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► **To cite this version:**

Sufia Hena, Leo Gutierrez, Jean-Philippe Croué. Removal of metronidazole from aqueous media by *C. vulgaris*. *Journal of Hazardous Materials*, 2020, 384, pp.121400. 10.1016/j.jhazmat.2019.121400 . hal-03488732

HAL Id: hal-03488732

<https://hal.science/hal-03488732>

Submitted on 21 Jul 2022

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Removal of metronidazole from aqueous media by *C. vulgaris*

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25 **Highlights**

26

27 1. Metronidazole is efficiently removed during algal treatment.

28 2. Metronidazole is removed via sorption onto the biomass.

29 3. Metronidazole showed toxicity to *C. vulgaris* at stationary phase of life cycle.

30 4. The removal efficiency of *C. vulgaris* decreased with increase of antibiotic.

31 **Abstract**

32 This current study investigated the removal of metronidazole from aqueous media by *C.*
33 *vulgaris*. Two different initial sizes of inoculum (0.05 and 0.1mg/L) were tested for a wide
34 concentration range of metronidazole (1-50 μ M). The effect of metronidazole concentrations on
35 biomass production was studied for 20 days. The exopolymeric substances (EPS) were
36 quantified and correlated with the removal of antibiotics from aqueous media. Specifically, MDZ
37 stimulated the production of EPS in *C. vulgaris*, which played the major role in the adsorption of
38 this antibiotic. Also, metronidazole significantly influenced the zeta potential of *C. vulgaris* in
39 the test cultures, indicating a change in surface characteristics. This decrease in surface negative
40 charge caused auto-flocculation phenomena at a stationary phase. Chronic and acute toxicity
41 experiments showed that metronidazole was harmful to *C. vulgaris* at stationary phase. Results
42 from this study would advance our knowledge on the treatment of metronidazole-contaminated
43 waters with *C. vulgaris* as a green technology-oriented process.

44

45 **Keywords:** Adsorption; *C. vulgaris*; EC50; metronidazole

46

47 1. Introduction

48 The excessive use of pharmaceuticals and their improper disposal pose risks to the environment
49 and public health [1]. Especially, antibiotics (i.e., widely used for preventing bacterial diseases in
50 humans and livestock) are continuously introduced in natural aquatic environments [2, 3].
51 Despite their low concentrations, antibiotics potentially affect the quality of drinking water,
52 human health, and ecosystems [4]. The residual antibiotics occurring in aquatic environments
53 would lead to the emergence of resistance among targeted and non-targeted strains of
54 microorganisms [5-7]. Also, surface waters contaminated with antibiotics expand the variety of
55 resistant microorganisms and the transfer of genetic material between multidrug-resistant
56 bacteria [8, 9].

57 Metronidazole is a first-generation derivative of 5-nitroimidazole drugs developed for
58 therapeutic uses [10, 11]. Metronidazole is an effective prescription medication used for treating
59 several infectious diseases caused by anaerobic bacteria and protozoa in humans and livestock
60 [12, 13], showing minimal side effects and a broad spectrum of applications [14]. In general,
61 antibiotics have a relatively short half-life in open environments (e.g., water bodies or soils) [2];
62 however, metronidazole is highly persistent in nature due to its high solubility [15] and low
63 biodegradability [16]. Also, the removal of metronidazole from water and wastewater during the
64 treatment train is not efficient [17]. Deblonde et al., [18], reported a mere 38.9% removal of
65 metronidazole from a wastewater treatment plant. Nevertheless, because of its high toxicity and
66 mutagenic effect on targeted and non-targeted microorganisms in the environment [12], it is
67 highly necessary to develop efficient protocols and (bio) technologies to remove this antibiotic
68 from aqueous media.

69 Several methods have been reported for the removal of metronidazole from aqueous media.
70 Briefly, chemical processes such as reduction [19], Fenton and photo-Fenton process [20], UV-
71 photolysis [21], and physical treatments (e.g., adsorption) [22, 23] have been mainly tested.
72 However, high energy and chemical consumptions, as well as the generation of secondary waste
73 are the main constraints for their successful (economic) implementation [24, 25]. On the other
74 hand, Rivera-Utrilla et al., [22] had reported that microorganisms in wastewater treatment plants
75 cannot efficiently degrade metronidazole. Activated sludge process is the most common
76 biological treatment method to remove most of the drugs from aqueous media; however, this
77 process fails to efficiently remove most of the antibiotics [26]. In general, antibiotics are not
78 readily biodegradable [27]. Antibiotics are mainly removed by sorption onto activated sludges in
79 wastewater treatment plants [4, 28]. However, antibiotics desorb from the activated sludge to the
80 aqueous media during its reuse in aeration tanks to maintain a fixed concentration of activated
81 microorganisms [29]. According to Grandclement et al., [30] wastewater treatment plants using
82 activated sludge are not specifically designed to remove antibiotics.

83 Simultaneously, the activated sludge in wastewater treatment plants promotes the occurrence of
84 antibiotic-resistant bacteria due to the exposure of bacteria to antibiotics present in wastewater
85 [31]. Therefore, the efficient treatment of antibiotics present in wastewater to ensure biosafety
86 for humans and their environment is a difficult task [32].

87 The use of microalgae as green technology is a viable option to remove antibiotics from water.
88 Moreover, microalgal cultures mitigate atmospheric CO₂ while the biomass can be used for
89 biofuel feedstocks. Briefly, microalgae are known for efficiently removing xenobiotic
90 compounds from water, including a range of pharmaceuticals. Garcia-Rodríguez et al., [33]
91 studied the role of microalga *Spyrogira* sp. in photolysis degradation and the contribution of light

92 in the total removal of six antibiotics from the sulfonamides and tetracyclines families. Du et al.,
93 [34] investigated the response of green algae *Chlorella pyrenoidosa* towards the removal of
94 ceftazidime antibiotic. Guo and Chen [2] showed the removal of cephalosporins from
95 wastewater using *Chlorella pyrenoidosa*. Conversely, Magalhães et al., [16] unsuccessfully
96 attempted to remove metronidazole from water using *Microcystis protocystis* (cyanobacteria).
97 From these previous studies, chlorophytes have clearly shown more resistant to stress conditions
98 during water treatment. *Chlorella sp.* is a chlorophyte which has been extensively used for the
99 removal of antibiotics such as 7-amino cephalosporanic acid [32], cephalosporins [2],
100 norfloxacin [35], levofloxacin [36], and enrofloxacin [37]. However, to the best of our
101 knowledge, metronidazole removal using *C. vulgaris* has not yet been reported.

102 This current study has investigated the metronidazole removal capacity of *C. vulgaris* and the
103 dominant mechanisms involved in the removal. The impact of metronidazole at different
104 concentrations on the growth rate of *C. vulgaris* was examined. Additionally, the tolerance and
105 chronic toxicity (EC50) of *C. vulgaris* to this antibiotic as a function of its stage of life cycle was
106 investigated. Finally, the exo-polymeric substances (EPS) production by *C. vulgaris* during
107 exposure to metronidazole, its influence on the surface properties of *C. vulgaris*, and its role
108 during the removal of this antibiotic were comprehensively studied. Results from this study will
109 advance our knowledge of the treatment of metronidazole-contaminated waters with *C. vulgaris*.

110 The protocols followed in the current investigation can be extended to the treatment of different
111 antibiotics-contaminated aquifers using green technology-oriented processes. Simultaneously, a
112 dual-approach can be used in the system to treat wastewater and to produce valuable biomass as
113 feedstock for biofuel, through anaerobic digestion. This latter process would aim to completely
114 degrade any antibiotic residue [61].

115 2. Materials and Methods

116 2.1. *Chemical reagents*

117 Metronidazole (HPLC grade; >99% purity) was obtained from Sigma-Aldrich. All the chemicals,
118 solvents, and reagents used were of analytical grade. A stock solution of 1mM metronidazole
119 (MDZ) was prepared with 30% methanol and 70% MilliQ water (v/v) and stored in a dark
120 airtight glass vial at 4°C for no longer than 30 days for further experiments.

121

122 2.2. *Algae Culturing and batch experiments*

123 *Chlorella vulgaris* (*C. vulgaris*) was obtained from CSIRO Marine Research Hobart, Tasmania.
124 *C. vulgaris* was re-cultured in 2 L conical flasks with a working volume of 1L in BG11 media
125 (Table 1), at 25±4°C. The cultures were exposed to fluorescent lamps to provide the irradiance of
126 120 µmol photon /m²/s until the stationary phase was attained.

127 Two sets of batch experiments were conducted. The freshly prepared culture media were
128 inoculated with two different sizes of inoculum of *C. vulgaris* for obtaining 0.05 g/L and 0.5 g
129 L⁻¹ biomass (i.e., dry biomass vs optical density calibration curve) in 3 L of Erlenmeyer flasks
130 with 2 L of working volume. The cultures were spiked with increasing concentration of MDZ (1-
131 50 µM) except for the control cultures of each batch. All experiments were conducted in
132 triplicate.

133

134 2.3. *Determination of biomass and chlorophyll-a*

135 The determination of the biomass of the test and control cultures was conducted every day by
136 measuring the optical density at 680 nm as described in [38]. A batch study was conducted to
137 evaluate the specific growth rate using the following formula;

138
$$\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)$$

139 Where, N_1 and N_2 are biomass concentrations (mg L^{-1}) at day t_1 and t_2 , respectively, during the
140 exponential phase of growth. All experiments were conducted in triplicate.

141 The extraction of chlorophyll-a and its quantification was conducted as reported by Xiong et al.,
142 [39]. A 10 mL of *C. vulgaris* was centrifuged at 4500 rpm for 10 min. The supernatant was
143 discarded and the pellet was collected. The pellet was re-suspended in 10 mL of methanol:water
144 (9:1; v/v) and incubated in 20 mL centrifuge tubes for 10 min. The incubated culture was
145 centrifuged again at 4500 rpm for 10 min. The absorbance of the supernatant was measured at
146 665 nm and 652 nm and the chlorophyll-a concentration of the extract was calculated using the
147 following equation:

148
$$\text{Chlorophyll-a } (\mu\text{g L}^{-1}) = 16.29 \times A_{665} - 8.54 \times A_{652}$$

149 Where, A_{665} and A_{652} are the absorbance of the supernatant at wavelength 665 nm and 652 nm,
150 respectively.

151

152 2.4. Toxicity of MDZ for *C. vulgaris*

153 The toxicity of MDZ was calculated as the Effective Concentration (EC50) of the stressor
154 (MDZ) at which 50% of the growth rate of *C. vulgaris* was inhibited. The inhibition in growth
155 was calculated as a function of chlorophyll-a content. To determine the chronic toxicity of MDZ,
156 a batch experiment was conducted at different concentrations of MDZ (65-90 μM) with an initial
157 biomass concentration of 0.1 g L^{-1} . The microalgae were exposed to antibiotic for 240 hours (10
158 days). However, for acute toxicity experiments, a 0.1 g L^{-1} culture at stationary phase (20 days
159 old culture) was spiked with 1-50 μM antibiotics for 96 hours. The growth rate was calculated in
160 terms of chlorophyll-a ($\mu\text{g L}^{-1}$).

