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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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6

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11

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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20 biochar-derived carbonaceous nanomaterials (RSB-CN) and sorghum straw biochar-derived
21 carbonaceous nanomaterials (SSB-CN) were utilised as probes for the evaluation of their
22 fluorescent properties and the detection of heavy metal ions. The BCN fluorescence
23 quenching and fluorescence recovery was tested with lead (Pb^{2+}), nickel (Ni^{2+}), copper (Cu^{2+})
24 and mercury (Hg^{2+}). Principal component analysis (PCA) and discriminant analysis were
25 used to differentiate among heavy metal ions in water samples. The BCN from different
26 feedstocks had different characteristics and produced different interactions with heavy metal
27 ions. DMB-CN had the highest quenching for Hg^{2+} and Ni^{2+} while SSB-CN and RSB-CN
28 responded best to Cu^{2+} and Pb^{2+} , respectively. The fluorescence quenching was modelled
29 using linear and empirical functions. PCA and discriminant analysis used the quenching
30 measurements to differentiate heavy metal ions in aqueous system. A key result was that the
31 discriminant analysis had a 100 % accuracy to detect Pb^{2+} , 66 % for Ni^{2+} and Cu^{2+} , and 33 %
32 for Hg^{2+} . This study has shown that biochar-derived carbonaceous nanomaterials could be
33 used in heavy metal ions sensing applications. This is the first step in the development of a
34 fast and accurate method for the detection of heavy metal ions in waters using
35 environmentally friendly BCN.

36

37 **Keywords:** Biochar; Carbonaceous nanomaterials; Heavy metal ions, Fluorescence sensors,
38 Quenching, Multivariate analysis

39

40 1 INTRODUCTION

41 Agro-industry is the core of local and regional economies around the world. However,
42 industries producing and processing agricultural raw materials generate millions of tons of

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 μM 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

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 (Ni²⁺) (Fisher Scientific), Copper sulphate (Cu²⁺), Mercury
137 Chloride (Hg²⁺), Lead Nitrate (Pb²⁺), 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

142 **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 $^\circ\text{C}$ 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 $^\circ\text{C}$ 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

161 **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**).

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

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 μ M. 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 μ M.
192 The fluorescence was measured after vigorously mixing. The fluorescence recovery was
193 calculated using **Equation 2**.

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

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**.

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

200 where, F_{EDTA} is the fluorescence after the EDTA addition, F_{Metal} is the fluorescence after the
201 addition of 50 μ M 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 μ M fluorescence reduction (FR %), 50 μ M 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 = $-0.053\text{pH} + 1.1523$ 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 = $-0.0286 \text{ pH} + 1.1397$ with $R^2 = 0.9994$).

254

255 3.2 Heavy metal ions quenching assays

256 3.2.1 Quenching experiment using a constant concentration of heavy metal ions

257 **Figure. 2** describes the quenching experiment using heavy metal ions at 50 μM and 50 ppm
258 of BCN. All the BCN were quenched by the four heavy metal ions tested. However, the
259 quenching level varied between metal ions and BCN. Cu^{2+} produced the highest quenching in
260 all the tested material, with an average fluorescence quenching across the individual BCN
261 samples of $40 \pm 2.3 \%$, followed by Pb^{2+} ($22 \pm 4.9 \%$), Ni^{2+} ($18 \pm 6.5 \%$) and Hg^{2+} (13 ± 5.2
262 $\%$). All heavy metal ions quenched DMB-CN fluorescence by at least 20 %. DMB-CN
263 achieved the largest fluorescence reduction percentage (FR %) in Hg^{2+} (22 %) and Ni^{2+} (30.3
264 $\%$). In contrast, SSB-CN showed strong FR % of 43 % with Cu^{2+} while showing weak
265 reduction for Ni^{2+} (10.3 %) and Hg^{2+} (6.8 %) and the lowest for Pb^{2+} (16.2 %). RSB-CN
266 fluorescence was strongly quenched by Cu^{2+} (39.4 %) and Pb^{2+} (28.5 %), intermediately by
267 Ni^{2+} (17.4 %) and weakly by Hg^{2+} (10 %). For all heavy metal ions, the highest quenching
268 was achieved by a different BCN (Cu^{2+} SSB-CN, Pb^{2+} DMB-CN, Ni^{2+} RSB-CN and Hg^{2+}
269 DMB-CN). The full quenching experiment statistical analyses, including analysis of variance
270 (ANOVAs) and the differences among means tests (Duncan's test), are in the supplementary
271 material. In all heavy metal ions, the ANOVAs evidenced differences among the BCN (p -
272 $value = 0.0004$). Therefore, it was possible to test the differences among the means using the
273 Duncan's test (**Table. 1**). Duncan's test demonstrated that SSB-CN (42.8 %) was similar to
274 DMB-CN and different from RSB-CN. Pb^{2+} generated the highest quenching in RSB-CN
275 (28.5 %) and DMB-CN (24.8 %) fluorescence, which were statistically similar. As DMB-CN
276 had the highest FR % for Ni^{2+} and Hg^{2+} and the Duncan's test determined it was significantly
277 different from the other BCN, it can be used for future individual heavy metal ions analysis.

