

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

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Evaluation of the effectiveness, safety, and feasibility of 9 potential biocides to disinfect acidic landfill leachate from algae and bacteria / Farinelli, G.; Giagnorio, M.; Ricceri, F.; Giannakis, S.; Tiraferri, A.. - In: WATER RESEARCH. - ISSN 0043-1354. - 191:(2021), p. 116801. [10.1016/j.watres.2020.116801]

*Availability:*

This version is available at: 11583/2862758 since: 2021-01-18T16:59:55Z

*Publisher:*

Elsevier Ltd

*Published*

DOI:10.1016/j.watres.2020.116801

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

4  
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20 **Highlights**

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

26

## 27 **ABSTRACT**

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

47

48

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

51

## 52 1. Introduction

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

65 Well-established solutions include processes like ozonation. Ozone must be produced in situ and  
66 it is thus associated with higher operational costs; also, it can generate bromate, a carcinogenic DBP,  
67 in the presence of bromide (Shah et al., 2015). The other commonly employed biocides have each  
68 advantages and disadvantages. For example, owing to its optimum cost-efficiency ratio, hypochlorite  
69 represents one of the most applied bactericidal compounds (Fukuzaki 2006). Nevertheless,  $\text{ClO}^-$   
70 leads to the formation of THMs and it is an irritant for the mucous membranes when concentrated in  
71 water (Fukuzaki 2006; Gómez-López et al., 2013; Lee and Huang 2019). Promising alternatives were  
72 developed during the last decade, among which chlorine dioxide and peracetic acid are the most  
73 successful examples. Even if these two compounds are effective biocides against a wide spectrum of  
74 microorganisms, recent studies demonstrated that the former can generate chlorite and chlorate, both  
75 harmful DBPs, together with traces of tribromomethane, while the latter represents a potential source  
76 of THMs in the presence of bromide and NOM in water (Al-Otoum et al., 2016; Domínguez Henao

77 et al., 2018; Stevens 1982; Xue et al., 2017; Yang et al., 2013). All the above-mentioned compounds  
78 rely on oxidative processes in order to attain disinfection. Other compounds can work as biocides  
79 through different mechanisms, for example, the couple methylisothiazolinone / chloro-  
80 methylisothiazolinone (MIT), as well the 2,2-dibromo-2-cyanoacetamide (DBNPA) (Kahrilas et al.,  
81 2015). Nevertheless, the literature lacks studies related to these compounds and to the formation of  
82 DBPs once they are used for disinfection of contaminated water streams.

83 In addition to the compounds discussed so far, reductants may find an application in water  
84 disinfection. For instance, they can potentially affect the photosynthetic mechanism, thus limiting the  
85 growth of algae species (Beekley and Hoffman 1981; Shimazaki and Sugahara 1979; Wodzinski et  
86 al., 1978; Yang et al., 2004). Reducing agents include sulfuric compounds, such as sulfur dioxide,  
87 bisulfite, and sulfite. The ability of sulfites to inhibit the growth of some bacterial groups has been  
88 reported (Irwin et al., 2017). In particular, sulfites are promising compounds for industrial  
89 applications, owing to their ease of storage, safety (they are not as explosive as oxidants), lack of  
90 toxicity for humans and the environment, and low capital and operational costs (Farinelli et al.,  
91 2019).

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

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

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

118

## 119 **2. Materials and methods**

### 120 **2.1. Chemicals, reagents, and water matrices used in the study**

121 The biocides investigated in this study were: (i) oxidants, namely, hypochlorite (HClO), peracetic  
122 acid (PAA), chlorine dioxide (ClO<sub>2</sub>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>); (ii)  
123 electrophilic, namely, DBNPA and MIT; (iii) reductants, specifically, sulfite and metabisulfite. All  
124 the chemicals were used as received. Except for chlorine dioxide, which was purchased from Apura  
125 Srl (Brescia, Italy), all the other compounds were purchased from Sigma-Aldrich (Milano, Italy).  
126 MIT was prepared by mixing methylisothiazolinone and chloro-methylisothiazolinone at the  
127 appropriate concentration in an aqueous solution in a volume ratio of 1 to 3. For disinfection and  
128 DBP generation experiments, individual biocides were dosed at the target concentrations (*vide infra*)  
129 in 15 mL of water samples. All the water chemistry analyses were performed at a private external  
130 laboratory (Natura Srl, Naples, Italy), using methods EPA 5030 C 2003 and EPA 8260 D 2018.

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

143 **Table 1.** Main characteristics of the contaminated groundwater from analysis of the samples and historical  
 144 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-NH <sub>4</sub> <sup>+</sup>	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 ± 0.6 × 10 <sup>6</sup>
pH		2.8

145

## 146 2.2 *Microalgae sampling and counting*

147 Samples for microalgae quantification were collected in real water samples before disinfection  
 148 and 2 h after the addition of each disinfectant. To quantify the algae concentration, a counting  
 149 chamber was employed (Paul Marienfield GmbH & Co, Lauda-Königshofen, Germany). The  
 150 chamber is equipped with an optical microscope (Renishaw, UK). The concentration of algae cells  
 151 suspended in the samples was determined by multiplying the average number of cells observed in the  
 152 microscope images by the relevant area, the chamber depth, and the dilution factor. The nominal  
 153 statistical error of the counting chamber is defined according to the formula: error = 1/*n*, where *n* is  
 154 the number of counted microalgae cells. In the counting of the initial concentration of microalgae,

155 the nominal error was roughly 1.4%. When only a few cells are counted in the chamber, the error is  
156 of the same order of magnitude of the measured value: therefore, for values of the removal efficiency  
157 equal or larger than 90%, its absolute value is no longer relevant and the disinfection should be  
158 considered nearly complete. In all the other cases, the nominal error was below 10%.

### 159 **2.3. Bacterial methods: LB, saline solution, and bacterial suspension preparation**

160 Luria Bertani (LB) broth was prepared by completely dissolving tryptone (10 g), yeast extract (5  
161 g), and sodium chloride (10 g) in 1 L of deionized water. A slightly hypertonic saline medium was  
162 also prepared by dissolving sodium chloride (8 g) and potassium chloride (0.8 g) in 1 L of deionized  
163 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  
164 HCl. Before use, the LB and saline mixtures were autoclaved for 15 min at 15 psi at a temperature of  
165 121 °C.

