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1 **MULTIVARIATE ANALYSIS OF BIOCHAR-DERIVED CARBONACEOUS**

2 **NANOMATERIALS FOR DETECTION OF HEAVY METAL IONS IN AQUEOUS**

3 **SYSTEMS**

4

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12 **ABSTRACT**

13 This article focuses on implementing multivariate analysis to evaluate biochar-derived

14 carbonaceous nanomaterials (BCN) from three different feedstocks for the detection and

15 differentiation of heavy metal ions in aqueous systems. The BCN were produced from dairy

16 manure, rice straw and sorghum straw biochar using our NanoRefinery process. The

17 NanoRefinery process transforms biochar into advanced nanomaterials using

18 depolymerisation/chemical oxidation and purification of nanomaterials using solvent

19 extraction. Dairy manure biochar-derived carbonaceous nanomaterials (DMB-CN), rice straw

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biochar-derived carbonaceous nanomaterials (RSB-CN) and sorghum straw biochar-derived

carbonaceous nanomaterials (SSB-CN) were utilised as probes for the evaluation of their

fluorescent properties and the detection of heavy metal ions. The BCN fluorescence

quenching and fluorescence recovery was tested with lead (Pb^{2+}), nickel (Ni^{2+}), copper (Cu^{2+})

and mercury (Hg^{2+}). Principal component analysis (PCA) and discriminant analysis were

used to differentiate among heavy metal ions in water samples. The BCN from different

feedstocks had different characteristics and produced different interactions with heavy metal

ions. DMB-CN had the highest quenching for Hg^{2+} and Ni^{2+} while SSB-CN and RSB-CN

responded best to Cu^{2+} and Pb^{2+}, respectively. The fluorescence quenching was modelled

using linear and empirical functions. PCA and discriminant analysis used the quenching

measurements to differentiate heavy metal ions in aqueous system. A key result was that the

discriminant analysis had a 100 % accuracy to detect Pb^{2+}, 66 % for Ni^{2+} and Cu^{2+}, and 33 %

for Hg^{2+}. This study has shown that biochar-derived carbonaceous nanomaterials could be

used in heavy metal ions sensing applications. This is the first step in the development of a

fast and accurate method for the detection of heavy metal ions in waters using

environmentally friendly BCN.

1   INTRODUCTION

Agro-industry is the core of local and regional economies around the world. However,

industries producing and processing agricultural raw materials generate millions of tons of

2

43   by-products and waste which are, in the majority, unexploited. Rice straw, sorghum straw

44   and dairy manure biochar are residues generated from agro-industrial activities seeking

45   transformative processes to create added-value products (Beltrán-Ramírez et al., 2019).

46   Worldwide, the estimated rice production for 2018 was 769 million tons (FAO, 2018).

47   Therefore, the worldwide production of rice straw is above 1 billion tons as each ton of rice

48   produces 1.5 tons of rice straw (FAO, 2018; Satlewal et al., 2018). Sorghum is the fifth most

49   important grain with a worldwide production of 85.2 million tons (Cuevas et al., 2018). Each

50   ton of sorghum results in 1.3 tons of sorghum straw creating a worldwide production of

51   sorghum straw of more than 100 million tons (Cuevas et al., 2018; Kim and Dale, 2004).

52   Similarly, animal manure is one of the largest wastes produced by the agro-industry. In

53   Europe alone, domesticated animals produce more than 1500 million tons, including 1284

54   million tons of cattle manure (Guo et al., 2010; Holm-Nielsen et al., 2009). The enormous

55   amounts of residues overwhelm traditional uses, such as field fertilisation, and present an

56   opportunity for novel transformation processes able to produce added-value products from

57   agro-industrial wastes.

58

59   Worldwide, the focus of agro-industrial waste management has evolved from disposal to the

60   use of these residues as raw materials for the production of chemicals, energy or materials

61   (Burg et al., 2018; Plácido and Zhang, 2018; Plácido et al., 2019). In keeping with this shift

62   toward sustainability, waste-to-energy effectively provides organic waste treatment,

63   sustainable energy generation and resource recovery. One of these waste-to-energy methods

64   is biomass thermal conversion through gasification or pyrolysis processes. Both processes

65   produce synthesis gas (syngas, 13–85 %), biooil (5–75 %), and biochar (10–30 %) (Hsieh et

66   al., 2015). Syngas and biooil can be used to produce biofuels, electricity and heating, all of

67   which are readily marketable with supply well matched to demand (Gao et al., 2017).

68 Pyrolysis and gasification of dairy manure, sorghum straw and rice straw are the subject of

69 current research. Dairy manure has been transformed using bench and pilot scale gasifiers

70 (Nam et al., 2016) with sand from the cattle bedding (Nam et al., 2017) and in co-feeding

71 with cotton gin trash and microalgae (Hanif et al., 2016). Similarly, sorghum and rice straw

72 thermal conversion have been transformed using pyrolysis in a pressurised reactor (Santos

73 and Capareda, 2016), fluidised bed gasification (Maglinao Jr et al., 2015), a bench-scale

74 auger pyrolysis (Nam et al., 2015) and acid pre-treated rice straw pyrolysis (Hsieh et al.,

75 2015). In contrast, current added-value applications for biochar such as the production of

76 activated carbons (Jin et al., 2016) or soil amendment do not have the capacity to absorb the

77 huge quantity of this material associated with the transformation of agro-industrial wastes.

