1 **Microalgae biochar-derived carbon dots and their application in**

2 **heavy metal sensing in aqueous systems**

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11 **ABSTRACT**

12 This research seeks a coupled solution for managing the large amounts of biochar produced

13 by microalgae biofuels production, and the necessity for novel, economic and accurate heavy

14 metal sensing methods. Therefore, this study evaluated the transformation of microalgae

15 biochar (MAB) into carbon dots (Cdots) and their subsequent application as heavy metal ion

16 sensors in aqueous systems. The experimental phase included the transformation of MAB

17 into microalgae biochar-derived carbon dots (MAB–Cdots), MAB–Cdot characterisation and

18 the evaluation of the MAB–Cdots as transducers for the detection of four heavy metal ions

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19   (Pb^{2+}, Cu^{2+}, Cd^{2+}, and Ni^{2+}). MAB–Cdot fluorescence was stable over a wide range of pH

20   and resistant to photo-bleaching, making them suitable as fluorescence probes. The MAB–

21   Cdot fluorescence was quenched by all of the metal ions and displayed different quenching

22   levels. Depending upon the ions involved, MAB–Cdots were used to detect the presence of

23   heavy metal ions from concentrations of 0.012 μM up to 2mM by measuring the reduction in

24   fluorescence intensity. Neutral and slightly alkaline pHs were optimal for Cu^{2+} Ni^{2+} and Pb^{2+}

25   heavy metal quenching. To quantify the concentration of the heavy metal ions, linear and

26   logarithmic functions were used to model the MAB–Cdots fluorescence quenching. The

27   sensing mechanism was determined to be reversible and purely collisional with some

28   fluorophores less accessible than the others. This work demonstrated the ability to produce

29   Cdots from microalgae biochar, examined their application as a transducer for detecting

30   heavy metal ions in aqueous systems and paves the way for novel sensing systems using

31   MAB-Cdots.

32

33   **Keywords:** Biochar; Carbon dots; Microalgae; Heavy metal ion; Fluorescence sensors;

34   Quenching; Renewable nanomaterials

35

36   **1   INTRODUCTION**

37   During the last decade, interest in using microalgae as a feedstock for biofuel production has

38   increased. The significance of microalgae biofuel production is based on microalgae's

39   productivity (microalgae produce 2 to 10-times more biomass than that produced by

40   terrestrial crops), ability to capture inorganic carbon as CO_2, use of marginal land for their

41   cultivation, and significant lipid accumulation (4-60%) (Nam et al., 2016; Unkefer et al.,

42   2017). The production of biofuels from microalgae thermal conversion produced bio-oil

43   products with improved heating values and lower oxygen/carbon ratios (Maguyon and

44   Capareda, 2013; Maguyon-Detras and Capareda, 2017; Nam et al., 2017). Although

45   microalgae upgraded bio-oil products have similar properties to some liquid fuels, bio-oil

46   production corresponds to only 17 to 43% of the initial biomass. The remaining biomass is

47   transformed into syngas (8 to 25%) and biochar (34 to 63%) (Maguyon and Capareda, 2013).

48   Assuming a microalgae pond of 20.000 m^2 and a microalgae production of 600 kg of

49   microalgae per day (Richardson et al., 2010), the thermal conversion of this biomass will

50   produce between 79 and 140 tons of waste, or microalgae biochar, per year. The significant

51   amount of biochar produced during this process is one of the hurdles in the industrialisation

52   of the microalgae thermal conversion technology. To unlock the economic potential of this

53   process, it is crucial to develop novel products and processes that can create a diverse

54   portfolio of value-added products from the massive amounts of biochar.

55

56   As the necessity for developing biochar products has become imperative, novel processes

57   have been reported for the transformation of biochar into different add-value products such as

58   nanosilica, humic substances and carbonaceous nanomaterials (Genuino et al., 2017; Placido

59   et al., 2016; Plácido and Capareda, 2015). A promising class of nanomaterials is optically

60   active carbon dots (Cdots). Cdots are zero-dimensional carbon materials with advantageous

61   physical characteristics for applications such as optoelectronic and photonics (Ryu et al.,

62   2013). Cdots have high solubility, outstanding photostability, favourable biocompatibility

63   and low toxicity. In recent years, these materials have been fundamental in developing novel

64   sensing methods for detecting different types of pollutants such heavy metal ions (Gao et al.,

65   2017; Nair et al., 2017; Warrier and Kharkar, 2018).