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

176

177

178 **2.4. Determination of the minimum inhibitory concentration (MIC) of *E. coli*, and**  
179 **determination of bacterial disinfection kinetics by biocides**

180 The minimum inhibitory concentration (MIC) is typically defined as “the minimal concentration  
181 of an antimicrobial necessary to inhibit the growth of a target microorganism”. In this study, we  
182 adapted the broth dilution assay for the determination of MIC with *E. coli* as target microorganism,  
183 and with the biocides in question instead of antimicrobials used in common MIC testing. As a proxy  
184 of *E. coli* concentration, the optical density of the samples was considered, measured by a  
185 spectrophotometer at 600 nm wavelength (OD<sub>600</sub>). MIC is an intrinsic characteristic of each biocide  
186 and depends also on the type of bacteria used as an evaluation strain. To determine this parameter for  
187 the different biocides investigated in this study, a falcon tube was filled with a  $7-8 \times 10^8$  CFU/mL  
188 suspension of *E. coli* in LB (OD<sub>600</sub>=1±0.1). Each biocide was spiked in the suspension at the target  
189 concentration and the falcon tube was then incubated for 24 h at 37 °C. Both positive and negative  
190 controls (*i.e.*, no biocide, and no bacteria, respectively) were always performed to ensure that no  
191 contamination occurred. The absorbance at 600 nm wavelength was then measured to determine the  
192 optical density, followed by calibration with suspensions of known concentrations determined by  
193 plating and counting. The presence of biocides can result to cell lysis, oxidation, and/or overall  
194 reduction of the cellular materials that cause light absorbance measured by the spectrophotometer  
195 when the bacteria are intact. There was a biocide concentration that caused significant reduction in  
196 the optical density; to avoid underestimating MIC, we selected as MIC the lowest biocide  
197 concentration that maintained optical density at a similar level to the initial spiking (final OD<sub>600</sub> after  
198 24 h = 1±0.2), before a 50% decrease in optical density and conversion to a “destructive  
199 concentration” from further addition of the biocide. In this manner, we can assume that bacterial  
200 growth was surely arrested at the chosen MIC level.

201 Furthermore, kinetics disinfection experiments were performed in real contaminated groundwater  
202 by addition of the various biocides. An appropriate amount of LB *E. coli* suspension was added to

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

## 212 **2.5. Methodology of life cycle analysis (LCA).**

213 The environmental burden from the production of 1 kg of the three most interesting biocides (*i.e.*,  
214 MIT, hydrogen peroxide, and metabisulfite; *vide infra*) were evaluated by life cycle analysis using  
215 OpenLCA 1.10 software, which incorporates the Ecoinvent 3.5 database. Three different  
216 methodologies were employed to conduct the environmental assessments: ReCiPe, Cumulative  
217 Energy Demand (CED), and IPCC2013. In the case of ReCiPe, the environmental analysis was  
218 modeled on both the endpoints and the midpoints, the latter presented as normalized values on the  
219 total impact. The CED approach was applied to assess the required energy expressed as the primary  
220 energy demand, while IPCC2013 analysis with a timeframe of 20 years was performed to assess the  
221 global warming potential of the production of the three biocides. While hydrogen peroxide is present  
222 in the Ecoinvent database, the impacts of metabisulfite and MIT were determined by modeling their  
223 most common synthesis approach. For metabisulfite, the reaction between SO<sub>2</sub> and NaOH was  
224 considered, while the protocol for MIT production was based on literature reports (Taubert et al.,  
225 2002).

