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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, 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 (ClO₂), hydrogen peroxide (H₂O₂), and persulfate (K₂S₂O₈); (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 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 ± 0.6 × 10 ⁶
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 OD₆₀₀) and a concentration of roughly 10⁹ 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 (OD₆₀₀). 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 (OD₆₀₀=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 OD₆₀₀ 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_2 and NaOH was
224 considered, while the protocol for MIT production was based on literature reports (Taubert et al.,
225 2002).

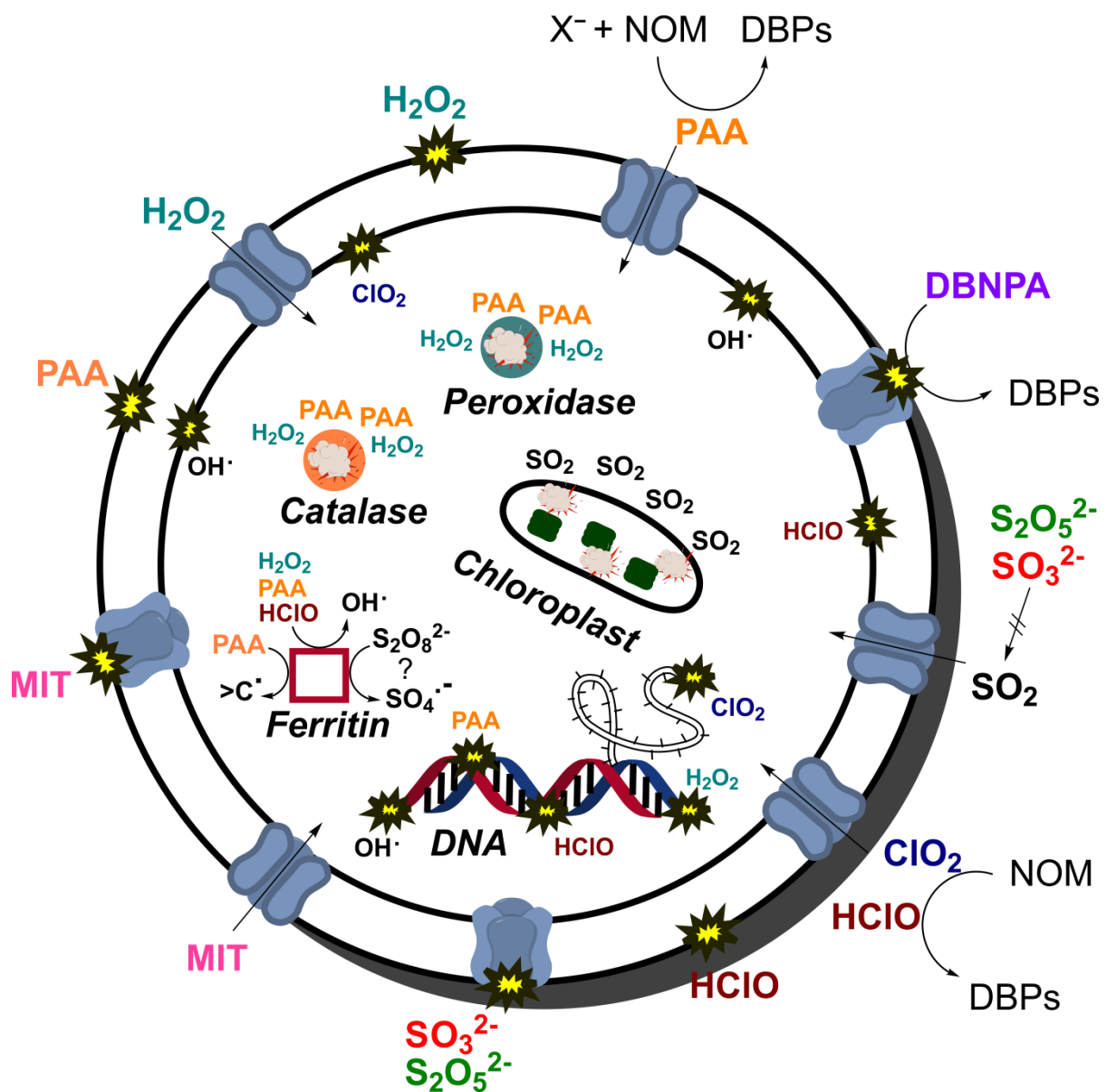
226 3. Results and Discussion

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, ClO₂,
231 H₂O₂, 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, ClO₂, H₂O₂, 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

