Evaluation of the effectiveness, safety, and feasibility of 9 potential biocides to disinfect acidic landfill leachate from algae and bacteria

Evaluation of the Effectiveness, Safety, and Feasibility of 9 Potential Biocides to Disinfect Acidic Landfill Leachate from Algae and Bacteria

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Highlights

- Efficacy of different biocides for disinfection against algae and *E. coli* is evaluated
- Organohalogens are quantified following disinfection of landfill leachate
- Biocides have a threshold dosage under which organohalogens form below detectability
- Disinfection mechanism partly explains biocide action with organohalogen formation
- Metabisulfite is identified as a safe, eco-friendly, and effective biocide

ABSTRACT

This study evaluates 9 biocides as disinfectants against microbiological contaminants, specifically, microalgae and *E. coli*, while assessing their safety and environmental impact. Specifically, the biocide effectiveness and corresponding generation of halogenated compounds is assessed in a real contaminated groundwater receiving acidic leachate from a phosphogypsum landfill. Oxidizing agents are investigated, namely, hypochlorite, peracetic acid, hydrogen peroxide, chlorine dioxide, and persulfate, together with electrophilic biocides, namely, 2,2-dibromo-2-cyanoacetamide and (chloro-) methylisothiazolinone. In addition, a novel disinfection approach is assessed by applying reducing agents, namely, sulfite and metabisulfite. The disinfection mechanism and the formation of halogenated compounds are discussed on the basis of the mode of action and of the molecular structure of each biocide. Overall, the results show that an optimal dosage of the biocides exists to minimize the formation of harmful compounds in water while maximizing disinfection, especially for hypochlorite and peracetic acid. This dosage was between 0.03 mM and 0.15 mM depending on the biocide. The safety of electrophilic biocides is found to be associated to their molecular structure rather than their mode of action. Hydrogen peroxide, MIT, and metabisulfite are the most promising disinfectants in the contaminated groundwater matrix of interest since no halogenated by-products are detected upon successful disinfection, while they are able to completely inactivate bacteria and remove over the 80% of microalgae in the selected matrix. In particular, metabisulfite represents a highly promising biocide, owing to its low environmental and health impacts, as well as economic feasibility (estimated reagent cost ~0.002 € per cubic meter of treated water).

Keywords: biocides; microalgae; landfill leachate; disinfection; disinfection by-products; metabisulfite

1. Introduction

The European Union defines biocides as substances “intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism” (EU 1998). Despite the potential risks for humans and for the environment, biocides are deployed in a variety of activities, including sanitation, the textile industries, and water treatment (Holah et al., 2002; Rutala and Weber 2004; Stewart et al., 2001; Windler et al., 2013). Within the water treatment industry, they are usually applied as individual reagents with antifouling and disinfection purposes (Fujioka et al., 2020; Griebe and Flemming 1998; Maillard 2005). However, disinfection processes based on biocide addition may lead to the formation of harmful disinfection by-products (DBPs), such as halogenated compounds (*e.g.*, trihalomethanes, THMs), which are often known or suspected carcinogenic compounds (Jones et al., 2012; Richardson et al., 2007; Shah et al., 2015). Therefore, the correct application and management of biocides is crucial for the implementation of safe water technologies.

Well-established solutions include processes like ozonation. Ozone must be produced in situ and it is thus associated with higher operational costs; also, it can generate bromate, a carcinogenic DBP, in the presence of bromide (Shah et al., 2015). The other commonly employed biocides have each advantages and disadvantages. For example, owing to its optimum cost-efficiency ratio, hypochlorite represents one of the most applied bactericidal compounds (Fukuzaki 2006). Nevertheless, ClO^- leads to the formation of THMs and it is an irritant for the mucous membranes when concentrated in water (Fukuzaki 2006; Gómez-López et al., 2013; Lee and Huang 2019). Promising alternatives were developed during the last decade, among which chlorine dioxide and peracetic acid are the most successful examples. Even if these two compounds are effective biocides against a wide spectrum of microorganisms, recent studies demonstrated that the former can generate chlorite and chlorate, both harmful DBPs, together with traces of tribromomethane, while the latter represents a potential source of THMs in the presence of bromide and NOM in water (Al-Otoum et al., 2016; Domínguez Henao et al., 2018; Stevens 1982; Xue et al., 2017; Yang et al., 2013). All the above-mentioned compounds rely on oxidative processes in order to attain disinfection. Other compounds can work as biocides through different mechanisms, for example, the couple methylisothiazolinone / chloro-methylisothiazolinone (MIT), as well the 2,2-dibromo-2-cyanoacetamide (DBNPA) (Kahrilas et al., 2015). Nevertheless, the literature lacks studies related to these compounds and to the formation of DBPs once they are used for disinfection of contaminated water streams.

In addition to the compounds discussed so far, reductants may find an application in water disinfection. For instance, they can potentially affect the photosynthetic mechanism, thus limiting the growth of algae species (Beekley and Hoffman 1981; Shimazaki and Sugahara 1979; Wodzinski et al., 1978; Yang et al., 2004). Reducing agents include sulfuric compounds, such as sulfur dioxide, bisulfite, and sulfite. The ability of sulfites to inhibit the growth of some bacterial groups has been reported (Irwin et al., 2017). In particular, sulfites are promising compounds for industrial applications, owing to their ease of storage, safety (they are not as explosive as oxidants), lack of toxicity for humans and the environment, and low capital and operational costs (Farinelli et al., 2019).

Finding an inexpensive and easy-to-deploy biocide, which concurrently is effective, eco-friendly, and does not contribute to DBPs generation, is a challenging task. This is especially true in the treatment of complex water and wastewater streams, such as those laden with organic materials and dissolved solids. Relevant examples of such effluents are landfill leachate and mine drainage. For example, the leachate of phosphogypsum landfills create an acidic environment, rich of phosphate nutrients and halides (Chernysh et al., 2018). Acidophilic microalgae can easily adapt and grow in this environment (Gross 2000; Hirooka et al., 2017). Although acidic conditions generally kill bacteria, Gut et al., (2006) observed that a significant salt concentration in the water matrix, chloride content in particular, plays a key role in the activity of proton membrane pumps that allows the survival of bacteria. Moreover, Jordan et al., (1999) and de Jonge et al., (2003) showed that some

Escherichia coli (*E. coli*) sub-populations can reach physiological equilibrium even at pH values as low as 2-3. The possible presence of different microorganisms and the complexity of such matrix make this type of contaminated water a perfect target for an investigation aimed at the evaluation of traditional as well as innovative biocides.

In this work, the disinfection effectiveness of different biocides is evaluated against both microalgae and *E. coli*, while their safety is assessed by quantifying the formation of harmful halogenated by-products. Specifically, 9 biocides acting through three different disinfection mechanisms are evaluated, as potential reagents applied individually in wastewater treatment. Along with the most common biocides commercially available, exploiting mainly oxidation, a detailed discussion is reported for electrophilic biocides, while a new disinfection route exploiting reducing agents is assessed for the first time. In order to evaluate the applicability of the studied solutions, the biocide behavior is evaluated in real groundwater contaminated with phosphogypsum landfill leachate. A brief discussion of the disinfection mechanism is provided for each biocide, with the goal to help the interpretation of the biocidal action and the consequent formation of halogenated compounds in the target water matrix. Finally, the most promising biocides are evaluated in terms of environmental impacts and cost of application.