161 *2.5. Extraction and analysis of Exo-polymeric substances (EPS)*

162 Samples of 50 mL of *C. vulgaris* cultures at late exponential phase (i.e., which started
163 approximately from day 10, Fig 1) were kept in the fridge at 4°C to settle the cells. The
164 supernatants were decanted and used for EPS analysis, while the settled cells were re-suspended
165 in 50 mL of MilliQ water in a beaker. The culture was stirred for 2 hours at 1000 rpm using a
166 magnetic stirrer to extract the EPS from the cell wall of *C. vulgaris*. Then, the cells were
167 separated by centrifugation at 1000 rpm for 10 minutes. The second supernatants were collected
168 for analysis of bound EPS. The pellets of the centrifuged biomass were dried at 60°C. The EPS
169 were extracted at every alternate day starting from day 10 until day 20.

170 30 mL of all supernatants were used for measuring the dry weight of EPS. Every supernatant of
171 the tests and control cultures of age 10 days and beyond were used for EPS determination. 30 mL
172 of supernatants were dried in an oven at 60°C until a stable weight was achieved to calculate the
173 ratio of the dry weight of EPS to biomass.

174

175 *2.6. Zeta potential*

176 The Zeta (ζ) potentials of *C. vulgaris* from the test (low and high inoculum spiked with 5 μ M
177 MDZ) and control cultures were analyzed on every alternate day from day 0 till day 20 using a
178 Zetasizer (Malvern). 10mL of cultures were collected from the Erlenmeyer flasks. The biomass
179 was allowed to settle down. The supernatants were discarded and biomass was re-suspended in
180 MilliQ water. The suspended *C. vulgaris* was pipetted in a folded cuvette. The zeta potential was
181 measured 5 times and the average and standard deviation were calculated.

182

183 *2.7. Analysis of MDZ*

184 Samples of 5 mL from test bioreactors were collected and filtered by a syringe filter (0.45 μm)
185 and analyzed using an HPLC equipped with UV/VIS detector (Agilent 1100 series, USA) at 320
186 nm. A 250 mm*4.6 mm C18 5- μm reverse phase HPLC column (AlltimaTM, GRACE) was used
187 and the mobile phase consisted of 0.1% phosphoric acid solution and methanol (80:20 v/v) with
188 a 1 mL min⁻¹ flow rate. To analyze the removal mechanism and removal efficiency of MDZ via
189 *C. vulgaris*, flocculated biomasses of microalgae were collected from the bottom of a bioreactor
190 (i.e., a random selection was conducted from a 10 μM MDZ high biomass bioreactor on day 20)
191 in triplicate. The biomasses were washed thrice with 5 mL MilliQ to remove the unbounded
192 molecules of MDZ from biomass using filter paper (GF/C; 1.2 μm) and the filtrates were
193 collected in HPLC vial for analysis after every wash. The absence of MDZ peak after third wash
194 approved the adequacy of three washes of collected biomasses for further steps of analysis. The
195 washed biomasses were collected on glass Petri dishes separately and vacuum dried at 60°C in
196 temp controlled oven for 24 hours till they achieved constant weight. 0.5 mg of dried biomass
197 was suspended into 20 mL of ethyl acetate followed by sonication for 15 minutes and
198 centrifuging at 12,000 rpm for 10 minutes [16]. The biomass was separated from ethyl acetate
199 using a syringe filter (GF/C; 1.2 μm). The filtrate was vacuum dried at 40°C, followed by
200 diluting the remaining residue in 20 mL fresh culture media. The final diluted residue (i.e.,
201 expecting desorbed MDZ in solution) was filtered with a 1.2 μm GF/C syringe filter and
202 collected in HPLC vial for analysis. The results were used to elucidate the MDZ removal
203 efficiency of *C. vulgaris* and the mechanism involved during the removal process.

204

205 3. Results and discussion:

206 3.1. Effects of MDZ on the growth of *C. vulgaris*:

207 Two sizes of inoculum (0.05 and 0.5 g L⁻¹) were selected to study the effect of MDZ on *C.*
208 *vulgaris* concentration in solution. These results were compared with those of control culture. As
209 depicted in Fig 1a-b (Refer to Table S1a-b for standard deviations), the growth of the cultures
210 can be divided into three phases. The early exponential phase was calculated for 10 days from
211 the beginning of the culture due to the highest growth rate during this period for all tests and
212 control culture regardless of inoculum size. Interestingly, the specific growth rates of *C. vulgaris*
213 during the early exponential phase were almost similar for cultures with the same inoculum size
214 but different concentrations of MDZ (Table 2). Clearly, there was no impact of MDZ on *C.*
215 *vulgaris* during this early exponential phase. Remarkably, there was no removal of MDZ from
216 any of the test bioreactors during the early exponential phase (Figure 2a-b, Table S2a-b). During
217 the late exponential phase (following the first 10 days of growth), the biomass increased with
218 similar specific growth rate for the same inoculum size. These results indicate that MDZ does not
219 impact the specific growth rate of the culture. However, MDZ hampered the duration of the late
220 exponential growth phase, where MDZ decreased the surface charge of the cells causing them to
221 flocculate and settling down (Fig 3).

222 The duration of the late exponential phases was different in different test bioreactors. The longest
223 duration was observed for control cultures as 8 days; nevertheless, the control culture of high
224 inoculum attained a lower specific growth rate than control culture and low inoculum (Table 2).
225 The test cultures with low inoculum remained in late exponential phase for 5-6 days, while high
226 inoculum test cultures showed the shortest duration of 2-3 days. The longer late exponential
227 phase for low inoculum cultures might be due to: 1) a lower concentration of microalgal cells
228 present in the media than higher inoculum bioreactors for the same amount of nutrients, and 2)
229 late auto-flocculation in low inoculum cultures. The late auto-flocculation in low inoculum test

230 bioreactors caused biomass to remain in suspension media for a longer time and hence increased
231 the duration of the late exponential phase. The concentrations of MDZ start decreasing during
232 late exponential phase in all cases regardless of inoculum sizes (0.05 and 0.5 g L⁻¹) or initial
233 concentrations (1-50 μM) of MDZ as shown in Fig 2a-b. These results confirmed that MDZ
234 played a major role in auto-flocculation as further explained in next section. In all test
235 bioreactors, the stationary phase did not last for more than 24 hours, which was then followed by
236 a quick decline in biomass content. The reason for this rapid decrease in suspended biomass was
237 due to auto-flocculation; however, the removal of MDZ continued even during the decline in
238 biomass phase.

239

240 3.2. *Effect of MDZ on zeta potential of C. vulgaris:*

241 Fig 3 revealed the electronegative zeta potential of *C. vulgaris* from control culture and test
242 bioreactor spiked with 5 μM MDZ (i.e., which was randomly selected as a representative case).
243 In both cases, the electronegative zeta potential decreased with the age of the culture.
244 Throughout the growth cycle of the microalgae, the concentration of the functional groups
245 present on the cell membrane and cell wall changes and influences the surface properties of the
246 cells [40]. However, during the active growth phase the lower intercellular interaction caused a
247 higher net electronegative zeta potential [41]. At late exponential growth phase, the metabolism
248 of the cells and the mobility decreased. Simultaneously, the changes on the cell surface caused
249 intercellular interactions which resulted in a lower electronegative zeta potential [41]. Xia et al.,
250 [42] reported that the decrease in the electronegative zeta potential of the *C. vulgaris* was also
251 due to the increase in the effective (hydrodynamic) size of the cells (i.e., cell with bound EPS) at

252 a later stage of its life cycle. Decho and Gutierrez [43] considered EPS as an extended part of the
253 cell.

254 Fig 3 showed that the electronegative zeta potential of the *C. vulgaris* decreased more
255 significantly in the test bioreactors than their respective control cultures. Among the test
256 bioreactors, the cultures with low inoculum size showed a higher electronegative zeta potential
257 than the high inoculum size culture at day 12 and beyond. The high inoculum culture contained
258 more biomass, which attained a stationary phase earlier than low inoculum cultures. Since the
259 EPS forms at late exponential or stationary phase of life cycle [43], high inoculum cultures start
260 forming EPS earlier than the low inoculum culture (Table 3).

261 As mentioned earlier, the net negative zeta potential of the microalgal cell decreased due to
262 change in functional groups present on the cell wall, which would be directly associated with the
263 metabolic conditions of the cells [40]. However, in the current study, the additional decrease in
264 zeta potential compared to their control cultures could be due to the charge dispersion interaction
265 of MDZ with algal cells (discussed in section 3.4) as well as the increase in the effective size of
266 the cell. The effective size of the cells would be larger in test cultures compared to their
267 respective control cultures due to the formation of bound EPS onto the cells in test cultures
268 (Table 3).

269 A drop in zeta potential in the test cultures indicated a decrease in the repulsive electrostatic
270 forces between the cells which led to auto-flocculation. The auto-flocculation takes place when
271 van der Waals forces overcome the repulsive electrostatic forces [44]. In this study, the repulsive
272 electrostatic forces were most likely decreased due to the adsorption of EPS around the cells and
273 also because of its interaction with MDZ in its scaffolds.

274

275 3.3. Effect of MDZ on the EPS production of *C. vulgaris*

276 EPS is produced by microalgae at a later stage of life cycle [43]. Microalgae produce EPS for
277 cell protection as a waste of metabolic processes [45]. There are two types of EPS produced by
278 *C. vulgaris*; a) EPS soluble (free) in media, and b) loosely bound (adsorbed) EPS which assists in
279 auto-flocculation of cells [46]. EPS is generally discussed as a three-dimensional architecture
280 scaffold providing space for trapping foreign compounds and slowing the diffusion rate, physical
281 stability of cells, digestion of xenobiotic substances, and communication between cells (i.e.,
282 quorum sensing) [43].

283 Control culture produced more soluble (free) EPS than bound (adsorbed) EPS (Table 3). This
284 result explains why control culture cells did not undergo auto-flocculation. However, in test
285 bioreactors the auto-flocculation phenomenon was common. A slight increase in bound EPS was
286 observed with an increase of MDZ concentration in both test cultures (low and high inoculum).
287 The bioreactor spiked with 1 μM MDZ produced significantly less EPS ($47.7 \pm 1.23 \text{ mg g}^{-1} \text{ DW}$
288 of *C. vulgaris*) than that with 5 μM ($151.6 \pm 3.45 \text{ mg g}^{-1} \text{ DW}$ of *C. vulgaris*), which indicated
289 that MDZ stimulated the EPS production in *C. vulgaris*. However, concentrations higher than 5
290 μM MDZ produced almost the same amount of EPS, which varied from 151.6-145.5 mg g^{-1} dry
291 weight (DW) and 139.3-146.1 mg g^{-1} DW for low and high biomass bioreactors, respectively.
292 These results revealed that 5 μM MDZ was the optimum concentration of antibiotics to produce
293 maximum EPS for this specific system.