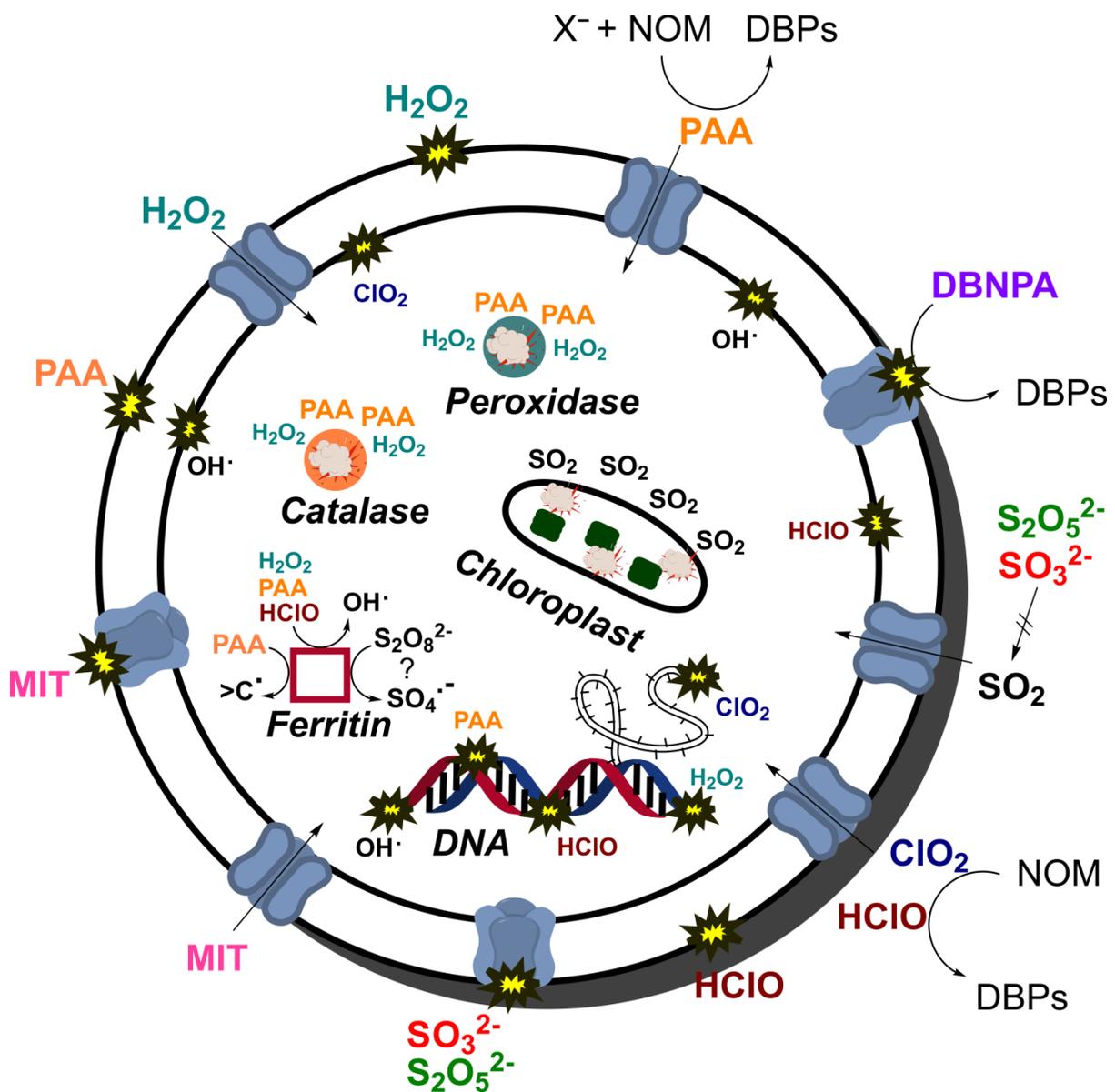
## 226 **3. Results and Discussion**

### 227 **3.1. Disinfection targets and principal characteristics of the studied biocides**

228 Before the analysis of the efficacy of each disinfectant, we present a succinct summary of their  
229 main characteristics. The mode of action of the 9 biocides investigated in this work is depicted in  
230 Figure 1 and briefly summarized in Table 2 together with each molecular structure. HClO, ClO<sub>2</sub>,  
231 H<sub>2</sub>O<sub>2</sub>, and PAA can penetrate the phospholipidic membrane through passive diffusion, thus these  
232 biocides disinfect through an intracellular action by acting on enzymatic processes and by attacking  
233 the DNA purine bases and the internal cytoplasmic membrane (Fukuzaki 2006; Ghanbari et al.,  
234 2021; Giannakis et al., 2016; Kitis 2004; Maillard 2002; Ofori et al., 2017; Tutumi et al., 1973; Xia  
235 et al., 2017). Moreover HClO, ClO<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, and PAA can also damage the outer cellular membrane  
236 (Feng et al., 2020; Fukuzaki 2006; Kitis 2004). Persulfate is active toward microorganism  
237 disinfection mostly if activated by transition metals (Wang et al., 2019; Xia et al., 2017). An  
238 estimation of the oxidation efficiency of an oxidant biocide can be estimated from a thermodynamic  
239 standpoint by comparing the redox potential of the reagent with that of bacteria strains (the *E. coli*  
240 redox potential is generally +0.45 to +0.72 V at pH 7 vs. SHE) (Ruales-Lonfat et al., 2015).  
241 Therefore, all the biocides with a redox potential higher than +0.45 to +0.72 V at pH 7 will be able to  
242 damage *E. coli* bacteria through oxidation.

243 The two electrophilic biocides, namely, DBNPA and MIT, disinfect by means of both  
244 extracellular and intracellular actions. DBNPA is mostly active by damaging the external cellular  
245 membrane through an electrophilic addition to the nucleophilic groups of membrane proteins  
246 (Campa et al., 2019; Kahrilas et al., 2015). MIT is active also by inhibiting internal enzymatic  
247 processes (Kahrilas et al., 2015; Maillard 2002; Williams 2006). Literature lacks a detailed  
248 explanation on the disinfection mechanism of metabisulfite. On the basis of previous reports and of  
249 its molecular structure, it is reasonable to assume that metabisulfite can potentially alter the

250 photosynthetic process of microalgae by releasing  $\text{SO}_2$  in solution and that it can damage the cellular  
 251 membrane in the same fashion of an electrophilic biocide (Beekley and Hoffman 1981; Shimazaki  
 252 and Sugahara 1979; Wodzinski et al., 1978; Yaganza et al., 2004; Yang et al., 2004), but its  
 253 intracellular activity is still under debate. A more exhaustive discussion of the mechanism of  
 254 disinfection and DBPs generation mode for each of the 9 biocides is provided in the Supporting  
 255 Information (SI).



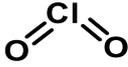
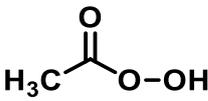
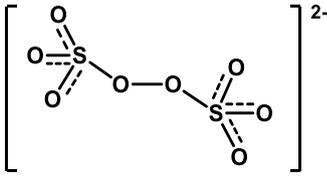
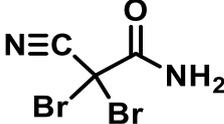
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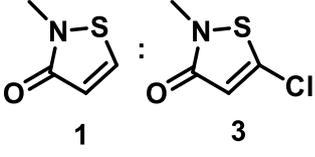
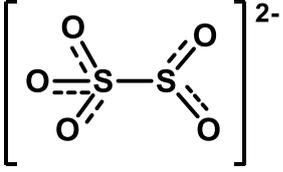
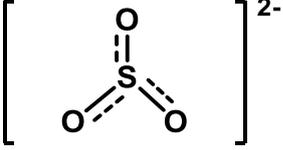
257 **Fig. 1.** Mode of action of the biocides investigated in this study. Please note that chloroplasts are present only

258

in algae.