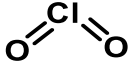
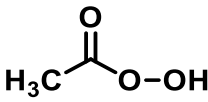
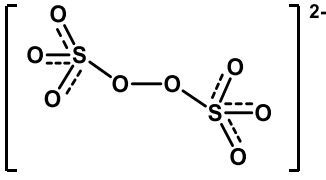
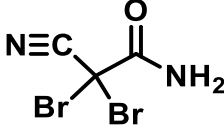
250 photosynthetic process of microalgae by releasing SO_2 in solution and that it can damage the cellular
 251 membrane in the same fashion of an electrophilic biocide (Beekley and Hoffman 1981; Shimazaki
 252 and Sugahara 1979; Wodzinski et al., 1978; Yaganza et al., 2004; Yang et al., 2004), but its
 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).

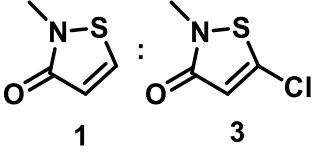
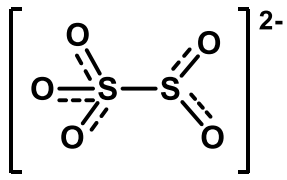
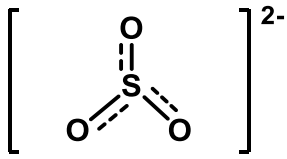


256

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"> 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 ($\text{HClO} + \text{Fe}^{2+} \rightarrow \text{HO}^\cdot + \text{Cl}^- + \text{Fe}^{3+}$) 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)		<ul style="list-style-type: none"> Intracellular action (and possible extracellular action). Inhibition of protein synthesis and enzymatic processes. Addition to internal cytoplasmic membrane ClO₂⁻, ClO₃⁻ generation through oxidation of Cl⁻. Slight THMs generation through electrophilic addition to NOM. 	O	0.95
Hydrogen peroxide, H ₂ O ₂ (Maillard 2002)	HO-OH	<ul style="list-style-type: none"> Intracellular/Extracellular action. Inhibition of peroxidase activity. Internal Fenton process. ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}^\cdot + \text{OH}^- + \text{Fe}^{3+}$) 	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"> Intracellular/Extracellular action. Inhibition of peroxidase activity. Internal “Fenton”-type process ($\text{C}_2\text{H}_4\text{O}_3 + \text{Fe}^{2+} \rightarrow \text{HO}^\cdot + \text{C}_2\text{H}_3\text{O}_2^- + \text{Fe}^{3+}$) Easier generation of OH[·] than H₂O₂. Possible THMs & HACs generation through oxidation of Cl⁻ (or Br⁻) to HClO (or HBrO) which binds NOM. 	O	1.4
Persulfate, S ₂ O ₈ ²⁻ (Wang et al., 2019; Xia et al., 2017)		<ul style="list-style-type: none"> Extracellular action (and possible intracellular action until otherwise proven). Activated in presence of transition metals (<i>i.e.</i>, Fe, Mn and Cu) or light. Sulfate radical generation (SO₄^{·-}). Possible THMs & HACs generation through radical processes. 	O	2.1
DBNPA (Campa et al., 2019; Kahrilas et al., 2015)		<ul style="list-style-type: none"> Extracellular action. Addition to -SH and -NH groups of membrane proteins. THMs release through nucleophilic acyclic substitution reaction. 	E	N.A.