294

295 3.4. Removal of MDZ

296 MDZ was analyzed from day 0 until day 20 in test bioreactors. The removal of MDZ by *C.*
297 *vulgaris* from aqueous media under different conditions is shown in Figure 2a-b. MDZ was not

298 removed until the early exponential growth phase. This result proved that MDZ did not adsorb
299 onto the cell surface of *C. vulgaris* possibly due to the low log K_{ow} (-2.3) value of MDZ. Also,
300 the pH of the *C. vulgaris* culture varied between 9-10, where MDZ is expected to behave as a
301 neutral molecule [47]. Therefore, electrostatic interactions between MDZ and negatively-charged
302 *C. vulgaris* would not be expected. However, π -electron of the heterocyclic aromatic ring of the
303 MDZ molecule would interact with different sites of EPS (Figure 4). EPS are highly hydrated
304 complex polymers mainly composed of polysaccharides, proteins, and DNA [48]. The CH group
305 of the polysaccharides form CH- π bond with the π -electrons of aromatic compounds [49, 50].
306 Consequently, the adsorption of MDZ molecules in the scaffold of the EPS matrix would involve
307 van der Waals interaction between π -electron of the heterocyclic aromatic ring of the MDZ
308 molecule and the hydrogen atom of CH group of the polysaccharides to form a CH- π bond.
309 Another possible mechanism would involve OH- π bond with OH groups of saccharides.
310 However, EPS has a complex structure where the OH groups of polysaccharides involved in
311 intermolecular interactions, might have no free OH to make bonds with MDZ [49]. Nishio [51]
312 has reported that saccharides are overall polar in nature but include some hydrophobic moieties
313 depending upon the orientation of OH groups, which have an affinity to form hydrogen bonds
314 with hydrophobic compounds [49]. The hydrophobic imidazole ring in MDZ would form OH- π
315 bond with OH groups of hydrophobic moieties. Previous studies have reported that MDZ
316 interacts with its π electron of heterocyclic aromatic ring to form π - π dispersion bonds with other
317 aromatic compounds [22]. Protein containing aromatic amino acids would be expected to favor
318 the formation of π - π dispersion bonds for MDZ to interact with EPS. Sanvordeker et al., [52]
319 confirmed that the alkyl side chain of MDZ participates in the binding to proteins. Besides, EPS

320 contains a diverse range of functional groups, including amine, carboxylate, phosphate, carbonyl,
321 amide, and hydroxyl which can form H-bonds with MDZ.

322 The removal of MDZ started after day 10 in all cases regardless of MDZ or biomass
323 concentration. Remarkably, MDZ removal and EPS formation occurred almost simultaneously.
324 These results indicated that the MDZ might be removed by EPS solely by adsorption
325 mechanism. Neither metabolites nor antimicrobial biotransformation products were observed in
326 HPLC chromatogram; suggesting that the EPS of *C. vulgaris* might not contain any metabolizing
327 enzyme for MDZ. Therefore, it is postulated that the decrease in MDZ concentration in the
328 bioreactors would only be associated with adsorption phenomena, which eventually played a role
329 in increasing the concentration of EPS. As a control experiment, bound EPS was extracted from
330 10 μ M high biomass bioreactor, sonicated for 15 minutes, followed by filtration and analysis,
331 where the presence of MDZ was clearly recorded. A bioreactor has a complex system of
332 suspended and flocculated biomass; thus, achieving an accurate mass balance of MDZ was not
333 possible. However, these results showed that 0.69 mg MDZ adsorbed per 100 mg of dry weight
334 of EPS (i.e., corresponding to 59% of initial DMZ) in a 10 μ M MDZ-high biomass bioreactor
335 after 20 days of incubation. The small molecular size of MDZ would favor its adsorption into the
336 scaffold of EPS, while the concentration gradient would play a major role in the diffusion of
337 MDZ into EPS [53].

338 A comparison between the MDZ removal of low and high biomass-containing bioreactor
339 revealed that the longer the late exponential growth phase, the lower the removal efficiency.
340 Specifically, a longer late exponential growth phase delayed the EPS production. In the case of 5
341 μ M MDZ bioreactors, the low biomass reactor was in active phase until day 15, while the active
342 or growth phase was over on day 12 in the high biomass reactor. Taking into account both

343 biomass and EPS, it was clear that EPS initiated the adsorption; however, the higher the biomass,
344 the better was the removal.

345 *3.5. Effect of initial concentrations of MDZ in culture media*

346 Wastewaters from different geographic locations contain different concentrations of
347 pharmaceuticals. Consequently, it is important to examine the MDZ removal from bioreactor as
348 a function of the initial concentration of antibiotic. Two sets of bioreactors with lower and higher
349 biomass concentrations were spiked with different initial concentrations (1-50 μM) of MDZ as
350 shown in Figure 5. In general, the efficiency of bioreactors decreased with an increase in the
351 initial concentration of MDZ for a fixed period of time. The adsorption phenomena in live
352 biological adsorbent are complex processes, especially when the adsorbate had to stimulate the
353 production of adsorbent. As depicted in Fig.5, only 50.80% of MDZ was removed in a low
354 biomass reactor initially containing 1 μM of MDZ. However, MDZ was completely removed in
355 both low as well as high biomass reactors originally containing 5 μM MDZ in 20 and 18 days of
356 experiments, respectively. Nevertheless, concentrations higher than 5 μM of MDZ showed the
357 same trend as the higher the initial concentration of antibiotic, the lower the removal (%) under
358 constant conditions (same inoculum size and incubation period).

359 It is evident from Table 3 that the EPS production increases with increasing concentration of
360 MDZ in culture; however, this becomes almost constant above 5 μM MDZ. Probably, this was
361 the reason why 1 μM MDZ in low inoculum size test culture was not able to completely remove
362 MDZ, whereas a 5 μM MDZ in low as well as in high inoculum size test cultures were capable
363 to completely remove MDZ from culture media.

364

365 *3.6. Mechanism of MDZ removal*

366 The removal of MDZ from the bioreactor was confirmed by analyzing the liquid phase using
367 HPLC, where a decrease in concentration was recorded. The absence of new chromatographic
368 peaks during the course of the experiments and the respective analysis validated the hypothesis
369 that *C. vulgaris* might not participate in any biodegradation mechanism. To confirm the
370 mechanism, ethyl acetate was used to desorb the adsorbed MDZ from the flocculated biomass.
371 The sole presence of an MDZ peak from the desorbed sample confirmed that MDZ was only
372 removed by sorption mechanism. These results showed that 0.93 mg of MDZ was adsorbed in 1
373 g dry weight of *C. vulgaris*. However, comparing this result with those obtained for MDZ
374 removal per 1 g of dry weight of EPS for the same test bioreactor on the same day (i.e. 0.69 mg
375 MDZ/100 mg of dry weight of EPS in 10 μ M MDZ high biomass bioreactor after 20 days of
376 incubation; as mentioned in section 3.4) the amount recovered from dry weight of *C. vulgaris*
377 showed a 9.13% loss in mass balance of MDZ. This decrease in the amount of MDZ suggested
378 its diffusion into the microalgal cell.

379 It is a general observation that dead microalgae cells have a higher adsorption capacity than live
380 cells [54]. Peng et al., [55] demonstrated that live cells of freshwater algae: *S. obliquus* and *C.*
381 *pyrenoidosa* adsorbed less progesterone and norgestrel in aqueous media than their dead
382 biomass. Yu et al., [56] explained that the better adsorption capacity of dead biomass was due to
383 loss of permeability control when the cell membrane structures were destroyed.

384 In this study, the adsorption capacity of live cells with EPS has been compared with live cells
385 without EPS. The first 10 days of culture (Figure 2 (a) and (b)) were considered as cells without
386 EPS while after day 10 cells started to form EPS (especially, attached or bonded) which were
387 considered as live cells with EPS. It is clear from Figure 2 (a) and (b) that there was no removal
388 of MDZ during the initial 10 days (live cells without EPS). The removal of MDZ started with the

389 EPS formation (i.e. after day 10 in all test bioreactors), which proved that live cells with EPS
390 played a major role in MDZ removal.

391 However, to confirm if suspended, attached, or both types of EPS were participating in removing
392 MDZ from water, the removal of MDZ from water was compared against the EPSs (suspended,
393 attached, and total EPS) obtained from 1 μ M MDZ test bioreactors (i.e., selected as a
394 representative case) of low and high inoculum (Figure 6). As depicted in Figure 6, it was
395 observed that the attached EPS showed the best fit (i.e., expressed by the highest R^2 value)
396 compared to the other two EPSs (R^2 of suspended EPS=0.49, and R^2 of total EPS=0.88). This
397 high value of correlation coefficient ($R^2 = 0.95$) of the attached EPS indicated a dominant
398 contribution of MDZ removal and good consistency between the 3D scaffold of the attached EPS
399 and the adsorbed antibiotic.

400

401 3.7. EC_{50} of *C. vulgaris* for MDZ

402 The most common toxicity assay is to calculate the effective concentration (EC_{50}) of a stressor
403 (i.e., metronidazole) at which 50% of the growth rate of an organism (microalgae) is inhibited.
404 Photosynthesis is an essential process for the survival of microalgae, which is related to growth
405 and several other metabolic activities. Thus, any disruption in the growth of microalgae during
406 stressors exposure is related to the changes in chlorophyll (Fig. 7a-b). Antibiotics damage
407 thylakoid and chloroplast lipids as they disrupt membranes and other organelles in cells; thus,
408 destroying the photosystem II (PSII) and consequently chlorophylls and carotenoids [57]. Low
409 chlorophyll content, as a stress response to pharmaceuticals, has been previously observed in
410 microalgae *Tetraselmis chui*, *Isochrysis galbana*, and *C. pyrenoidosa* in the presence of
411 thiamphenicol, florfenicol, and chloramphenicol [58].

412 Two approaches were followed to study the growth inhibition in *C. vulgaris* by MDZ; chronic
413 and acute toxicity of MDZ. The chronic toxicity assay was conducted to assess its adverse effects
414 on an organism as the result of long term exposure, while the acute toxicity is performed for
415 short term exposure. The chronic toxicity of MDZ on *C. vulgaris* was only observed when the
416 antibiotic is added during its growth phase, while acute toxicity was visible when the antibiotic
417 came in contact with microalgal cells at its stationary state. The results showed that the chronic
418 toxicity EC50 for *C. vulgaris* was 83.64 μM (Figure 8a), while for acute toxicity it was 13.63
419 μM (Figure 8b). The growth inhibition of *C. vulgaris* exposed to increasing concentrations of
420 MDZ (i.e., as measured by the chlorophyll-a yield) is shown in Figure 7a-b for chronic and acute
421 toxicity, respectively.

422 According to Commission of the European Community (1996), pollutants with an $\text{EC}_{50} > 100 \text{ mg}$
423 L^{-1} fall under the category of harmless substances, while those with EC_{50} from 10-100 mg L^{-1}
424 are included under the harmful category. Also, substances with EC_{50} between 1-10 mg L^{-1} , and
425 $\text{EC}_{50} < 1 \text{ mg L}^{-1}$ are considered as very toxic. Results from this study indicate that MDZ is
426 harmful to *C. vulgaris* (Figures 8a-b).