259 **Table 2.** Molecular structure, mode of action (MOA), and mechanism of DBPs generation for each biocide  
 260 investigated in this study. In the fourth column, “oxidizing” (O), “electrophilic” (E), and “reductant” (R) refer  
 261 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	<ul style="list-style-type: none"> <li>• HClO prevails at pH 2.8.</li> <li>• Formation of chloramines thwarted at pH 2.8</li> <li>• If present, dominant chloramine species trichloramine and dichloramine with low disinfecting effect</li> <li>• Intracellular/Extracellular action.</li> <li>• Internal “Fenton-like” process (<math>\text{HClO} + \text{Fe}^{2+} \rightarrow \text{HO}^\cdot + \text{Cl}^- + \text{Fe}^{3+}</math>)</li> <li>• Addition to purine base of DNA and nucleophilic sites of internal organelles.</li> <li>• THMs and HACs generation through electrophilic addition to organic material.</li> </ul>	O	1.4
Chlorine dioxide, ClO <sub>2</sub> (Maillard 2002; Ofori et al., 2017)		<ul style="list-style-type: none"> <li>• Intracellular action (and possible extracellular action).</li> <li>• Inhibition of protein synthesis and enzymatic processes.</li> <li>• Addition to internal cytoplasmic membrane</li> <li>• ClO<sub>2</sub><sup>-</sup>, ClO<sub>3</sub><sup>-</sup> generation through oxidation of Cl<sup>-</sup>.</li> <li>• Slight THMs generation through electrophilic addition to NOM.</li> </ul>	O	0.95
Hydrogen peroxide, H <sub>2</sub> O <sub>2</sub> (Maillard 2002)	HO—OH	<ul style="list-style-type: none"> <li>• Intracellular/Extracellular action.</li> <li>• Inhibition of peroxidase activity.</li> <li>• Internal Fenton process. (<math>\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}^\cdot + \text{OH}^- + \text{Fe}^{3+}</math>)</li> <li>•</li> </ul>	O	1.8
Peracetic acid, PAA (Ghanbari et al., 2021; Kahrilas et al., 2015; Kitis 2004; Tutumi et al., 1973)		<ul style="list-style-type: none"> <li>• Intracellular/Extracellular action.</li> <li>• Inhibition of peroxidase activity.</li> <li>• Internal “Fenton”-type process (<math>\text{C}_2\text{H}_4\text{O}_3 + \text{Fe}^{2+} \rightarrow \text{HO}^\cdot + \text{C}_2\text{H}_3\text{O}_2^- + \text{Fe}^{3+}</math>)</li> <li>• Easier generation of OH<sup>•</sup> than H<sub>2</sub>O<sub>2</sub>.</li> <li>• Possible THMs &amp; HACs generation through oxidation of Cl<sup>-</sup> (or Br<sup>-</sup>) to HClO (or HBrO) which binds NOM.</li> </ul>	O	1.4
Persulfate, S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> (Wang et al., 2019; Xia et al., 2017)		<ul style="list-style-type: none"> <li>• Extracellular action (and possible intracellular action until otherwise proven).</li> <li>• Activated in presence of transition metals (<i>i.e.</i>, Fe, Mn and Cu) or light.</li> <li>• Sulfate radical generation (SO<sub>4</sub><sup>•-</sup>).</li> <li>• Possible THMs &amp; HACs generation through radical processes.</li> </ul>	O	2.1
DBNPA (Campa et al., 2019; Kahrilas et al., 2015)		<ul style="list-style-type: none"> <li>• Extracellular action.</li> <li>• Addition to -SH and -NH groups of membrane proteins.</li> <li>• THMs release through nucleophilic acyclic substitution reaction.</li> </ul>	E	N.A.

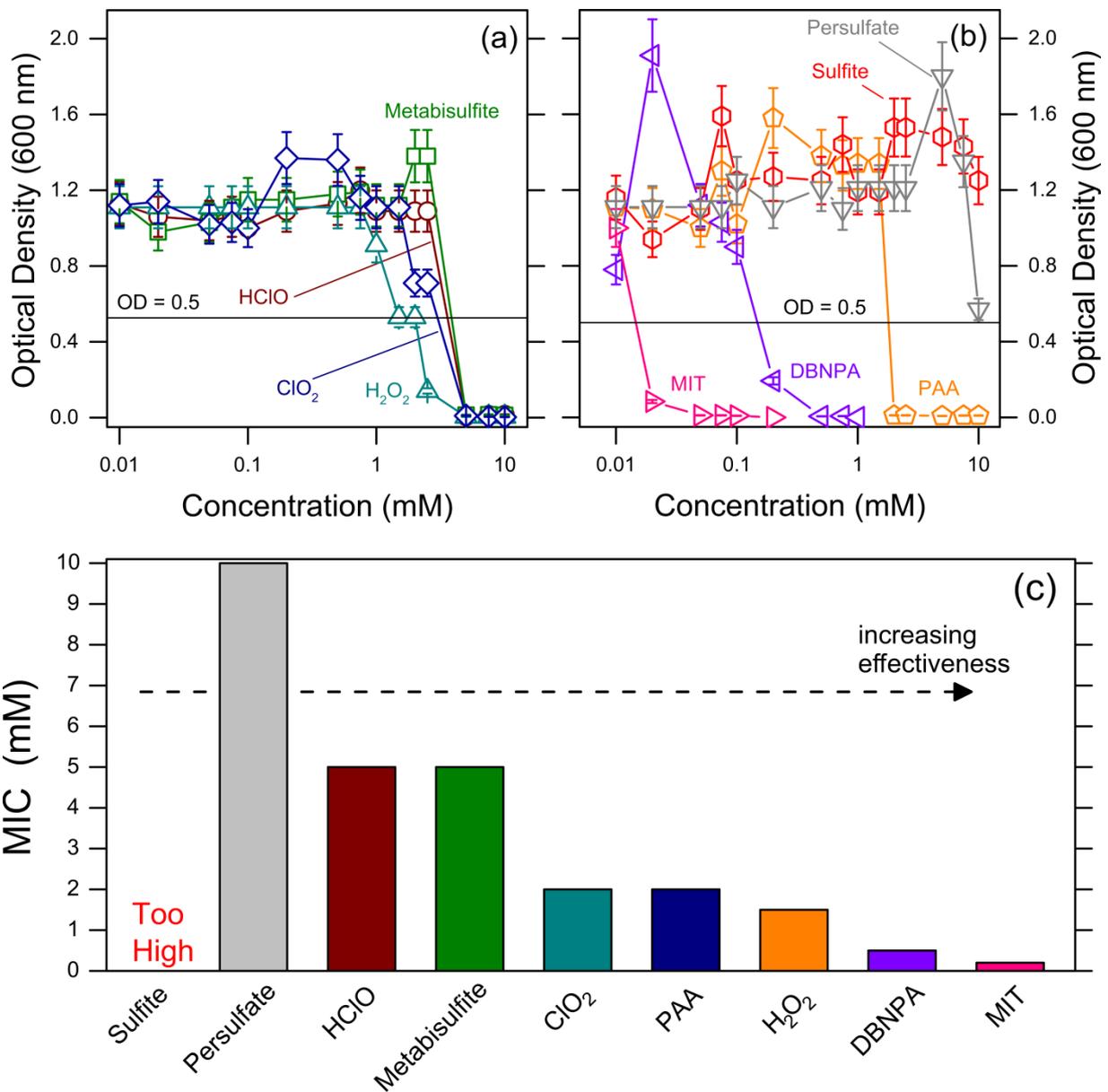
MIT (Kahrilas et al., 2015; Maillard 2002; Williams 2006)		<ul style="list-style-type: none"> <li>• Extracellular and intracellular action.</li> <li>• Addition to -SH and -NH groups of membrane proteins.</li> <li>• Inhibition of enzymatic processes.</li> <li>• Internal radical processes.</li> <li>• No THMs &amp; HACs generation mechanism detected.</li> </ul>	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)		<ul style="list-style-type: none"> <li>• Extracellular and possible intracellular action through reductant activity.</li> <li>• Possible extracellular action electrophilic addition.</li> <li>• No THMs &amp; HACs generation mechanism detected.</li> </ul>	R/E	N.A.
Sulfite, $SO_3^{2-}$ (Beekley and Hoffman 1981; Shimazaki and Sugahara 1979; Wodzinski et al., 1978; Yang et al., 2004)		<ul style="list-style-type: none"> <li>• Extracellular</li> <li>• Possible intracellular action through reductant activity.</li> <li>• Possible extracellular action electrophilic addition.</li> <li>• No THMs &amp; HACs generation mechanism detected.</li> </ul>	R/E	N.A.

