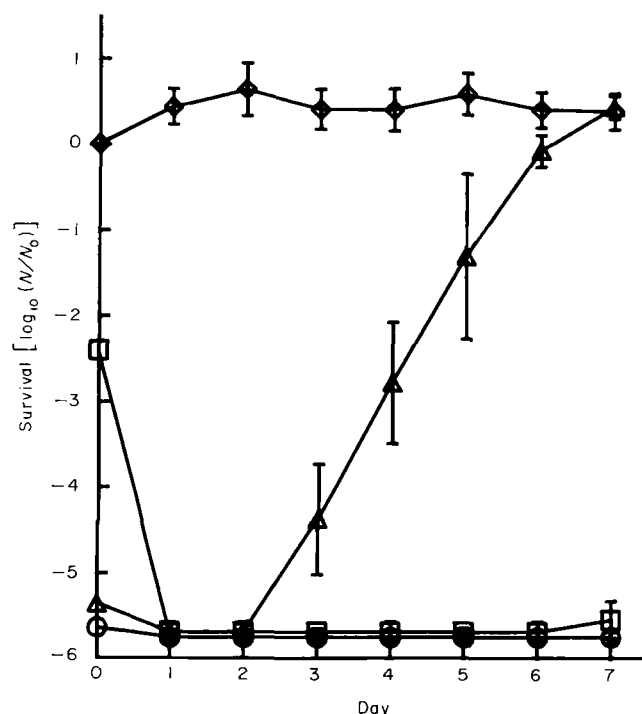


Fig. 8 Regrowth of *Pseudomonas cepacia* following disinfection with iodine (Δ), Cu/Ag ($\square$), iodine plus Cu/Ag ($\circ$) or without treatment ($\diamond$). Disinfection conditions were as for Fig. 7

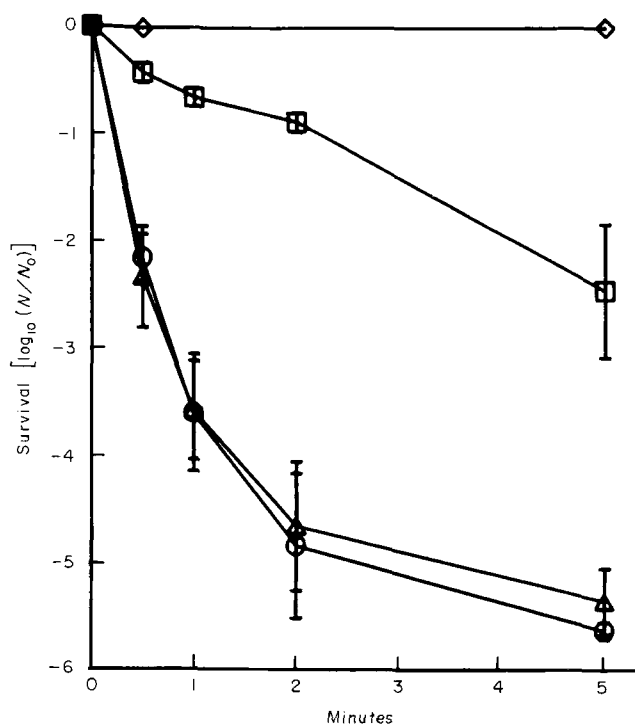


Fig. 7 Survival of PBW grown *Pseudomonas cepacia* following treatment with 500 ppb iodine (Δ), 400 ppb Cu/44 ppb Ag ions ($\square$), Cu/Ag plus iodine ($\circ$) or without treatment ($\diamond$)

et al. 1988; Landeen *et al.* 1989a; Yahya *et al.* 1989a, b). Similar results were obtained in simulated swimming pool tests (Landeen *et al.* 1980b; Yahya *et al.* 1990). The results presented above confirm these findings and show that low levels of copper and silver ions enhanced the activity of halogens when a culture of *Ps. cepacia* was grown either in rich medium or under nutrient restriction. Cu/Ag-treated water plus iodine, at concentrations of 100 ppb copper/11 ppb silver and 200 ppb iodine, were more effective in reducing cell numbers than either iodine or metal ions alone, regardless of the antecedent growth conditions of the culture. In this study, cultures grown on a relatively rich medium (R2A agar) compared with those grown under nutrient restriction (PBW) did not exhibit differences in disinfection sensitivities that have been observed before for cultures grown in brain-heart infusion or PBW (Pyle & McFeters 1989).