78

79 Research is now beginning to investigate transformation into nano- and micro-biochar via

80 physical processes and application to the removal of pollutants and the production of

81 electrode materials (Li et al., 2017; Liu et al., 2018; Naghdi et al., 2017; Zeng et al., 2018)

82 and the use of biochar depolymerisation for the transformation of biochar into different

83 added-value products. Humic and fulvic acids were generated as a product of the chemical

84 depolymerisation of cotton gin trash (CGT) biochar and municipal solid waste (MSW)

85 biochar (Genuino et al., 2017; Plácido and Capareda, 2015) via biochar depolymerisation,

86 nano-silica was obtained from CGT biochar (Plácido and Capareda, 2015). The production of

87 carbon-based nanomaterials is the most recent development in the application of biochar

88 chemical depolymerisation. Placido et al. have recently reported the production and

89 purification of carbon dots (Cdots) from microalgae biochar (Plácido et al., 2019). The

90 physicochemical properties and biological compatibility of Cdots have enabled their use as

91 bioimaging probes and the initial assessments for their application as environmental sensors.

92

93   Cdots and other carbonaceous nanomaterials applied as heavy metal ions sensors have been

94   produced from different feedstocks including chitosan, egg whites, lotus root, orange peels,

95   DNA, pigeon waste, and chocolate (Gogoi et al., 2015; Gu et al., 2016; Liu et al., 2016; Song

96   et al., 2015; Tiong et al., 2015; Ye et al., 2017; Zhang et al., 2015). Heavy metal ions

97   detection using Cdots is based on fluorescence quenching where the metal ions interact with

98   the Cdot groups, reducing their fluorescence. The typical detection range for heavy metal

99   ions includes values from below the minimum allowed by the environmental regulation (ppb

100   or nM) to the toxic concentrations for different organisms (50-100 μM). The quenching

101   effect is dependent on the specific metal ion-Cdot interaction, which differs for each

102   combination of Cdot and metal ion. Egg white Cdots were quenched by Fe^{3+} ions but not by

103   other metal ions (Zhang et al., 2015). DNA-derived Cdots were quenched by 20 uM of Hg^{2+}

104   and Ag^+ ions (Song et al., 2015). Similarly, Hg^{2+} ions were the most significant quenching

105   agent with orange peel Cdots (Gu et al., 2016) and pigeon waste Cdots (Ye et al., 2017).

106   Although these Cdots were produced from renewable sources, their production occurred as a

107   stand-alone process not linked directly with bioenergy production. The BCN evaluated in this

108   work were produced from processes optimised for energy production and, therefore,

109   represent added-value products from agro-industrial waste.

110

111   Biochar-derived carbonaceous nanomaterials (BCN) evidenced different levels of interaction

112   with heavy metal ions. However, some BCN can interact with several heavy metal ions,

113   showing low selectivity (Plácido et al., 2019). Therefore to improve the heavy metal ion

114   selectivity, it is necessary to move beyond the use of a single BCN methodologies to a

115   combination of different BCN with multivariate statistics. The aim of this study was to evaluate

116   the fluorescent properties of BCN from three different biochars (dairy manure, rice straw and

117   sorghum straw) as fluorescence probes for detecting heavy metal ions, and implementing

5

118 statistical multivariate analyses as a tool to differentiate heavy metal ions in aqueous systems.

119 This new understanding should help to improve predictions of BCN applications in heavy metal

120 sensing.

121

122

123 **2   MATERIALS AND METHODS**

124 **2.1   Substrate**

125 Dairy manure, rice straw and sorghum straw biochars were used as substrates for BCN

126 production.. Sorghum straw biochar was produced in a fluidised bed/pyrolysis process at 500

127 °C for 30 minutes (Maglinao Jr et al., 2015). Whereas, rice straw biochar was produced in a

128 pyrolysis process using a batch pressure reactor at 500 °C for 30 minutes (Series 4580

129 HP/HT, *Parr* Instrument Company, Moline, IL) (Nam et al., 2015). Dairy manure biochar was

130 produced in a fluidised bed/gasification process at 700 °C (Nam et al., 2016). All biochars were

131 sieved using a 1 mm mesh, after which the biochars were kept in a closed container at

132 environment temperature (20–25 °C) until use.

133

134 **2.2   Chemicals**

135 All chemicals were analytical grade: Potassium permanganate (KMnO4) (Alfa Aesar), Acetone

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

137 Chloride (Hg^{2+}), Lead Nitrate (Pb^{2+}), sodium dihydrogen phosphate, disodium hydrogen

138 phosphate and Ethylenediaminetetraacetic Acid (EDTA) Tetrasodium Salt (Sigma-Aldrich).