MIT (Kahrilas et al., 2015; Maillard 2002; Williams 2006)		<ul style="list-style-type: none"> • 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)		<ul style="list-style-type: none"> • 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)		<ul style="list-style-type: none"> • Extracellular • Possible intracellular action through reductant activity. • Possible extracellular action electrophilic addition. • No THMs & HACs generation mechanism detected. 	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

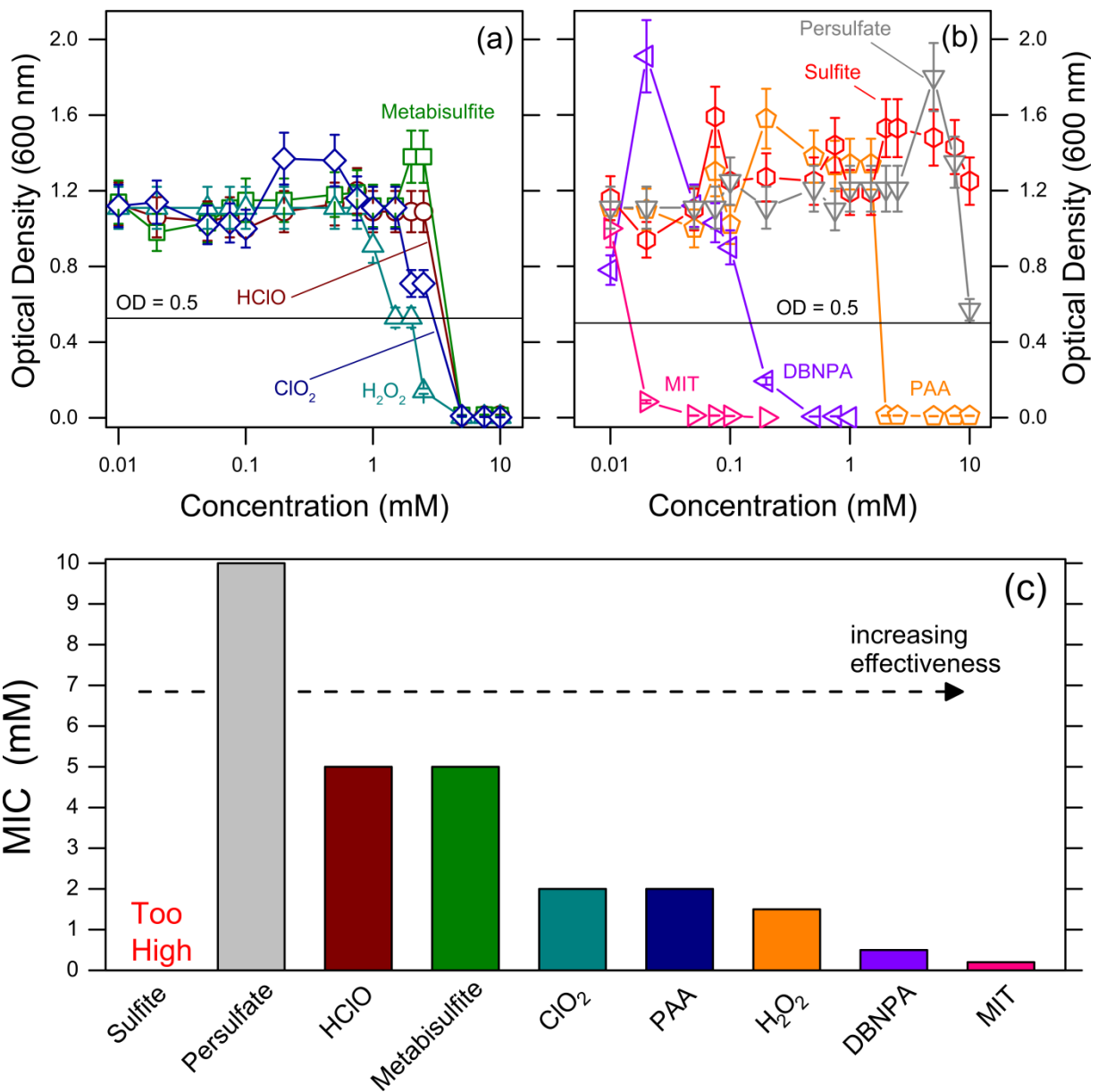
270 observed. No MIC value was found for sulfite in the biocide concentration range investigated in this

271 study, that is, 0-10 mM, and a high MIC value (~ 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.

