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Chlorine Demand and Inactivation of Fungal Propagules

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Conidia of filamentous fungi, vegetative yeast cells, and coliform bacteria were tested to determine their chlorine demand and their sensitivity to chlorine inactivation. Levels of chlorine demand for the various conidia, yeast, and coliforms were, respectively, 3.6×10^{-9} to 3.2×10^{-8}, 1.2×10^{-9} to 8.0×10^{-9}, and 2.5×10^{-11} to 6.3×10^{-10} mg of chlorine per propagule. Preliminary evidence suggests that the chlorine demand per propagule increases as the number of propagules per milliliter decreases. In general, conidia showed greatest resistance to chlorine inactiviation, followed by the yeast and coliforms. Inactivation by chlorine was influenced by pH, with inactivation (chlorine activity) falling in the order pH 5 > 7 > 8.

Historically, little attention has been paid to fungi in water distribution systems. Recently it has been reported that fungi can pass through sand filters and survive disinfection of water with chlorine (17). The presence of fungi in a water source has implications for the effectiveness of disinfection and other operations of treatment facilities as well as for possible reactions with chlorine to form chlorinated organic compounds in the finished water. Fungi in potable water distribution systems may have direct effects on human health (allergenic or toxigenic species), contribute to the occurrence of nosocomial infections in compromised individuals, contaminate foodstuffs during processing or preparation, and interfere with potable water distribution either directly or through deteriorative action on gasket or joint materials. Fungi may enter a water distribution system during construction, by passing through treatment processes (17), by means of leaks into the system, or from air in contact with water stored in distribution system reservoirs.

Although there have been many studies dealing with the effect of chlorine on bacterial spores and vegetative cells (8, 9, 12, 18, 23), protozoan cells and cysts (4, 5, 11), viruses (7, 21), and algae (13), there have been no studies, except some work on *Candida* species (6, 8, 9), on the sensitivity of fungi to chlorine. With the exception of some work on protozoa (5), there also is very little information on chlorine demand of microorganisms. This investigation dealt with the chlorine demand of fungi (conidia and yeast cells) isolated from water distribution systems. The influence of pH on chlorine inactivation of conidia and yeast cells was also investigated.

MATERIALS AND METHODS

Source, isolation, and maintenance of microorganisms. Of the numerous fungi isolated from three distribution systems supplied from small public ground water systems in Chester County, Pennsylvania, *Aspergillus fumigatus*, *A. niger*, a *Cladosporium* sp., *Cryptococcus laurentii*, *Penicillium oxalicum*, *Rhodotorula glutinis*, and *R. rubra* were chosen for this study.

Water samples were collected from residential taps in clean, sterile polypropylene bottles to which 0.1 ml of a 10% (wt/vol) sodium thiosulfate solution was added before autoclaving to reduce any free residual chlorine in the water. The taps were flushed for approximately 3 min before sampling. The fungi were separated from 50-ml portions of the samples by the membrane filter procedure (3, 19), using sterile 50-ml syringes, 47-mm Nuclepore polycarbonate filter holders, and Millipore type HA (0.45 μm) filters. Control samples, consisting of sterilized tap water, were also filtered. The filters were then transferred to a selective fungal medium, consisting of Sabouraud dextrose agar amended with rose bengal (33.3 μg/ml) and streptomycin (80.0 μg/ml), and scored daily for a period of 2 weeks. Of the 135 samples collected, 60 (45%) were positive for fungi. Typically, counts ranged between 1 to 10 fungal propagules per 50-ml sample. Fungi were maintained and cultured for experiments on Sabouraud dextrose agar.

Isolated fungal colonies were then checked for purity and transferred to slants of Sabouraud dextrose agar. Identification of the isolated fungi was accomplished through the use of various taxonomic guides and monographs (2, 14, 15, 20, 22).

For comparative purposes, three coliform strains were also tested for their chlorine demand and inactivation by chlorine. *Escherichia coli* was obtained from the American Type Culture Collection (ATCC 11775). *Citrobacter freundii* and *Enterobacter cloacae* were isolated on m-Endo medium (Difco Laboratories) from

the same distribution systems as the fungi, using the procedures described in *Standard Methods for the Examination of Water and Wastewater* (1). Bacterial identifications were performed by means of the API 20E system (Analytab Products). Bacteria were maintained and cultured for experiments on tryptic soy agar.