It is possible that the composition of the rich medium may be of significance, since R2A agar is a complex medium with lower nutrient concentrations than brain-heart infusion. Bacterial cell envelope constituents and properties may vary according to the composition and strength of the growth medium (Brown & Williams 1985). The ionic composition of the medium may influence cell

sensitivity to silver ions (Brown & Anderson 1968). The presence of plasmids in *E. coli* can affect membrane protein constituents, attachment to surfaces, and sensitivity to chlorine or copper (Goodson & Rowbury 1986; Hicks & Rowbury 1986, 1988). Thus, the sensitivity of bacteria to disinfection depends not only on the nature of the environment but also on the types and genetic complement of bacteria present and their ability to adhere to surfaces.

Higher concentrations of both metal ions and iodine plus metal ions reduced cell numbers more quickly than lower levels, but no better than high concentrations of iodine alone after 5 min. Bacterial suspensions exposed to metal ions or metal ions plus iodine exhibited inactivation responses that were generally less biphasic with time than those observed with iodine alone. In addition, bacterial regrowth occurred in samples that contained only iodine, indicating that a small resistant population remained and was able to multiply after disinfection. Samples that contained Cu/Ag-treated water exhibited no regrowth. These observations suggest that the use of copper and silver provides an effective antibacterial residual while iodine alone may not. This may be related to differences in the mode of action of halogens (Dychdala 1983; Gottardi 1983) and heavy metal ions (Hugo & Russell 1982; Grier 1983) and thus possible differences in bacterial recovery from disinfectant injury.

Treatment with copper and silver ions combined with low concentrations of chlorine has been proven effective for the disinfection of simulated swimming pools containing natural body flora and urine (Yahya *et al.* 1990). This form of combined treatment has been recommended for water disinfection in other situations such as spa pools, hot tubs and cooling towers. In these circumstances it may be possible to achieve adequate disinfection at low levels of chlorine which do not annoy bathers or cause excessive corrosion of plumbing materials. Our results for disinfection with Cu/Ag ions and iodine or chlorine suggest that the addition of electrolytically generated Cu and Ag ions to water systems where low concentrations of these metals are permissible may facilitate the control of microbes in the system by ensuring adequate disinfection and by preventing post-disinfection regrowth in the water storage and distribution network.

Copper is frequently found in potable water, since it has been used in water treatment and originates from both natural sources and plumbing materials (Domek *et al.* 1984). Silver has been shown to have little effect on mammalian cells at the concentrations at which it is effective against bacteria (Berger *et al.* 1976). Thus, provided the concentrations of Cu and Ag are within the prescribed limits for potable water quality, the use of these metals in combination with low levels of chlorine or iodine may be an alternative to current drinking water treatment procedures.

For example, in emergency potable water treatment when the metal-treated water will be consumed for limited periods of time, this treatment should be more reliable than current methods which frequently rely on chlorine or iodine alone. In addition, this form of treatment might remedy situations where excessive numbers of coliform bacteria are detected in treated water distribution systems.

One of the objections to the use of copper and silver for water disinfection has been that the metal tends to concentrate on surfaces and this limits the availability of ions for disinfection in the liquid phase. As has been pointed out by Yahya *et al.* (1990), this characteristic of these metals may inhibit or slow down biofilm formation, as it is intended to do in point-of-use carbon filters (Grier 1983). It might also enhance halogen disinfection in situations where biofilms are already present, such as in water distribution systems with recurrent contamination when biofouling is thought to contribute to the problem (LeChevallier *et al.* 1990). Further, this could be an advantage in the prevention of surface contamination in emergency water treatment kits.

The results presented above and published data related to the use of Cu and Ag in combination with a halogen show that these agents may be reliable forms of water treatment to control bacteria which are adapted to nutrient restricted conditions, in the presence of organic materials, and when biofilms occur. This suggests that disinfection by combining these agents may have wider application than has been previously thought.

ACKNOWLEDGEMENTS

We thank Moyasar Yahya, from the University of Arizona, for his help with the protocol used for the chlorine plus Tarn-Pure experiments. We also gratefully acknowledge Shelley Watters for advice and technical assistance. This research was supported by Tarn-Pure USA, and the National Aeronautics and Space Administration (contract NAG 9-241).

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