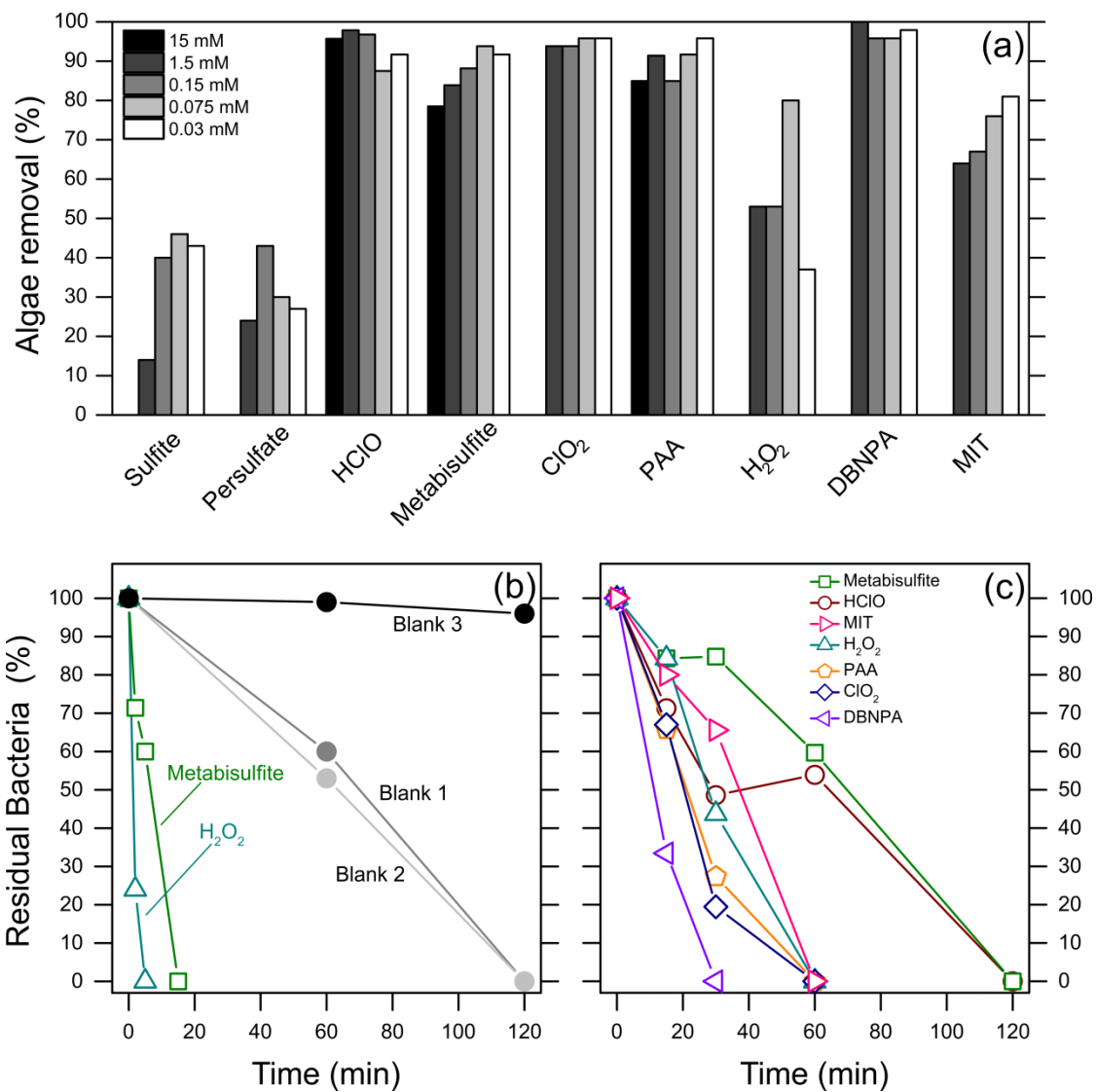


276
 277 **Fig. 2.** Minimum inhibitory concentration (MIC) of the biocides toward *E. coli* inactivation. (a, b) Results of
 278 light absorbance of the *E. coli* suspension as a function of biocide concentration. The lines are only intended
 279 as guides for the eye. Initial spiking to OD₆₀₀ = 1 ± 0.1 was carried out, and the results depict the OD₆₀₀
 280 measured after 24 h. The control samples (no biocide addition) resulted in the OD₆₀₀ 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, H₂O₂, ClO₂, 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, ClO₂ 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 ClO₂ 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 ₂	PAA	H ₂ O 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