Preparation of chlorine demand-free water and glassware. Chlorine demand-free (CDF) water was prepared by adding sodium hypochlorite to deionized distilled water to achieve a free chlorine level of approximately 7 mg/liter. The water was stored in the dark for 24 h and then exposed to approximately 24 h of sunlight to inactivate the excess chlorine. Inactivation of the chlorine was confirmed by titration on a Fischer and Porter amperometric titrator.

CDF glassware was prepared by placing glassware in a vat of concentrated sodium hypochlorite for 24 h. The glassware was then rinsed five times with CDF water and allowed to air dry.

Sensitivity of fungi and coliforms to chlorine inactivation. Fungal conidia, yeast cells, or coliforms were gently scraped off of agar slants and washed three times with CDF water. One milliliter of the conidia, yeast, or coliform suspension (typical concentrations per ml were 1.0×10^5 to 5.0×10^6 for conidia, 10^5 to 10^6 for yeast, and 10^6 to 10^7 for coliforms) was then added to a 250-ml flask containing 99 ml of Sorenson phosphate buffer (made up to pH 5, 7, or 8) and adjusted initially to various levels of free chlorine with sodium hypochlorite. Use of the three pH values allowed for a comparison of the fungicidal activity of a range of ratios of hypochlorous acid to hypochlorite ion. The flasks were then placed on a rotary motion shaker (180 oscillations/min; Ederbach Corp.) and incubated in the dark at 25 ± 2°C.

After 10, 30, and 60 min, one flask at each pH was removed from the shaker; 1 ml was withdrawn from each flask and diluted appropriately, and the viable propagules remaining were titered, in triplicate, by either the pour plate (yeast and coliforms) or the spread plate (conidia) method on either eosin-methylene blue agar (coliforms) or Sabouraud dextrose-rose bengal agar (yeast and conidia). Any remaining chlorine was inactivated by incorporation of sodium thiosulfate (0.25% [wt/vol]) in the initial dilution blanks. At the same time, the free and total chlorine remaining was measured with the amperometric titrator. Uninoculated controls were included to measure chlorine losses not due to propagule demands.

Determination of the chlorine demand of fungal conidia, yeast cells, and coliforms. Chlorine demand of the various propagules was determined after a 60-min exposure to the chlorine. Chlorine demand per propagule was calculated by substracting the amount of free chlorine remaining after 60 min from the initial free concentration and dividing by the titer of propagules added. It should be noted that no attempt was made to maintain the initial chlorine concentration as this would preclude determinations of chlorine demands.

RESULTS AND DISCUSSION

All of the fungal conidia studied, as well as the vegetative cells (yeast and bacteria), exhibited a demand for free chlorine. This chlorine demand continued over the 60-min time course of the experiment, as indicated by the decline in free chlorine concentrations. The decline continued for all organisms studied, even when complete spore or cell inactivation had occurred (at 10 or 30 min), thus suggesting that the total chlorine demand of microorganisms is in excess of concentrations required for complete inactivation. This relationship is illustrated in Table 1. There was no significant loss of free chlorine in the uninoculated controls, verifying that the systems were CDF.

The free chlorine demand for the various conidia, yeasts, and coliforms was determined. In general, the chlorine demands for conidia, yeast, and coliforms were 3.6×10^{-9} to 3.2×10^{-8}, 1.2×10^{-9} to 8.0×10^{-9}, and 2.5×10^{-11} to 6.3×10^{-10} mg of chlorine per propagule, respectively, after 1 h of exposure.

Preliminary evidence also indicated that there is a relationship between the number of propagules per milliliter and the chlorine demand per propagule (Table 2). The relationship suggests

TABLE 1. Reduction of free chlorine concentration by fungal propagules and coliform bacteria at pH 7

Organism	Free chlorine concentration (mg/liter) at:				Chlorine demand (mg per cell or conidium) after 60 min
	Initially	10 min	30 min	60 min	
A. fumigatus conidia	10	8.4[a]	8.4	8.0	1.2×10^{-8}
A. niger conidia	3	1.4	0.98	0.74	3.2×10^{-8}
Cladosporium sp. conidia	2	1.7	1.6[a]	1.6	3.6×10^{-9}
P. oxalicum conidia	2	0.95	0.80	0.65	5.9×10^{-9}
C. laurentii cells	2	1.3[a]	1.3	1.2	8.0×10^{-9}
R. glutinis cells	2	0.50	0.10[a]	0.03	2.6×10^{-9}
R. rubra cells	2	0.23	0.12[a]	0	2.4×10^{-9}
C. freundii cells	1	0.30	0.07	0.04	2.5×10^{-11}
E. cloacae cells	2	0.80[a]	0.70	0.51	1.7×10^{-10}
E. coli cells	1	0.45	0.40	0.25	6.3×10^{-10}

[a] Indicates time at which all conidia or cells were inactivated. No indication means some survival after 60 min of exposure.