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

140 Millipore) was employed in all the procedures.

141

2.3 Biochar-derived carbonaceous nanomaterials preparation

143 The biochar depolymerisation reaction was as follows: 10 wt % solutions of $KMnO_4$ were

144 mixed with biochar (5 wt %) in 125 mL Erlenmeyer flasks. The depolymerisation was

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

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

147 at 5000 rpm for 20 minutes at room temperature to separate the liquid (supernatant) and solid

148 phases (remaining biochar). The liquid phase (supernatant), the biochar-derived carbonaceous

149 nanomaterial (BCN) rich phase, was mixed with acetone until the production of a second liquid

150 phase. Then, the two liquid phases (upper and lower) were separated by centrifugation at 5000

151 rpm for 20 minutes (Legend RT, Sorvall). The upper phase was withdrawn and later roto-

152 evaporated (miVAc Quattro concentrator, Genevac) until dry. After weighing, the dried upper

153 phase was resuspended in deionised and filtered water, and ultrasonicated for 1 minute at 50

154 % amplitude (200W) (Branson, Emerson). The BCN were obtained after repeating the organic

155 solvent precipitation process two additional times. The extracted BCN were suspended in water

156 and kept at 4 °C until use. The BCN fluorescence emission and excitation spectra and the

157 stability studies were performed in a Hitachi F2500 spectrophotometer. The stability of the

158 BCN fluorescence was measured at different pHs (pH 3 to 10) and exposure times (0 to 1 h),

159 the photobleaching measurements were obtained under constant excitation during 1 hour.

160

2.4 Heavy metal ions quenching assays

162 Two quenching experiments were performed in this work. First, an evaluation of the

163 quenching ability of the individual BCN (50 ppm) using a defined concentration of 50 μM of

164 each heavy metal ion. This concentration was selected as it is widely used in heavy metal

165 ions quenching studies with nanomaterials (Essner et al., 2016; Hou et al., 2015). Second, an

166 evaluation of the quenching ability of mixed BCN (50 ppm) using heavy metal ion

167 concentrations from 0.0125 μM up to 50 μM. The range utilised was selected to include the

168 minimum limit for these ions and concentrations reported in wastewaters effluents. Stock

169 solutions of the metal salts were prepared at concentrations of at least 25 mM and for BCN at

170 concentrations of 1000 ppm. All the solutions were prepared using deionised and 0.22 µM

171 filtered water. The quenching studies utilised 1 mL BCN solutions of 50 ppm diluted from

172 the 1000 ppm solutions. The metal solutions were added to the cuvette containing BCN

173 starting from 0.0125 μM up to 50 μM. Fluorescence spectra were collected after each heavy

174 metal aliquot addition. The reduction in fluorescence was calculated as Fluorescence

175 reduction percentage (FR %) (see **Equation 1**).

176 $Fluorescence\ reduction\ \% = \left(\frac{BCN\ FL_0 - BCN\ FL_{HMt}}{BCN\ FL_0} \right) \times 100$ **Equation 1**

177 Where *BCN FL$_0$* is the BCN fluorescence without heavy metal addition and *BCN FL$_{HMt}$*

178 corresponds to the BCN fluorescence after a specific concentration of heavy metal was

179 added. All experiments were performed in triplicate and the initial quenching experiment

180 results were analysed using the SAS Software University Edition. To understand the

181 quenching mechanisms Stern–Volmer plots were constructed plotting *BCN FL$_0$/ BCN FL$_{HMt}$*

182 (F$_0$/F) vs metal ion concentration.

183
184 **2.5   Fluorescence quenching mechanism**

185 The metal quenching mechanisms studies utilised BCN solutions of 50 ppm diluted from the

186 1000 ppm solutions. The BCN solutions (50 ppm) fluorescence was measured at the

187 maximum excitation wavelengths for each BCN (DMB-CN (330 nm), SSB-CN (310 nm),

188 and RSB-CN (315 nm)). Then, the metal ions solutions were added to the previously

189 measured solution of BCN (50 ppm) to reach a concentration of 50 µM. The fluorescence of

190 the metal ion/BCN solution was measured. Afterwards, EDTA solution was added to the

191 metal ion/BCN solution to reach EDTA concentrations of 5, 10, 25, 50, 100, and 200 µM.

192 The fluorescence was measured after vigorously mixing. The fluorescence recovery was

193 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 BCN fluorescence. Additionally, the fluorescence restoration percentage (F.Res. %)

197 that measure the amount of fluorescence recovered after the metal reduction was calculated

198 using **equation 3**.

199 $Fluorescence\ restoration\ \% = \frac{F_{EDTA} - F_{Metal}}{F_0 - F_{Metal}} \times 100$ **Equation 3**

200 where, F_{EDTA} is the fluorescence after the EDTA addition, F_{Metal} is the fluorescence after the

201 addition of 50 µM of heavy metal ions and F_0 is the initial BCN fluorescence.

202

203 **2.6   Statistical Multivariate analysis**

204 Multivariate analysis was employed to evaluate the use of BCN as a tool for heavy metal

205 discrimination. Six different variables were utilised two alphabetical (Metal and BCN) and

206 four numerical (25 and 50 µM fluorescence reduction (FR %), 50 µM EDTA fluorescence

207 recovery and 100 μM EDTA F.Res %). As 50 μM EDTA fluorescence recovery and 100 μM

208 EDTA F.Res % were not normally distributed, the variables were transformed using a power

209 to ten and power squared transformations, respectively. The PROC PRINCOMP statement

210 (SAS Software University Edition) was employed for the PCA calculations using the data

211 correlation matrix. The PROC DISCRIM statement (SAS Software University Edition) was

212 employed to create a discrimination rule to identify heavy metal ions using the variables

213 employed in the PCA. A second set of data was constructed to evaluate the accuracy of the

214 discrimination rule.

