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1 **Comparative study of the characteristics and fluorescent**
2 **properties of three different biochar derived-carbonaceous**
3 **nanomaterials for bioimaging and heavy metal ions sensing**

4
5 J. Plácido^{1*}, S. Bustamante-López^{1,2}, K.E. Meissner², D.E. Kelly¹ and S.L. Kelly^{1*}

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7 *1 Institute of Life Science (ILS 1), Swansea University Medical School, Swansea University,*
8 *Swansea, SA2 8PP, Wales, UK*

9 *2 Department of Physics, Centre for NanoHealth, Swansea University, Swansea, SA2 8PP,*
10 *Wales, UK*

11
12 **ABSTRACT**

13 Three types of biochar (microalgae, rice straw and sorghum straw) from biomass thermal
14 conversion production were tested for producing biochar-derived carbonaceous nanomaterials
15 (BCN). BCN were obtained after using chemical depolymerisation and solvent extraction,
16 NanoRefinery process. Microalgae biochar-derived carbonaceous nanomaterials (MAB-CN),
17 rice straw biochar-derived carbonaceous nanomaterials (RSB-CN) and sorghum straw biochar-
18 derived carbonaceous nanomaterials (SSB-CN) were characterised using spectroscopic and

* Corresponding authors:

Address: Institute of Life Science 1, Medical School, Swansea University, Swansea SA2 8PP, UK

Phone: +44 1792 503430 / +44 1792 662207

Fax: +44 1792 503430

Correspondance emails: j.e.placidoescobar@swansea.ac.uk / s.l.kelly@swansea.ac.uk

19 microscopic techniques. This characterisation evidenced significant differences among the
20 three BCN with MAB-CN exhibiting greater structural differences compared to RSB-CN and
21 SSB-CN. Biocompatibility, cellular uptake, and cellular localisation were evaluated using
22 three yeast species, *Saccharomyces cerevisiae*, *Candida albicans*, and *Yarrowia lipolytica*.
23 While all BCN were biocompatible, the degree of biocompatibility for each species was
24 dependent on pH, BCN concentration and BCN type. Additionally, BCN were evaluated as
25 transducers for the detection of 12 heavy metal ions. MAB-CN, RSB-CN, and SSB-CN had
26 different responses to the 12 heavy metal ions. The SSB-CN/Cu (II) and the MAB-CN/Zn (II)
27 combinations evidenced selectivity over the other metal ions with these combinations having
28 limits of detection of 0.0125 μM and 9 μM , respectively. The results from this research pave
29 the way for BCN novel applications for bioimaging and heavy metal ions sensing probes.

30

31 **Keywords:** Biochar; Carbonaceous nanomaterials; Heavy metal ions sensing; Bioimaging;
32 Fluorescence probes;

33

34 1 INTRODUCTION

35 To achieve their objectives for growth, jobs and sustainability, the energy strategies of many
36 governments around the world include, as a major component, the use of biomass as a
37 sustainable source of electricity, heating, and biofuels. By 2030, the European Union aims to
38 generate at least 27% of energy from renewable energy, and a minimum share of advanced
39 biofuels of at least 6.8% [1]. Likewise, by 2030, the United States expects to sustainably
40 produce 1 billion dry tons of non-food biomass and use them to expand the bioeconomy,
41 contributing \$260 billion and 1.1 million jobs to the US economy [2]. To achieve these goals,

42 a fundamental shift toward increased production of biofuels and renewable energy from
43 biomass is required. Therefore, the current technologies for biomass transformation need to
44 reach further levels of sophistication to maximise the value derived from biomass feedstocks
45 and by-products obtained from their transformation [1,2].

46

47 Thermal conversion is one of the most important techniques for biofuels and bioenergy
48 production. Gasification and pyrolysis processes are two core thermal conversion processes.
49 Gasification is conducted at temperatures higher than 700 °C, ambient or high pressure, and
50 reduced oxygen concentration. Pyrolysis is conducted at lower temperatures (400–600 °C),
51 under higher pressure, and without oxygen. Both processes generate synthesis gas (syngas, 13–
52 85%), biooil (5–75%), and biochar (10–30%). Syngas can be employed directly to generate
53 electricity (combustion) or liquid fuels using the Fischer-Tropsch process [3]. Biooil can be
54 upgraded to generate liquid biofuels or chemicals [4]. Biochar’s principal applications are soil
55 amendment [5-7] and activated carbons [8,9].

56

57 The upsurge in the worldwide goals for biofuels and bioenergy production will raise the
58 number of industrial processes using thermal conversion for biomass transformation. Syngas
59 and biooil are high value products employed for energy and biofuel generation, and their
60 production rise can be easily managed. In contrast, the current lack of biochar applications
61 makes it difficult to manage the massive amounts of biochar associated with the worldwide
62 increase in thermal conversion processes. Therefore, it is critical to find new processes for the
63 transformation of biochar into value-added products.