427 Besides the efficiency of *C. vulgaris* for removing DMZ from wastewater, the biomass generated
428 during this treatment would be a valuable feedstock for producing biofuels. Algal biomass has
429 been commonly used as a soil conditioner, plant fertilizer, and animal feed [30]. However, the
430 harvested biomass of *C. vulgaris* would not be recommended as a fertilizer because of its MDZ
431 content. A scheme for biofuel production using MDZ-mediated water is proposed in Figure 9.
432 Rawat et al., [59] have previously demonstrated the production of biogas using algal biomass
433 through anaerobic digestion. The temperatures of these processes vary from 350-800°C [60].

434 Remarkably, MDZ completely decomposes at 180°C [61], which is significantly lower than the
435 abovementioned lowest temperature of anaerobic digestion processes.

436 4. Conclusions

437 In the current study, *C. vulgaris* was used as a novel treatment to remove metronidazole from
438 aqueous media. These results indicated that MDZ stimulated the production of EPS in *C.*
439 *vulgaris*, which played a major role in the adsorption of this antibiotic. *C. vulgaris* displayed an
440 excellent removal capacity for the target antibiotic at 5µM concentration within the range of 0.05
441 to 0.5g L⁻¹ inoculum of *C. vulgaris*. These results showed that MDZ was only removed by
442 adsorption mechanism onto the scaffold of the EPS, which slowly diffused into the cells. The
443 removal efficiency decreased with increasing initial concentration of antibiotics. The tolerance of
444 *C. vulgaris* towards MDZ depended upon the stage of the life cycle, and on the exposure time to
445 the antibiotic. However, MDZ is marked as a harmful pollutant to *C. vulgaris*. Nevertheless, the
446 recovery of biofuel from the harvested biomass through anaerobic digestion shows promising for
447 future biofuel applications.

448 Competing interest

449 The authors declare no competing interest.

450 References:

- 451
452 [1] D. Kang, Q. Zhao, Y. Wu, C. Wu, W. Xiang, Removal of nutrients and pharmaceuticals and personal care
453 products from wastewater using periphyton photobioreactors, *Bioresour. Technol.* 248 (2018) 113–119.
454
- 455 [2] R. Guo, J. Chen, Application of alga-activated sludge combined system (AASCS) as a novel treatment to remove
456 cephalosporins, *Chem. Eng. J.* 260 (2015) 550–556
- 457 [3] B. Yang, J. Zuo, P. Li, K. Wang, X. Yu, M. Zhang, Effective ultrasound electrochemical degradation of
458 biological toxicity and refractory cephalosporin pharmaceutical wastewater. *Chem. Eng. J.* 287 (2016) 30–37.
459
- 460 [4] A. Hom-Diaz, Z.N. Norvill, P. Blaquez, T. Vicent, B. Guieysse, Ciprofloxacin removal during secondary
461 domestic wastewater treatment in high rate algal ponds, *Chemosphere* 180 (2017) 33–41.
462

- 463 [5] J. Tanwar, S. Das, Z. Fatima, S. Hameed, Multidrug resistance: an emerging crisis. *Interdiscip. Perspect. Infect.*
464 *Dis.* (2014):541340. doi: 10.1155/2014/541340
465
- 466 [6] C. Bouki, D. Venieri, E. Diamadopoulos, Detection and fate of antibiotic resistant bacteria in wastewater
467 treatment plants: a review. *Ecotoxicol. Environ. Saf.* 91 (2013) 1–9.
- 468 [7] X.H. Wang, A.Y. Lin, Phototransformation of cephalosporin antibiotics in an aqueous environment results in
469 higher toxicity. *Environ. Sci. Technol.* 46 (2012) 12417–12426.
470
- 471 [8] V. Singh, “Antimicrobial resistance,” in *Microbial Pathogens and Strategies for Combating Them: Science,*
472 *Technology and Education*, 1 (2013) 291–296.
473
- 474 [9] M. Popeda, E. Pluciennik, A.K. Bednarek, “Proteins in cancer resistance,” *Postępy Higieny i Medycyny*
475 *Doświadczalnej*, 68 (2014) 616–632.
476
477
- 478 [10] M.J. Ahmed, S.K. Theydan, Microporous activated carbon from Siris seed pods by microwave-induced KOH
479 activation onidazole adsorption, *J. Anal. Appl. Pyrolysis* 99 (2013) 101- 109.
480
- 481 [11] L.A. Dunn, A.G. Burgess, K.G. Krauer, L. Eckmann, P. Vanelle, M.D. Crozet, F.D. Gillin, P. Uproft, J.A.
482 Uproft, A new-generation 5-nitroimidazole can induce highly metronidazole-resistant *Giardia lamblia In vitro*, *Int.*
483 *J. Antimicrob. Agents* 36 (2010) 37-42.
484
- 485 [12] A. Bendesky, D. Menéndez, P. Ostrosky-Wegman, Is metronidazole carcinogenic? *Mutation Res.* 511 (2002)
486 133-144.
487
- 488 [13] M. Sakamoto, K. Takeba, T. Sasamoto, T. Kusano, H. Hayashi, S. Kanai, M. Kanda, T. Nagayama,
489 Determination of dimetridazole, metronidazole and ronidazole in salmon and honey by liquid chromatography
490 coupled with tandem mass spectrometry. *Shokuhin Eiseigaku Zasshi*, 52 (2011) 51-58.
491
- 492 [14] A. Hernandez Ceruelos, L.C. Romero-Quezada, J.C. Ruvalcaba Ledezma, L. Lopez Contreras, Therapeutic uses
493 of metronidazole and its side effects: an update, *Eur Rev Med Pharmacol Sci.* 23 (2019) 397-401.
494
- 495 [15] W. Cheng, M. Yang, Y. Xie, B. Liang, Z. Fang, E.P. Tsang, Enhancement of mineralization of metronidazole
496 by the electro-Fenton process with a Ce/SnO₂-Sb coated titanium anode, *Chem. Eng. J.* 220 (2013) 214-220.
497
- 498 [16] S.M.S. Magalhães, C.M. Brêtas, J.M. Brêtas, G.A. Pianetti, M.W. Franco, F.A.R. Barbosa, Toxic
499 concentrations of metronidazole to *Microcystis protocystis*, *Braz. J. Biol.*, 74 (2014) 120-124.
500
- 501 [17] Q. Jiang, H.H. Ngo, L.D. Nghiem, F.I. Hai, W.E. Price, J. Zhang, S. Liang, L. Deng, W. Guo, Effect of
502 hydraulic retention time on the performance of a hybrid moving bed biofilm reactor-membrane bioreactor system for
503 micropollutants removal from municipal wastewater, *Bioresour Technol.* (2017)
504 Doi.org/10.1016/j.biortech.2017.09.114
505
- 506 [18] T. Deblonde, C. Cossu-Leguille, P. Hartemann, Emerging pollutants in waste water: A review of the literature,
507 *Int. J. Hygiene and Environ. Health* 214 (2011) 442-448
- 508 [19] Z. Fang, J. Chen, X. Qiu, X. Qiu, W. Cheng, L. Zhu, Effective removal of antibiotic metronidazole from water
509 by nanoscale zero-valent iron particles, *Desalination* 268 (2011) 60–67.
510
- 511 [20] H. Shemer, Y.K. Kunukcu, K.G. Linden, Degradation of the pharmaceutical Metronidazole via UV, Fenton and
512 photo-Fenton processes, *Chemosphere* 63 (2006) 269–276.
513

- 514 [21] R.F. Dantas, O. Rossiter, A.K.R. Teixeira, A.S.M. Simoes, V.L. da Silva, Direct UV photolysis of propranolol
515 and metronidazole in aqueous solution, *Chem. Eng. J.* 158 (2010) 143–147.
516
- 517 [22] J. Rivera-Utrilla, G. Prados-Joya, M. Sanchez-Polo, M.A. Ferro-García, M.I. Bautista-Toledo, Removal of
518 nitroimidazole antibiotics from aqueous solution by adsorption/bioadsorption on activated carbon, *J. Hazard. Mater.*
519 170 (2009) 298–305.
520
- 521 [23] D.H. Carrales-Alvarado, R. Ocampo-Perez, R. Leyva-Ramos, J. Rivera-Utrilla, Removal of antibiotic
522 metronidazole by adsorption on various carbon materials from aqueous phase, *J. Colloid Interface Sci.* 436, (2014)
523 276–285.
524
- 525 [24] V. Homem, L. Santos, Degradation and removal methods of antibiotics from aqueous matrices – a review, *J.*
526 *Environ. Manage.* 92 (2011) 2304–2347
527
- 528 [25] W. Li, Y. Shi, L. Gao, J. Liu, Y. Cai, Occurrence and removal of antibiotics in a municipal wastewater
529 reclamation plant in Beijing, China, *Chemosphere* 92 (2013) 435–444.
530
- 531 [26] H.Y. Kim, J. Jeon, S. Yu, M. Lee, T.H. Kim, S.D. Kim, Reduction of toxicity of antimicrobial compounds by
532 degradation processes using activated sludge, gamma radiation, and UV, *Chemosphere* 93 (2013) 2480–2487.
533
- 534 [27] P. Verlicchi, M. Al Aukidy, E. Zambello, Occurrence of pharmaceutical compounds in urban wastewater:
535 removal, mass load and environmental risk after a secondary treatment —a review. *Sci Total Environ* 429 (2012)
536 123–55.
537
- 538 [28] F. Polesel, K. Lehnberg, W. Dott, S. Trapp, K.V. Thomas, B.G. Plosz, Factors influencing sorption of
539 ciprofloxacin onto activated sludge: Experimental assessment and modelling implications. *Chemosphere* 119 (2015)
540 105–111.
- 541 [29] F. Polesel, H.R. Andersen, S. Trapp, B.G. Plosz, Removal of Antibiotics in Biological Wastewater Treatment
542 Systems—A Critical Assessment Using the Activated Sludge Modeling Framework for Xenobiotics (ASM-X).
543 *Environ. Sci. Technol.* 50 (2016) 10316–10334.
544
- 545 [30] C. Grandclement, I. Seyssiecq, A. Piram, P.W.W. Chung, G. Vanot, N. Tiliacos, N. Roche, P. Doumenq, From
546 the conventional biological wastewater treatment to hybrid processes, the evaluation of organic micropollutant
547 removal: A review, *Water Res.* 111 (2017) 297–317.
548
- 549 [31] Y. Che, Y. Xia, L. Liu, A.D. Li, Y. Yang, T. Zhang, Mobile antibiotic resistome in wastewater treatment plants
550 revealed by Nanopore metagenomic sequencing, *Microbiome*, 7 (2019) <https://doi.org/10.1186/s40168-019-0663-0>
- 551
- 552 [32] W.Q. Guo, Z. He-Shan, L. Shuo, D. Juan-Shan, F. Xiao-Chi, Y. Ren-Li, W. Qing-Lian, R. Nan-Qi, C. Jo-Shu
553 Removal of cephalosporin antibiotics 7-ACA from wastewater during the cultivation of lipid-accumulating
554 microalgae. *Bioresour. Technol.* 221 (2016) 284–290.
555
- 556 [33] A. Garcia-Rodríguez, V. Matamoros, C. Fontàs, V. Salvadó, The influence of light exposure, water quality and
557 vegetation on the removal of sulfonamides and tetracyclines: A laboratory-scale study, *Chemosphere* 90 (2013)
558 2297–2302.
559
- 560 [34] Y. Du, Y. Feng, R. Guo, J. Chen, Enhancement by the artificial controlled culture for the algal treatment of
561 antibiotic ceftazidime: a three-step response performance and high-removal efficiency, *RSC Adv.*, 5 (2015) 72755-
562 72763
563
- 564 [35] J. Zhang, D. Fu, J. Wu, Photodegradation of Norfloxacin in aqueous solution containing algae. *J Environ Sci* 24
565 (2012) 743–749.