2. Materials and methods

2.1. Chemicals, reagents, and water matrices used in the study

The biocides investigated in this study were: (i) oxidants, namely, hypochlorite (HClO), peracetic acid (PAA), chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), and persulfate ($K_2S_2O_8$); (ii) electrophilic, namely, DBNPA and MIT; (iii) reductants, specifically, sulfite and metabisulfite. All the chemicals were used as received. Except for chlorine dioxide, which was purchased from Apura Srl (Brescia, Italy), all the other compounds were purchased from Sigma-Aldrich (Milano, Italy). MIT was prepared by mixing methylisothiazolinone and chloro-methylisothiazolinone at the appropriate concentration in an aqueous solution in a volume ratio of 1 to 3. For disinfection and DBP generation experiments, individual biocides were dosed at the target concentrations (*vide infra*) in 15 mL of water samples. All the water chemistry analyses were performed at a private external laboratory (Natura Srl, Naples, Italy), using methods EPA 5030 C 2003 and EPA 8260 D 2018.

Groundwater receiving leachate from a phosphogypsum landfill was directly obtained from the pumping wells in a contaminated site in the south of Italy and used as is. The main characteristics of the contaminated groundwater are summarized in Table 1. A significant concentration of microalgae is present, and an intermediate level of organic matter. The contaminated water did not contain trihalomethanes or other halogenated organic compounds at detectable concentrations. Disinfection of this wastewater is required as a first stage in the existing treatment train, to protect and enhance the subsequent coagulation process, accomplished through addition of polyelectrolytes, ferric chloride, and calcium hydroxide for the removal of a large part of heavy metals, radionuclides, phosphate, sulfate, and fluoride (Cui et al., 2020; Liang et al., 2009; Liu et al., 2019; Nielson and Smith 2005). A second disinfection phase is then present to reduce fouling and biofouling in the following ultrafiltration and reverse osmosis filtrations aimed at final effluent desalination (Fujioka et al., 2020; Griebe and Flemming 1998; Kim et al., 2009).

Table 1. Main characteristics of the contaminated groundwater from analysis of the samples and historical data obtained from the treatment plant management.

Parameter	Units	Average Value or Range
Chloride	mg/L	2690
Bromide	mg/L	6.4
Fluoride	mg/L	220-320
Sulfate	mg/L	3130
Phosphate	mg/L	2400
Nitrate	mg/L	60
Bicarbonate	mg/L	10
$N\text{-}NH_4^+$	mg/L	480-590
Iron	mg/L	5.4
Manganese	mg/L	6.2
Arsenic	mg/L	0.799
Cadmium	mg/L	1.55
Lithium	mg/L	<0.01
Nickel	mg/L	1.1
Lead	mg/L	0.004
Copper	mg/L	0.1
Zinc	mg/L	4.1
TOC	mg/L	58 ± 12
Microalgae	cells/mL	$1.76 \pm 0.6 \times 10^6$
pH		2.8

2.2 Microalgae sampling and counting

Samples for microalgae quantification were collected in real water samples before disinfection and 2 h after the addition of each disinfectant. To quantify the algae concentration, a counting chamber was employed (Paul Marienfield Gmbh & Co, Lauda-Königshofen, Germany). The chamber is equipped with an optical microscope (Renishaw, UK). The concentration of algae cells suspended in the samples was determined by multiplying the average number of cells observed in the microscope images by the relevant area, the chamber depth, and the dilution factor. The nominal statistical error of the counting chamber is defined according to the formula: error = $1/n$, where n is the number of counted microalgae cells. In the counting of the initial concentration of microalgae, the nominal error was roughly 1.4%. When only a few cells are counted in the chamber, the error is of the same order of magnitude of the measured value: therefore, for values of the removal efficiency equal or larger than 90%, its absolute value is no longer relevant and the disinfection should be considered nearly complete. In all the other cases, the nominal error was below 10%.

2.3. Bacterial methods: LB, saline solution, and bacterial suspension preparation

Luria Bertani (LB) broth was prepared by completely dissolving tryptone (10 g), yeast extract (5 g), and sodium chloride (10 g) in 1 L of deionized water. A slightly hypertonic saline medium was also prepared by dissolving sodium chloride (8 g) and potassium chloride (0.8 g) in 1 L of deionized water; the pH of this solution was adjusted to a value between 7 and 7.5 with 0.1 M NaOH and 0.1 M HCl. Before use, the LB and saline mixtures were autoclaved for 15 min at 15 psi at a temperature of 121 °C.

To prepare the bacterial suspension, a volume of 5 mL LB was pipetted into a 15 mL falcon tube. The *E. coli* strain was a wild-type isolate from urban secondary wastewater, obtained by isolation and selection of the microorganism on selective growth media. The *E. coli* bacterial inoculum was made from a pre-prepared master plate; a colony was dispersed in 5 mL LB by mixing in a vortex machine for 30 s. Following this step, the falcon tube was placed in a temperature-controlled incubator at 37 °C for 8 h under gentle agitation by circular motion. Subsequently, 2.5 mL of the suspension was diluted in 250 mL of LB and left for 15 h in the incubator to achieve stationary growth phase (3.5-5.5 OD_{600}) and a concentration of roughly 10^9 colony forming units (CFU)/mL; the detailed procedure of bacterial preparation and purification was published elsewhere (Giannakis et al., 2013).

2.4. *Determination of the minimum inhibitory concentration (MIC) of E. coli, and determination of bacterial disinfection kinetics by biocides*

The minimum inhibitory concentration (MIC) is typically defined as "the minimal concentration of an antimicrobial necessary to inhibit the growth of a target microorganism". In this study, we adapted the broth dilution assay for the determination of MIC with *E. coli* as target microorganism, and with the biocides in question instead of antimicrobials used in common MIC testing. As a proxy of *E. coli* concentration, the optical density of the samples was considered, measured by a spectrophotometer at 600 nm wavelength (OD_{600}). MIC is an intrinsic characteristic of each biocide and depends also on the type of bacteria used as an evaluation strain. To determine this parameter for the different biocides investigated in this study, a falcon tube was filled with a 7-8×10^8 CFU/mL suspension of *E. coli* in LB (OD_{600}=1±0.1). Each biocide was spiked in the suspension at the target concentration and the falcon tube was then incubated for 24 h at 37 °C. Both positive and negative controls (*i.e.*, no biocide, and no bacteria, respectively) were always performed to ensure that no contamination occurred. The absorbance at 600 nm wavelength was then measured to determine the optical density, followed by calibration with suspensions of known concentrations determined by plating and counting. The presence of biocides can result to cell lysis, oxidation, and/or overall reduction of the cellular materials that cause light absorbance measured by the spectrophotometer when the bacteria are intact. There was a biocide concentration that caused significant reduction in the optical density; to avoid underestimating MIC, we selected as MIC the lowest biocide concentration that maintained optical density at a similar level to the initial spiking (final OD_{600} after 24 h = 1±0.2), before a 50% decrease in optical density and conversion to a "destructive concentration" from further addition of the biocide. In this manner, we can assume that bacterial growth was surely arrested at the chosen MIC level.

Furthermore, kinetics disinfection experiments were performed in real contaminated groundwater by addition of the various biocides. An appropriate amount of LB *E. coli* suspension was added to the matrix to obtain a concentration of bacteria of roughly 10^6 CFU/mL. The biocides were added individually to the matrix and samples were collected at regular intervals. Upon sampling, serial dilutions were immediately made in saline medium and the resulting suspensions were spread onto agar plates. After 1 day of incubation at 37 °C, the CFUs were counted to determine the concentration of cultivable cells. A concentration of biocides equal to 0.03 mM was chosen for kinetics disinfection tests, this being the lowest value determined for the removal of microalgae from real groundwater samples (*vide infra*). The same disinfection experiments were also performed with metabisulfite and hydrogen peroxide at each respective MIC (*vide infra* for MIC values determined by experiments).

