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Inactivation of three genera of dominant fungal spores in groundwater using chlorine dioxide: Effectiveness, influencing factors, and mechanisms

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ABSTRACT

Fungi in aquatic environments received more attention recently; therefore, the characteristics of inactivation of fungal spores by widely used disinfectants are quite important. Nonetheless, the inactivation efficacy of fungal spores by chlorine dioxide is poorly known. In this study, the effectiveness of chlorine dioxide at inactivation of three dominant genera of fungal spores isolated from drinking groundwater and the effects of pH, temperature, chlorine dioxide concentration, and humic acid were evaluated. The inactivation mechanisms were explored by analyzing the leakage of intracellular substances, the increase in extracellular adenosine triphosphate (ATP), deoxyribonucleic acid (DNA), and proteins as well as the changes in spore morphology. The kinetics of inactivation by chlorine dioxide fitted the Chick-Watson model, and different fungal species showed different resistance to chlorine dioxide inactivation, which was in the following order: *Cladosporium* sp.>*Trichoderma* sp. > *Penicillium* sp., which are much more resistant than *Escherichia coli*. Regarding the three genera of fungal spores used in this study, chlorine dioxide was more effective at inactivation of fungal spores than chlorine. The effect of disinfectant concentration and temperature was positive, and the impact of pH levels (6.0 and 7.0) was insignificant, whereas the influence of water matrices on the inactivation efficiency was negative. The increased concentration of characteristic extracellular substances and changes of spore morphology were observed after inactivation with chlorine dioxide and were due to cell wall and cell membrane damage in fungal spores, causing the leakage of intracellular substances and death of a fungal spore.

1. Introduction

Fungi in aquatic environments have received more attention recently. Several researchers have reported the occurrence of filamentous fungi in water (Gottlich et al., 2002; Hageskal et al., 2006; Hinzelin and Block, 2008; Pereira et al., 2009; Oliveira et al., 2013; Goncalves et al., 2006). One research group (Hageskal et al., 2006) described widespread occurrence of filamentous fungi in Norwegian drinking water distribution and found that fungi can survive water treatment. Fungi are reported to occur widely in different water sources, including surface water (Pereira et al., 2010), groundwater (Oliveira et al., 2016), and spring water (Pereira et al., 2009). Moreover, they can also be detected in the drinking water distribution system (Sammon et al., 2010), tap water (Hageskal et al., 2006), and even bottled mineral water (Cabral and Pinto, 2002). With respect to the fungal species, *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp. and *Candida* spp. are the most common (Pereira et al., 2009, 2010).

A mass outbreak of filamentous fungi in water may cause a series of problems, mainly (1) can cause taste and odor problems (Hageskal et al., 2009); (2) can produce mycotoxins causing a toxic reaction, skin allergy, asthma, and hypersensitivity pneumonitis (Curtis et al., 2004); (3) may produce visible particles and high turbidity (Paterson and Lima, 2005); and (4) can form a biofilm,

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which requires a higher concentration of disinfectants (Siqueira, 2011).

Detection of fungi in water is reported often (Gottlich et al., 2002; Hageskal et al., 2006; Hinzelin and Block, 2008; Pereira et al., 2009; Oliveira et al., 2013; Goncalves et al., 2006; Pereira et al., 2010; Oliveira et al., 2016); however, there are only a few reports on the control of fungi in water (Pereira et al., 2013; Nourmoradi et al., 2012; Savi and Scussel, 2014; Sisti et al., 2012; Al-Gabr et al., 2014). For example, the efficacy of two chemical coagulants (aluminum sulfate and ferric chloride) and three filtration media (sand, activated carbon, and ceramic granules) for the control of *Aspergillus flavus* in surface water was tested by one group (Al-Gabr et al., 2014), and they found that the extent of overall removal of fungi was probably not sufficient because the very low numbers levels of fungal cells remaining in the processed water could regrow in large numbers as biofilms in water systems. Other researchers (Pereira et al., 2013) investigated the effectiveness of free chlorine at inactivating various fungal species (*Cladosporium tenuissimum*, *Cladosporium cladosporioides*, *Phoma glomerata*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Penicillium griseofulvum*, *and Penicillium citrinum*) and found that different species show varying resistance to chlorination. Generally, chlorination is not considered effective enough to inactivate fungi at the concentration used in drinking water treatment (Sisti et al., 2012; Kanzler et al., 2008). Some investigators (Nourmoradi et al., 2012) studied the inactivation efficiency of ultraviolet (UV) irradiation towards *Aspergillus* spp. and found that 4 log inactivation of *A.* fumigatus, *A.* niger and *A.* flavus at a density of 1000 colony-forming units (CFU)/mL is achieved at UV fluence of 12.45, 16.6, and 20.75 mJ/cm^2, respectively, indicating that UV irradiation can effectively inactivate *Aspergillus* spores in water. The combination of UV and chlorine was found to be more effective than UV or chlorination alone (Al-Gabr et al., 2013). Ozonation for inactivation of fungi at a concentration of 60 μmoL/mL has been tested for different contact periods, and researchers revealed that ozone treatment was effective in the inactivation of fungi (Savi and Scussel, 2014).