TABLE 2. Chlorine demand after a 60-min exposure to chlorine at pH 7 as a function of spore or cell titer

No. of spores or cells per ml	Chlorine demand (mg/spore or cell) of:[a]	
	A. niger	*R. glutinis*
80–90	6.25×10^{-6}	4.44×10^{-7}
800–900	8.75×10^{-7}	2.00×10^{-7}
8,000–9,000	1.25×10^{-7}	1.36×10^{-7}
80,000–90,000	4.38×10^{-8}	0.53×10^{-7}

[a] *A. niger* was exposed to 10 mg of chlorine per liter and *R. glutinis* to 2 mg of chlorine per liter.

that as the number of propagules per milliliter decreases, the chlorine demand per propagule is increased. The significance of this observation is not clear at this time. Inasmuch as the number of propagules isolated per 50-ml sample was lower than the numbers used in this experiment, the chlorine demand of a propagule in a water distribution system might be higher than the values presented.

Depending upon the initial chlorine concentration, a portion of the free chlorine was converted to an iodide-displaceable combined species. The titer of this combined chloride increased over the 60-min time course of the experiment. The exact nature and role of these combined chloride species requires further investigation.

It seems clear that conidia of filamentous fungi and yeast cells are present in finished water or in a water distribution system. Maintenance of a free chlorine residual in the system is one method of inactivating the fungi before they can become established. However, the free chlorine residual in the distribution system declines with time, and the fungal propagules, as well as other microorganisms, exert a chlorine demand themselves. If enough propagules to consume all of the residual chlorine are introduced into the system, then some will survive and may become established in joints, in pits on the walls of mains, in sediment in the bottom of mains, or in any other suitable habitat which they may encounter.

The resistance of the various fungal propagules to chlorine inactivation was also investigated. Conidia of the four filamentous fungi (*A. fumigatus*, *A. niger*, a *Cladosporium* sp., and *P. oxalicum*) showed greatest resistance to chlorine, followed by the yeast and the coliforms (Table 3). *A. niger* exhibited the greatest resistance, with some conidia surviving a 10-min exposure to residual chlorine levels greater than 6.7 mg/liter (initial concentration, 10 mg/liter) at all pH values. *A. fumigatus* conidia survived, at all pH values, a 60-min exposure to residual chlorine levels greater than 1.9 mg/liter (initial concentration, 3 mg/liter), and *P. oxalicum* conidia survived a 60-min exposure to residual levels greater than 0.64 mg/liter (initial concentration, 2 mg/liter). Conidia of the *Cladosporium* sp. showed less resistance than other conidia, surviving only a 10-min exposure to residual chlorine levels greater than 1.7 mg/liter (initial concentration, 2 mg/liter) at pH 7 and 8, with no survival at pH 5. With the exception of *C. laurentii*, the yeast showed some resistance to an initial concentration of 2 mg of chlorine per liter, and *R. rubra* showed some resistance to an initial concentration of 3 mg of chlorine per liter at pH 8. The coliforms showed some survival (1%) to a 60-min exposure to residual chlorine levels greater than 0.04 mg/liter (initial concentration 1 mg/liter) at all pH values and no survival to a 10-min exposure to residual levels greater than 0.51 mg/liter (initial concentration 2 mg/liter).

Although the size of the conidium or cell probably plays some role in the chlorine demand, it is interesting to note that the greater the resistance to chlorine inactivation, the greater the chlorine demand.

The increased resistance of the fungal conidia, as opposed to the vegetative yeast and bacterial cells, to chlorine exposure was consistent with findings that fungal spores show a greater degree of resistance to environmental chemical agents than do vegetative cells (10, 16). Studies with bacterial spores (18) also have showed their greater resistance to chlorine than the vegetative cells. The increased resistance of the yeast to chlorine, as opposed to the coliforms, is in agreement with findings of others and has been reported to be due to differences in the permeability of the cells to chlorine (8, 9).