66

67   The detection of heavy metal ions in aqueous systems is critical as heavy metal ions pose

68   serious threats to the environment and human health. Anthropomorphic activities have

69   increased the risk of heavy metal contamination and the occurrence of environmental issues,

70   creating the need for on-site, quick and accurate sensing methods for heavy metal ions

71   (Gogoi et al., 2015). Therefore, novel sensing techniques need to be able to detect heavy

72   metal ions below and above the regulation limits (Cd^{2+} 0.005 mg/L, Cu^{2+} 1.3 mg/L, Pb^{2+}

73   0.015 mg/L, Ni^{2+} 0.1 mg/L). The principle for heavy metal detection using Cdots is

74   fluorescence quenching, where the metal ions interact with the Cdots fluorophores groups

75   and reduce their fluorescence. The quenching effect is dependent on the metal ion-Cdot

76   interaction, which is different for each type of Cdot and metal ion (Gogoi et al., 2015; Gu et

77   al., 2016; Liu et al., 2016b; Song et al., 2015; Tiong et al., 2015; Ye et al., 2017; Zhang et al.,

78   2015). For instance, chicken egg white Cdots were only quenched by Fe^{3+} ions (Zhang et al.,

79   2015) while DNA-derived Cdots were quenched by both Hg^{2+} (20 μM) and Ag^+ (20 μM) ions

80   (Song et al., 2015). The fluorescence quenching of Cdots in the latter case was linear between

81   0 and 0.5 μM, and 0 and between 10 μM for Hg^{2+} and Ag^+, respectively. Similarly, Hg^{2+} ions

82   had the most significant quenching effect on orange peel Cdots (Gu et al., 2016), pigeon

83   feather Cdots, pigeon manure Cdots, pigeon egg white Cdots and pigeon egg yolk Cdots (Ye

84   et al., 2017). Pigeon feathers Cdots were quenched by Fe^{3+} and Ag^+ while pigeon manure

85   Cdots were quenched by Fe^{3+} and Cu^{2+} (Ye et al., 2017). Chocolate Cdots were quenched by

86   Pb^{2+} with a linear relationship between 0 and 2 μM (Liu et al., 2016b). Clearly, differences in

87   the Cdot production process and input material influence the chemical structure and sensing

88   capabilities of the diverse array of Cdots.

89

90 While Cdots have been intentionally synthesised from microalgae biomass, this is the first

91 report of Cdots derived from microalgae biochar (MAB-Cdots) and evaluated in heavy metal

92 ion detection. Cdots produced from microalgae biomass have been used as fluorescent

93 bioimaging probes for *Arabidopsis thaliana* and human breast cancer MCF-7 cells (Guo et

94 al., 2017; Zhang et al., 2017). However, these microalgae–derived Cdots have not been

95 evaluated for sensing heavy metal ions in aqueous systems. Thus, the need to evaluate the

96 diversity of MAB-Cdot response to heavy metal ion exposure across a range of heavy metal

97 ions. The use of MAB-Cdots and their further application as a heavy metal sensor is a

98 coupled solution for managing the large amounts of biochar produced by biofuels production,

99 and the necessity for novel, economic and accurate heavy metal sensing methods. Therefore,

100 the aim of this article was to evaluate microalgae biochar from biofuels production as a raw

101 material for MAB-Cdots production and the assessment of the potential of this advanced

102 nanomaterial as a transducer for the detection of 4 heavy metal ions (Pb^{2+}, Cu^{2+}, Cd^{2+} and

103 Ni^{2+}) in aqueous systems. The article includes the production and characterisation of MAB-

104 Cdots as well as their evaluation as heavy metal ions sensors via fluorescence quenching.

105

2   MATERIALS AND METHODS

2.1   Substrate

108 Microalgae biochar (MAB) was used as a substrate for the production of microalgae biochar–

109 derived carbon dots (MAB–Cdots). Dr Sergio Capareda and his Bio-Energy Testing and

110 Analysis Laboratory (BETA Lab) at Texas A&M University kindly donated the MAB. MAB

111 was produced from ground and dried *Nannochloropsis oculata* in a pyrolysis process using a

112 batch pressure reactor at 500 °C for 30 min (Series 4580 HP/HT, Parr Instrument Company,

113   Moline, IL). After collecting the MAB from the pressure reactor, it was crushed using a mortar

114   and sieved using a 1 mm mesh.

115

116   **2.2   Chemicals**

117   All chemicals were analytical grade: Potassium permanganate ($KMnO_4$) (Alfa Aesar), Acetone

118   (Acros Organics), Nickel sulphate (Ni^{2+}) (Fisher Scientific), Copper sulphate (Cu^{2+}), Cadmium

119   sulphate (Cd^{2+}), Lead Nitrate (Pb^{2+}), sodium dihydrogen phosphate, disodium hydrogen

120   phosphate and Ethylenediaminetetraacetic Acid Tetrasodium (EDTA) salt (Sigma-Aldrich).

121   Deionised and filtered (Milli-Q ultrapure water system with a 0.22 µm filter, Merck Millipore)

122   water was used in all the procedures.