In various studies, chlorine dioxide has been reported to be an effective disinfectant for inactivation of bacteria, viruses and protozoa (Vicuna-Reyes et al., 2008; Lim et al., 2010; Christian et al., 2001). To the best of our knowledge, there are few reports on the inactivation of fungi with chlorine dioxide (Ma et al., 2017). Furthermore, the existing studies on inactivation of fungi by means of various disinfectants have mainly focused on the inactivation effectiveness and kinetics, whereas information about the mechanism of inactivation of fungi has been rarely reported.

In sum, the objectives of this study were to 1) investigate the efficiency and kinetics of chlorine dioxide at inactivation of three dominant genera of fungal spores isolated from drinking groundwater; 2) examine the influence of several operating parameters, such as chlorine dioxide concentration, pH, temperature, humic acid, and chemical characteristics of the water; 3) identify the mechanism of chlorine dioxide-driven inactivation of fungal spores by monitoring the leakage of intracellular substances and observing the morphological changes.

2. Materials and methods

2.1. Isolation and identification of filamentous fungi

Untreated groundwater samples were regularly collected from northwest China between October 2015 and October 2016 and then stored and analyzed according to the procedures described below. Water samples were collected into sterile, 1-L polyethylene bottles and kept at 4 °C during transportation and storage. Analyses were conducted within 24 h. Travel and field blanks samples containing sterilized, ultrapure water were included in all analyses. The travel blanks remained unopened during the sampling trip, whereas the field blanks were open during sample collection (Pereira et al., 2013). No fungal growth was detected in either of the blanks.

Membrane filtration and plating method (Pereira et al., 2010) were used to study and isolate fungi from groundwater in northwest China. For the membrane filtration, 100 mL of water samples were passed through membrane filters with a 0.45-μm pore size. After that, the filters were transferred to the center of dichloran rose bengal chloramphenicol (DRBC) agar. All the plates were incubated at 27 °C in the dark and checked every 2 days for a maximum of 15 days. The identification of fungi was based on their macroscopic and microscopic characteristics (Crous et al., 2009), and molecular biological analysis was based on its ITS1-ITS4 regions (Pereira et al., 2010; Wen et al., 2017).

The selected fungal isolates in the present study were *Penicillium polonicum* (a representative of *Penicillium* spp.), *Trichoderma harzianum* (a representative of *Trichoderma* spp.), and *Cladosporium cladosporioides* (a representative of *Cladosporium* spp.) owing to their presence in groundwater (Hageskal et al., 2006; Pereira et al., 2009; Wen et al., 2017).

2.2. Preparation of conidial and bacterial suspensions

The inactivation of fungi was examined with fungal spores because they are biologically important, more easily produced than mycelia, and have higher resistance (Braga et al., 2015). Filamentous fungi were grown on the DRBC agar in a constant temperature incubator at 27 °C for 5–7 days. After that, the fungal spores were washed off with sterile PBS from the DRBC plate into a 10-mL centrifugal tube, then the spores and mycelia were separated by filtration (a 10-μm filter). Finally, the fungal spores were further purified by centrifugation (3000 rpm, 5 min) (Pereira et al., 2013). The concentration of spores was determined using a counting chamber (Neubauer) (Pereira et al., 2013) and was maintained at a target concentration of 10^5–10^6 CFU/mL. Control experiments also have been made, which indicates the concentration of fungal spore in PBS solution is stable (Fig. S1).

E. coli was chosen as a representative of bacteria to study the efficiency of inactivation of bacteria by chlorine dioxide. *E. coli* (ATCC25922) was inoculated into 100 mL liquid nutrient broth medium and incubated in a shaking incubator at 37 °C, 120 rpm, for 24 h. The medium was centrifuged at 6000 rpm for 10 min, the supernatant was discarded, and the precipitate was washed three times and diluted with sterile PBS to a target concentration of 10^5–10^6 CFU/mL.

2.3. Chlorine dioxide generation and determination

Chlorine dioxide was produced by mixing 50 mL of 4% potassium peroxodisulfate ($K_2S_2O_8$, 2 g in 50 mL Milli-Q water) with 50 mL of 8% sodium chlorite ($NaClO_2$, 4 g in 50 mL Milli-Q water) (Huber et al., 2005). The stock solution concentration of chlorine dioxide was determined using spectrophotometry at 358 nm with the molar absorption coefficient of 1200 M^{-1} cm^{-1} (Huber et al., 2005). The concentration of residual chlorine dioxide after inactivation of fungal spores was determined by a continuous iodometric method (HJ 551-2016, 2016; Huang, 2000).

2.4. Inactivation procedures

All the glassware used in the inactivation experiments was soaked overnight in sodium hypochlorite, rinsed with ultrapure water, and sterilized at 121 °C for 30 min. The initial concentration