215

216 **3   RESULTS**

217 **3.1   Biochar-derived carbonaceous nanomaterials fluorescence stability**

218 **Figure. 1a** describes the maximum fluorescence intensity and the wavelength associated with

219 that fluorescence for different excitation wavelengths. DMB-CN fluorescence intensity

220 increased as the excitation wavelength increased from 280 to its maximum at 330 nm before

221 decreasing. RSB-CN and SSB-CN fluorescence intensity increased between excitation

222 wavelengths of 280 and 310 nm. SSB-CN fluorescence intensity reduced at higher excitation

223 wavelengths. RSB-CN behaved differently than the other BCN as the fluorescence intensity

224 was almost constant between 310 and 340 nm before decreasing. The BCN maximum

225 emission wavelengths exhibited small increases between 280 and 340 nm with linear slopes

226 between 290 and 330 nm for DMB-CN (0.18 nm/nm), 300 and 340 nm for SSB-CN (0.20

227 nm/nm) and between 290 and 310 nm for RSB-CN (0.35 nm/nm). In all BCN, the maximum

228 emission wavelength exhibited a considerable shift to longer wavelength after an excitation

229 wavelength of 350 nm with slopes between 3 and 4-times greater than at the shorter

230   excitation wavelengths. The excitation wavelength corresponding to the highest emission was

231   310, 315 and 340 nm for SSB-CN, RSB-CN and DMB-CN, respectively.

232

233   **Figure. 1b** shows how the BCN normalised fluorescence intensity reduced after 1 hour

234   exposure at the peak excitation wavelength. DMB-CN (excitation wavelength 330 nm)

235   showed the largest reduction of 8 % followed by RSB-CN (excitation wavelength 315 nm)

236   with a 2.3 % reduction and SSB-CN (excitation wavelength 310 nm) with a 1.85 % reduction.

237   In addition to differences in the magnitude of fluorescence reduction after 1 hour, the

238   dynamics of the reduction were also different. DMB-CN fluorescence reduced at all times.

239   RSB-CN decreased during the first 20 minutes before stabilising. SSB-CN fluorescence

240   increased during the first 5 minutes before decreasing during the next 35 minutes and finally

241   stabilising. These results demonstrate that BCN are fairly resistant to photobleaching and

242   should be stable enough for repeated measurements (Song et al., 2015; Ye et al., 2017).

243

244   **Figure. 1c** describes the modifications in the BCN fluorescence at different pH. All BCN

245   displayed a fluorescence intensity increase with the pH reduction . The largest DMB-CN

246   fluorescence increase occurred between pH 8 and pH 7 where there was a 10 % increase.

247   Between pH 7 and pH 3, the fluorescence intensity increased less than 2 %. In RSB-CN, the

248   pH reduction between pH 8 and pH 3 produced a linear increase in the RSB-CN fluorescence

249   intensity (40 %) that was fit by a linear regression (Fluorescence = -0.053pH + 1.1523 with

250   R^2 = 0.9838). SSB-CN emission fluorescence increased almost 10 % between pH 8 and 4.

251   However, at pH 3 the fluorescence reduced 6 % compared to pH 4 fluorescence. Between, pH

252   8 and 5 the SSB-CN fluorescence and pH relationship were fit by a linear regression

253   (Fluorescence = -0.0286 pH + 1.1397 with R^2 = 0.9994).

254

3.2 Heavy metal ions quenching assays

3.2.1 Quenching experiment using a constant concentration of heavy metal ions

Figure. 2 describes the quenching experiment using heavy metal ions at 50 μM and 50 ppm of BCN. All the BCN were quenched by the four heavy metal ions tested. However, the quenching level varied between metal ions and BCN. Cu^{2+} produced the highest quenching in all the tested material, with an average fluorescence quenching across the individual BCN samples of 40 ± 2.3 %, followed by Pb^{2+} (22 ± 4.9 %), Ni^{2+} (18 ± 6.5 %) and Hg^{2+} (13 ± 5.2 %). All heavy metal ions quenched DMB-CN fluorescence by at least 20 %. DMB-CN achieved the largest fluorescence reduction percentage (FR %) in Hg^{2+} (22 %) and Ni^{2+} (30.3 %). In contrast, SSB-CN showed strong FR % of 43 % with Cu^{2+} while showing weak reduction for Ni^{2+} (10.3 %) and Hg^{2+} (6.8 %) and the lowest for Pb^{2+} (16.2 %). RSB-CN fluorescence was strongly quenched by Cu^{2+} (39.4 %) and Pb^{2+} (28.5 %), intermediately by Ni^{2+} (17.4 %) and weakly by Hg^{2+} (10 %). For all heavy metal ions, the highest quenching was achieved by a different BCN (Cu^{2+} SSB-CN, Pb^{2+} DMB-CN, Ni^{2+} RSB-CN and Hg^{2+} DMB-CN). The full quenching experiment statistical analyses, including analysis of variance (ANOVAs) and the differences among means tests (Duncan's test), are in the supplementary material. In all heavy metal ions, the ANOVAs evidenced differences among the BCN (*p-value* = 0.0004). Therefore, it was possible to test the differences among the means using the Duncan's test (**Table. 1**). Duncan's test demonstrated that SSB-CN (42.8 %) was similar to DMB-CN and different from RSB-CN. Pb^{2+} generated the highest quenching in RSB-CN (28.5 %) and DMB-CN (24.8 %) fluorescence, which were statistically similar. As DMB-CN had the highest FR % for Ni^{2+} and Hg^{2+} and the Duncan's test determined it was significantly different from the other BCN, it can be used for future individual heavy metal ions analysis.