64

65 In recent years, new processes for the transformation of biochar into different added-value
66 products have been reported. Humic and fulvic acids were generated as a product of the
67 chemical and biological depolymerisation of cotton gin trash (CGT) biochar [10,11]. Similarly,
68 humic substances were generated via alkaline depolymerisation of municipal solid waste
69 (MSW) biochar. This work optimised and modelled humic acid production from MSW biochar
70 using an artificial neural network [12]. CGT biochar chemical depolymerisation produced
71 nano-silica as an additional material from biochar [11].

72

73 The production of carbon-based nanomaterials is one of the most recent developments in the
74 production of add-value products from bioenergy production biochar. Placido et al. [13]
75 recently reported the production and purification of carbonaceous nanomaterials from
76 microalgae biochar by using chemical depolymerisation and solvent extraction
77 (NanoRefinery). These nanomaterials were evaluated as a transducer for the detection of heavy
78 metal ions in aqueous systems. The fluorescence emitted by the microalgae biochar-derived
79 carbonaceous nanomaterials (MAB-CN) was quenched by four heavy metal ions, Ni (II), Pb
80 (II), Cd (II), and Cu (II). The MAB-CN fluorescence reduction was dependent on the heavy
81 metal ion concentration.

82

83 Biomass thermal conversion uses several feedstocks and diverse types of production processes.
84 Therefore, the resulting biochar from these diverse processes have various chemical structures
85 and properties. Carbon dots (Cdots) and other carbonaceous nanomaterials (CN) produced
86 from other carbonaceous sources exhibit diverse physicochemical properties and variable
87 biocompatibility [14-17]. Therefore, CN generated from different types of biochar are
88 predicted to have diverse structures and properties. The effect of different feedstocks and

89 production processes on the structure and properties of biochar-derived carbonaceous
90 nanomaterials (BCN) has not yet been studied. The objective of this research was to study three
91 types of biochar ((microalgae, rice straw and sorghum straw)) for the production of BCN, and
92 compare/contrast their physicochemical properties as well as their application as bioimaging
93 fluorescent probes and as transducers for heavy metal ions detection in aqueous systems.

94

95 **2 MATERIALS AND METHODS**

96 **2.1 Substrate**

97 Microalgae, rice straw and sorghum straw biochars were the initial substrates for BCN
98 production. Dr Sergio Capareda and his laboratory Bio-Energy Testing and Analysis
99 Laboratory (BETA Lab) at Texas A&M University kindly donated all the biochars. Sorghum
100 straw biochar (SSB) was obtained from sorghum straw in a fluidised bed/pyrolysis process at
101 500 °C for 30 min. Whereas, rice straw biochar (RSB) and microalgae biochar (MAB) were
102 obtained in a pyrolysis process using a batch pressure reactor at 500 °C for 30 min (Series 4580
103 HP/HT, Parr Instrument Company, Moline, IL). After collecting the biochars from the reactor,
104 they were crushed using a mortar and sieved using a 1 mm mesh.

105

106 **2.2 Chemicals**

107 All chemicals were analytical grade: Potassium permanganate (KMnO₄) (Alfa Aesar),
108 Acetone (Acros Organics), potato dextrose broth (PDB) medium (ForMedium). The heavy
109 metal ions included: Nickel sulphate (Ni(II)) (Fisher Scientific), Copper sulphate (Cu (II)),
110 Cadmium sulphate (Cd (II)), Lead Nitrate (Pb (II)), Cobalt nitrate (Co (II)) (Sigma–Aldrich),
111 Barium chloride (Ba (II)) (Sigma–Aldrich), lithium acetate (Li (I)) (Sigma–Aldrich), iron

112 sulphate (Fe(II)) (Sigma–Aldrich), manganese chloride (Mn (II)) (Acros Organics), zinc
113 sulphate (Zn (II)) (Sigma–Aldrich), silver nitrate (Ag (I)) (Sigma–Aldrich), sodium molybdate
114 (Mo (VI)) (Sigma–Aldrich). Deionised and filtered (Milli–Q ultrapure water system with a
115 0.22 µm filter, Merck Millipore) water was utilised in all the procedures.