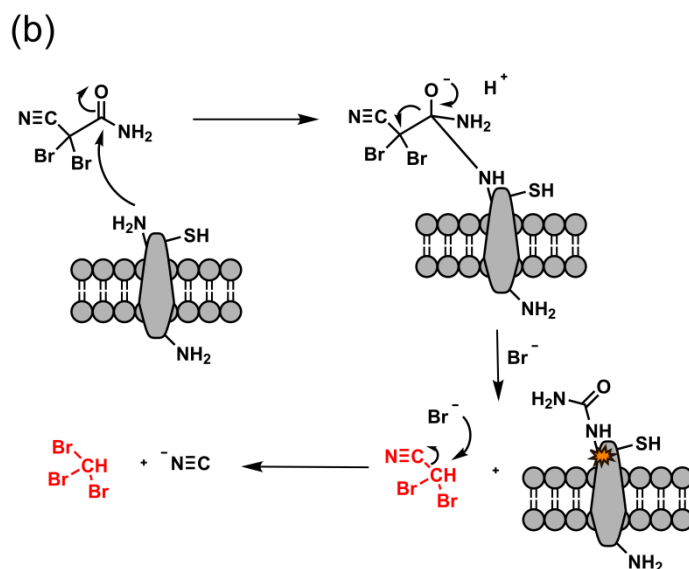
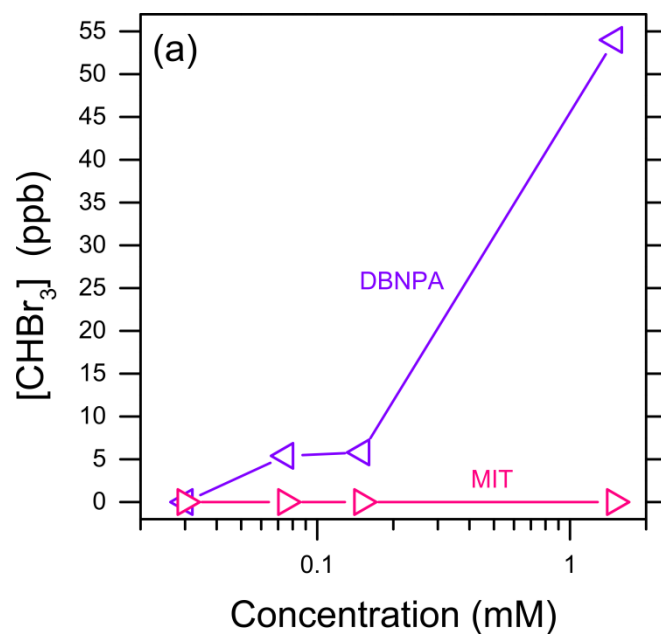
346 “N.A.”: test not performed. “LoQ”: limit of reliable detection.