278

3.2.2 *Quenching experiment using variable concentrations of heavy metal ions*

The fluorescence reduction (FR %) as a function of the heavy metal concentration was

evaluated for the BCN. The fluorescence spectra of the quenching experiments for the three

BCN and heavy metal ions are included in the supplementary material. All BCN / heavy

metal ion combinations exhibited a reduction in the fluorescence when the heavy metal ion

concentration increased. DMB-CN, RSB-CN and SSB-CN quenching percentage followed a

trend similar to the 50 µM experiments, showing high quenching for Cu^{2+}, followed by Pb^{2+},

Ni^{2+} and Hg^{2+}. The FR % was modelled to analyse the effect of heavy metal concentration in

BCN fluorescence quenching. **Table. 2** describes the fitting of the quenching experiments.

Cu^{2+} concentration and SSB-CN FR % had a linear correlation at the majority of the

concentrations evaluated. In contrast, DMB-CN had a combination of a linear and a power

correlation and RSB-CN had two power functions. The limit of detection (LOD) for SSB-

CN/Cu^{2+} and RSB-CN/Cu^{2+} was 0.55 µM, whereas for DMB-CN/Cu^{2+} the LOD was 0.3 µM.

The correlations between Pb^{2+} concentration and BCN FR % were described using power

and linear functions. However, the three materials fitted a power function between 5 and 50

µM. The three trend lines were similar in the power term. However, the scaling factor

multiplying the concentration was greater in the BCN with greater quenching (RSB-CN) and

smaller in the BCN with low quenching (SSB-CN) (**Table. 2**). The LOD for the BCN/Pb^{2+}

were 0.4, 3 and 2 µM for MAB-CN, SSB-CN and RSB-CN, respectively. Ni^{2+} quenching was

modelled using power function but with differences in the power and scalar terms. DMB-CN

had the highest power and scalar terms which also described the highest quenching observed

in this material. RSB-CN FR % was modelled using the same equation for all the range of

concentrations. Similar to $Pb^{2+,}$ The LOD for the BCN/Ni^{2+} were 0.3, 3 and 2 µM for MAB-

CN, SSB-CN and RSB-CN, respectively. Hg^{2+} quenching was the lowest of all the heavy

13

303 metal ions and was modelled using power and logarithmic functions for all BCN. DMB-CN

304 and RSB-CN were modelled with power functions for all the range of concentrations and, as

305 the other equations, high quenching was associated with greater scalar and power values. As

306 SSB-CN had the lowest quenching, the data was the only BCN well fitted by a logarithmic

307 function (**Table 2**). The LOD for the BCN/Hg^{2+} were 0.6, 4.5 and 8.5 µM for MAB-CN,

308 SSB-CN and RSB-CN, respectively. The vast majority of the fitting curves had an $R^2 \sim 0.99$.

309 This high correlation motivated the investigation into the quantification and discrimination of

310 heavy metal ion concentration using mixed BCN.

311

312 **3.3   Quenching mechanism**

313 The Stern-Volmer plots for BCN fluorescence quenching by heavy metal ions were utilised

314 to evaluate the fluorescence quenching mechanisms (**Figure. 3**). RSB-CN and SSB-CN

315 fluorescence quenched with Cu^{2+} were the only Stern-Volmer plots with a linear correlation

316 (**Figure. 3a** and **Table. 3**). A linear Stern-Volmer plot indicates collisional quenching that

317 can be characterised using the Stern-Volmer equation $F_0/F = 1 + K_{SV}[Q]$, where Ksv is the

318 Stern-Volmer quenching constant. RSB-CN had a K_{SV} of 0.0162 L.µMol^{-1}, and SBB-CN had

319 a K_{SV} of 0.017 L.µMol^{-1}. The differences in the K_{SV} explained the higher quenching

320 produced by Cu^{2+} in SSB-CN than RSB-CN. The Stern-Volmer plots for the other

321 combinations of metal ions and BCN types had a nonlinear behaviour with a downward

322 curvature (**Figure. 3a, b, c, d**). Such curves are representative of pure collisional quenching

323 when some of the fluorophores are less accessible than others (Ke et al., 2018; Warrier and

324 Kharkar, 2018). As downwards non-linear Stern-Volmer plots are not fully understood and

325 modelled, the equations from **Table. 2** and **Equation 3** were combined to describe the

326 behaviour of F_0/F with the change of metal ions concentration for downwards non-linear

327 Stern-Volmer plots. The empirical model utilised two constant terms, a scalar and an

328 exponent, which are associated with the heavy metal ions concentration (**Table. 3**). The

329 empirical model described the behaviour of F_0/F with significant accuracy as the majority of

330 the equations had an R^2 above 0.99. As the model is an empirical approach, it was not

331 possible to define a correlation between the terms in the equations and measurable properties

332 from interactions between heavy metal ions and BCN. However, the values of the scalar and

333 power terms were similar in some BCN/heavy metal ion interactions. RSB-CN's scalar term

334 is similar in all the F_0/F plots with nonlinear downwards curves (0.029-0.033) and all the Pb^{2+}

335 plots had a similar exponent term. DMB-CN had the most diverse combination of scalar and

336 power terms as almost all of them were different.