566
567 [36] J.Q. Xiong, M.B. Kurade, B.H. Jeon, Biodegradation of Levofloxacin by an acclimated freshwater microalga,
568 *Chlorella vulgaris*. Chem. Eng. J., 313 (2017) 1251-1257.
569
570 [37] J.Q. Xiong, M.B. Kurade, B.H. Jeon, Ecotoxicological effects of enrofloxacin and its removal by monoculture
571 of microalgal species and their consortium, Environ. Pollut. 226 (2017) 486-493.
572
573 [38] S. Hena, N. Fatihah, S. Tabassum, N. Ismail, Three stage cultivation process of facultative strain of *Chlorella*
574 *sorokiniana* for treating dairy farm effluent and lipid enhancement, Water Res. 80 (2015) 346-356.
575
576 [39] J.Q. Xiong, M.B. Kurade, R.A.I. Abou-Shanab, M.K. Ji, J. Choi, J.O. Kim, B.H. Jeon, Biodegradation of
577 carbamazepine using freshwater microalgae *Chlamydomonas mexicana* and *Scenedesmus obliquus* and the
578 determination of its metabolic fate, Bioresour. Technol. 205 (2016) 183-190.
579
580 [40] X. Zhang, P. Amendola, J.C. Hewson, M. Sommerfeld, Q. Hu, Influence of growth phase on harvesting of
Chlorella zofingiensis by dissolved air flotation. Bioresour Technol. 116 (2012) 477-84.
581
582 [41] M.K. Danquah, B. Gladman, N. Moheimani, G.M. Forde, Microalgal growth characteristics and subsequent
influence on dewatering efficiency. Chem. Eng. J. 151(2009) 73-78.
583
584 [42] L. Xia, R. Huang, Y. Li, S. Song, The effect of growth phase on the surface properties of three oleaginous
585 microalgae (*Botryococcus* sp. FACGB-762, *Chlorella* sp. XJ-445 and *Desmodesmus bijugatus* XJ-231). Front.
Microbiol. 8 (2017) doi: 10.3389/fmicb.2017.00922
586
587 [43] A.W. Decho, T. Gutierrez, Microbial Extracellular Polymeric Substances (EPSs) in Ocean Systems Systems.
Front. Microbiol. 8 (2017) doi: 10.3389/fmicb.2017.00922.
588
589 [44] R. Henderson, S.A. Parsons, B. Jefferson, The impact of algal properties and pre-oxidation on solid-liquid
separation of algae. Water Res. 42 (2008) 1827-1845.
590
591 [45] R. Xiao, Y. Zheng, Overview of microalgal extracellular polymeric substances (EPS) and their applications,
592 Biotechnol Adv. 15 (2016) 1225-1244.
593
594 [46] S. Salim, N.R. Kosterink, N.D. Tchetskoua Wacka, M.H. Vermuë, R.H. Wijffels, Mechanism behind auto-
595 flocculation of unicellular green microalgae *Ettlia texensis*, J.Biotechnol. 174 (2014) 34-38.
596
597 [47] E. Caliskan, S. Gokturk, Adsorption characteristics of sulfamethoxazole and metronidazole on activated
598 carbon, Separation Sci. Technol. 45 (2010) 244-255.
599
600 [48] H.C. Flemming, J. Wingender, The biofilm matrix. Nat. Rev. Microbiol. 8 (2010) 623-633.
601
602 [49] J.L. Asensio, A. Arda, F.J. Canada, J. Jimenez-Baebro, Carbohydrate-aromatic interactions. Acc. Chem. Res.
603 46 (2013) 946-954.
604
605 [50] M. Kumari, R.B. Sunoj, P.V. Balaji, Exploration of CH- π mediated stacking interactions in saccharide:
aromatic residue complexes through conformational sampling. Carbohydr. Res. 361 (2012) 133-140.
606
607 [51] M. Nishio, The CH/ π hydrogen bond in chemistry. Conformation, supramolecules, optical resolution and
interactions involving carbohydrates. Phys. Chem. Chem. Phys. 13 (2011) 13873-13900.
608
609 [52] D.R. Sanvordeker, Y.W. Chien, T.K. Lin, H.J. Lambert, Binding of metronidazole and its derivatives to plasma
610 proteins: An assessment of drug binding phenomenon, J. Pharmaceutical Sci. 64 (1975)
611 Doi.org/10.1002/jps.2600641111.

- 612 [53] A.W. Decho, "Localization of quorum sensing by extracellular polymeric substances (EPS): considerations of
613 in situ signaling," in *The Physical Basis of Bacterial Quorum Communication*, ed. S. J. Hagen (New York, NY:
614 Springer), (2015) doi: 10.1007/978-1-4939-1402-9_6
- 615 [54] Y. Tao, W. Li, B. Xue, J. Zhong, S. Yao, Q. Wu, Different effects of copper (II), cadmium (II) and phosphate
616 on the sorption of phenanthrene on the biomass of cyanobacteria, *J. Hazard. Mater.* 261 (2013) 21–28.
617
- 618 [55] F.Q. Peng, G.G. Ying, B. Yang, S. Liu, H.J. Lai, Y.S. Liu, Z.F. Chen, G.J. Zhou, Biotransformation of
619 progesterone and norgestrel by two freshwater microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*):
620 transformation kinetics and products identification. *Chemosphere* 95 (2014) 581–588.
621
- 622 [56] Y. Yu, Y. Zhou, Z. Wang, O. Lopez Torres, R. Guo., J. Chen, Investigation of the removal mechanism of
623 antibiotic ceftazidime by green algae and subsequent microbial impact assessment. *Scientific Report*, (2017)
624 DOI:10.1038/s41598-017-04128-3
625
- 626 [57] A. Magdaleno, M.E. Saenz, A.B. Juárez, J. Moretton, Effects of six antibiotics and their binary mixtures on
627 growth of *Pseudokirchneriella subcapitata*, *Ecotoxicol. Environ. Safety* 113 (2015) 72–78.
628
- 629 [58] H.T. Lai, J.H. Hou, C.I. Su, C.L. Chen, Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of
630 algae *Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui*, *Ecotoxicol. Environ. Saf.* 72 (2009) 329-
631 334.
632
- 633 [59] R. Rawat, K. Ranjith, T. Mutanda, F. Bux, Dual role of microalgae: Phycoremediation of domestic wastewater
634 and biomass production for sustainable biofuels production, *Appl. Energy*, 88 (2011) 3411-3424.
635
- 636 [60] D. Vamvuka, Bio-oil, solid and gaseous biofuels from biomass pyrolysis processes-an overview, *International*
637 *J. Energy Res.* 35 (2011) 835-862.
638
- 639 [61] N.A.B. de Souza, A.C.D. Medeiros, A.F.O. Santos, R.O. Macedo, Thermal stability of metronidazole drug and
640 tablets, *Journal of Thermal and Calorimetry*, 72 (2003) 535-538.

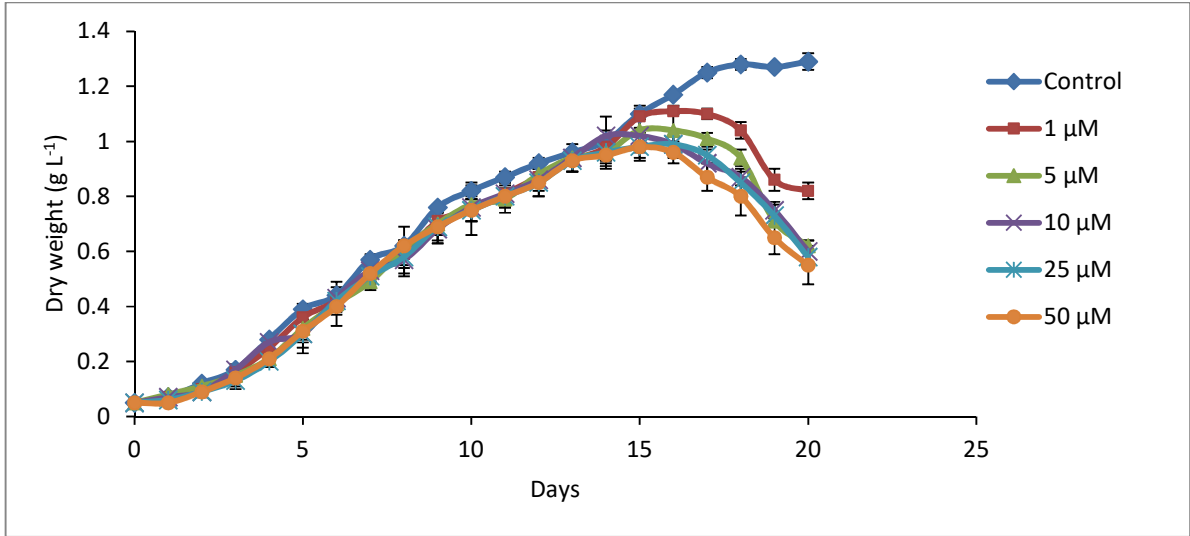


Figure 1a: Growth curve of low inoculum size of *C. vulgaris* for control and test cultures at different concentrations (1-50 μM) of MDZ

- The data are the mean of three replicates.
- Refer Supplementary Table 1a for the data and SD