116

117 **2.3 Biochar-derived carbonaceous nanomaterials preparation**

118 The biochar depolymerisation reaction was as follows: 10% solutions of KMnO_4 were mixed
119 with biochar (5%) in 125 mL Erlenmeyer flasks. The depolymerisation was performed at 120
120 °C for 1 h at 15 psi in an autoclave (Med 12, Selecta) [11]. After the chemical depolymerisation,
121 the biochar solutions were centrifuged at 5000 rpm for 20 min at room temperature to separate
122 the liquid and solid phases. The liquid phases were filtered using 0.22 µm filters (Millex) and
123 refrigerated at 4 °C until use. The depolymerised biochar (solid phase) was dried in a
124 convection oven at 105 °C for 24 h. The liquid phase was purified by repeated solvent
125 extraction. Acetone was mixed with the liquid phase until the production of a second liquid
126 phase [18,19]. The phases were separated by centrifugation at 5000 rpm for 20 min (Legend
127 RT, Sorvall). The upper phase was withdrawn and roto-evaporated (miVAc Quattro
128 concentrator, Genevac) until dry. After weighing, the solids were re-suspended in ultrapure
129 water and ultrasonicated for 1 minute at 50% amplitude (200 W) (Branson, Emerson). The
130 BCN were obtained after repeating the organic solvent precipitation process two additional
131 times. The extracted BCN were suspended in water and kept at 4 °C until use.

132

133 **2.4 Biochar-derived carbonaceous nanomaterials characterisation**

134 The BCN were characterised with diverse spectroscopic and microscopic techniques. The BCN
135 solutions were diluted to lower concentrations to facilitate characterisation. The fluorescence
136 emission and excitation spectra of the BCN were determined on a Hitachi F2500
137 spectrophotometer. FT-IR spectra were collected using a Frontier FT-IR spectrophotometer
138 with sampler (PerkinElmer) from 4000–600 cm^{-1} . The FT-IR spectra were analysed with
139 Spectragryph software version 1.1 (Spectroscopy Ninja). UV-Vis absorption spectra were
140 recorded using a U3310 spectrophotometer (Hitachi). Atomic force microscopy (AFM) images
141 were captured on the BioScope AFM (BrukerCorporation) in ScanAssistant mode (tip radius
142 nominal 2 nm and maximum 12 nm) and image analysis was performed using the Bruker
143 NanoScope software package v8.15 (Bruker Corporation). For AFM imaging, the BCN were
144 diluted to 100 ppm, filtered through a 0.2 μm filter and dried on mica substrate. The BCN size
145 and zeta potential in solution were obtained using the Zetasizer Nano ZS (Malvern). The
146 measurements were performed using 0.2 μm filtered solutions in a DTS1070 cell, with water
147 as dispersant (Refractive Index: 1.330) and a BCN refractive index of 2.418 [20]. The size and
148 zeta potential were obtained using the instrument's software.

149

150 **2.5 Biocompatibility studies**

151 The biocompatibility of the Biochar-derived carbonaceous nanomaterials (BCN) was studied
152 in three yeast species: *Saccharomyces cerevisiae* AH22, *Candida albicans* SC 5314, and
153 *Yarrowia lipolytica* (ATCC 46483). The yeast growth curve studies were performed in a
154 Bioscreen C (Oy Growth Curves Ab Ltd). The instrument assessed five BCN concentrations
155 (50, 100, 250, 500, 1000 ppm) in wells with 200 μL of PDB and 100 μL of 1×10^5 cells mL^{-1}
156 inoculum with 3 replicates for each treatment. The cell concentration change in each well was

157 evaluated via optical density change at a wavelength of 600 nm for 72 h and 30 °C. The growth
158 curves were also evaluated using BCN at pH 10, 7, and 3.

159

160 **2.6 Cell Imaging**

161 **2.6.1 Biochar-derived carbonaceous nanomaterials bioimaging**

162 The capabilities of BCN for cell bioimaging were tested in three yeast species (*S.*
163 *cerevisiae*, *C. albicans*, and *Y. lipolytica*). Yeast species were initially cultured in PDB for 24
164 h at 30 °C and then inoculated with a BCN concentration of 250 ppm for 2 h at 30 °C. After
165 incubation, the samples were centrifuged at 1000 rpm and washed with fresh PDB. This process
166 was repeated twice. Finally, the samples were re-suspended in PDB at 1:10 of the original
167 volume. After washing, the cells were imaged using confocal microscopy using a Zeiss LSM
168 710 confocal system with Zeiss AXIO Observer Z1 inverted microscope stand with transmitted
169 light (HAL), Illuminator HXP 120C and laser illumination sources. The images were collected
170 under bright field and 405 nm fluorescence excitation.