262

### 263 3.2. Disinfection efficacy

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

274 concentrations to inhibit bacterial growth. Figure 2c summarizes the MIC values determined for all  
 275 the biocides.

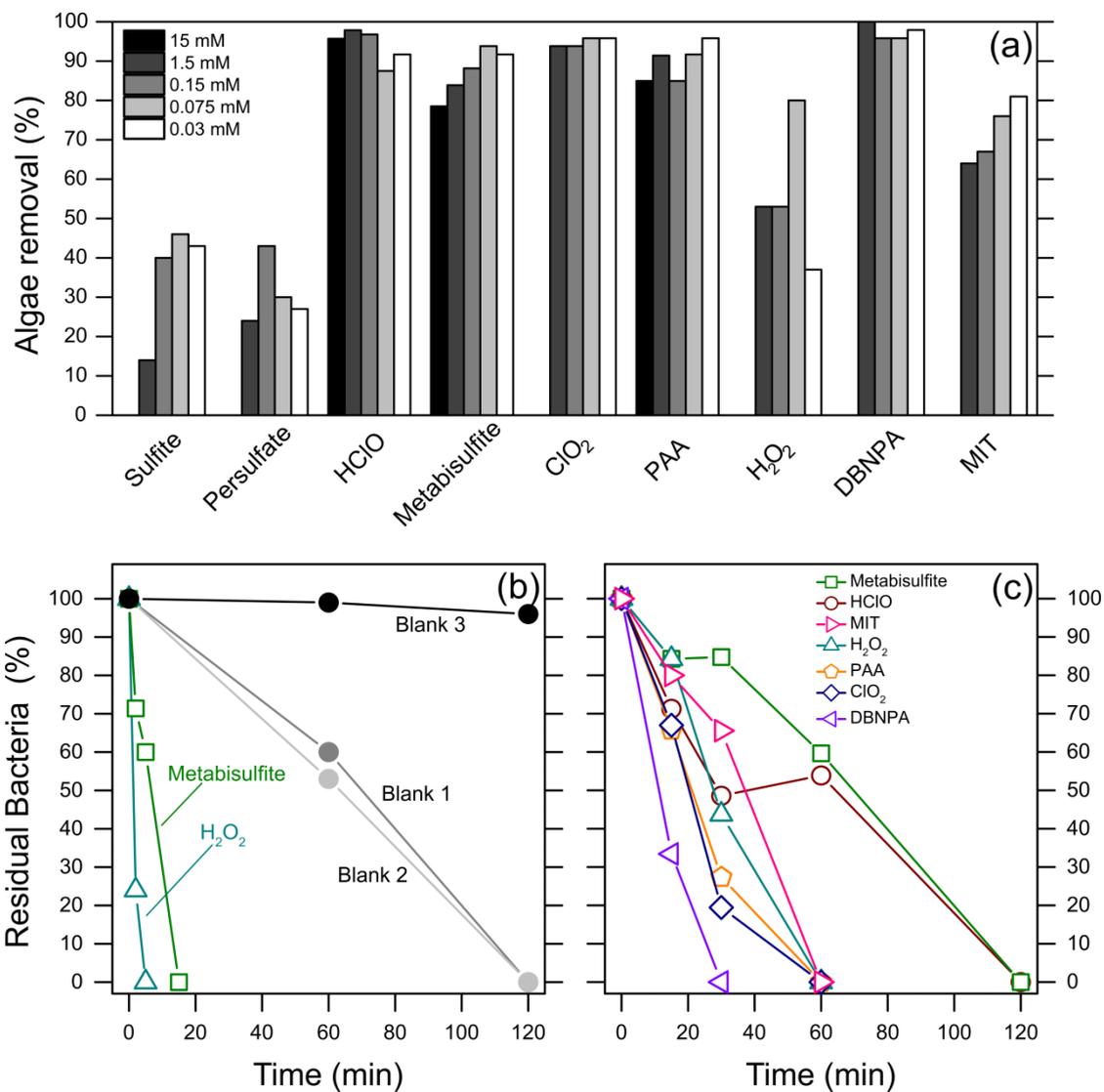


276  
 277 **Fig. 2.** Minimum inhibitory concentration (MIC) of the biocides toward *E. coli* inactivation. (a, b) Results of  
 278 light absorbance of the *E. coli* suspension as a function of biocide concentration. The lines are only intended  
 279 as guides for the eye. Initial spiking to OD<sub>600</sub> = 1 ± 0.1 was carried out, and the results depict the OD<sub>600</sub>  
 280 measured after 24 h. The control samples (no biocide addition) resulted in the OD<sub>600</sub> range = 3.5-5.5 at the  
 281 stationary growth phase. (c) Summary of MIC values. No MIC values could be determined for sulfite and  
 282 persulfate up to 10 mM biocide concentration.

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

299 Several algae species, such as *Chlorella*, adapt well in acidic environments (Gross 2000; Hirooka  
300 et al., 2017). On the contrary, *E. coli* cannot typically survive below pH 4 (Conner and Kotrola  
301 1995). The water matrix effect on *E. coli* viability was investigated to understand this behavior and  
302 to isolate the effect of biocides from that of pH. The results of *E. coli* cultivability from the three  
303 blanks indicate that the acidity of the contaminated groundwater (pH 2.8) is responsible for a  
304 significant bacterial inactivation within 2 h (Figure 3a). Therefore, a biocide can only be considered  
305 effective toward bacteria disinfection in this matrix if it inactivates *E. coli* faster than 2 h. Clearly,  
306 metabisulfite and hydrogen peroxide were able to quickly inactivate *E. coli* at their respective MIC.  
307 On the other hand, metabisulfite was not as quickly effective at a low concentration of 0.03 mM

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



311  
 312 **Fig. 3.** Disinfection of microalgae and *E. coli* in the real contaminated groundwater matrix. (a) Removal rates  
 313 of microalgae at varying biocide concentration. (b, c) Bacteria disinfection kinetics. In (b), metabisulfite and  
 314 hydrogen peroxide were added at their MIC. Blank 1 shows the matrix effect on *E. coli* viability; blank 2  
 315 shows the matrix effect on *E. coli* viability by buffering the suspension at pH 7 before plating; blank 3 shows  
 316 the matrix effect on *E. coli* viability by buffering the suspension at pH 7 before biocide addition. In (c), *E. coli*  
 317 disinfection results obtained with a biocide concentration of 0.03 mM are shown. The lines are only intended  
 318 as guides for the eye. The standard deviation is equal or lower than 25% for each of the data reported in (b, c).