123

124   **2.3   Preparation of microalgae biochar–derived carbon dots**

125   The biochar depolymerisation reaction was as follows: 10% solutions of $KMnO_4$ were mixed

126   with microalgae biochar (5%) in 125 mL Erlenmeyer flasks. The depolymerisation was

127   performed at 120 °C for 1 hour at 15 psi in an autoclave (Med 12, Selecta) (Plácido and

128   Capareda, 2015). After the chemical depolymerisation, the biochar solutions were centrifuged

129   at 5000 rpm for 20 min (Legend RT, Sorvall) at room temperature to separate the liquid and

130   solid phases. Acetone was mixed with the liquid phase until the production of a second liquid

131   phase (Han et al., 2017; Varisco et al., 2017). The phases were separated by centrifugation at

132   5000 rpm for 20 min (Legend RT, Sorvall). The upper phase was withdrawn and roto-

133   evaporated (miVAc Quattro concentrator, Genevac) until dry. After weighing, the solids were

134   resuspended in ultrapure water and ultrasonicated for 1 minute at 50% amplitude (200W)

135   (Branson, Emerson). The MAB–Cdots were obtained from the resuspended solids after

136 repeating the organic solvent precipitation process twice. The extracted MAB–Cdots were

137 suspended in water and kept at 4 °C until use.

138

2.4 Characterisation of microalgae biochar-derived carbon dots

140 The MAB–Cdots were characterised with different spectroscopic and microscopic techniques.

141 The fluorescence emission and excitation spectra of the MAB–Cdots were obtained on a

142 Hitachi F2500 spectrophotometer. The stability of the MAB–Cdots fluorescence was measured

143 at different pHs (pH 3 to 10) and exposure times (0 to 1h). FT–IR spectra were collected using

144 a Frontier FT-IR spectrophotometer with sampler (PerkinElmer) from $4000-600$ cm^{-1} and the

145 spectra analyses were performed using the Spectragryph software version 1.1 (Spectroscopy

146 Ninja). UV-Vis absorption spectra were recorded using a U3310 spectrophotometer (Hitachi).

147 Atomic force microscopy (AFM) images were captured on the BioScope AFM

148 (BrukerCorporation.) in ScanAssistant mode and the image analysis was performed using the

149 Brucker NanoScope software package v8.15 (Bruker Corporation), the particles were diluted

150 to 100 ppm, filtered through a 0.2 μM filter and placed on mica substrate for AFM imaging.

151 The MAB–Cdot size, zeta potential, and conductivity in solution were obtained by dynamic

152 light-scattering (DLS) and laser doppler velocimetry (LDV) with the Zetasizer Nano ZS

153 (Malvern). The measurements were performed using 0.2 μm filtered solutions in a DTS1070

154 cell with water as a dispersant (Refractive Index: 1.330) and assuming a MAB–Cdot refractive

155 index of 2.418 (Nguyen et al., 2016).

156

2.5 Heavy metal ions quenching assays

158 Stock solutions of the metal ions were prepared at concentrations of at least 25 mM and for

159 MAB–Cdots at concentrations of 1000 ppm. All the solutions were prepared using deionised

160 and 0.22 µM filtered water. The metal titration quenching studies utilised MAB–Cdot

161 solutions of 50 ppm diluted from the 1000 ppm solutions. The metal solutions were added to

162 the cuvette containing MAB–Cdots starting from 0.0125 µM up to 20 mM. The range utilised

163 was selected to include the minimum limit for these metals and concentrations reported on

164 wastewaters effluents. Fluorescence spectra were collected after each heavy metal aliquot

165 was added. The reduction in fluorescence was calculated as fluorescence reduction

166 percentage (%) (see Equation 1).

167 $Fluorescence\ reduction\ \% = \left(\frac{FL_0 - FL_{HMt}}{FL_0} \right) \times 100$ **Equation 1**

168 Where FL_0 is the MAB–Cdot fluorescence without the addition of heavy metal ions and

169 FL_{HMt} corresponds to the MAB–Cdot fluorescence after a specific concentration of heavy

170 metal was added. In the presence of Pb^{2+}, concentrations above 2 mM produced significant

171 scattering and prevented accurate measurement. For Cu^{2+} and Ni^{2+}, it was possible to reach a

172 concentration of 20 mM without generating significant scattering. To evaluate the pH effect

173 on MAB–Cdot heavy metal quenching, MAB–Cdots were diluted in buffers of citrate or

174 phosphate (5 mM) at pH 3, 4, 5, 6, 7, and 8 to reach a MAB–Cdot concentration of 50 ppm.