171

172 **2.6.2 Bioimage Processing**

173 To evaluate and identify differences in the fluorescence emitted by the BCN in the yeast
174 cells, the images were analysed using the ImageJ software version 1.50i (Wayne Rasband,
175 National Institutes Of Health, USA) and the SAS[®] Studio software 3.71 (University Edition,
176 SAS Institute Inc., Cary, NC, USA). The analysis in image J software was performed on three
177 separate images of each combination of yeast species and BCN type. Each image was
178 processed to calculate the corrected total cell fluorescence (CTCF) through cell selection,
179 fluorescence and area measurement, background correction, and CTCF calculation. The CTCF

180 was the response variable for the statistical analysis. As CTCF distribution did not follow a
181 normal distribution, a $Y=X^{1/4}$ variable transformation was performed. The transformed variable
182 was analysed in a two way non-balanced ANOVA because the yeast species and BCN type
183 combinations had different sample sizes. the yeast species and BCN type were used as factors,
184 and the three yeast species (*S. cerevisiae*, *C. albicans*, and *Y. lipolytica*) and the three BCN
185 types (SSB-CN, RSB-CN and MAB-CN) as levels for each factor. The unbalanced ANOVA
186 was calculated with the PROC GLM from the SAS[®] Studio software 3.71 (University edition,
187 SAS Institute Inc., Cary, NC, USA).

188

189 **2.7 Heavy metal ions quenching assays**

190 Stock solutions of the metal ions were prepared at concentrations of at least 25 mM and for
191 BCN at concentrations of 1000 ppm. All the solutions were prepared using deionised and
192 0.22 μM filtered water. The metal ions titration quenching studies utilised BCN solutions of
193 50 ppm diluted from the 1000 ppm solutions. The fluorescence of the BCN solution was
194 measured and then the metal ions solutions were added to the cuvette containing BCN (50
195 ppm) to reach a concentration of 50 μM . Then, the fluorescence of metal/ BCN solution was
196 measured. The reduction in fluorescence was calculated as fluorescence reduction percentage
197 (%) (see Equation 1). Metal ions titration quenching studies were determined using the metal
198 ions with highest effect in the BCN fluorescence. Cu (II) and Hg(II) were used at
199 concentrations from 0.0125 μM to 50 μM . Whereas, Zn (II) was prepared at concentrations
200 between 0.0125 μM to 1000 μM . The concentration range was selected to include the
201 minimum regulatory limit for these metal ions and concentrations reported on wastewaters
202 effluents. The heavy metal ion solution was added to the cuvette containing BCN starting
203 from 0.0125 μM up to 50 μM or 1000 μM . Fluorescence spectra were collected after each

204 heavy metal ion aliquot was added. The reduction in fluorescence was calculated as
205 fluorescence reduction percentage (%) (see **Equation 1**).

$$206 \text{ Fluorescence reduction \%} = \left(\frac{FL_0 - FL_{HMt}}{FL_0} \right) \times 100 \quad \text{Equation 1}$$

207 Where FL_0 is the BCN fluorescence without the addition of heavy metal ions and FL_{HMt}
208 corresponds to the BCN fluorescence after a specific concentration of heavy metal ions was
209 added.

210

211 **3 RESULTS**

212 **3.1 Biochar chemical depolymerisation**

213 The chemical depolymerisation of MAB, RSB and SSB produced modification of their
214 chemical structure. These modifications were followed by FT-IR spectroscopy (**Figure 1**). The
215 three non-depolymerised biochar spectra display similar bands between 400 and 500 cm^{-1} , 900
216 and 1200 cm^{-1} , 1700 and 1250 cm^{-1} and 2800 and 3000 cm^{-1} . A signal at 455 cm^{-1} was shared
217 by all the non-depolymerised biochars and it was associated with the presence of silica in the
218 biochar. The silica found in MSB and RSB was explained by the composition of the raw
219 material, which has significant amounts of silica in their composition [21,22]. In contrast, the
220 presence of silica in SSB was explained by the presence of remaining bed material from the
221 fluidised bed pyrolysis process [23,24]. The MAB peaks at 873 and 1415 cm^{-1} were more
222 intense than RSB and SSB. These signals demonstrated a structure with greater amounts of
223 aromatic compounds in MAB than that of RSB and SSB. Likewise, the RSB and SSB peaks at
224 775, 1027 and 1415 cm^{-1} demonstrated a structure rich in carbon molecules linked to oxygen
225 and hydrogen atoms. The loss of intensity and sharpness in the peaks related to carbon linkages,

226 such as 775, 873, 1078, 1415, 2920 and 2851 cm^{-1} , evidenced modification of the biochars'
227 structure and release of carbonaceous compounds into the liquid phase.