337

338 To assess the strength of the interactions between BCN and the heavy metal ions, EDTA was

339 used to evaluate the fluorescence recovery (**Figure. 4**). The fluorescence significantly

340 recovered in all BCN after EDTA addition with the amount of fluorescence recovery

341 dependent on the EDTA concentration and the heavy metal type. For Pb^{2+} and Cu^{2+}, 50 μM

342 of EDTA was the minimum concentration to reach a fluorescence recovery (F/F_0) above 0.9.

343 Ni^{2+} and Hg^{2+} required 100 μM of EDTA to reach a F/F_0 above 0.9. The EDTA concentration

344 increase did not produce a continuous increase in the fluorescence recovery. In fact, the

345 fluorescence did not change with the addition of EDTA concentrations below 25 μM. The

346 differences in EDTA concentration to reach significant fluorescence recovery indicate

347 differences in the BCN quenching sites for each metal and differences in the binding energies

348 between BCN and heavy metal ions. The F.Res % was greater in Cu^{2+} followed by Pb^{2+}, Ni^{2+}

349 and Hg^{2+}. DMB-CN had the highest F.Res % in three of the four ions indicating a lower

350 binding energy between the DMB-CN groups and the heavy metal ions. In contrast, SSB had

351　the strongest interactions with Pb^{2+}, Ni^{2+} and Hg^{2+} where the F.Res % was between 23 % and

352　75 %.

353

354　**3.4　Statistical Multivariate analysis**

355　*3.4.1　Principal component analysis*

356　Six different variables, two alphabetical (Metal and BCN) and four numerical (25 and 50 μM

357　F.R%, F_0/F 50 μM EDTA and F.Res % 100 μM EDTA), were evaluated to facilitate the

358　detection and discrimination of heavy metal ions using BCN. The initial step in the

359　multivariate analysis was PCA. In this case, PCA was calculated from the numerical

360　variables and discriminated using the alphanumerical variables (**supplementary material**).

361

362　Two initial principal components were selected as the main components for the analysis

363　because PC 1 (69.75 %) and PC 2 (23.05 %) explained 92.8 % of the variation in the

364　experiment (**Figure. 5a**). The scree plot (**Figure. 5b**) confirmed this selection as PC 1 and

365　PC 2 also had the highest eigenvalues. The component pattern plot (**Figure. 5c**) shows that

366　all variables positively correlate with PC 1 and uniformly with three variables (F.Res % 100

367　μM EDTA, 25 and 50 μM F.R %). In contrast, F_0/F 50 μM EDTA correlated highly and

368　positively with PC 2. In PC 2, the other variables did not have a strong correlation and were

369　positive and negative. The variables correlation matrix indicated (**Supplementary material**)

370　a strong correlation between 50 μM F.R % and F.Res % 100 μM EDTA (0.821), 50 μM F.R

371　% and 25 μM F.R % (0.9561), and 25 μM F.R % and F.Res % 100 μM EDTA (0.7318). F_0/F

372　50 μM EDTA did not have a strong correlation with any of the other variables as the

373　correlation was below 0.37. The variables did not have a negative correlation in any case.

374   **Figure. 5d** and **5e** depict the component scores plots for PC1 and PC2 grouped by metal and

375   by BCN, respectively.

376

377   When the components scores were grouped by metal, Cu^{2+} and Pb^{2+} groups were easily

378   identified in **Figure. 5d**. Cu^{2+} have similar scores with all the BCN, whereas the other metal

379   ions scores were more diffuse. The Pb^{2+} group was easily identified and SSB-CN/ Pb^{2+} scores

380   are closer to all the SSB-CN scores than the other Pb^{2+} scores. In contrast, Hg^{2+} and Ni^{2+} were

381   mixed, making the metal ion grouping difficult. Although Hg^{2+} was mixed with Ni^{2+}, the

382   Hg^{2+} distribution in the plot was more compact than Ni^{2+}. On the other hand, the component

383   scores grouped by BCN exhibited a distribution across the entire plot (**Figure. 5e**). The effect

384   of the BCN material interactions were easier to observe in **Figure. 5e**. The SSB-CN formed

385   two clusters one including Pb^{2+} Hg^{2+} and $Ni^{2+,}$ and other one with Cu^{2+}. A cluster of DMB-

386   CN was observed with Hg^{2+} and Ni^{2+}. RSB-CN scores did not cluster, but the scores were

387   distributed through the PC 1 axis. The clusters observed in DMB-CN and SSB-CN were

388   responsible of the lack of clarity grouping Hg^{2+} and Ni^{2+}. The results from the PCA

389   demonstrate that it is possible to use fluorescence measurement from BCN to explore the

390   differences and similitudes in the interactions between heavy metal ions and BCN. In the

391   future, it will be important to evaluate the effect of other BCN to improve the ability to group

392   and discriminate heavy metal ions.