175 Each metal solution was added to the cuvette containing MAB–Cdots to reach metal

176 concentration of 50 µM. The fluorescence was collected before and after the addition of the

177 heavy metal solution. The effect of buffer strength on MAB–Cdot/ heavy metal ions

178 quenching was evaluated using 5 different concentrations of buffer phosphate pH 7 (2.5, 5,

179 10, 25, 50, 100 mM), MAB–Cdots were diluted in the buffers at 50 ppm. Each metal solution

180 was added to the cuvette containing MAB–Cdots to reach metal concentration of 50 µM. The

181 fluorescence reduction was calculated using **Equation 1.**

182
183 **2.6 Fluorescence quenching mechanism**

184 To understand the quenching mechanisms Stern–Volmer plots were constructed plotting F_0/F

185 versus metal ions concentrations. Stock solutions of metal ions were prepared at

186 concentrations of 100 mM, MAB–Cdots at concentrations of 1000 ppm and EDTA at

187 concentrations of 100 mM. The metal quenching mechanisms studies utilized MAB–Cdot

188 solutions of 50 ppm diluted from the 1000 ppm solutions. The fluorescence of the MAB-

189 Cdots solution was measured and then the metal solutions were added to the cuvette

190 containing MAB–Cdots (50 ppm) to reach a concentration of 50 μM. The fluorescence of

191 metal/MAB–Cdots solution was measured, followed byaddition of EDTA to a final

192 concentration of 100 μM and after vigorous mixing fluorescence was measured. The

193 fluorescence recovery was calculated using equation 2.

194 $$Fluorescence\ recovery = \frac{F}{F_0}$$ **Equation 2**

195 Where F is the fluorescence after the addition of the heavy metal or the EDTA and F_0 is the

196 initial fluorescence of the MAB–Cdots.

197

198 **3 RESULTS**

199 **3.1 Characterisation of microalgae biochar-derived carbon dots**

200 The AFM microscopy describes the MAB–Cdot morphology (**supplementary material,**

201 **Figure 1a**). The particle height was normally distributed with an average height of 4.7±0.9

202 nm and the height ranged between 2.9 and 7.3 nm. The MAB–Cdots had a lateral dimension

203 of 68±25 nm with a range between 38 and 153 nm. The MAB–Cdots had an average

204 hydrodynamic diameter of 175.5 nm, this diameter is close to the maximum lateral dimension

205   of the AFM. The MAB–Cdots had a zeta potential of -39.9 mV, which describes negatively

206   charged molecules with moderate stability. A negative zeta potential facilitates interactions

207   with positively charged particles such as heavy metal ions. MAB–Cdot FTIR spectra

208   (**supplementary material, Figure 1c**) had several signals from different chemical linkages,

209   the majority of these signals were associated with the presence of carbon linkages (1561,

210   1413, 1667, 2957, 2933, 2871, 719 and 648 cm^{-1}, **supplementary material, Figure 1c**).

211   Bonds associated with aromatic carbons were the strongest signals (1561, 1413 cm^{-1}) and C–

212   O and/or C=O linkages, C–H linkages, and aromatic linkages were more than 50% of the

213   peaks identified. Additionally, MAB–Cdot FTIR spectra exhibited signals associated with

214   sulphur, nitrogen and silica linkages. The peaks associated with aromatic, carboxyl and

215   hydroxyl linkages were reported in Cdots from lignocellulosic material (Wang et al., 2017)

216   and have been associated with the interaction between other carbonaceous materials and

217   pollutants such heavy metal ions.

218

219   **3.2   Fluorescence stability of microalgae biochar-derived carbon dots**

220   As the interaction between MAB-Cdots and heavy metal ions is measured through the MAB-

221   Cdot fluorescence reduction, it is important to characterise the fluorescent behaviour under

222   different conditions. To understand the stability of the MAB–Cdot fluorescence, MAB–Cdot

223   emission and excitation spectra were measured while modifying the pH, excitation and

224   emission wavelengths, and time of exposure (**Figure 1**). MAB–Cdot fluorescence was

225   observed for excitation wavelengths from the UV and extending until no emission was

226   observed beyond 600 nm excitation. While generally consistent in the middle of the

227   excitation range with a single emission peak at approximately 398 nm, the shape of the

228   spectrum was different at an excitation wavelength of 280 nm. At the long excitation

229   wavelengths, the shape remained generally constant but with a shifted centre/decreased

230   intensity (**Figure 1a**). Under excitation between 280 and 350 nm, the normalised emission

231   curves narrowed without significantly changing the maximum emission wavelength of

232   approximately 398 nm. Whereas under excitation between 350 and 450 nm, the maximum

233   emission wavelength shifted, but the width remained constant. As the difference between

234   excitation and emission wavelengths was not constant between 280 and 350 nm, the Stokes

235   shift varied from 218 nm (280/498) to 148 (350/498) while it remained constant at 50 nm

236   from 350 to 450 nm (**Figure 1b**). As the maximum emission was achieved at an excitation

237   wavelength of 330 nm this wavelength was used in the further experiments. The MAB–Cdot

238   fluorescence was strong, stable and almost constant over a wide range of pH (**Figure 1c**).