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348 Notably, metabisulfite, H₂O₂, 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, H₂O₂, 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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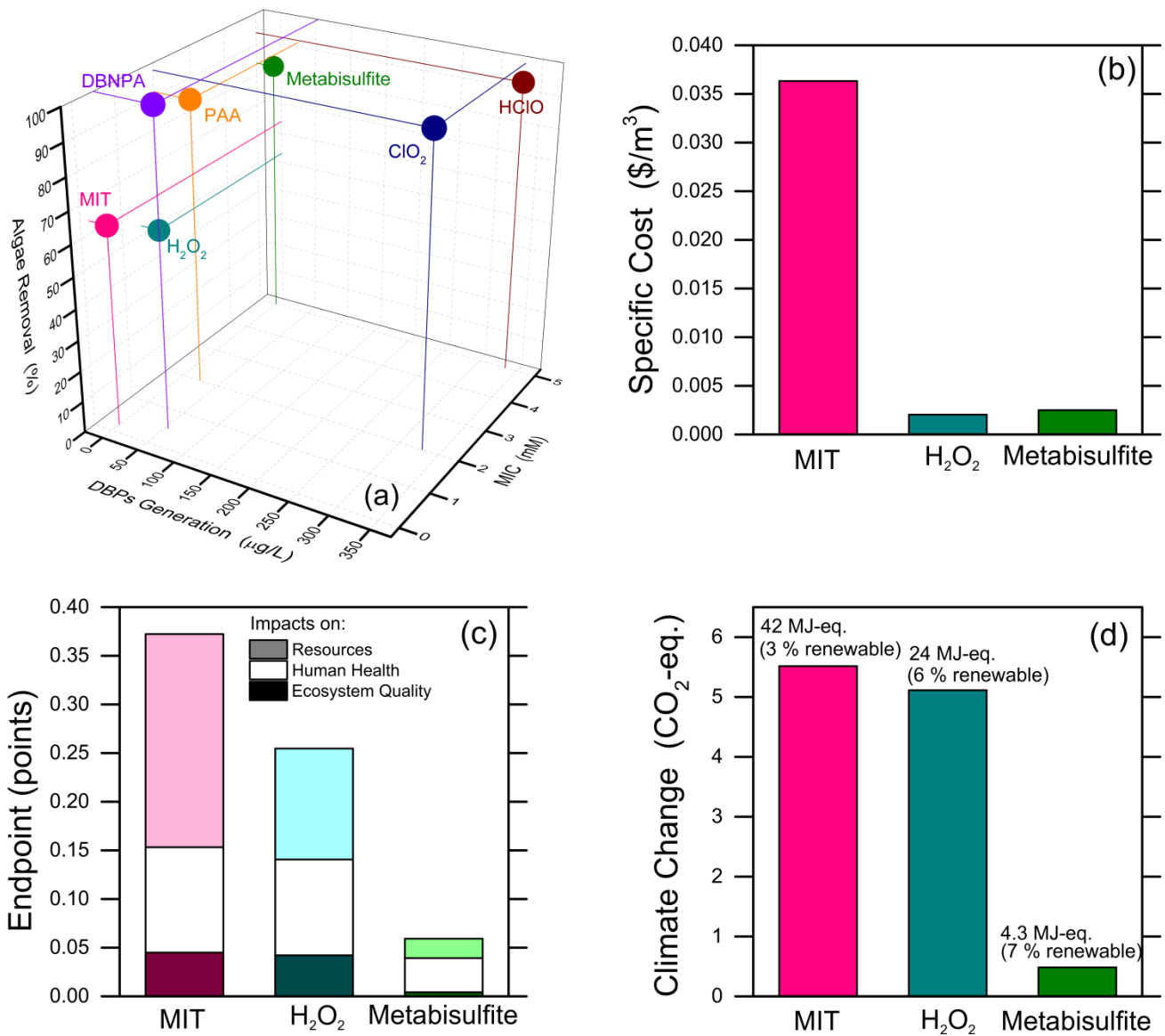
3.4. Metabisulfite, H₂O₂, and MIT: logistics of implementation.

Metabisulfite, H₂O₂, and MIT resulted equally safe in terms of DBPs generation, but they are different in terms practical utilization (*e.g.*, storage, safety). Indeed, H₂O₂ 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 H₂O₂ 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, H₂O₂
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 m³ 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), H₂O₂ (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 \$/m³)
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 CO₂ emission, which
428 is the major parameter for climate change evaluations in LCA analyses. A more notable production
429 of CO₂ 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 H₂O₂
432 also involves a relatively large amount of CO₂ 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 SO₂ 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; ClO₂;
447 H₂O₂; 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, ClO₂,
453 DBNPA, MIT, or metabisulfite. H₂O₂ 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, H₂O₂, 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

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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.

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685 **Graphical Abstract**