319 These data lead to an important conclusion: when working with a complex water matrix such as  
320 that examined in this study, each of the following biocide may be employed at low dose when only  
321 microalgae removal is required: PAA, H<sub>2</sub>O<sub>2</sub>, ClO<sub>2</sub>, DBNPA, MIT, HClO, and metabisulfite. On the  
322 other hand, in the presence of *E. coli* or other persistent/surviving bacteria behaving in a similar way,  
323 the utilization of HClO and metabisulfite requires larger concentration to achieve both an effective  
324 algae removal and a suitable antibacterial activity.

### 325 3.3. *Generation of disinfection by-products*

326 The formation of disinfection by-products was determined following addition of biocides in the  
327 real groundwater matrix. As expected, ClO<sub>2</sub> generated THMs, and in particular tribromomethane (a  
328 carcinogenic compound), at all the investigated concentrations. The formation of this halogenated  
329 compound upon employment of ClO<sub>2</sub> is consistent with reports in the literature (Al-Otoum et al.,  
330 2016; Gómez-López et al., 2013). As expected, also the use HClO and PAA induced the formation of  
331 halogenated by-products (Dell'Erba et al., 2007; Domínguez Henao et al., 2018; Shah et al., 2015;  
332 Xue et al., 2017), specifically at biocide concentrations of 15, 1.5, and 0.15 mM (see Table 3 and SI  
333 for the complete set of analysis). Tribromomethane was detected as the most prevalent DBP. In  
334 particular, in the case of PAA, the concentration of halogenated by-products sharply increased with  
335 increasing disinfectant dose. This behavior indicates the involvement of PAA itself as a primary  
336 source in halogenated by-products formation (Table 2 and SI provide a more exhaustive discussion  
337 on the generation of DBPs related to the nine biocides). However, THMs or other halogenated  
338 compounds were not detected at concentrations of biocides equal to 0.075 or 0.03 mM, even though  
339 both HClO and PAA still maintained a suitable disinfectant efficiency. Therefore, 0.075 mM  
340 represents the threshold safety-related dose for HClO and PAA application to the water matrix in  
341 examination. These results suggest that it should be possible to find a threshold dose for any matrix  
342 at which adequate disinfection occurs without the detectable formation of halogenated compounds  
343 when employing of HClO and PAA.

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

	Sulfite	Persulfate	HClO	Metabisulfite	ClO <sub>2</sub>	PAA	H <sub>2</sub> O <sub>2</sub>	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

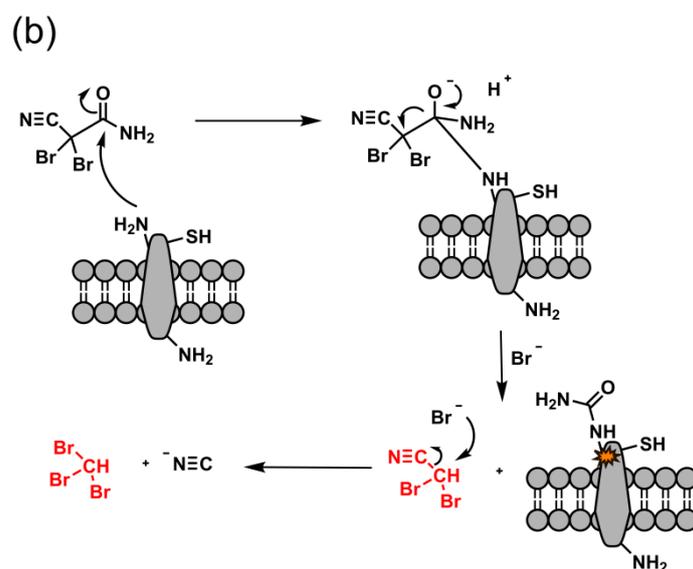
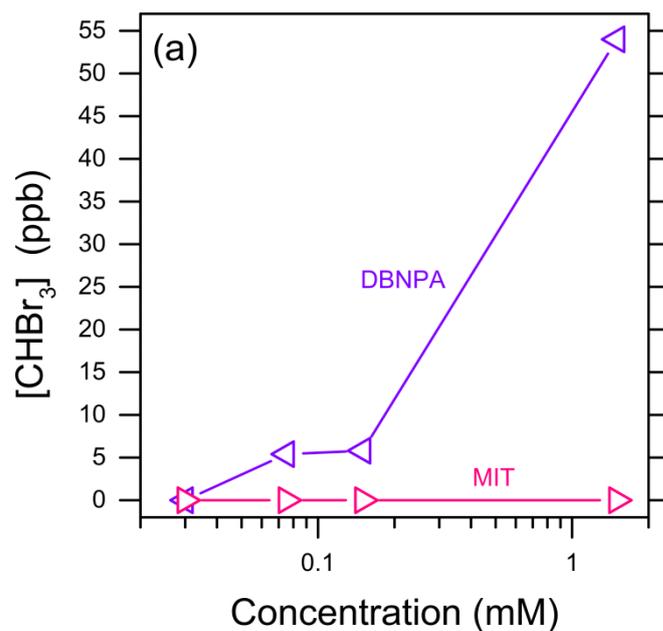
346 “N.A.”: test not performed. “LoQ”: limit of reliable detection.

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348 Notably, metabisulfite, H<sub>2</sub>O<sub>2</sub>, MIT, sulfite, and persulfate did not generate any trace of  
 349 halogenated compounds, even at the upper limits of the biocide concentration range. The behavior of  
 350 sulfite and persulfate may be merely ascribed to their low disinfection activity toward bacteria and  
 351 algae, which limited the formation of by-products and prevented the formation of halogenated  
 352 compounds. Ultimately, we surmise that metabisulfite, H<sub>2</sub>O<sub>2</sub>, and MIT are the most interesting  
 353 biocides of the study, since they maintained high disinfection efficiency toward microorganisms,  
 354 without generating DBPs.