2.5. Methodology of life cycle analysis (LCA).

The environmental burden from the production of 1 kg of the three most interesting biocides (*i.e.*, MIT, hydrogen peroxide, and metabisulfite; *vide infra*) were evaluated by life cycle analysis using OpenLCA 1.10 software, which incorporates the Ecoinvent 3.5 database. Three different methodologies were employed to conduct the environmental assessments: ReCiPe, Cumulative Energy Demand (CED), and IPCC2013. In the case of ReCiPe, the environmental analysis was modeled on both the endpoints and the midpoints, the latter presented as normalized values on the total impact. The CED approach was applied to assess the required energy expressed as the primary energy demand, while IPCC2013 analysis with a timeframe of 20 years was performed to assess the global warming potential of the production of the three biocides. While hydrogen peroxide is present in the Ecoinvent database, the impacts of metabisulfite and MIT were determined by modeling their most common synthesis approach. For metabisulfite, the reaction between SO_2 and NaOH was considered, while the protocol for MIT production was based on literature reports (Taubert et al., 2002).

3. Results and Discussion

3.1. Disinfection targets and principal characteristics of the studied biocides

Before the analysis of the efficacy of each disinfectant, we present a succinct summary of their main characteristics. The mode of action of the 9 biocides investigated in this work is depicted in Figure 1 and briefly summarized in Table 2 together with each molecular structure. HClO, ClO_2, H_2O_2, and PAA can penetrate the phospholipidic membrane through passive diffusion, thus these biocides disinfect through an intracellular action by acting on enzymatic processes and by attacking the DNA purine bases and the internal cytoplasmic membrane (Fukuzaki 2006; Ghanbari et al., 2021; Giannakis et al., 2016; Kitis 2004; Maillard 2002; Ofori et al., 2017; Tutumi et al., 1973; Xia et al., 2017). Moreover HClO, ClO_2, H_2O_2, and PAA can also damage the outer cellular membrane (Feng et al., 2020; Fukuzaki 2006; Kitis 2004). Persulfate is active toward microorganism disinfection mostly if activated by transition metals (Wang et al., 2019; Xia et al., 2017). An estimation of the oxidation efficiency of an oxidant biocide can be estimated from a thermodynamic standpoint by comparing the redox potential of the reagent with that of bacteria strains (the *E. coli* redox potential is generally +0.45 to +0.72 V at pH 7 vs. SHE) (Ruales-Lonfat et al., 2015). Therefore, all the biocides with a redox potential higher than +0.45 to +0.72 V at pH 7 will be able to damage *E. coli* bacteria through oxidation.

The two electrophilic biocides, namely, DBNPA and MIT, disinfect by means of both extracellular and intracellular actions. DBNPA is mostly active by damaging the external cellular membrane through an electrophilic addition to the nucleophilic groups of membrane proteins (Campa et al., 2019; Kahrilas et al., 2015). MIT is active also by inhibiting internal enzymatic processes (Kahrilas et al., 2015; Maillard 2002; Williams 2006). Literature lacks a detailed explanation on the disinfection mechanism of metabisulfite. On the basis of previous reports and of its molecular structure, it is reasonable to assume that metabisulfite can potentially alter the photosynthetic process of microalgae by releasing SO_2 in solution and that it can damage the cellular membrane in the same fashion of an electrophilic biocide (Beekley and Hoffman 1981; Shimazaki and Sugahara 1979; Wodzinski et al., 1978; Yaganza et al., 2004; Yang et al., 2004), but its intracellular activity is still under debate. A more exhaustive discussion of the mechanism of disinfection and DBPs generation mode for each of the 9 biocides is provided in the Supporting Information (SI).

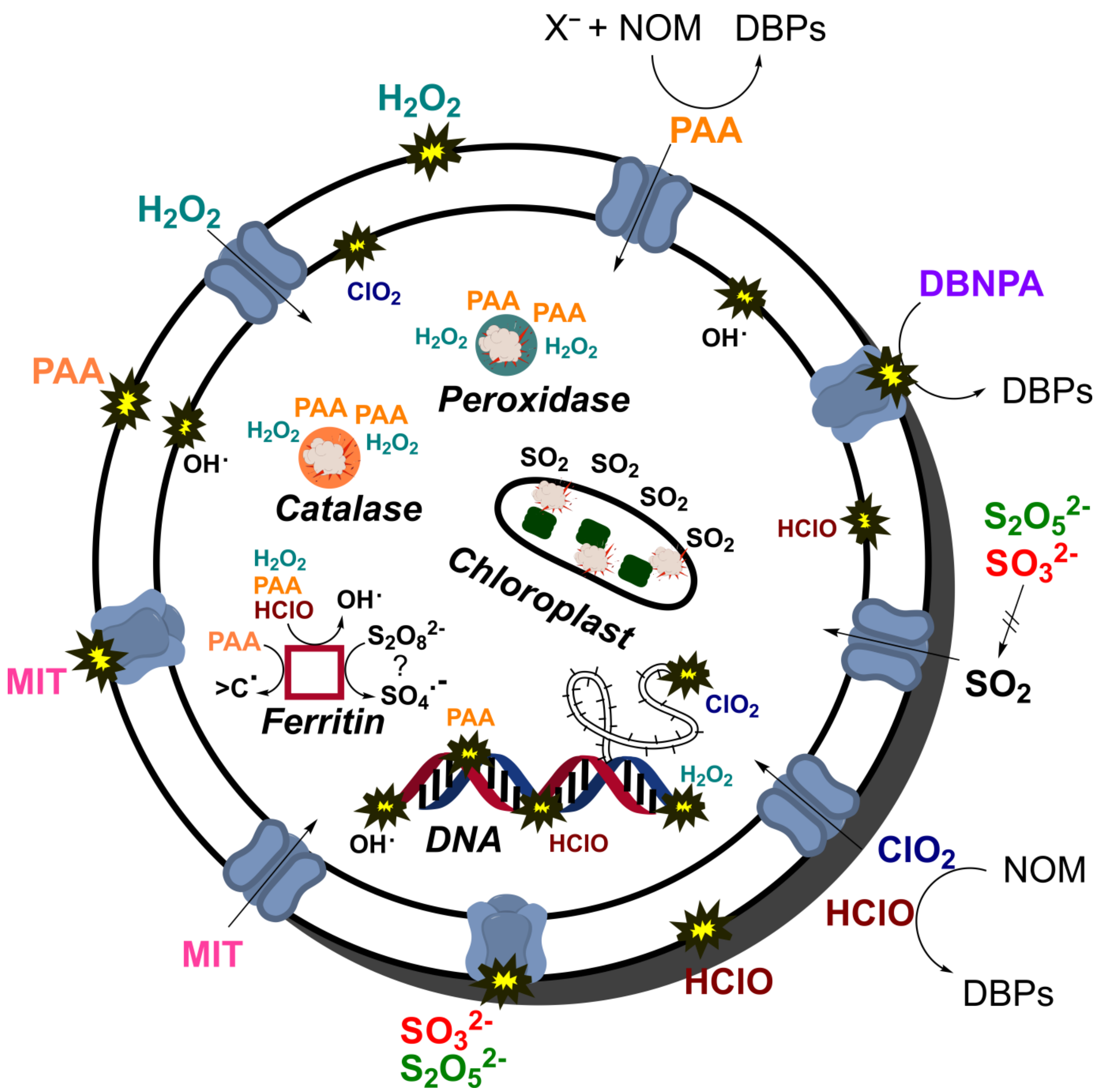
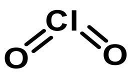
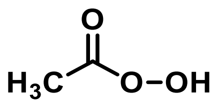
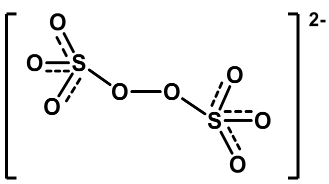
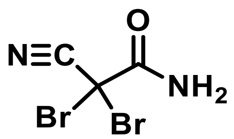
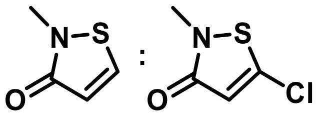
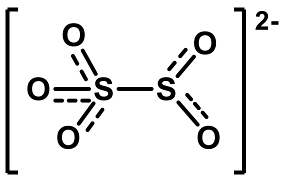
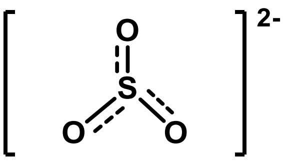


Fig. 1. Mode of action of the biocides investigated in this study. Please note that chloroplasts are present only in algae.