Inasmuch as one of our primary interests was in generating free chlorine demand data, high conidia and cell titers and chlorine concentrations were selected to ensure measurable changes in cell numbers and chlorine concentrations. These high cell numbers most likely explain the survival of coliforms to an initial concentration of 1 mg of chlorine per liter, although no attempt was made to determine whether this was due to the effect of physical shielding (clumping), the reduction of chlorine concentrations to sublethal levels before complete inactivation, or some other mechanism. In no case were net survivals in excess of 1%.

The pH of the buffer solution was a factor in the disinfectant action of the chlorine. At pH 5 (99.7% hypochlorous acid, 0.3% hypochlorite ion), the greatest inactivation of conidia and vegetative cells usually occurred. This was followed in decreasing chlorine activity by pH 7 (75.2% hypochlorous acid, 24.8% hypochlorite ion) and 8 (23.3% hypochlorous acid, 76.7% hypochlorite ion) (Table 3). These data tend to indicate that hypochlorous acid is more active

TABLE 3. Influence of various chlorine concentrations on the inactivation of fungal propagules and coliform bacteria at pH 5, 7, and 8 after 10 and 60 min of exposure

Organism	Inactivation at an initial chlorine concentration (mg/liter) of:[a]														
	1			2			3			5			10		
	pH 5	pH 7	pH 8	pH 5	pH 7	pH 8	pH 5	pH 7	pH 8	pH 5	pH 7	pH 8	pH 5	pH 7	pH 8
A. fumigatus conidia		ND[b]			ND		99/99	99/99	99/99	100/100	100/100	99/100	100/100	100/100	100/100
A. niger conidia		ND		50/96	71/99	61/99	76/99	79/99	77/90	99/100	99/100	99/100	99/100	99/100	99/100
Cladosporium sp. conidia	99/99	99/99	99/99	100/100	99/100	99/100	100/100	100/100	100/100		ND			ND	
P. oxalicum conidia		ND		99/99	99/99	98/99	100/100	99/100	99/100	100/100	100/100	99/100	100/100	100/100	100/100
C. laurentii vegetative cells	99/99	99/99	98/99	100/100	100/100	100/100		ND			ND			ND	
R. glutinis vegetative cells	98/99	98/99	98/99	100/100	99/100	99/100	100/100	100/100	100/100		ND			ND	
R. rubra vegetative cells	80/99	65/95	12/91	100/100	99/100	96/99	100/100	100/100	99/99	100/100	100/100	100/100		ND	
C. freundii	99/99	99/99	99/99	100/100	100/100	100/100		ND			ND			ND	
E. cloacae	99/99	99/99	99/99	100/100	100/100	100/100		ND			ND			ND	
E. coli ATCC 11775	99/99	99/99	99/99	100/100	100/100	100/100		ND			ND			ND	

[a] Inactivation was measured as a ratio of the percent inactivated at 10 min to the percent inactivated at 60 min.
[b] ND, Not determined at that chlorine concentration.

than the hypochlorite ion in inactivating the conidia and cells. Similar results have been reported elsewhere for bacteria (9), protozoa (11), and viruses (7). It should also be noted that in the absence of chlorine there was no significant difference in the survival of the conidia or vegetative cells at the various pH values.

In conclusion, it appears that, due to their resistance to chlorine inactivation, fungal conidia are likely to survive conventional water treatment and that colonization of distribution systems is very possible and will occur even in the presence of large (0.4 to 0.5 mg/liter) chlorine residual concentrations. When fungi are present in water distribution systems, they can reduce the chlorine residual and, if sewage were introduced by a cross-connection, pathogen survival would be more likely and of longer duration. In addition, colonies of fungal growth appear likely to provide or share environments where bacterial or other microbial populations might flourish if disinfectant residuals are the only, otherwise, limiting factor.

Recently, both pathogenic and aflatoxin-producing fungi have been isolated from water distribution systems (W. D. Rosenzweig, H. A. Minnigh, and W. O. Pipes, unpublished data). The added possibility of colonization by these organisms with their resultant intrinsic health hazard should be further addressed, as should the contribution of fungal colonies to the formation of halogenated organic compounds.

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