239   These results indicated that MAB–Cdots could be used at different pH without generating a

240   significant change in their fluorescent properties. MAB–Cdot photo-stability was evaluated

241   by exposing MAB–Cdots to a continuous UV-Light illumination for 1 h with fluorescence

242   collection every 5 min. The MAB–Cdots fluorescence intensity decreased less than 8% after

243   1 h (**Figure 1d**). These results showed that the MAB–Cdots are photobleaching resistant over

244   the period of normal sample measurement.

245

246   **3.3 Heavy metal ions quenching assay**

247   To evaluate the applicability of MAB–Cdots to fluorescence-based metal ion sensing,

248   fluorescence quenching experiments were performed with Pb^{2+}, Cu^{2+}, Cd^{2+} and Ni^{2+} (**Figure**

249   **2**). The four metal ions quenched the fluorescence of the MAB–Cdots. However, the

250   quenching level and the metal concentration necessary to obtain quenching varied from metal

251   ion to metal ion. Pb^{2+} was able to quench the fluorescence of MAB–Cdots up to 30% using

252   around 2 mM of Pb^{2+}. At Pb^{2+} concentrations greater than 2 mM, MAB–Cdots generated

253 significant scattering and prevented extension of the measurement range. Pb^{2+} concentration

254 and fluorescence reduction were linearly correlated using a logarithmic linearisation from

255 0.012 μM and 5 μM (**Figure 2a,** embedded figure). Cu^{2+} achieved the greatest quenching,

256 producing a fluorescence reduction of more than 60% at 20 mM (**Figure 2b**). The Cu^{2+}

257 concentration and the MAB–Cdot fluorescence reduction were linearly correlated between 1

258 μM and 10 mM using a logarithmic linearisation (**Figure 2b,** embedded figure). Cd^{2+}

259 produced the lowest fluorescence quenching with a maximum fluorescence reduction of less

260 than 10% (**Figure 2c**). Here, the quenching varied from 0.0125 μM up to 50 μM above which

261 the fluorescence reduction saturated at around 8%. The Cd^{2+} concentration and fluorescence

262 reduction were linearly correlated using a logarithmic linearisation between 0.01 μM and 1

263 μM. After Cu^{2+}, the Ni^{2+} quenching was the second largest quenching, producing a

264 fluorescence reduction of nearly 50% at 20 mM (**Figure 2d**). Similar to Cu^{2+}, the Ni^{2+}

265 concentration and MAB–Cdot fluorescence reduction was linearly correlated using a

266 logarithmic linearisation between 3 μM and 10 mM (**Figure 2d,** embedded figure). **Table 1**

267 describes the fitting parameters obtained from the heavy metal ions quenching experiments.

268 MAB–Cdots could detect the broadest concentration range for Cu^{2+} and Ni^{2+}. Pb^{2+} and Cd^{2+}

269 had the lowest concentrations (0.012 μM) with a response to MAB–Cdots. As Cd^{2+} did not

270 produce significant quenching, additional tests were not performed for this metal.

271

272 Buffer solutions from pH 3 to 8 were used to evaluate the effect of pH on the MAB–Cdot

273 fluorescence quenching from 50 μM of metal ions (**Figure 3a**). In Cu^{2+} and Pb^{2+}, the use of

274 buffer solutions with lower pH produced a decrease in the quenching. The metal most

275 affected by buffering was Pb^{2+}, where the maximum quenching in a pH 6 buffer solution

276 (4.35%) decreased by 66% when compared to the quenching obtained in ultrapure water

277 (14%). With Cu^{2+}, the maximum quenching in buffer solution (15%) was approximately half

278 that obtained in ultrapure water (27%). At low acidic pH, the quenching effect produced by

279 the metal ions decreased by 85% to 95% (Cu^{2+} and Ni^{2+} 85% and Pb^{2+} 93%) when compared

280 with the ultrapure water experiment and decreased by 73% to 85% when compared with the

281 maximum quenching obtained with a buffer solution. The highest quenching was obtained at

282 neutral and slightly basic or acid pH. However, the optimum pH was different for each metal

283 ion (Cu^{2+} 7, Ni^{2+} 8 and Pb^{2+} 6). This process can be explained by the interaction among

284 MAB–Cdot chemical groups, heavy metal ions and the buffer solution. Ni^{2+} reached the same

285 level of quenching at pH 8 as with the experiment using ultrapure water. In all metals, low

286 acidic pH was associated with low fluorescence quenching and was also associated with

287 carboxyl and hydroxyl groups.