Table 2. Molecular structure, mode of action (MOA), and mechanism of DBPs generation for each biocide investigated in this study. In the fourth column, “oxidizing” (O), “electrophilic” (E), and “reductant” (R) refer to the main MOA of each biocide. The last column presents the redox potential for each oxidant biocide.

Biocide and main references	Molecular structure	Mechanisms of disinfection and of DBPs generation	MOA	E_0 (V vs. SHE; pH 7)
Hypochlorite, HClO (Fukuzaki 2006; Maillard 2002)	Cl—OH	• HClO prevails at pH 2.8. • Formation of chloramines thwarted at pH 2.8 • If present, dominant chloramine species trichloramine and dichloramine with low disinfecting effect • Intracellular/Extracellular action. • Internal “Fenton-like” process ($HClO + Fe^{2+} \rightarrow HO^{\cdot} + Cl^{-} + Fe^{3+}$) • Addition to purine base of DNA and nucleophilic sites of internal organelles. • THMs and HACs generation through electrophilic addition to organic material.	O	1.4
Chlorine dioxide, ClO_2 (Maillard 2002; Ofori et al., 2017)		• Intracellular action (and possible extracellular action). • Inhibition of protein synthesis and enzymatic processes. • Addition to internal cytoplasmic membrane • ClO_2^-, ClO_3^- generation through oxidation of Cl^-. • Slight THMs generation through electrophilic addition to NOM.	O	0.95
Hydrogen peroxide, H_2O_2 (Maillard 2002)	HO–OH	• Intracellular/Extracellular action. • Inhibition of peroxidase activity. • Internal Fenton process. ($H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^{-} + Fe^{3+}$) •	O	1.8
Peracetic acid, PAA (Ghanbari et al., 2021; Kahrilas et al., 2015; Kitis 2004; Tutumi et al., 1973)		• Intracellular/Extracellular action. • Inhibition of peroxidase activity. • Internal “Fenton”-type process ($C_2H_4O_3 + Fe^{2+} \rightarrow HO^{\cdot} + C_2H_3O_2^{-} + Fe^{3+}$) • Easier generation of $OH^{\cdot}$ than H_2O_2. • Possible THMs & HACs generation through oxidation of Cl^- (or Br^-) to HClO (or HBrO) which binds NOM.	O	1.4
Persulfate, $S_2O_8^{2-}$ (Wang et al., 2019; Xia et al., 2017)		• Extracellular action (and possible intracellular action until otherwise proven). • Activated in presence of transition metals (*i.e.,* Fe, Mn and Cu) or light. • Sulfate radical generation ($SO_4^{\cdot-}$). • Possible THMs & HACs generation through radical processes.	O	2.1
DBNPA (Campa et al., 2019; Kahrilas et al., 2015)		• Extracellular action. • Addition to -SH and -NH groups of membrane proteins. • THMs release through nucleophilic acylic substitution reaction.	E	N.A.

MIT (Kahrilas et al., 2015; Maillard 2002; Williams 2006)	 1 : 3	• Extracellular and intracellular action. • Addition to -SH and -NH groups of membrane proteins. • Inhibition of enzymatic processes. • Internal radical processes. • No THMs & HACs generation mechanism detected.	E	N.A.
Metabisulfite, $S_2O_5^{2-}$ (Beekley and Hoffman 1981; Shimazaki and Sugahara 1979; Wodzinski et al., 1978; Yaganza et al., 2004; Yang et al., 2004)		• Extracellular and possible intracellular action through reductant activity. • Possible extracellular action electrophilic addition. • No THMs & HACs generation mechanism detected.	R/E	N.A.
Sulfite, SO_3^{2-} (Beekley and Hoffman 1981; Shimazaki and Sugahara 1979; Wodzinski et al., 1978; Yang et al., 2004)		• Extracellular • Possible intracellular action through reductant activity. • Possible extracellular action electrophilic addition. • No THMs & HACs generation mechanism detected.	R/E	N.A.

3.2. Disinfection efficacy

As a first step to understand the capability of the various biocides to inactivate *E. coli* bacteria, we benchmarked their efficacy under the most unfavorable conditions by assessing their minimum inhibitory concentration (MIC), with the main results summarized in Figure 2. In Figure 2a and 2b, we present the optical density (OD_{600}) as a proxy of bacterial concentration, vs. the biocide concentration. A decrease in OD_{600} indicates destruction of the cell, hence for each biocide the MIC was identified as the lowest concentration above which significant decrease in optical density was observed. No MIC value was found for sulfite in the biocide concentration range investigated in this study, that is, 0-10 mM, and a high MIC value (~10 mM) was found for persulfate. Therefore, these compounds were not used for further investigation on disinfection against *E. coli*. The most effective biocides were MIT, DBNPA, H_2O_2, and PAA, in this order, as they required the lowest concentrations to inhibit bacterial growth. Figure 2c summarizes the MIC values determined for all the biocides.