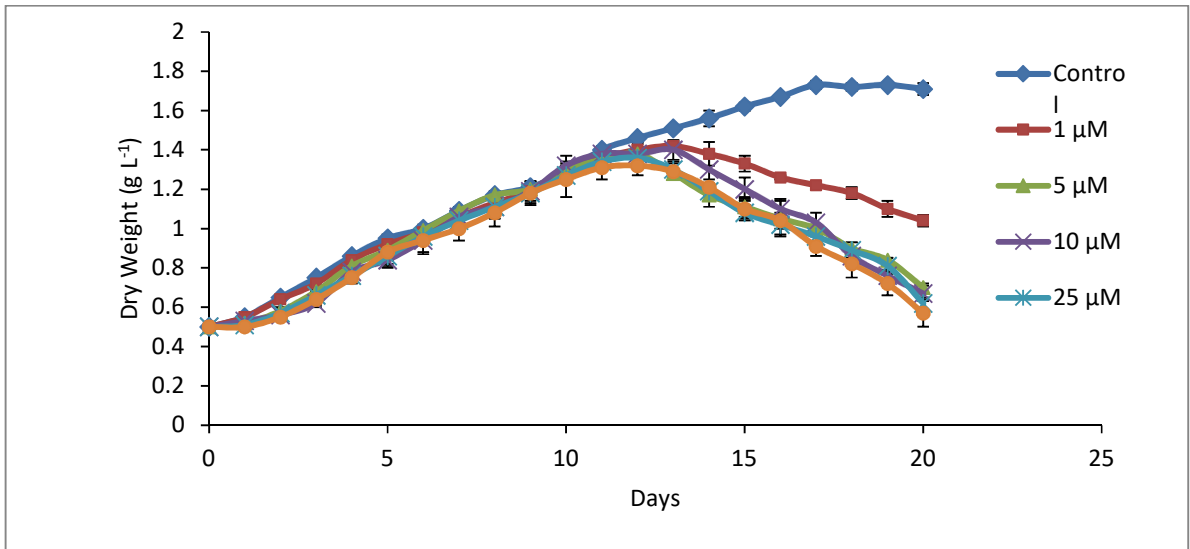


Figure 1b: Growth curve of high inoculum size of *C. vulgaris* for control and test cultures at different concentrations (1-50 μM) of MDZ

- The data are the mean of three replicates.
- Refer Supplementary Table 1b for the data and SD

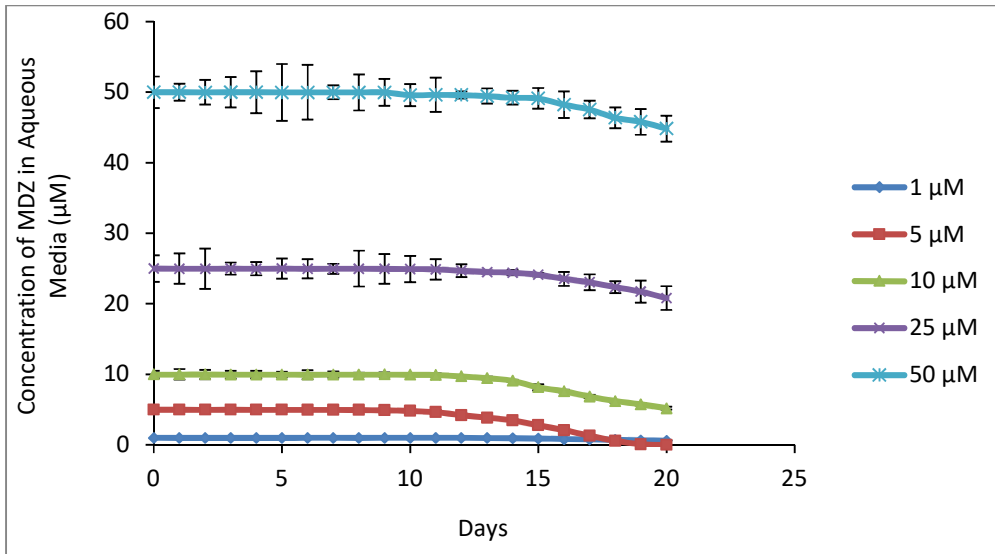


Figure 2a: Evolution of MDZ concentration during treatment using low inoculum size of *C. vulgaris* containing different concentration (1-50 μM) of MDZ in aqueous media

- The data are the mean of three replicates.
- Refer Supplementary Table 2a for the data and SD

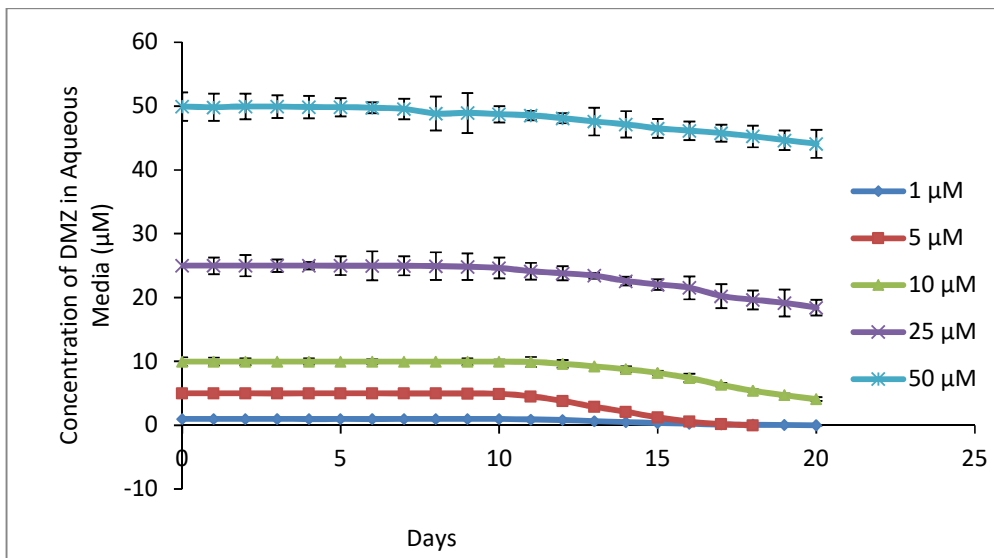


Figure 2b: Evolution of MDZ concentration during treatment using high inoculum size of *C. vulgaris* containing different concentration (1-50 μM) of MDZ in aqueous media

- The data are the mean of three replicates.
- Refer Supplementary Table 2a for the data and SD

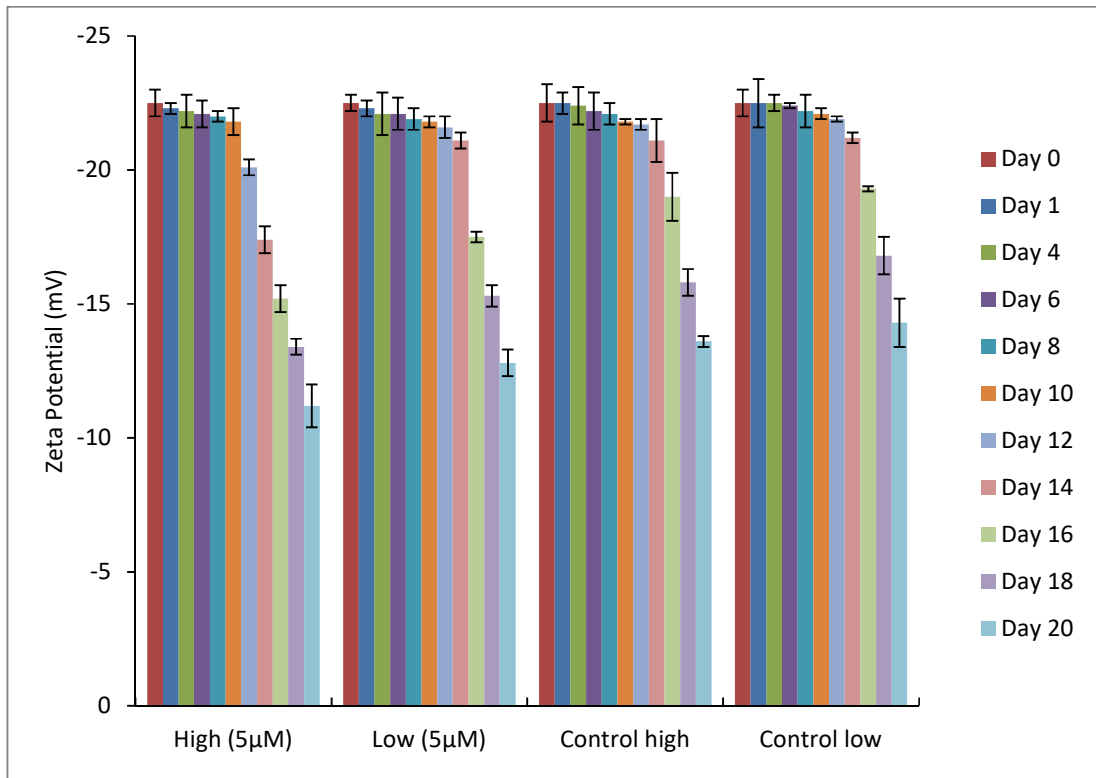
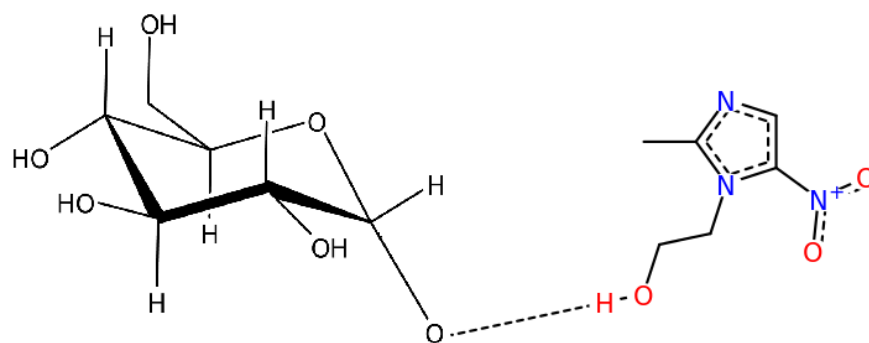
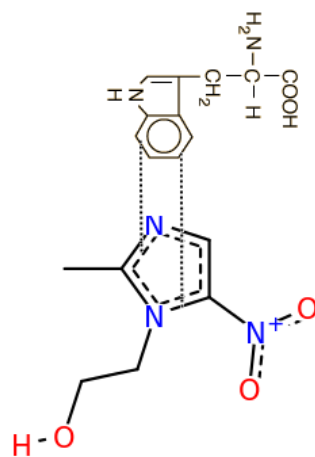


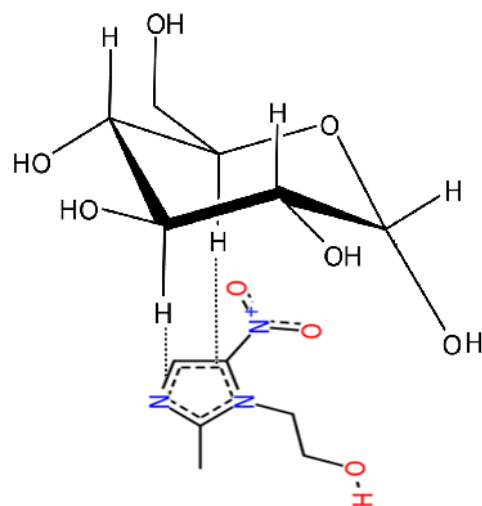
Figure 3: Zeta potential of low and high inoculum size of *C. vulgaris* in control culture and culture spiked with 5 µM MDZ on alternate days