288

289 As buffer solutions reduced the fluorescence quenching, the buffer capacity was evaluated to

290 identify their effect on MAB–Cdot fluorescence quenching (**Figure 3b**). Similar to the pH

291 assays, the initial MAB–Cdot fluorescence was not influenced by changes of buffer strength

292 (2.5-100mM) (data was not included). MAB–Cdot fluorescence quenched by Pb^{2+} was

293 highly reduced by the addition of all amounts of buffer. After the initial reduction in

294 fluorescence from the addition of low concentrations of buffer phosphate, higher

295 concentrations did not produce any further fluorescence reduction. Similarly, increased buffer

296 strength generated a negative effect on the MAB–Cdot fluorescence quenching in Cu^{2+} and

297 Ni^{2+}, the change from 2.5 mM to 100 mM produced a reduction in the quenching ability of

298 Cu^{2+} and Ni^{2+} of 10 and 5%, respectively. When compared with the quenching performed

299 with ultrapure water, the quenching ability of Cu^{2+} and Ni^{2+} at 100 mM reduced almost 64

300 and 60 %, respectively. Although in all metal ions, 100 mM generated the lowest

301 fluorescence reduction.

3.4 Quenching mechanism

The Stern-Volmer plots for the MAB–Cdot fluorescence quenching by heavy metal ions were used to evaluate the fluorescence quenching mechanisms (**Figure 4**). In all cases, the relationship between fluorescence quenching and the metal ions concentration were nonlinear with a downward curvature. Such non-linear Stern-Volmer curves are characteristic of pure collisional quenching where some of the fluorophores/quenching sites are less accessible than others (Ke et al., 2018; Warrier and Kharkar, 2018). Thus, the MAB–Cdot Stern-Volmer plots indicate a system having accessible and inaccessible quenching sites. Therefore, some of the excited quenching sites in the MAB–Cdot structure experienced contact with the metal ion facilitating the transition to the ground state, whereas other inaccessible quenching sites maintained their excited state as they could not interact with the heavy metal ions. The inaccessible quenching sites can be hidden in the MAB–Cdot structure due to MAB–Cdot interactions or by other constituents in the MAB–Cdots. The most efficient quencher was Cu^{2+} followed by Ni^{2+} and Pb^{2+}. The differences observed in the Stern-Volmer plots indicated significant differences in the interactions between MAB–Cdots and the metal ions

To understand the strength of the interactions between MAB–Cdots and the metal ions, EDTA was used to evaluate whether it is possible to recover the fluorescence after metal ion addition (**Figure 5**). As shown in the embed image, it was possible to completely recover the initial MAB–Cdot fluorescence in the system after using 100 µM of EDTA (2:1 EDTA:Metal ions). A *t-test* was used to demonstrate the MAB-Cdots fluorescence recovery after EDTA addition (**Supplementary material, Table 1**). The full fluorescence recovery demonstrated that the metal ions had a weaker interaction with MAB-Cdots as compared to EDTA.

326 Therefore, a chelating agent with greater binding energies for the metal ions, such as EDTA,

327 strips the metal ions from the MAB–Cdot quenching sites and enables the

328 fluorophores/quenching sites to be re-used.

329

330 **4 DISCUSSION**

331 This is the first article reporting the production of Cdots from biochar originated from

332 microalgae thermal conversion looking to couple advanced materials production with

333 environmental sensing. As a potential application for this value-added product, the use of

334 MAB-Cdots as a transducer for the detection of heavy metal ions was explored. Previously,

335 Cdots have been produced from raw microalgae as a primary product and effectively used as

336 a fluorescent bioimaging probe (Guo et al., 2017; Zhang et al., 2017). Compared with the

337 methods previously reported for the primary production of microalgae Cdots, the method

338 reported in this article not only provides a value-added product from industrial waste, but is

339 also a simpler and faster approach using a two-step method with 4 h of processing time to

340 produce MAB–Cdots. The optical properties of the MAB–Cdots, such as photobleaching

341 resistance, pH stability and negative zeta potential, support their use as a detection probe for

342 heavy metal ions (Gu et al., 2016; Liu et al., 2016b; Ye et al., 2017). Photobleaching is the

343 alteration of a fluorophore that makes it permanently unable to fluoresce. Although the MAB-

344 Cdots fluorescence reduced after one hour of continuous exposure to UV-Light (330 nm), the

345 fluorescence decreased 10% of the original value. This makes MAC-Cdots more resistant to

346 photobleaching than other organic fluorophores (e.g. FITC) which completely lose their

347 fluorescence after seconds to minutes of continuous illumination exposure (Mahmoudi et al.,

348 2011). Similar to MAB-Cdots, photobleaching resistance is a common property in other

349 renewably produced Cdots used for heavy metal ion sensing such as Cdots derived from

350   feather wastes (Ye et al., 2017) and DNA derived fluorescent bio-dots (Song et al., 2015).