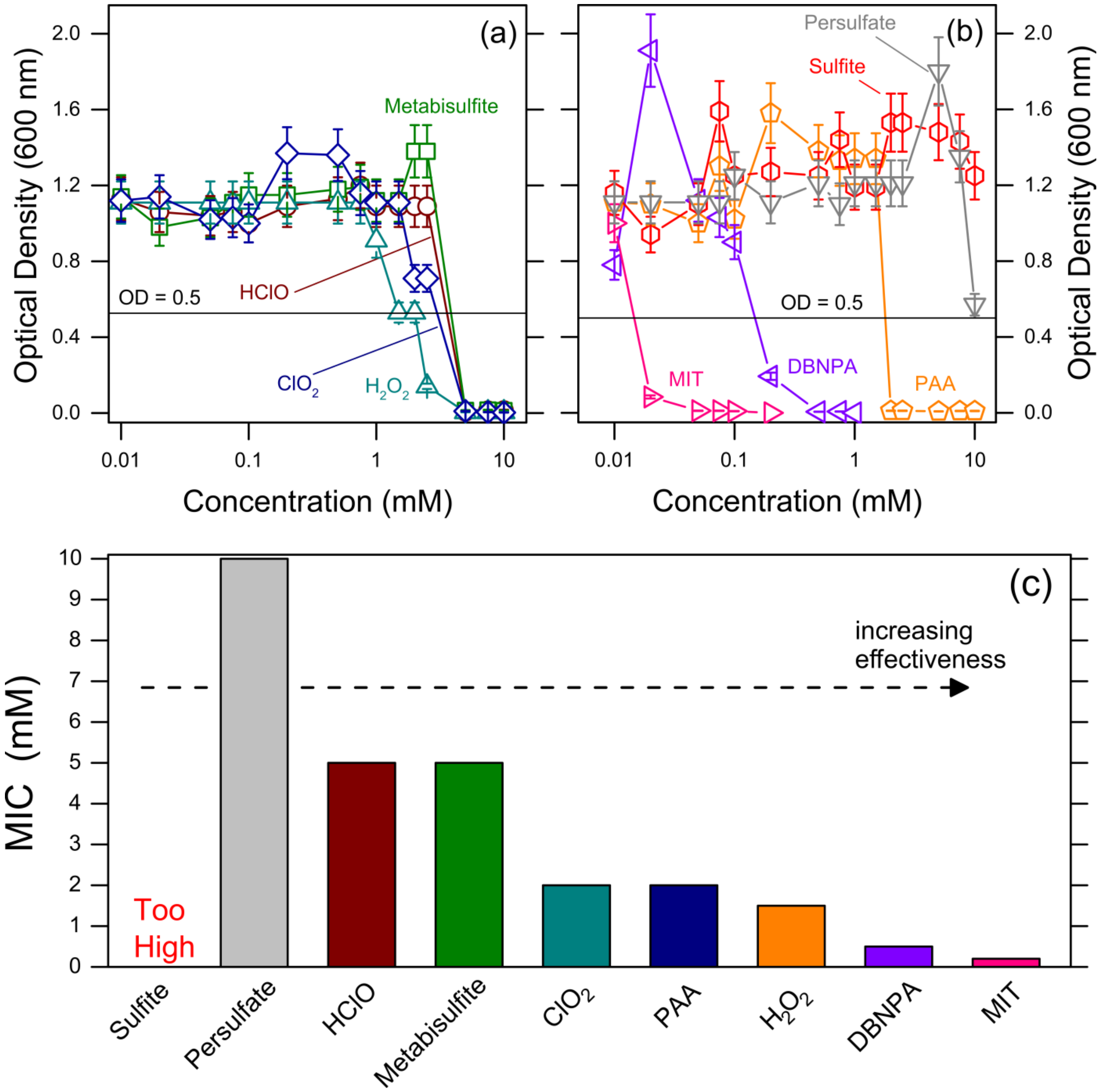


Fig. 2. Minimum inhibitory concentration (MIC) of the biocides toward *E. coli* inactivation. (a, b) Results of light absorbance of the *E. coli* suspension as a function of biocide concentration. The lines are only intended as guides for the eye. Initial spiking to OD_{600} = 1±0.1 was carried out, and the results depict the OD_{600} measured after 24 h. The control samples (no biocide addition) resulted in the OD_{600} range = 3.5-5.5 at the stationary growth phase. (c) Summary of MIC values. No MIC values could be determined for sulfite and persulfate up to 10 mM biocide concentration.

Figure 3a reports the values of microalgae removal percentage achieved by employing the different biocides at varying concentrations in the real groundwater samples. Consistent with the results observed with bacteria and presented in Figure 2, sulfite and persulfate showed the lowest biocidal activity also towards microalgae. Indeed, the activity of persulfate for both microbiological and chemical decontamination has been clearly shown only if this reagent is activated by transition metals (Boukari et al., 2011; Venieri et al., 2020; Wang et al., 2019; Xia et al., 2017). The literature instead lacks reports about the disinfection efficacy of sulfite. In general, no evident increase in algae removal rate was observed by increasing the concentrations of biocides. Actually, metabisulfite and sulfite showed a similar, inverse trend of increasing disinfection efficacy by reducing the reagent concentration, which suggests that these reagents may undergo “suicidal”, self-inhibition reactions at large concentrations. Furthermore, MIT showed the same inverse trend with concentration. This compound is known to have a slow biocidal activity; moreover, it is highly susceptible to oxidation (Williams 2006). It is thus possible that, at high concentration, this biocide reacted faster with the oxygen freely present in the solution (~0.3 mM) than with the microbiological target. Overall, it was found that the lowest concentration of 0.03 mM would allow near maximization of algae removal while minimizing the amount of required MIT reagent.

Several algae species, such as *Chlorella*, adapt well in acidic environments (Gross 2000; Hirooka et al., 2017). On the contrary, *E. coli* cannot typically survive below pH 4 (Conner and Kotrola 1995). The water matrix effect on *E. coli* viability was investigated to understand this behavior and to isolate the effect of biocides from that of pH. The results of *E. coli* cultivability from the three blanks indicate that the acidity of the contaminated groundwater (pH 2.8) is responsible for a significant bacterial inactivation within 2 h (Figure 3a). Therefore, a biocide can only be considered effective toward bacteria disinfection in this matrix if it inactivates *E. coli* faster than 2 h. Clearly, metabisulfite and hydrogen peroxide were able to quickly inactivate *E. coli* at their respective MIC. On the other hand, metabisulfite was not as quickly effective at a low concentration of 0.03 mM

(Figure 3c). Except also for HClO, all the other biocides were instead able to inactivate bacteria at a fast rate even at this low dose, which was also found to be the optimal concentration for microalgae removal, as stated above (Figure 3a).