355 Another interesting result obtained in this study is the behavior of DBNPA and the formation of  
 356 tribromomethane as a disinfection by-product. Specifically, the concentration of tribromomethane in

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



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**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.

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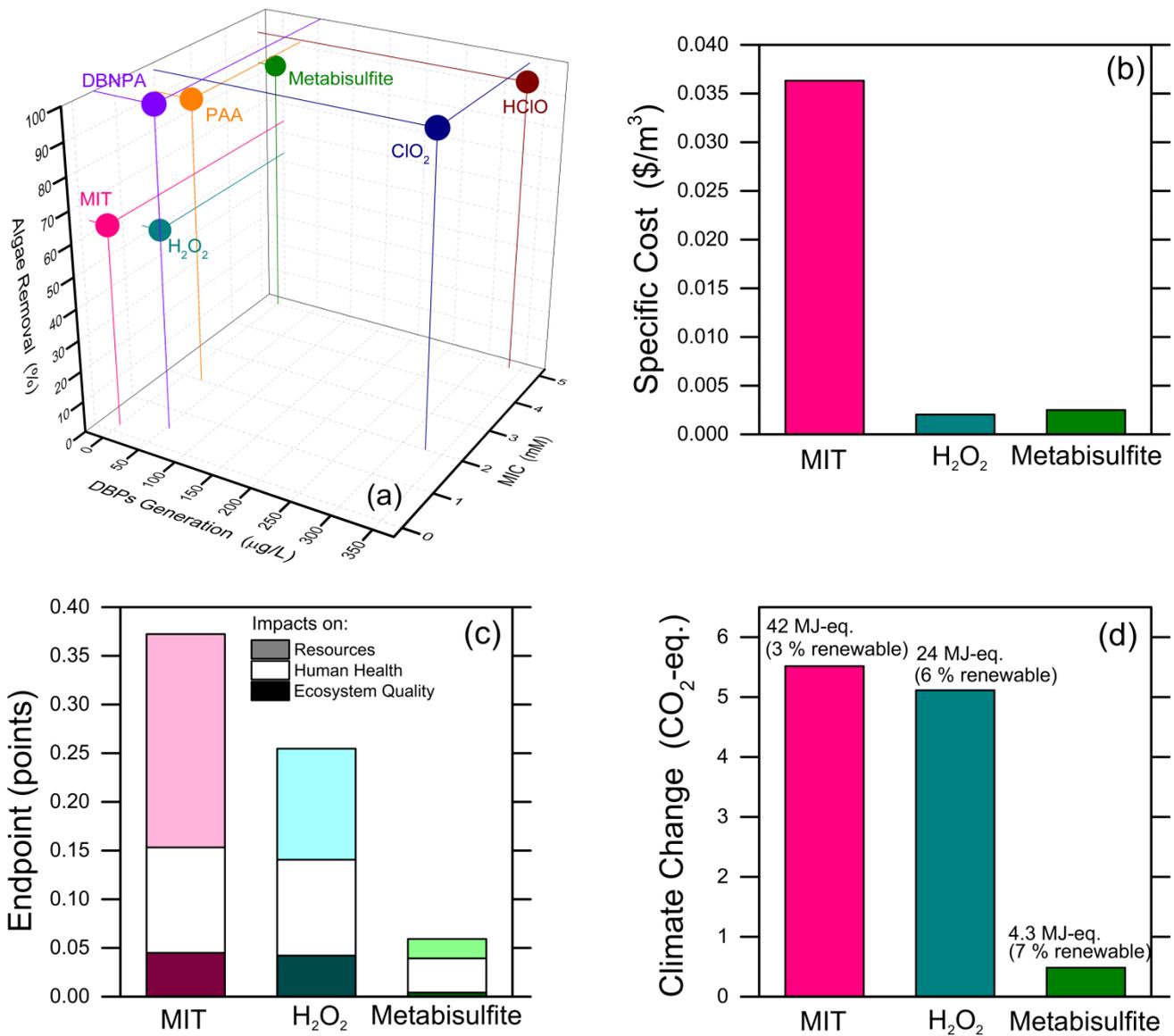
### 3.4. Metabisulfite, H<sub>2</sub>O<sub>2</sub>, and MIT: logistics of implementation.

Metabisulfite, H<sub>2</sub>O<sub>2</sub>, and MIT resulted equally safe in terms of DBPs generation, but they are different in terms practical utilization (*e.g.*, storage, safety). Indeed, H<sub>2</sub>O<sub>2</sub> and MIT are well known in the literature as effective disinfectants (Giannakis et al., 2016; Kahrilas et al., 2015; Williams

380 2006); however, they present some practical limitations compared to metabisulfite. Firstly, the  
381 storage of large amounts of H<sub>2</sub>O<sub>2</sub> is dangerous because of its explosive characteristics (Schreck et al.,  
382 2004). Besides, the utilization of hydrogen peroxide is strongly discouraged before membrane  
383 desalination systems, due to the possible degradation of the membrane when exposed to oxidizing  
384 agents (da Silva et al., 2006; Kang et al., 2007; Korolkov et al., 2014). On the other hand, MIT is at  
385 both an allergenic and a cytotoxic compound (Burnett et al., 2010; Castanedo-Tardana and Zug  
386 2013; Groot and Weyland 1988; Hannuksela 1986). Metabisulfite, although rarely studied for  
387 applications similar to that of this study, may be the safest biocide overall, also consistent with the  
388 hypothesis of its disinfection mechanism (Table 1 and Figure 1).

### 389 **3.5. Environmental impacts, economic analysis, and overall review of biocides**

390 A summary of the efficacy and DBPs generation potential of the biocides used for the  
391 disinfection of leachate-contaminated groundwater is reported in Figure 5a. In this three dimensional  
392 plot, a dot is associated with each biocide, with the exclusion of persulfate and sulfite. The values of  
393 MIC, microalgae removal rate at biocide concentration of 1.5 mM, and average formation of  
394 halogenated compounds were considered to perform this summary analysis. An ideal biocide that is  
395 both highly effective and does not induce the production of harmful DBPs would sit in the top left  
396 corner of the graph. Chlorine-based disinfectants are instead found on the right side of the graph,  
397 because they are associated with the production of significant concentrations of harmful DBPs. The  
398 safest biocides in this respect were MIT, hydrogen peroxide, and metabisulfite. Of these three, H<sub>2</sub>O<sub>2</sub>  
399 and especially MIT were found to be highly effective against *E. coli* (*i.e.*, low MIC) and slightly less  
400 so to remove microalgae. A larger concentration of metabisulfite may instead be necessary to  
401 achieve the same disinfection efficacy of the other two biocides against both algae and bacteria.