351   The photobleaching observed in the MAB-Cdots did not influence individual quenching

352   measurements because the MAB-Cdot exposure to UV-Vis light in this process was less than

353   30 seconds for each the initial and quenching measurement. In fact, this level of

354   photobleaching resistance enables multiple measurements of this system over time without

355   introducing measurement error.

356

357   The different quenching levels and dynamics registered by each heavy metal ion can be

358   associated with chemical, electronic and vibrational properties (Shtepliuk et al., 2017). As

359   evidenced by the FT-IR spectra, MAB–Cdot structure is rich in C–O, C=O and C-OH

360   linkages, indicating similarities to graphene oxide dots and their interactions with heavy

361   metal ions. Although work on other Cdots has pointed to the presence of amino groups as

362   responsible for the interaction with heavy metal ions specially Cu^{2+} (Liu et al., 2016a), MAB-

363   Cdots did not have a significant presence of amino groups in their structure (supplementary

364   material). The MAB–Cdot/heavy metal ion interactions involve MAB–Cdots functional

365   groups with unshared electron pairs capable of forming coordination linkages with the heavy

366   metal ions. The most important groups are carboxylic acids and phenolic OH. Carboxylic and

367   phenolic groups are hard donors with electrostatic forces and entropy gains driving their

368   interactions with heavy metal ions. The high affinity between carboxylic acids and Pb^{2+},

369   Cu^{2+}, and Ni^{2+} ions is well known and has been proved in other material systems such as

370   humic substances (Perelomov et al., 2018), (Zhou et al., 2018)hydrogels and acetic acid films

371   (Xu et al., 2016).

372   **Table 2** compares MAB-Cdots with different renewably produced Cdots used for detecting

373   heavy metal ions in terms of the production method, the heavy metal ion detection, the

374 detection range and the limit of detection. The advantage of using residual biochar from the

375 bioenergy industry introduces the possibility of coupling bioenergy and nanomaterials

376 production from waste. In contrast, all the production methods for other renewable Cdots

377 produce the Cdots as the primary product and require significant energy input into the

378 process. Additionally, biochar-derived carbon dots can be produced from the bioenergy

379 production from several other feedstocks beside microalgae. Pb^{2+}, Cu^{2+} and Ni^{2+} all

380 significantly quenched MAB–Cdots. In contrast, Hg^{2+} and Pb^{2+} are the most common heavy

381 metal ions with significant quenching in other renewably produced Cdots. The advantage of

382 significant quenching by only one or two types of heavy metal ions is the reduction of

383 selectivity issues. The complexity of selectivity in MAB–Cdots need to be addressed in the

384 future. On the plus side, MAB-Cdots were quenched by both Ni^{2+} and Cu^{2+}. Ni^{2+} did not

385 significantly quench other renewably produced Cdots, and Chitosan hydrogels-Cdots were

386 the only known Cdots to be quenched by Cu^{2+}. The detection ranges of heavy metal ions

387 were generally wider for the MAB-Cdots than the other renewable Cdots. As shown in **Table**

388 **2**, this wide range will allow their use in highly polluted environments (mine pollution or

389 wastewaters effluents) (1 mg/L to 500 mg/L) and in low polluted waters for detecting heavy

390 metal ions close to the permitted concentrations (Cd^{2+} 0.005 mg/L, Cu^{2+} 1.3 mg/L, Pb^{2+} 0.015

391 mg/L, Ni^{2+} 0.1 mg/L). The limits of detection for MAB-Cdots were similar to those reported

392 by other renewably produced Cdots, below 0.1 μM. Similar to other renewably produced

393 Cdots, MAB-Cdots were affected by pH and buffer strength. At an acidic pH, the MAB–Cdot

394 hydroxyl (–OH) and carboxyl linkages (–COOH) shift from the charged form (–OH⁻ and –

395 COO⁻) into an uncharged form (–OH and –COOH). These changes in MAB–Cdot charge

396 reduce the interaction points between the heavy metal ions and MAB-Cdots altering the

397 quenching effect produced by the heavy metal ions.

398

399 The high affinity between heavy metal ions and MAB–Cdots also causes selectivity issues.

400 Therefore, it is necessary to improve the MAB–Cdot selectivity for developing a sensing tool

401 with an accurate determination and discrimination between heavy metal ions. Following

402 published work in the field, the MAB–Cdot selectivity can be improved through a number of

403 different approaches. First, the inclusion of phosphorous or nitrogen groups in the Cdot

404 structure improved the selectivity in pidgeon feather derived Cdots (Ye et al., 2017) and lotus

405 root derived Cdots (Gu et al., 2016). Second, coupling a secondary set of materials to

406 improve the selectivity has been shown in different types of Cdots or hydrogels (Gogoi et al.,

407 2015). Third, the inclusion of an additional set of measurements complementing the

408 fluorescence quenching and use multivariate statistics for heavy metal discrimination has

409 been an effective approach (Lippolis et al., 2018).