228

229 The non-depolymerised MAB FT-IR spectrum included ten signals at 455, 705, 873, 1027,
230 1415, 1574, 2851, and 2920 cm^{-1} . The strongest signals corresponded to 450, 873, 1027, and
231 1415 cm^{-1} . The strong and sharp signals observed at 873 cm^{-1} (aromatic C–H), 1415 cm^{-1} (C=O,
232 C–C ring stretch), 2851 cm^{-1} (C–H aliphatic) and 2920 cm^{-1} (C–H aromatic and unsaturated)
233 were the key signatures of the MAB spectra. In contrast, the depolymerised MAB spectrum
234 exhibited three bands (400 to 700, 800 to 1200, and 1200 to 1700 cm^{-1}) dominating the peak
235 profile. The 400 to 700 cm^{-1} band contained a new strong peak at 415 cm^{-1} connected with
236 potassium presence (K–OH). The bands of 800 to 1200, and 1200 to 1700 cm^{-1} shared signals
237 with the non-depolymerised MAB. The chemically depolymerised MAB carbon associated
238 peaks (873, 1027, 1415 and 1574 cm^{-1}) showed considerable reduction in the intensity and
239 sharpness of the peaks. In a like manner, the carbon related peaks at 2851 and 2020 cm^{-1} were
240 also reduced considerably. The reduction in the carbon related peaks demonstrates the
241 reduction in carbonaceous linkages resulting from the depolymerisation process, and the
242 possible release of carbonaceous compounds to the liquid phase.

243

244 The non-depolymerised RSB FT-IR spectrum had eight peaks at 455, 775, 873, 1078, 1415,
245 1574, 2851 and 2920 cm^{-1} . The most intense band associated with carbon linkages was the
246 band between 900 and 1200 cm^{-1} with a maximum at 1078 cm^{-1} (C–OH hydroxyl). The band
247 between 1700 and 1250 cm^{-1} contained two strong signals, 1415 cm^{-1} (C=O, C–C ring stretch)
248 and 1574 cm^{-1} (C=O, COO^-). The aromatic signals at 873, 2851 and 2920 cm^{-1} were present,
249 but were less intense than MAB. However, the signal at 775 cm^{-1} was sharper and more intense.

250 On the other hand, the chemically depolymerised RSB spectrum had three principal changes
251 in their spectra compared with the non-depolymerised biochar. First, a significant increase
252 between 400 and 600 cm^{-1} with a max at 415 cm^{-1} (K–OH). Second, an intensity reduction in
253 the band between 800 and 1200 cm^{-1} . Third, the disappearance of the signal at 775 cm^{-1} . The
254 aromatic signals at 873, 1415, 1574, 2851 and 2920 cm^{-1} decreased significantly between
255 spectra, although they were still observed in the depolymerised RSB spectrum. The RSB
256 depolymerisation reaction produced a reduction of intensity in the signals associated with
257 carboxyl, hydroxyl and methyl linkages, indicating possible release of this type compounds
258 into the liquid phase.

259

260 The SSB spectrum had nine peaks at 455, 775, 873, 1078, 1320, 1415, 1574, 2851 and 2920
261 cm^{-1} . The most intense peaks were 455, 1078, 1415 and 1574 cm^{-1} . The 455 cm^{-1} signal is
262 associated with potassium linkages and the final three are correlated with carbon linkages
263 between aromatic carbons and with substituents such as hydroxyl, carboxyl, or ester. In contrast
264 to non-depolymerised SSB, the chemically depolymerised SSB spectrum had four significant
265 changes. First, a significant rise between 400 and 600 cm^{-1} with a max at 415 cm^{-1} (K–OH).and
266 two shoulders. Second, an intensity reduction in the band between 850 and 1200 cm^{-1} including
267 a shift in the maximum signal wavenumber from 1078 cm^{-1} to 1027 cm^{-1} . Third, the complete
268 reduction of the signals at 873, 1078, 2851 and 2920 cm^{-1} . Fourth, a significant reduction of
269 the signals at 775, 1415 and 1574 cm^{-1} . Aromatic, carboxylic and hydroxyl linkages
270 participated the most in the depolymerisation reaction, which indicates possible release of
271 compounds with these linkages into the liquid phase.

272

273 **3.2 Biochar-derived carbonaceous nanomaterials characterisation.**

274 The liquid phases obtained from the biochar depolymerisation were mixed with an organic
275 solvent sequentially until obtaining BCN. The liquid phases obtained differed among the three
276 biochars. The RSB and SSB generated a liquid with a dark brown colour while MAB produced
277 a dark orange liquid. After the purification process, all the BCN solutions had yellowish and
278 light brown colours. The BCN yield varied for each material, evidencing the effect in the initial
279 feedstock and the production process. The highest yield (BCN g/ Biochar g) was obtained by
280 MAB-CN (13%), followed by SSB-CN (7%) and RSB-CN (4%). Lower yields can be
281 increased by including more biochar depolymerisation cycles.