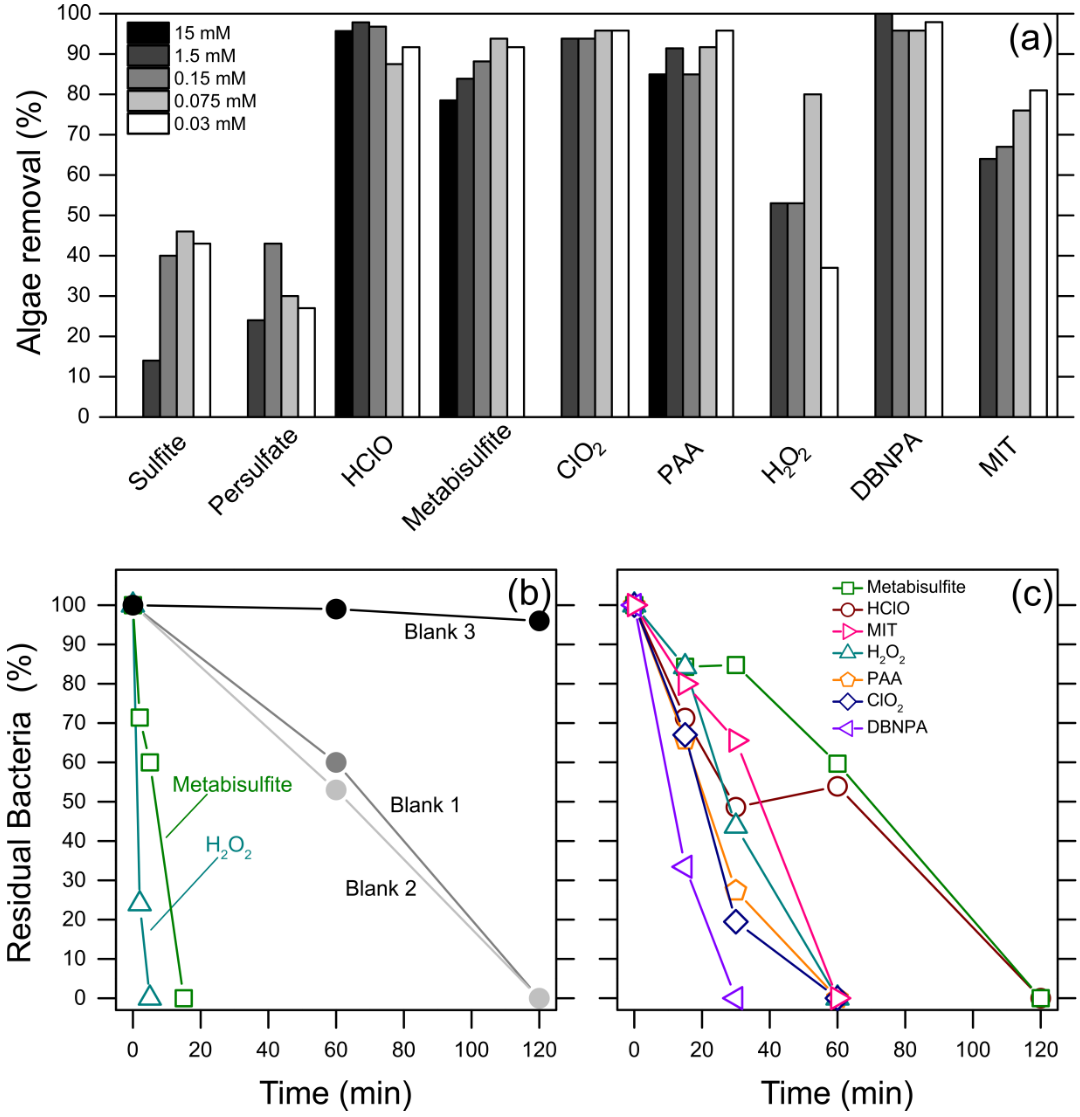


Fig. 3. Disinfection of microalgae and *E. coli* in the real contaminated groundwater matrix. (a) Removal rates of microalgae at varying biocide concentration. (b, c) Bacteria disinfection kinetics. In (b), metabisulfite and hydrogen peroxide were added at their MIC. Blank 1 shows the matrix effect on *E. coli* viability; blank 2 shows the matrix effect on *E. coli* viability by buffering the suspension at pH 7 before plating; blank 3 shows the matrix effect on *E. coli* viability by buffering the suspension at pH 7 before biocide addition. In (c), *E. coli* disinfection results obtained with a biocide concentration of 0.03 mM are shown. The lines are only intended as guides for the eye. The standard deviation is equal or lower than 25% for each of the data reported in (b, c).

These data lead to an important conclusion: when working with a complex water matrix such as that examined in this study, each of the following biocide may be employed at low dose when only microalgae removal is required: PAA, H_2O_2, ClO_2, DBNPA, MIT, HClO, and metabisulfite. On the other hand, in the presence of *E. coli* or other persistent/surviving bacteria behaving in a similar way, the utilization of HClO and metabisulfite requires larger concentration to achieve both an effective algae removal and a suitable antibacterial activity.

3.3. Generation of disinfection by-products

The formation of disinfection by-products was determined following addition of biocides in the real groundwater matrix. As expected, ClO_2 generated THMs, and in particular tribromomethane (a carcinogenic compound), at all the investigated concentrations. The formation of this halogenated compound upon employment of ClO_2 is consistent with reports in the literature (Al-Otoum et al., 2016; Gómez-López et al., 2013). As expected, also the use HClO and PAA induced the formation of halogenated by-products (Dell'Erba et al., 2007; Domínguez Henao et al., 2018; Shah et al., 2015; Xue et al., 2017), specifically at biocide concentrations of 15, 1.5, and 0.15 mM (see Table 3 and SI for the complete set of analysis). Tribromomethane was detected as the most prevalent DBP. In particular, in the case of PAA, the concentration of halogenated by-products sharply increased with increasing disinfectant dose. This behavior indicates the involvement of PAA itself as a primary source in halogenated by-products formation (Table 2 and SI provide a more exhaustive discussion on the generation of DBPs related to the nine biocides). However, THMs or other halogenated compounds were not detected at concentrations of biocides equal to 0.075 or 0.03 mM, even though both HClO and PAA still maintained a suitable disinfectant efficiency. Therefore, 0.075 mM represents the threshold safety-related dose for HClO and PAA application to the water matrix in examination. These results suggest that it should be possible to find a threshold dose for any matrix at which adequate disinfection occurs without the detectable formation of halogenated compounds when employing of HClO and PAA.

Table 3. Concentration of total THMs expressed in μg/L and other halogenated compounds upon disinfection of the contaminated groundwater through addition of the various biocides at different dosage.

	Sulfite	**Persulfate**	**HClO**	**Metabisulfite**	**ClO_2**	**PAA**	**H_2O_2**	**DBNPA**	**MIT**
	Total THMs (μg/L)								
15 mM	*N.A.*	*N.A.*	**312**	< LoQ	*N.A.*	**588**	< LoQ	*N.A.*	*N.A.*
1.5 mM	< LoQ	< LoQ	**329**	< LoQ	**335**	**12**	< LoQ	**54**	< LoQ
0.15 mM	< LoQ	< LoQ	**303**	< LoQ	**220**	< LoQ	< LoQ	**5.8**	< LoQ
0.075 mM	< LoQ	< LoQ	< LoQ	< LoQ	**13**	< LoQ	< LoQ	**5.4**	< LoQ
0.03 mM	< LoQ	< LoQ	< LoQ	< LoQ	**10**	< LoQ	< LoQ	< LoQ	< LoQ
	Other halogenated organic compounds (μg/L)								
15 mM	*N.A.*	*N.A.*	< LoQ	< LoQ	*N.A.*	**84**	< LoQ	*N.A.*	*N.A.*
1.5 mM	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	**13**	< LoQ	< LoQ	< LoQ
0.15 mM	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	**10**	< LoQ	< LoQ	< LoQ
0.075 mM	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
0.03 mM	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ

"N.A.": test not performed. "LoQ": limit of reliable detection.

Notably, metabisulfite, H_2O_2, MIT, sulfite, and persulfate did not generate any trace of halogenated compounds, even at the upper limits of the biocide concentration range. The behavior of sulfite and persulfate may be merely ascribed to their low disinfection activity toward bacteria and algae, which limited the formation of by-products and prevented the formation of halogenated compounds. Ultimately, we surmise that metabisulfite, H_2O_2, and MIT are the most interesting biocides of the study, since they maintained high disinfection efficiency toward microorganisms, without generating DBPs.

Another interesting result obtained in this study is the behavior of DBNPA and the formation of tribromomethane as a disinfection by-product. Specifically, the concentration of tribromomethane in water increased linearly with the dose of DBNPA (see Figure 4a), suggesting that this disinfectant itself is an important source of the related by-product. The formation of tribromomethane may be ascribed to the molecular structure of the biocide: it is reasonable to consider the release of a 2,2-dibromo-2-cyanomethyl group after the nucleophilic acyl substitution between the thiol or aminic residues of the membrane proteins and the disinfectant (see section on electrophilic biocides in the SI) (Campa et al., 2019; Kahrilas et al., 2015). Once in solution, the cyano- group can be replaced by a bromide present in the aqueous environment (~6.4 ppm, see Table 1), thus forming the most stable by-product, that is, tribromomethane (Figure 4b). At 0.03 mM biocide concentration, the formation of halogenated by-products was not detected. Given the reaction mechanism of the disinfectant, however, it is reasonable to assume that 2,2-dibromo-2-cyanomethane was still released, but in quantities that were too modest to react with the bromide and generate tribromomethane at detectable concentration. It is interesting to note that MIT belongs to the same biocide category of DBNPA (*i.e.*, electrophilic biocides), but that its employment was not associated with the formation of halogenated by-products (Figure 4a), potentially by virtue of the different molecular structure of the two biocides.