H bond between saccharide and MDZ



π - π dispersion bond between aromatic compound of protein (tryptophan) and MDZ



CH- π bond between saccharide and MDZ

Figure 4: Possible bonding between MDZ and components of EPS

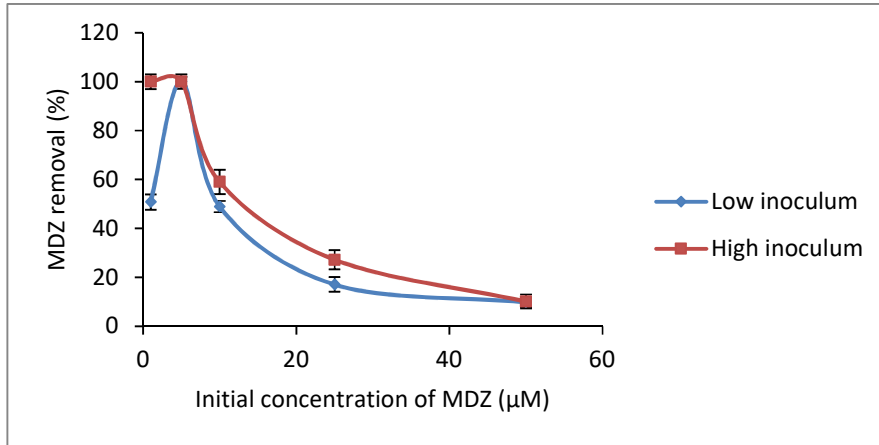


Figure 5: Effect of initial MDZ concentration on MDZ removal

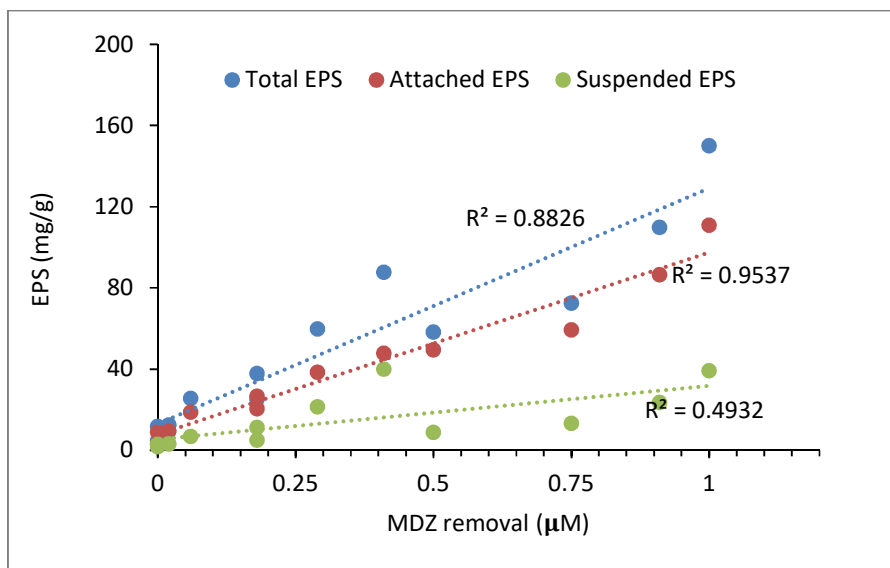


Figure 6: Correlations between the amount of attached, suspended and total EPS with MDZ removal from 1 µM test bioreactors with low and high inoculum from day 10 to day 20

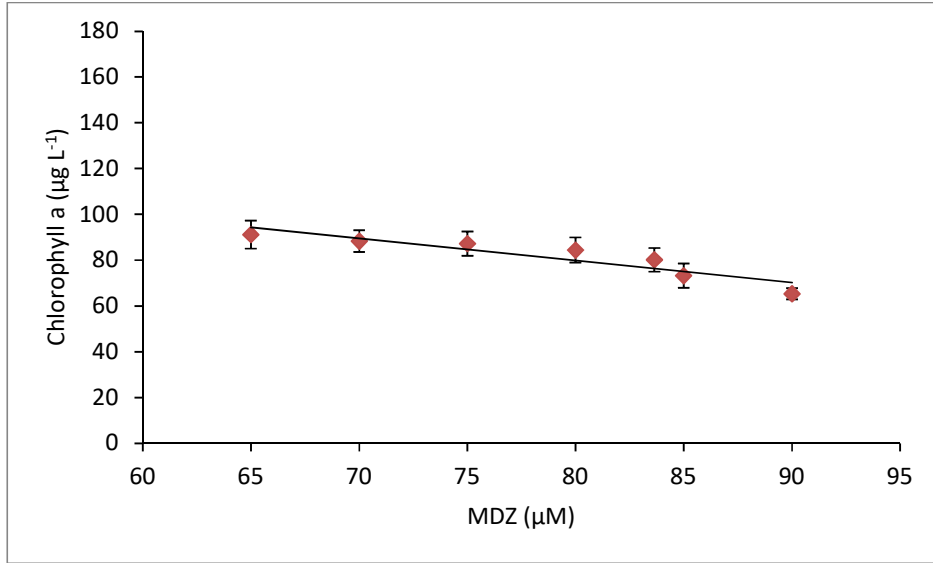


Figure 7a: Chlorophyll a in *C. vulgaris* during a 240-hour metronidazole chronic toxicity test. The control culture (culture without MDZ) possessed 160.22 µg L⁻¹ after 240 hours

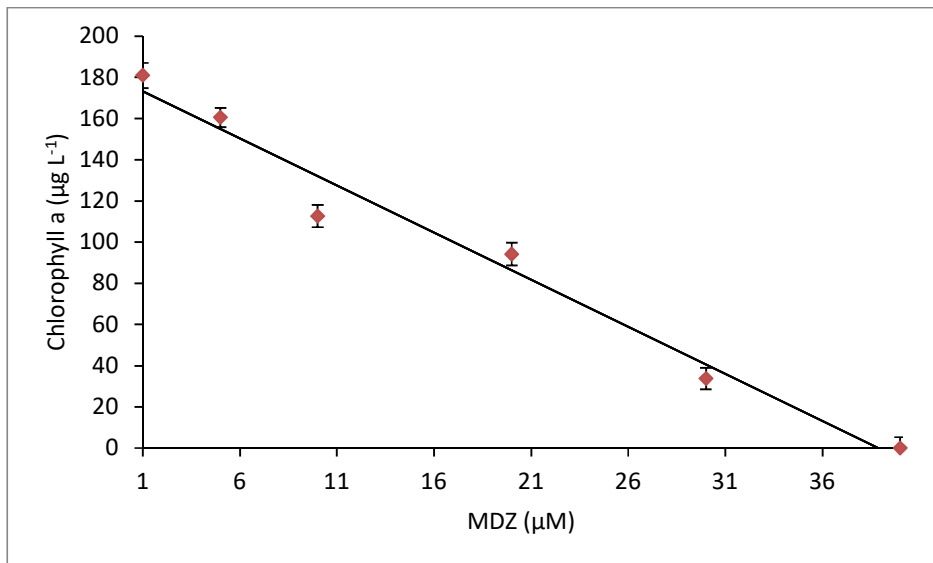


Figure 7b: Chlorophyll a in *C. vulgaris* during a 96-hour metronidazole acute toxicity test. The control culture (culture without MDZ) possessed 240.1 µg L⁻¹ after 96 hours

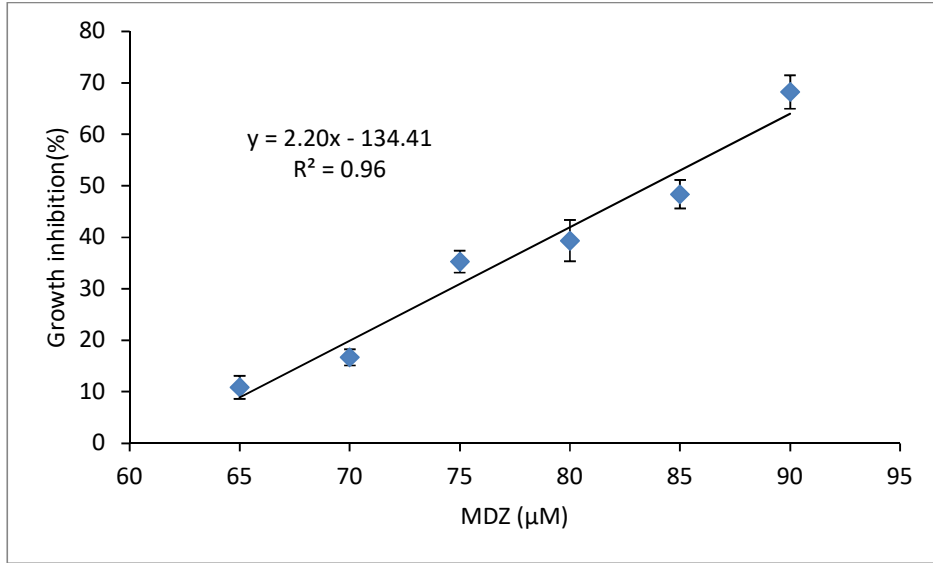


Figure 8a: Growth inhibition (%) of *C. vulgaris* by MDZ during chronic toxicity test.

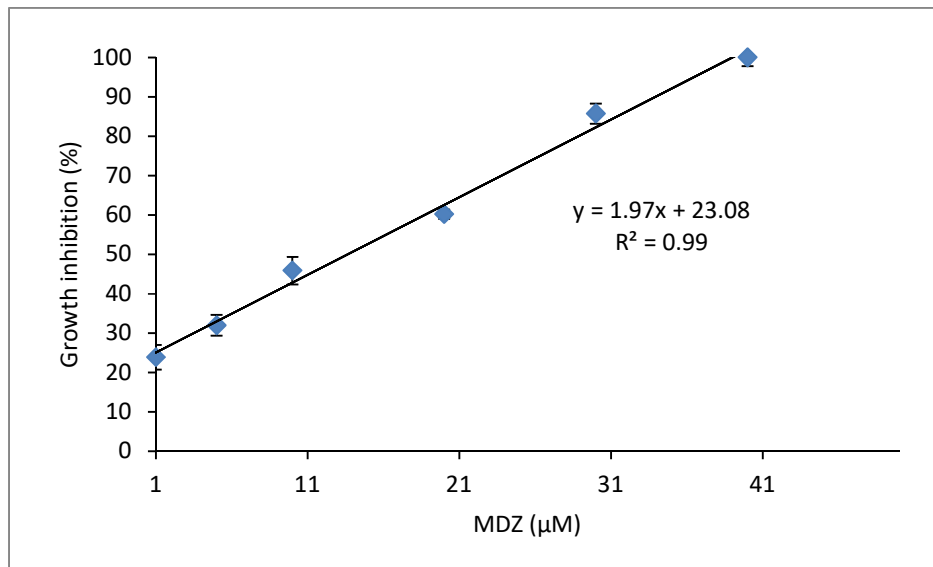


Figure 8b: Growth inhibition (%) of *C. vulgaris* by MDZ during chronic toxicity test