282 **Figure 2** illustrates the characterisation of the MAB-CN. AFM microscopy (**Figure 2a**) was
283 employed to study MAB-CN topography. The particles height had a normal distribution
284 confirmed by the Kolmogorov-Smirnov test (**Annex 1, Supplementary material**). The MAB-
285 CN had an average height of 4.7 ± 0.96 nm with a minimum height of 2.9 nm and a maximum
286 height of 7.3 nm (**Figure 2b**). The MAB-CN had a lateral dimension of 68 ± 25 nm, with the
287 smallest lateral dimension of 38 nm and the maximum lateral dimension of 153 nm. The AFM
288 section (diagonal line white line) described the height and distance among particles. The
289 section included particles of different heights, but in quantities similar to the height distribution
290 (**Figure 2c**). The spectroscopic characterisation was performed using fluorescence, UV-Vis
291 and FTIR spectroscopy. MAB-CN emission and excitation spectra at various pH. MAB-CN
292 exhibited their maximum excitation and emission wavelengths at 328 and 400 nm, respectively.
293 The particles emitted fluorescence when excited up to 450 nm, where an increase in the
294 excitation wavelength produced a reduction in the fluorescence emitted and a corresponding
295 increase in the emission wavelength (see **Annex 2, supplementary material**). The MAB-CN
296 pH studies exhibited a small variation ($\pm 2\%$) in the magnitude of the emitted fluorescence. In
297 contrast, the peak of excitation scan fluorescence (328 nm) decreased around 2% after each pH
298 unit reduction from pH 8 to 5. The maximum emission and excitation wavelengths were not

299 affected by the pH changes (see **Annex 2, supplementary material**). The MAB-CN' FTIR
300 spectrum (**Figure 2e**) indicated a mixture of chemical bonds (see **Annex 2, supplementary**
301 **material**). However, the majority of the wavenumbers and the strongest signals were
302 associated with the presence of carbon linkages (648, 719, 1413, 1561, 1667, 2957, 2933 and
303 2871 cm^{-1}). Bonds associated with aromatic carbons were the strongest signals (1561, 1413
304 cm^{-1}) with C–H bonds, C–O or C=O bonds and aromatic bonds comprised 62% of the
305 wavenumbers identified. Additionally, the MAB-CN FTIR spectra demonstrated the probable
306 presence of sulphur (1013 and 648 cm^{-1}), nitrogen (1377 cm^{-1}) and silica (753, 404 and 511
307 cm^{-1}) linkages. The hydrodynamic diameter and zeta potential in solution of MAB-CN (see
308 **Annex 2, supplementary material**) described molecules with a hydrodynamic diameter of
309 approximately 200 nm. The zeta potential described negatively charged molecules with
310 moderate stability (-39.9 mV).

311

312 **Figure 3** exhibits the spectroscopic and morphologic characterisation of RSB-CN. The AFM
313 images (**Figure 3a**) described a wide range of heights and lateral dimensions. The RSB-CN
314 average height was 6.7 ± 2.8 nm with a minimum height of 3.3 nm and a maximum height of
315 16 nm (**Figure 3b**). The particle height distribution did not fit a normal distribution (Annex 1,
316 Supplementary material). However, the majority of the RSB-CN heights (89%) were below 10
317 nm. The RSB-CN average lateral dimension was 95.8 ± 47.4 nm with a maximum of 319.7 nm
318 and a minimum of 45.1 nm. The AFM section (**Figure 3c**) described a horizontal section (white
319 line) in which it was possible to identify the different particles heights in the sample. RSB-CN
320 fluorescence spectra (**Figure 3d**) showed the maximum emission and excitation signals at 420
321 and 330 nm, respectively. The excitation spectra contained a series of small peaks that became
322 sharper with the pH reduction. At alkaline pH, the peaks formed a band from 300 to 350 nm,
323 with three peaks at 340, 330 and 313 nm where the 340 nm peak was largest. From pH 6 to pH

324 3, the strongest excitation peak was observed at 330 nm. The emission peak sharpness changed
325 with the pH reduction, but the maximum emission wavelength was located at 420 nm for all
326 pHs. The pH strongly influenced the emission and excitation fluorescence generated by RSB-
327 CN. The pH reduction created a 7.5% linear increase in both the emission and excitation
328 fluorescence for each pH unit reduced. The difference between the fluorescence emitted by
329 RSB-CN at pH 8 and pH 3 was almost 40% (**Annex 2, supplementary material**). The RSB-
330 CN FTIR spectrum (**Figure 3e**) had signals grouped in three large bands from 400 to 1100 cm^{-1} ,
331 from 1100 to 1800 cm^{-1} and from 2000 to 4000 cm^{-1} . The most intense signals were located
332 in the 1100 to 1800 cm^{-1} with three peaks at 1563 (C–C stretching, C=C aromatic stretching),
333 1393 ($-\text{COO}^-$ symmetrical vibrations), and 1367 cm^{-1} ($-\text{COOH}$). The 400 to 1100 cm^{-1} band
334 included half of the spectrum's peaks and diverse functional groups such as C–O and C=O
335 bonds, S–C bonds, aromatic signals, and Si–O bonds (see **Annex 2, supplementary material**).
336 The 2000 to 4000 cm^{-1} band comprised three wide signals with a flat peak revealing the
337 presence of OH and C–H linkages in the RSB-CN structure. The FTIR spectrum indicated
338 nanoparticles rich in aromatic structures with a significant amount of substituents especially,
339 carbonyl hydroxyl and methyl groups. The hydrodynamic diameter and zeta potential in
340 solution of RSB-CN (see **Annex 2, supplementary material**) described molecules with a
341 hydrodynamic diameter of approximately 200 nm and a large negative zeta potential (-65.8
342 mV) indicating particles with high stability in solution.