393

394   *3.4.2   Discriminant analysis*

395   The discriminant analysis aimed to determine the type of heavy metal ion in an aqueous

396   sample using the quenching results from SSB-CN, RSB-CN and DMB-CN. The discriminant

397   analysis had the same numeric variables utilised in the PCA (*F.R % 25 and 50 μM, F_0/F 50*

398 µM EDTA and *F.Res %* 100 µM EDTA). As the homogeneity of within covariance matrices

399 test had a chi-square value significant at the 0.001 level, the within covariance matrices were

400 used in the discrimination function. Therefore, a quadratic discriminant analysis (QDA) was

401 performed. **Table. 4** summarises the performance of the QDA regarding the metal ions

402 classification from the training database. From the training database, the prediction was 100

403 % accurate for Cu^{2+} and Pb^{2+} and 88.9 % accurate for Hg^{2+} and Ni^{2+}. The application of cross

404 validation exposed additional misclassifications for Cu^{2+} and Hg^{2+} where the model

405 prediction accuracy was 88.9 and 66.7 %, respectively. Pb^{2+} and Ni^{2+} kept the same accuracy

406 between the cross validation and the initial test. The total error count estimate for the cross

407 validation tests was slightly greater (0.1389) than the original run (0.0556).

408

409 In order to test the reliability of the QDA previously evaluated, a novel data set was collected

410 for completing a validation test beyond the training database. The novel samples included

411 results from the quenching of the four heavy metal ions and the three BCN. **Table. 5**

412 contains the scores of the validation test for the novel samples using the QDA obtained from

413 the initial dataset. In this case, the QDA maintained the 100 % accuracy for Pb^{2+}

414 determination between the original and the validation test. However, the accuracy decreased

415 in Cu^{2+}, Ni^{2+} and Hg^{2+} to 66.7 %, 66.7 % and 33.3 %, respectively. The most significant

416 issues were observed with Hg^{2+} where the QDA had significant issues differentiating Hg^{2+}

417 from Ni^{2+} using the validation data. The reduced accuracy with the validation data was

418 especially apparent with the DMB-CN results in which the QDA located Hg^{2+} as Ni^{2+} and

419 Ni^{2+} as Hg^{2+}. The similarities between these two metal ions in DMB-CN were also observed

420 in the PCA results (**Figure. 5**). Although the accuracy reduced between the initial and

421 validation data, the QDA was able to correctly predict the metal ions in 66 % of the occasions

422 from the validation set and the principal errors were associated with the discrimination

423   between Ni^{2+} and Hg^{2+}. This demonstrates that it is possible to utilise discrimination analysis

424   as a tool for the determination of specific heavy metal ions in water samples using BCN

425   quenching data.

426

427   **4   DISCUSSION**

428   The biochar-derived carbonaceous nanomaterials (BCN) from different feedstocks, rice

429   straw, sorghum straw and dairy manure, had different fluorescence characteristics. The peak

430   excitation wavelength differed among SSB-CN, DMB-CN and RSB-CN with values of 310,

431   330 and 315 nm, respectively. pH changes produced modifications in the fluorescence,

432   especially in RSB-CN and SSB-CN. The lack of changes in the DMB-CN fluorescence with

433   pH can be associated with the presence of aromatic rings attached to carboxyl and/or

434   hydroxyl groups.

435

436   Hg^{2+} and Ni^{2+} reached the highest quenching in DMB-CN (22 and 30 %), Cu^{2+} in SSB-CN

437   (42 %) and Pb^{2+} in RSB-CN (43 %). The different quenching obtained by each metal can be

438   associated with the BCN chemical, electronic and vibrational properties (Shtepliuk et al.,

439   2017). BCN and heavy metal ions interact using the C–O, C=O and C-OH groups in the BCN

440   structure, which were confirmed via FT-IR spectroscopy (Plácido et al., 2019). The unshared

441   electron pairs in these groups are capable of forming coordination linkages with heavy metal

442   ions. Carboxylic and phenolic groups drive the interactions with metal ions. The high affinity

443   between carboxylic acids and Pb^{2+}, Cu^{2+}, Ni^{2+} and Hg^{2+} ions has been reported in other

444   materials (Bala et al., 2007; Kaşgöz et al., 2006; Xu et al., 2016). The differences in the

445   interactions between BCN and heavy metal ions are associated with the amount of carboxyl,

19

446   phenolic and hydroxyl groups in the BCN structure (Tchaikovskaya et al., 2016), the adjacent

447   or surrounding groups to the quenching groups, and the location of the quenching groups.

448   The adjacent groups increase or reduce the electrons attraction (Smith, 2013) and steric

449   effects in these groups and the quenching groups can improve or reduce the interaction with

450   heavy metal ions (Chatterjee et al., 2005).