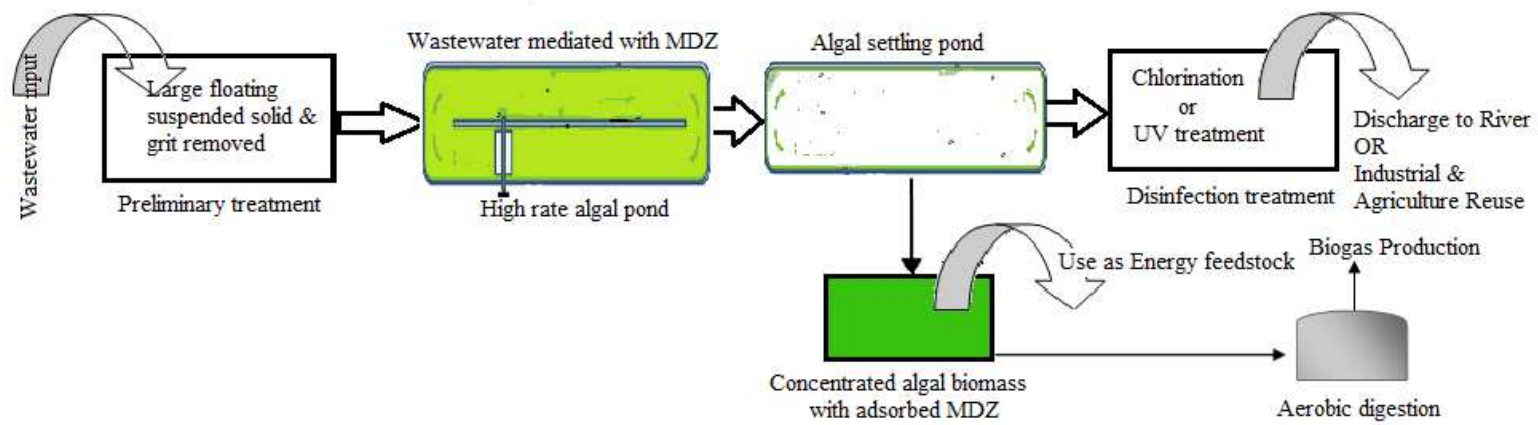


Figure 9: Proposed scheme for biogas production using MDZ-containing wastewater.

Table 1: Compositions of BG-11 media in 1L MilliQ

Chemicals	Weight (mg)
K_2HPO_4	75
KH_2PO_4	175
$MgSO_4 \cdot 7H_2O$	75
$NaNO_3$	250
$CaCl_2 \cdot 2H_2O$	25
$NaCl$	25
$Na_2EDTA \cdot 2H_2O$	50
$FeSO_4 \cdot 7H_2O$ in 0.1mL H_2SO_4	4.9
H_3BO_3	11.42
$ZnSO_4 \cdot 7H_2O$	1.412
$MnCl_2 \cdot 4H_2O$	0.232
$CuSO_4 \cdot 5H_2O$	0.252
$Co(NO_3)_2 \cdot 6H_2O$	0.08
$Na_2MoO_4 \cdot 2H_2O$	0.192

Table 2: Specific growth rate of *C. vulgaris* in control and test culture with different concentration of MDZ

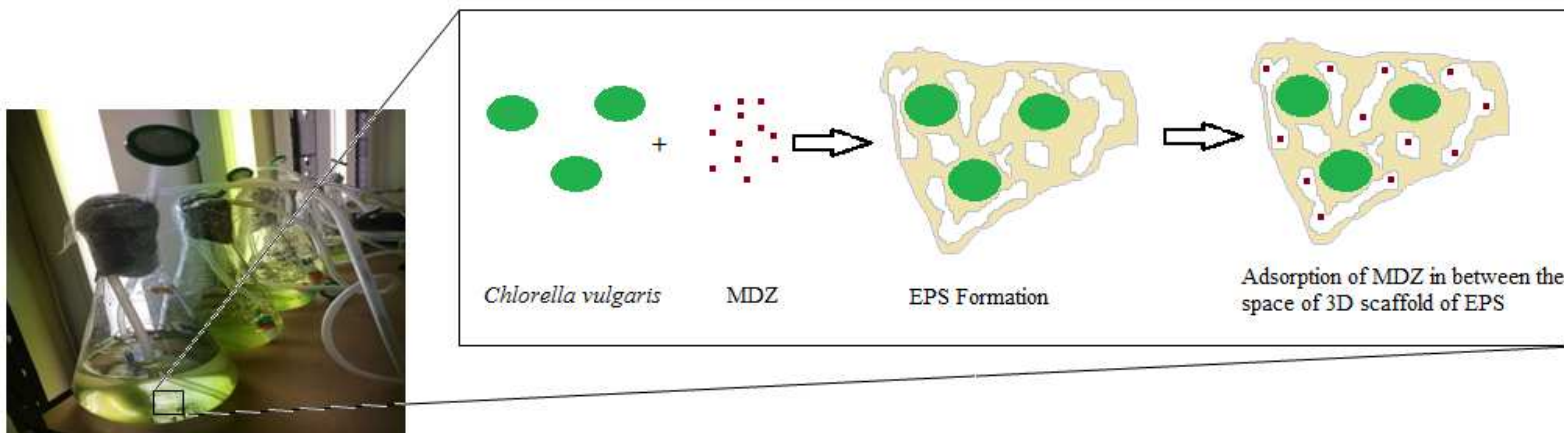
Concentration of MDZ	Low inoculum size		High inoculum size	
	Specific growth rate of early exponential phase (d ⁻¹)	Specific growth rate of late exponential phase (d ⁻¹)	Specific growth rate of early exponential phase (d ⁻¹)	Specific growth rate of late exponential phase (d ⁻¹)
Control (without MDZ)	0.279	0.055	0.095	0.036
1 μM	0.271	0.065	0.094	0.034
5 μM	0.273	0.060	0.096	0.029
10 μM	0.272	0.058	0.097	0.019
25 μM	0.271	0.046	0.093	0.035
50 μM	0.271	0.053	0.091	0.028

Table 3: EPS production in control culture and test bioreactors

Days	Control culture		Low inoculum bioreactors									
			1 μ M		5 μ M		10 μ M		25 μ M		50 μ M	
	EPS (S)*	EPS (A)*	EPS (S)	EPS (A)	EPS (S)	EPS (A)	EPS (S)	EPS (A)	EPS (S)	EPS (A)	EPS (S)	EPS (A)
10	2.31 \pm 0.11	1.45 \pm 0.09	1.62 \pm 0.33	3.01 \pm 0.08	1.71 \pm 0.08	5.54 \pm 0.16	1.76 \pm 0.63	5.12 \pm 0.53	1.97 \pm 0.16	5.62 \pm 0.11	1.88 \pm 0.09	6.32 \pm 1.66
12	7.40 \pm 0.26	7.92 \pm 0.22	2.89 \pm 0.78	8.84 \pm 0.65	2.19 \pm 0.22	15.33 \pm 1.11	2.11 \pm 0.08	15.54 \pm 0.38	2.21 \pm 0.36	15.78 \pm 0.76	2.08 \pm 0.23	16.34 \pm 1.89
14	20.52 \pm 0.46	14.91 \pm 0.28	6.79 \pm 0.54	18.77 \pm 0.54	4.23 \pm 0.23	37.87 \pm 1.29	4.19 \pm 0.43	37.1 \pm 0.97	4.56 \pm 0.69	38.2 \pm 1.06	4.21 \pm 0.25	38.33 \pm 1.27
16	34.82 \pm 0.25	19.28 \pm 0.78	11.19 \pm 1.09	26.54 \pm 0.43	7.88 \pm 0.18	76.45 \pm 2.66	7.12 \pm 0.65	77.15 \pm 1.86	7.19 \pm 0.46	75.29 \pm 1.64	6.89 \pm 0.61	73.32 \pm 2.76
18	42.95 \pm 1.67	30.65 \pm 0.64	21.32 \pm 1.68	38.41 \pm 0.79	20.65 \pm	113.62 \pm 3.54	19.9 \pm 1.06	111.3 \pm 1.69	14.06 \pm 0.87	121.23 \pm 1.39	18.88 \pm 0.28	110.12 \pm 3.18
20	48.76 \pm 1.86	37.50 \pm 2.09	39.98 \pm 1.87	47.72 \pm 1.23	33.67 \pm 1.26	146.1 \pm 2.34	31.04 \pm 0.38	142.32 \pm 2.21	24.76 \pm 1.11	148.99 \pm 2.43	30.03 \pm 0.53	139.31 \pm 5.16
High inoculum bioreactors												
10	4.27 \pm 0.22	2.11 \pm 0.13	3.12 \pm 0.08	9.32 \pm 0.65	3.25 \pm 0.11	12.25 \pm 1.07	3.29 \pm 0.38	12.11 \pm 1.09	3.41 \pm 0.09	12.43 \pm 1.66	3.52 \pm 0.33	13.11 \pm 0.87
12	9.16 \pm 0.31	8.99 \pm 0.76	4.89 \pm 0.18	20.36 \pm 0.23	4.22 \pm 0.18	26.48 \pm 1.76	4.15 \pm 0.79	25.54 \pm 1.24	4.55 \pm 0.97	26.98 \pm 1.45	4.23 \pm 0.27	27.23 \pm 1.56
14	29.12 \pm 1.55	21.34 \pm 1.56	8.77 \pm 0.54	49.42 \pm 1.43	6.78 \pm 0.16	55.15 \pm 1.18	7.12 \pm 0.68	57.75 \pm 1.76	7.67 \pm 0.76	56.67 \pm 2.12	7.45 \pm 0.87	58.43 \pm 0.98
16	37.45 \pm 1.42	34.77 \pm 1.82	13.23 \pm 0.21	59.23 \pm 1.54	11.32 \pm 0.64	107.92 \pm 2.45	12.34 \pm 0.45	78.92 \pm 2.06	12.65 \pm 1.14	76.78 \pm 2.32	10.54 \pm 1.09	76.67 \pm 1.67
18	48.72 \pm 2.64	46.34 \pm 2.44	23.45 \pm 0.67	86.43 \pm 2.69	14.43 \pm 0.98	121.4 \pm 2.27	13.98 \pm 0.88	119.12 \pm 2.46	21.33 \pm 1.12	110.43 \pm 2.34	13.78 \pm 0.95	118.34 \pm 2.65
20	54.66 \pm 1.98	51.86 \pm 1.87	39.18 \pm 2.11	110.9 \pm 2.45	26.76 \pm 1.17	151.6 \pm 3.45	25.08 \pm 1.11	148.78 \pm 2.65	32.65 \pm 1.45	140.01 \pm 4.11	24.44 \pm 1.22	145.55 \pm 3.56

(S)*—Suspended EPS; (A)*--Attached EPS; EPS were measured in mg g⁻¹ dry weight of *C. vulgaris*

Graphical Abstract:



Schematic diagram of MDZ removal in *Chlorella vulgaris* culture