343

344 **Figure 4** depicts the spectroscopic and morphologic characterisation of SSB-CN. The AFM
345 morphologic characterisation (**Figure 4a**) evidenced an average height of 2.5 ± 1.7 nm with a
346 minimum of 0.4 nm and a maximum of 9.2 nm. The particle height's distribution did not fit a
347 normal distribution (**Annex 1, Supplementary material**) as it was a positive skewed
348 distribution (skewness: 1.85) (**Figure 4b**). In this distribution, 90% of the particles had a height

349 below 5 nm and 50% below 2 nm. The lateral dimension average of the particles was $54.6 \pm$
350 43.5 nm with a minimum lateral dimension of 17.6 nm and a maximum lateral dimension of
351 223.3 nm. The AFM section analysis (**Figure 4c**), exhibits a horizontal section (white line)
352 with a majority of particles below 5 nm, corresponding with the height distribution. The
353 fluorescence spectra (**Figure 4d**) revealed the maximum excitation peak around 310 nm and
354 the maximum emission peak at 420 nm. A pH decrease caused an increase in SSB-CN
355 fluorescence of almost 10% between pH 8 and pH 4, with the increase linear between pH 8 and
356 5 (see **Annex 2, supplementary material**). pH 3 generated a 6% reduction in the emission
357 fluorescence versus pH 4. The excitation fluorescence increased with a reduction from pH 8 to
358 pH 5, and reduced from pH 4 and pH 3. The maximum emission wavelength was constant at
359 all pHs. Whereas, the maximum excitation wavelength shifted 8 nm at pH 3. The SSB-CN
360 FTIR spectrum (**Figure 4f**) had three bands at 400 to 1100, 1100 to 1800 and 2800 to 4000 cm^{-1}
361 ¹. The most intense signals were 1562 and 1395 cm^{-1} and the maximum peaks in the 1100 to
362 1800 band cm^{-1} . These peaks were associated with the presence of aromatic compounds and
363 carbonyl groups. The 400 to 1100 cm^{-1} band comprised wavenumbers correlated with
364 functional groups such as aromatic, carbonyl, C-H, C-S and O-Si (see **Annex 2,**
365 **supplementary material**). The band between 2800 and 4000 cm^{-1} contained two peaks,
366 indicating hydrogenation in the SSB-CN structure. The SSB-CN hydrodynamic diameter was
367 on average below 150 nm and the majority of the particles were in only one distribution peak
368 (see **Annex 2, supplementary material**). SSB-CN had a large negative zeta potential (-63
369 mV) indicating particles with high stability in solution .

370

371 SSB-CN and RSB-CN AFM images exhibited a more intersected configuration, which
372 resembled a honeycomb organisation. These levels of organisation can be related to chemical
373 interactions, such as between BCN itself or the mica and the BCN, or to BCN structural changes

374 associated with water removal. The fluorescence spectra provided one of the most significant
375 differences among the three BCN. The SSB-CN, RSB-CN and MAB-CN had Stokes shifts of
376 109 nm, 90 nm and 72 nm, respectively. The pH effect on the emission and excitation spectra
377 differed as well. In SSB-CN and RSB-CN, decreasing the pH increased the emission and
378 excitation fluorescence while MAB-CN were not affected by pH changes. The increase in the
379 fluorescence is likely associated with the structure of these nanomaterials since SSB-CN and
380 RSB-CN are richer in carboxylic and hydroxyl groups than MAB-CN. As these groups are
381 commonly identified as fluorophores for carbonaceous nanomaterials [25,26], changes in the
382 pH will modify the carboxylic and hydroxyl groups by producing dissociation and association
383 of the hydrogen atoms. As illustrated by the FTIR spectra, the BCN had an aromatic structure
384 with several types of substituents in their structure. The principal differences among the three
385 FTIR spectra were observed in the number and intensity of the peaks and shoulders between
386 400 and 1100 cm^{-1} and between 1200 and 1800 cm^{-1} . The three BCN shared the signals at 1561,
387 1008, 701, 646 and 620 cm^{-1} . All these signals are carbon bonds involved in aromatic rings,
388 carbonyl linkages and S-C linkages. These signals indicated the prominence of aromatic
389 groups in BCN structures, which is a constant component on Cdots from lignocellulosic
390 material [27]. A significant difference was observed between 1200 and 1500 cm^{-1} . MAB-CN
391 had a max peak at 1413 cm^{-1} with four shoulders. RSB-CN had two maximum peaks at 1393
392 and 1367 cm^{-1} without shoulders. SSB-CN had only a maximum signal at 1395 cm^{-1} with three
393 shoulders. Additionally, the relationship between the two peaks between 1200 and 1800 cm^{-1}
394 is another indicator of structural differences. In MAB-CN, the 1800 cm^{-1} peak was significantly
395 greater than the 1200 cm^{-1} peak, while in RSB-CN and SSB-CN both peaks have similar sizes.
396 RSB-CN and SSB-CN had a considerable number of common peaks, but with different
397 intensity and sharpness. The majority of uncommon signals in the RSB-CN spectrum were
398 from shoulders or bands associated with hydroxyl and C-H bonds (2800-2200, 1800-1900,