410

411 In all cases, the Stern-Volmer plots for the MAB–Cdot fluorescence quenching were

412 nonlinear with a downward curvature. This type of behaviour is different from that reported

413 by other renewably produced Cdots. Pidgeon feathers derived Cdots and orange peel derived

414 Cdots had linear behaviours that fit the traditional Stern-Volmer model ($F_0/F=K_{sv}[C] +1$).

415 Orange peel derived Cdots were linear at concentration similar to those used in the MAB–

416 Cdots. However, the Stern-Volmer linearity in pidgeon feather derived Cdots was only valid

417 at low concentrations. At low concentrations (0 to 5 μM), the Stern-Volmer plot for the

418 MAB–Cdots/Cu^{2+} quenching is linear, whereas Ni^{2+} and Pb^{2+} kept the non-linear downward

419 behaviour. In Stern-Volmer plots, linearity deviations may be associated with the quencher,

420 diffusion properties or the local concentration of the quencher around the quenching sites (Ke

421 et al., 2018; Warrier and Kharkar, 2018). Therefore, the affinity of the heavy metal ions for

422 carboxylic and phenolic OH groups are responsible for some of the MAB–Cdot fluorescence

423 quenching. The interaction sites not quenched can be associated to a lack of interaction with

424 the metal ions by steric effects or chemical repulsion.

425

426 The environmental sensors market is estimated to be worth 1.37 billion dollars and the

427 stringent environmental regulations will be pushing the development of more accurate and

428 portable sensors and less impact of human activities on the environment. Therefore, there is a

429 market for MAB-Cdot derived biosensors, produced from renewable. Future work will focus

430 on modelling the quenching process, improving the selectivity and accuracy of MAB-Cdots

431 and analysing the economics of integrating MAB-Cdots production with that of biofuels.

432

433 **5   CONCLUSIONS**

434 This article studied the use of microalgae biochar as a raw material to produce Microalgae

435 Biochar-derived Cdots and the use of this nanomaterial as a transducer for the detection of

436 heavy metals in aqueous systems. MAB–Cdots fluorescence was significantly quenched by

437 Pb^{2+}, Cu^{2+} and Ni^{2+}. The correlation between fluorescence reduction and metal ion

438 concentration was established for each metal ion including the limits of detection ($0.01\mu M$

439 Pb^{2+}, $0.1\mu M$ Cu^{2+} and $0.1\mu M$ Ni^{2+}) and range of detection ($0.01\mu M – 2mM$, Pb^{2+}; $1.5\mu M – 10$

440 mM, Cu^{2+} and $2.5\ \mu M –10$ mM, Ni^{2+}). The Stern-Volmer plots implied the presence of

441 accessible and inaccessible interaction sites in the MAB-Cdot structure while EDTA studies

442 suggested loose interactions and reversibility of the sensor system. This is the first step in the

443 development of a novel and sustainable method for environmental sensors from microalgae

444 biochar and the integration of nanomaterial production into microalgae biofuels production.

445

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FIGURES AND TABLES LIST

Table 1. Linearisation parameters for heavy metals quenching using MAB–Cdots

Table 2. Comparison between MAB-Cdots and previously reported renewably produced Cdots as heavy metal sensors.

Figure 1. a) MAB–Cdots emission spectra at different excitation wavelengths b) MAB–Cdots maximum fluorescence and max emission wavelength at different excitation wavelengths. b) c) MAB–Cdots fluorescence at different pHs d) MAB–Cdots fluorescence at different illumination times.

Figure 2. FL emission spectra of MAB–Cdots in the presence of different concentrations of heavy metals a) Pb^{2+} b) Cu^{2+} c) Cd^{2+} d) Ni^{2+}. Embedded images show the dependence of $(I_0-I)/I_0$ on the concentrations of heavy metals

Figure 3. MAB–Cdots FL-quenched efficiency $(I–I)/I0$ a) at different pHs b) at different phosphate buffer pH 7 concentrations

Figure 4. Stern-Volmer plots for MAB–Cdots quenching using 50 ppm of MAB–Cdots and different concentrations of metals (0.0125 µM to 20 mM) a) Cu^{2+} b) Ni^{2+} c) Pb^{2+}

Figure 5. MAB–Cdots fluorescence emission spectra FL in the absence and the MAB–Cdots fluorescence recovery, 50 µM of heavy metal and FL restoration in the presence of 100 µM of a) Cu^{2+} b) Ni^{2+} c) Pb^{2+}.