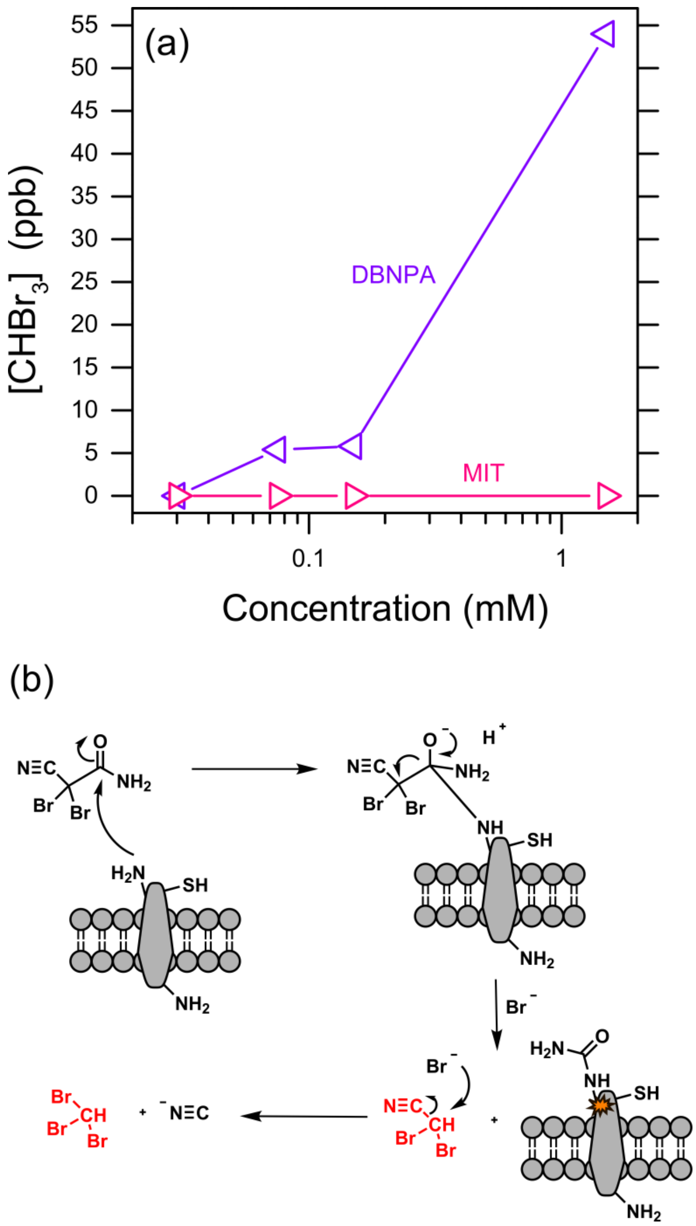


Fig. 4. (a) Trend of tribromomethane concentration in water as a function of DBNPA and MIT concentrations. The lines are only intended as guides for the eye. (b) Proposed scheme of DBNPA disinfection mechanism and tribromomethane generation.

3.4. Metabisulfite, H_2O_2, and MIT: logistics of implementation.

Metabisulfite, H_2O_2, and MIT resulted equally safe in terms of DBPs generation, but they are different in terms practical utilization (*e.g.*, storage, safety). Indeed, H_2O_2 and MIT are well known in the literature as effective disinfectants (Giannakis et al., 2016; Kahrilas et al., 2015; Williams

2006); however, they present some practical limitations compared to metabisulfite. Firstly, the storage of large amounts of H_2O_2 is dangerous because of its explosive characteristics (Schreck et al., 2004). Besides, the utilization of hydrogen peroxide is strongly discouraged before membrane desalination systems, due to the possible degradation of the membrane when exposed to oxidizing agents (da Silva et al., 2006; Kang et al., 2007; Korolkov et al., 2014). On the other hand, MIT is at both an allergenic and a cytotoxic compound (Burnett et al., 2010; Castanedo-Tardana and Zug 2013; Groot and Weyland 1988; Hannuksela 1986). Metabisulfite, although rarely studied for applications similar to that of this study, may be the safest biocide overall, also consistent with the hypothesis of its disinfection mechanism (Table 1 and Figure 1).

3.5. Environmental impacts, economic analysis, and overall review of biocides

A summary of the efficacy and DBPs generation potential of the biocides used for the disinfection of leachate-contaminated groundwater is reported in Figure 5a. In this three dimensional plot, a dot is associated with each biocide, with the exclusion of persulfate and sulfite. The values of MIC, microalgae removal rate at biocide concentration of 1.5 mM, and average formation of halogenated compounds were considered to perform this summary analysis. An ideal biocide that is both highly effective and does not induce the production of harmful DBPs would sit in the top left corner of the graph. Chlorine-based disinfectants are instead found on the right side of the graph, because they are associated with the production of significant concentrations of harfmul DBPs. The safest biocides in this respect were MIT, hydrogen peroxide, and metabisulfite. Of these three, H_2O_2 and especially MIT were found to be highly effective against *E. coli* (*i.e.*, low MIC) and slightly less so to remove microalgae. A larger concentration of metabisulfite may instead be necessary to achieve the same disinfection efficacy of the other two biocides against both algae and bacteria.