451

452   The FR % and the heavy metal ions concentration correlation fitted linear, power and

453   logarithmical functions. The type of function used in each BCN/heavy metal ion combination

454   was defined by the R^2 of the function. The majority of the functions had a R^2 above 0.99,

455   evidencing the future use of these functions for quantifying the amount of heavy metal ions in

456   aqueous systems. Differences between the functions enable discrimination and detection of a

457   specific heavy metal ion in a sample. The different Stern-Volmer plots had linear and non-

458   linear downward patterns. These patterns are associated with collisional quenching with the

459   linear pattern indicating pure collisional quenching with all the quenching sites are available,

460   and the non-linear downward pattern indicating pure collisional quenching where some

461   interaction groups are less accessible than others (Ke et al., 2018; Warrier and Kharkar,

462   2018). SSB-CN and RSB-CN interaction with Cu^{2+} were the only quenching sites following

463   the Stern-Volmer linear model. The other combinations of BCN and heavy metal ions

464   evidenced quenching sites with less or more accessibility. Therefore, some excited quenching

465   sites interacted with heavy metal ions, facilitating the transition to the ground state, while

466   other inaccessible quenching sites maintained their excited state. Linearity deviations are

467   associated to the quencher characteristics, quencher diffusion properties and the quenching

468   sites (Ke et al., 2018; Warrier and Kharkar, 2018). The BCN structure and location of the

469   quenching sites can produce different steric or repulsion effects to each quencher generating

470   the different downward curves. Empirical functions were employed to describe the Stern-

471 Volmer plots patterns (**Table. 3**). These equations accurately predicted the shape of the Stern-

472 Volmer plots. However, the physical or chemical significance of the two parameters,

473 exponent and scalar terms, is currently a research topic. As occurs in the traditional Stern-

474 Volmer model, these parameters should be associated with the characteristics of the quencher

475 and the quenching site. Future work will focus in the study of the BCN/heavy metal ion

476 interactions, and seek to develop a quenching model that can explain the physical and

477 chemical phenomena associated with the BCN fluorescence quenching by heavy metal ions.

478

479 The EDTA experiment showed differences in the strength of interactions among the

480 BCN/heavy metal ion parings. BCN interactions with Ni^{2+} and Hg^{2+} were stronger than Cu^{2+}

481 and Pb^{2+} as shown by the EDTA concentration needed to achieve fluorescence recovery

482 (F/F_0) above 0.9. Ni^{2+} and Hg^{2+} required double the concentration of EDTA compared to

483 Cu^{2+} and Pb^{2+}. Similar to the fluorescence recovery, the fluorescence restoration percentage

484 was lower in Ni^{2+} and Hg^{2+} than Cu^{2+} and Pb^{2+}. This measurement demonstrated that some of

485 the interactions were not fully reversible with the EDTA addition. The reduced reversibility

486 indicated a strong interaction between some of the quenching sites and the heavy metal ions.

487

488 Multivariate analyses were able to differentiate heavy metal ions in aqueous samples with

489 variable accuracy. Pb^{2+} was identified with a 100 % accuracy, followed by Cu^{2+} (66 %), Ni^{2+}

490 (66 %) and Hg^{2+} (33 %). The principal difficulty for the PCA and the discriminant analysis

491 was the differentiation between Hg^{2+} and Ni^{2+}. Neither multivariate test was able to achieve a

492 full separation between these two heavy metal ions. To improve the error observed in the

493 QDA, it is important to increase the number of samples in the training data. The use of other

494 quenching variables and utilisation of novel BCN can help discriminate more accurately the

495   differences between Hg^{2+} and Ni^{2+}. In the future, BCN/ heavy metal ion quenching

496   information data combined with multivariate statistics may provide a fast tool to detect a

497   larger number of heavy metal ions and to quantify the amounts of heavy metal ions in

498   aqueous systems.

499

500   Stringent environmental regulations for heavy metal ions will be pushing the development of

501   more accurate and portable detection methods. BCN derived heavy metal sensors and

502   detection methodologies can be integrated in the $1.37 billion environmental sensor market.

503   Using a BCN price of 1% of the carbon dots price in the market (129 £/kg), this product will

504   have 18-times more value than the original biochar (0.076 £/kg). Additionally, the

505   technologies and equipment required for the process are developed in different scales

506   facilitating the technical establishment of this process. Future work will focus on modelling

507   the quenching process, the initial steps for a portable sensor and the economics of integrating

508   nanomaterials production with that of pyrolysis and gasification facilities.

509

510   **5   CONCLUSIONS**

511   This article demonstrated the different optical and chemical properties of biochar-derived

512   carbonaceous nanomaterials (BCN) as fluorescence probes for heavy metal sensing. In

513   addition, this study lays the groundwork for future research into the use of the different

514   interactions between heavy metal ions and BCN as variables for multivariate analysis. Hg^{2+}

515   and Ni^{2+} reached the highest quenching in DMB-CN (22 and 30 %), Cu^{2+} in SSB-CN (42 %)

516   and Pb^{2+} in RSB-CN (43 %). The Stern-Volmer plots (F_0/F and heavy metal concentration)

517   had linear and non-linear downward patterns and these patterns were modelled using linear

functions (SSB-CN/Cu^{2+} and RSB-CN/Cu^{2+}) and empirical functions (power functions) to

describe the non-linear downward Stern-Volmer plots. The EDTA experiments demonstrated

different levels of interaction between the BCN and the metal ions as different EDTA

concentrations were necessary to recover the BCN fluorescence. PCA and discriminant

analysis demonstrated that is possible to combine the quenching measurements obtained from

the different BCN to determine and differentiate among heavy metal ions in aqueous system.

The discriminant analysis using validation dataset had a 100 % accuracy to detect Pb^{2+}, 66 %

for Ni^{2+} and Cu^{2+}, and 33 % for Hg^{2+}. This is a first step to utilise quenching data from BCN

and heavy metal ions to develop an easy and accurate sensor for heavy metal ions in aqueous

systems.

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8   FIGURES AND TABLE LIST