278

279 3.2.2 *Quenching experiment using variable concentrations of heavy metal ions*

280 The fluorescence reduction (FR %) as a function of the heavy metal concentration was
281 evaluated for the BCN. The fluorescence spectra of the quenching experiments for the three
282 BCN and heavy metal ions are included in the supplementary material. All BCN / heavy
283 metal ion combinations exhibited a reduction in the fluorescence when the heavy metal ion
284 concentration increased. DMB-CN, RSB-CN and SSB-CN quenching percentage followed a
285 trend similar to the 50 μM experiments, showing high quenching for Cu^{2+} , followed by Pb^{2+} ,
286 Ni^{2+} and Hg^{2+} . The FR % was modelled to analyse the effect of heavy metal concentration in
287 BCN fluorescence quenching. **Table. 2** describes the fitting of the quenching experiments.
288 Cu^{2+} concentration and SSB-CN FR % had a linear correlation at the majority of the
289 concentrations evaluated. In contrast, DMB-CN had a combination of a linear and a power
290 correlation and RSB-CN had two power functions. The limit of detection (LOD) for SSB-
291 CN/ Cu^{2+} and RSB-CN/ Cu^{2+} was 0.55 μM , whereas for DMB-CN/ Cu^{2+} the LOD was 0.3 μM .
292 The correlations between Pb^{2+} concentration and BCN FR % were described using power
293 and linear functions. However, the three materials fitted a power function between 5 and 50
294 μM . The three trend lines were similar in the power term. However, the scaling factor
295 multiplying the concentration was greater in the BCN with greater quenching (RSB-CN) and
296 smaller in the BCN with low quenching (SSB-CN) (**Table. 2**). The LOD for the BCN/ Pb^{2+}
297 were 0.4, 3 and 2 μM for MAB-CN, SSB-CN and RSB-CN, respectively. Ni^{2+} quenching was
298 modelled using power function but with differences in the power and scalar terms. DMB-CN
299 had the highest power and scalar terms which also described the highest quenching observed
300 in this material. RSB-CN FR % was modelled using the same equation for all the range of
301 concentrations. Similar to Pb^{2+} . The LOD for the BCN/ Ni^{2+} were 0.3, 3 and 2 μM for MAB-
302 CN, SSB-CN and RSB-CN, respectively. Hg^{2+} quenching was the lowest of all the heavy

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/Hg²⁺ 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 Cu²⁺ 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 K_{SV} is the
318 Stern-Volmer quenching constant. RSB-CN had a K_{SV} of $0.0162 \text{ L}\cdot\mu\text{Mol}^{-1}$, and SBB-CN had
319 a K_{SV} of $0.017 \text{ L}\cdot\mu\text{Mol}^{-1}$. The differences in the K_{SV} explained the higher quenching
320 produced by Cu²⁺ 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; Warriar 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 alphanumeric 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 Ni²⁺ and Hg²⁺. 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 Hg²⁺ and Ni²⁺ reached the highest quenching in DMB-CN (22 and 30 %), Cu²⁺ in SSB-CN
437 (42 %) and Pb²⁺ 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 Pb²⁺, Cu²⁺, Ni²⁺ and Hg²⁺ 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,

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; Warriar 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; Warriar 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 pairings. 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

518 functions (SSB-CN/Cu²⁺ and RSB-CN/Cu²⁺) and empirical functions (power functions) to
519 describe the non-linear downward Stern-Volmer plots. The EDTA experiments demonstrated
520 different levels of interaction between the BCN and the metal ions as different EDTA
521 concentrations were necessary to recover the BCN fluorescence. PCA and discriminant
522 analysis demonstrated that is possible to combine the quenching measurements obtained from
523 the different BCN to determine and differentiate among heavy metal ions in aqueous system.
524 The discriminant analysis using validation dataset had a 100 % accuracy to detect Pb²⁺, 66 %
525 for Ni²⁺ and Cu²⁺, and 33 % for Hg²⁺. This is a first step to utilise quenching data from BCN
526 and heavy metal ions to develop an easy and accurate sensor for heavy metal ions in aqueous
527 systems.

528

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536

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655

8 FIGURES AND TABLE LIST

Table 1. Duncan grouping form means of treatments for the 50 μM quenching experiments

Table 2. Equations modelling the interaction between fluorescence reduction (FR %) and heavy metal ions concentration.

Table 3. Stern-Volmer plots shape and equations for modelling BCN interactions with heavy metal ions.

Table 4. Overall classification performance (count and %) of the discriminant model, with and without cross validation (CV), for the four heavy metal ions.

Table 5. Validation test for the discriminant model

Figure 1. a) BCN fluorescence intensity (FL) and fluorescence wavelength (WL) at different excitation wavelengths, b) BCN fluorescence under constant illumination times c) BCN Fluorescence at different pHs. The BCN concentration was 1000 ppm and the arrows indicate the respective Y-axis

Figure 2. BCN fluorescence reduction percentage (FR %) using 50 μM of heavy metal ions. The bars are the standard deviation.

Figure 3. Stern-Volmer plots for BCN quenching using 50 ppm of BCN and different concentrations of heavy metal ions (0.0125 μM to 50 μM) a) Cu^{2+} b) Pb^{2+} c) Ni^{2+} d) Hg^{2+}

Figure 4. BCN fluorescence recovery at different EDTA concentrations (5 to 200 μM) a) Cu^{2+} b) Pb^{2+} c) Ni^{2+} d) Hg^{2+} . Embed images correspond to BCN fluorescence recovery percentage (F.R. %)

Figure 5. Principal component analysis a) Variance explained b) Scree plot c) Component pattern plot d) Component scores plot grouped by heavy metal ions e) Component scores plot grouped by BCN