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**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<sup>3</sup> 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.

411 On the basis of these observations, the economic and environmental impacts associated with the  
412 use of MIT, hydrogen peroxide, and metabisulfite were evaluated. Wholesale cost of reagents was  
413 assumed, and in particular 1200, 400, and 200 \$/ton for, respectively, MIT (14% w/w solution in  
414 water), H<sub>2</sub>O<sub>2</sub> (50% v/v solution in water), and sodium metabisulfite (97% purity). To calculate the  
415 cost of application to disinfect one cubic meter of contaminated groundwater, concentrations of 0.03  
416 mM, 0.075 mM, and 0.075 mM were considered in the effluent matrix, for the three biocides,  
417 respectively. These values are based on the results presented in Figure 3a and are thus associated  
418 with their effect against microalgae. Despite MIT should be dosed at the lowest concentration, the  
419 cost of its application would be the largest among the three biocides owing to its high market price;  
420 see Figure 5b. The use of hydrogen peroxide and metabisulfite would be economical (~0.0025 \$/m<sup>3</sup>)  
421 due to a combination of low price and medium concentration required in the matrix.

422 Considering the environmental impacts of the three biocides, MIT is associated with the largest  
423 burdens, mainly because this substance is toxic for the environment and for humans (Burnett et al.,  
424 2010; De Groot and Herxheimer 1989; Schnuch et al., 1998); see also Figure 5c and Figure S1 in the  
425 SI. Its production involves the reaction of five compounds, namely, acrylic acid, hydrogen sulfide,  
426 methanol, methylamine, and hydrogen chloride, thus the exploitation of a large amount of  
427 environmental resources. Figure 5d shows the environmental impacts related to CO<sub>2</sub> emission, which  
428 is the major parameter for climate change evaluations in LCA analyses. A more notable production  
429 of CO<sub>2</sub> is associated to the exploitation of MIT. This result can be rationalized considering the  
430 significant amount of energy required for the extraction, processing, and production of each of the  
431 compounds listed above and necessary for the synthesis of the final reagent. The production of H<sub>2</sub>O<sub>2</sub>  
432 also involves a relatively large amount of CO<sub>2</sub> release, as it takes place through anthraquinone auto-  
433 oxidation. In addition, the use of hydrogen peroxide poses problems of transport and storage,  
434 because this substance is unstable and may cause fire or explosion. On the contrary, the application  
435 of metabisulfite is not associated to particularly high energy requirements or environmental impacts.

436 This result stems from the fact that the electrolytic process aimed at the production of NaOH is well  
437 established, while SO<sub>2</sub> is a waste element resulting from metal extraction processes and its reuse  
438 implies a gain in terms of life cycle. Another important consideration can be drawn from the results  
439 reported in figure 5d: almost the totality of the energy supply currently derives from the exploitation  
440 of non-renewable energy sources in case of MIT and hydrogen peroxide production. A reduction of  
441 the reliance on fossil fuels and a subsequent exploitation of more green energy sources would be  
442 strongly beneficial especially in the case of the application of these two biocides, as it would result in  
443 the abatement of greenhouse gas emissions.

444

#### 445 **4. Conclusion**

446 This study investigated the performance and safety of 9 different biocides (HClO; PAA; ClO<sub>2</sub>;  
447 H<sub>2</sub>O<sub>2</sub>; persulfate; DBNPA; MIT; sulfite; metabisulfite) when employed for the abatement of algae  
448 and bacteria (*E. coli*) in a complex aqueous solution, specifically, leachate from a phosphogypsum  
449 landfill. Overall, the following conclusions can be drawn:

450 (i) Various biocides are effective in the removal of algae in acidic wastewater, including  
451 oxidizing compounds, electrophilic biocides, and reducing agents. In particular, algae disinfection  
452 rates larger than 80% were achieved even with a low addition (0.03 mM) of HClO, PAA, ClO<sub>2</sub>,  
453 DBNPA, MIT, or metabisulfite. H<sub>2</sub>O<sub>2</sub> required a dose of 0.075 mM to remove algae with a rate equal  
454 or larger than 80%.

455 (ii) MIT and DBNPA were the most effective biocides against *E. coli*, while metabisulfite and  
456 HClO required higher dosage to achieve similarly high removal rates. Therefore, no strong  
457 correlation between the removal rate against microalgae and against *E. coli* was found in this study  
458 for the various biocides. In terms of overall disinfection effectiveness, the two electrophilic biocides

459 showed the highest performance, while addition of sulfite and persulfate as individual reagents had  
460 no or little effect.

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

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

471 (v) The most favorable biocides within the water matrix analyzed in this study, considering  
472 simultaneously safety and effectiveness, were MIT, H<sub>2</sub>O<sub>2</sub>, and metabisulfite.

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

475 While the effectiveness and disinfection byproduct generation of the biocides were investigated  
476 in a specific contaminated matrix, the results of this study could be used as guidelines for the choice  
477 of the best biocide in different wastewaters. Oxidizing agents, such as PAA, should not be employed  
478 in water matrices with large concentrations of halides, especially bromide, due to the consequent  
479 likely generation of halogenated compounds. Moreover, this study indicates that an optimal biocide  
480 dose exists to maximize disinfection and safety; therefore, preliminary experiments should be  
481 performed to determine the correct biocide application in each matrix. Finally, our results suggest  
482 that unconventional reagents may be applied effectively for the abatement of microorganisms, such

483 as microalgae and *E. coli*, within complex water sources. In particular, metabisulfite is a promising  
484 new disinfectant, safer and more eco-friendly than traditional biocides. However, further studies are  
485 required to understand its disinfection mechanism in detail.

486

## 487 **Acknowledgements**

488 Alberto Tiraferri and Giulio Farinelli would like to thank Politecnico di Torino for financial  
489 support (58\_RRI19TIRALB). Stefanos Giannakis would like to acknowledge the Spanish Ministry  
490 of Science, Innovation and Universities (MICIU) for the Ramón y Cajal Fellowship (RYC2018-  
491 024033-I).

## 492 **Declaration of Competing Interest**

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

## 495 **Supplementary material**

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

497

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685 **Graphical Abstract**