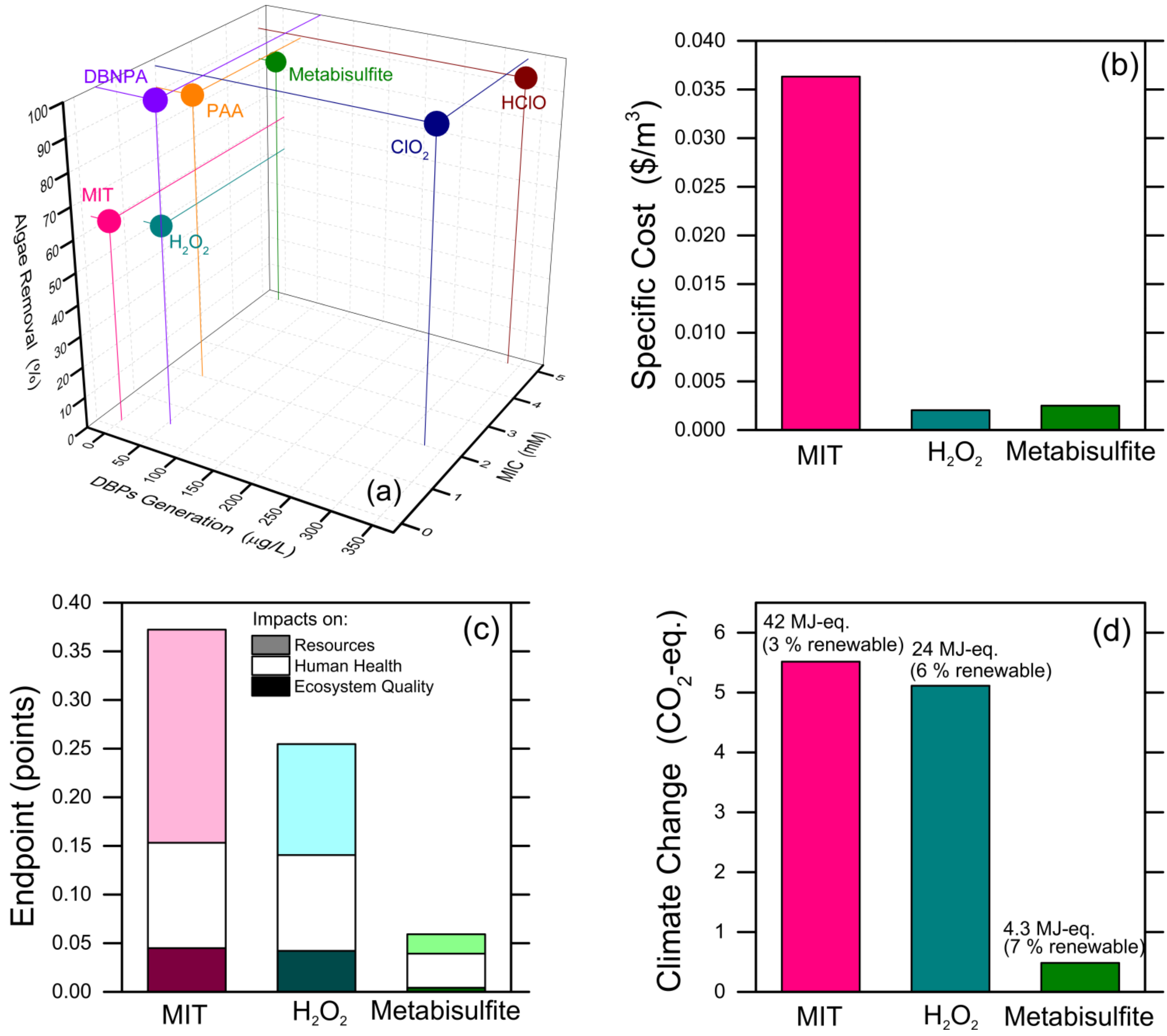


Fig. 5. Evaluation of the biocides in terms of performance, environmental impacts, and cost of use. (a) Summary of safety and disinfection efficacy, expressed as (x axis) DBP generation, (y axis) MIC, and (z axis) microalgae removal (at dosage of 1.5 mM). (b) Cost of deployment to treat one m^3 of wastewater, based on the optimal dosage found in this study. (c) Endpoint results of ReCiPe methodology in LCA; light shade, white, and dark shade colors refer to the categories "resources", "human health", and "ecosystem quality", respectively. (d) Results of IPCC2013 analysis and indication of the energy costs from CED analysis. In (b-d), only the three most promising biocides are presented, namely, MIT, hydrogen peroxide, and metabisulfite.

On the basis of these observations, the economic and environmental impacts associated with the use of MIT, hydrogen peroxide, and metabisulfite were evaluated. Wholesale cost of reagents was assumed, and in particular 1200, 400, and 200 $/ton for, respectively, MIT (14% w/w solution in water), H_2O_2 (50% v/v solution in water), and sodium metabisulfite (97% purity). To calculate the cost of application to disinfect one cubic meter of contaminated groundwater, concentrations of 0.03 mM, 0.075 mM, and 0.075 mM were considered in the effluent matrix, for the three biocides, respectively. These values are based on the results presented in Figure 3a and are thus associated with their effect against microalgae. Despite MIT should be dosed at the lowest concentration, the cost of its application would be the largest among the three biocides owing to its high market price; see Figure 5b. The use of hydrogen peroxide and metabisulfite would be economical (~0.0025 $\$/m^3$) due to a combination of low price and medium concentration required in the matrix.

Considering the environmental impacts of the three biocides, MIT is associated with the largest burdens, mainly because this substance is toxic for the environment and for humans (Burnett et al., 2010; De Groot and Herxheimer 1989; Schnuch et al., 1998); see also Figure 5c and Figure S1 in the SI. Its production involves the reaction of five compounds, namely, acrylic acid, hydrogen sulfide, methanol, methylamine, and hydrogen chloride, thus the exploitation of a large amount of environmental resources. Figure 5d shows the environmental impacts related to CO_2 emission, which is the major parameter for climate change evaluations in LCA analyses. A more notable production of CO_2 is associated to the exploitation of MIT. This result can be rationalized considering the significant amount of energy required for the extraction, processing, and production of each of the compounds listed above and necessary for the synthesis of the final reagent. The production of H_2O_2 also involves a relatively large amount of CO_2 release, as it takes place through anthraquinone auto-oxidation. In addition, the use of hydrogen peroxide poses problems of transport and storage, because this substance is unstable and may cause fire or explosion. On the contrary, the application of metabisulfite is not associated to particularly high energy requirements or environmental impacts.

This result stems from the fact that the electrolytic process aimed at the production of NaOH is well established, while SO_2 is a waste element resulting from metal extraction processes and its reuse implies a gain in terms of life cycle. Another important consideration can be drawn from the results reported in figure 5d: almost the totality of the energy supply currently derives from the exploitation of non-renewable energy sources in case of MIT and hydrogen peroxide production. A reduction of the reliance on fossil fuels and a subsequent exploitation of more green energy sources would be strongly beneficial especially in the case of the application of these two biocides, as it would result in the abatement of greenhouse gas emissions.

4. Conclusion

This study investigated the performance and safety of 9 different biocides (HClO; PAA; ClO_2; H_2O_2; persulfate; DBNPA; MIT; sulfite; metabisulfite) when employed for the abatement of algae and bacteria (*E. coli*) in a complex aqueous solution, specifically, leachate from a phosphogypsum landfill. Overall, the following conclusions can be drawn:

(i) Various biocides are effective in the removal of algae in acidic wastewater, including oxidizing compounds, electrophilic biocides, and reducing agents. In particular, algae disinfection rates larger than 80% were achieved even with a low addition (0.03 mM) of HClO, PAA, ClO_2, DBNPA, MIT, or metabisulfite. H_2O_2 required a dose of 0.075 mM to remove algae with a rate equal or larger than 80%.

(ii) MIT and DBNPA were the most effective biocides against *E. coli*, while metabisulfite and HClO required higher dosage to achieve similarly high removal rates. Therefore, no strong correlation between the removal rate against microalgae and against *E. coli* was found in this study for the various biocides. In terms of overall disinfection effectiveness, the two electrophilic biocides showed the highest performance, while addition of sulfite and persulfate as individual reagents had no or little effect.

(iii) Due to the possible generation of harmful disinfection by-products, HClO and PAA may not be employed for water disinfection with concentrations above a threshold value. Below this value, likely specific for each water matrix, these two biocides may represent effective and clean compounds for the abatement of algae and bacteria. In the complex water matrix investigated in this study, this threshold value was 0.075 mM.

(iv) The molecular structure of each biocide plays a key role in the disinfection process. A chief example is represented by the different behavior of DBNPA and MIT. Despite their analogous disinfection mechanisms (they are both electrophilic biocides), DBNPA induced the formation of tribromomethane during disinfection, while MIT acted as an effective biocide without generation of detectable levels of halogenated compounds.

(v) The most favorable biocides within the water matrix analyzed in this study, considering simultaneously safety and effectiveness, were MIT, H_2O_2, and metabisulfite.

(vi) In particular, metabisulfite represents a highly promising new biocide due to its low cost, low environmental impacts, and adequate efficacy against both microalgae and bacteria.

While the effectiveness and disinfection byproduct generation of the biocides were investigated in a specific contaminated matrix, the results of this study could be used as guidelines for the choice of the best biocide in different wastewaters. Oxidizing agents, such as PAA, should not be employed in water matrices with large concentrations of halides, especially bromide, due to the consequent likely generation of halogenated compounds. Moreover, this study indicates that an optimal biocide dose exists to maximize disinfection and safety; therefore, preliminary experiments should be performed to determine the correct biocide application in each matrix. Finally, our results suggest that unconventional reagents may be applied effectively for the abatement of microorganisms, such as microalgae and *E. coli*, within complex water sources. In particular, metabisulfite is a promising new disinfectant, safer and more eco-friendly than traditional biocides. However, further studies are required to understand its disinfection mechanism in detail.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

Supplementary material associated with this article can be found in the online version.

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Graphical Abstract