399 1688, 1617, 1438, and 880 cm^{-1}). The uncommon bands in the SSB-CN correlated with
400 aromatic C–H and S–O bonds. The presence of sulphur, nitrogen and silica bonds in all the
401 samples indicate that the BCN had a different composition than other carbonaceous
402 nanomaterials such as Cdots or graphene carbon dots but with similar optical properties as
403 other nanomaterials from lignocellulosic material [16]. All the BCN had moderate to high
404 negative zeta potential indicating their facility to interact with positive particles such as heavy
405 metal ions.

406

407 **3.3 Biocompatibility studies**

408 The effect of the BCN in the yeast growth is summarised in **Table 1**. Additionally, the growth
409 curves from the biocompatibility studies for each yeast species are in the **Annex 3** of the
410 supplementary material. At all pH, yeast species, and BCN types, concentrations of 100 ppm
411 or below did not generate significant changes in the yeasts' growth curves. MAB-CN produced
412 various effects in the three yeast species. MAB-CN did not modify the *Y. lipolytica* growth
413 curves at any pH or MAB-CN concentrations. In contrast, *S. cerevisiae* and *C. albicans*
414 evidenced modifications in their growth curves. *S. cerevisiae* growth was inhibited at pH 10
415 and concentrations above 100 ppm. The growth inhibition was correlated with the increase of
416 the MAB-CN concentration. At pH 7, a slight inhibition occurred at 500 and 1000 ppm.
417 However, the inhibition did not correlate with the MAB-CN concentration. At pH 4, the only
418 inhibition was observed at 1000 ppm and was similar to that observed at pH 7. *C. albicans* was
419 inhibited at 250, 500 and 1000 ppm at basic and neutral pH, 1000 ppm and 500 ppm generated
420 considerable inhibition. At pH 4, MAB-CN at 1000 ppm inhibited *C. albicans* growth.
421 However, the inhibition was less significant than the other pHs. In general, at acid pH the yeast
422 species experienced less inhibition.

423

424 RSB-CN exhibited an inhibitory effect at alkaline pH and concentrations of 500 ppm and 1000
425 ppm. *S. cerevisiae* and *Y. lipolytica* were partially inhibited at 500 ppm and completely
426 inhibited at 1000 ppm. In contrast, *C. albicans* was completely inhibited at both concentrations.
427 At neutral and acidic pH, the RSB-CN concentrations tested did not inhibit *C. albicans*, but the
428 log phase of the curves were less sharp with the pH rise. In *S. cerevisiae* and acidic pH, RSB-
429 CN did not produce inhibition at any concentration. At concentration above 250 ppm and
430 neutral pH, RSB-CN generated a low inhibition in *S. cerevisiae*. *Y. lipolytica* at neutral and
431 acid pH was not inhibited by any concentration of RSB-CN

432

433 SSB-CN was the most bio-compatible material, as *S. cerevisiae*, *C. albicans*, and *Y. lipolytica*
434 were not inhibited at any of the pHs and SSB-CN concentrations. The changes in the growth
435 curves patterns were associated with the pH changes instead of the concentration or presence
436 of SSB-CN. This evidenced SSB-CN's favourable characteristic as it can be used at any
437 concentrations at neutral and alkaline pHs without generating inhibition.

438

439 **3.4 BCN bioimaging**

440 **Figure 5** displays confocal fluorescence microscopy images recorded after 2 h of growth with
441 BCN. The image illustrated BCN uptake by the three yeast that depended on a combination of
442 BCN type and yeast. **Figure 5a** describes the effect of MAB-CN in the three yeast species. *S.*
443 *cerevisiae* exhibited a less intense signal with the fluorescence observed throughout the entire
444 cell. *C. albicans* fluorescence was localised in a cellular organelle for some cells and distributed
445 the entire cell possibly indicating multiple uptake/distribution processes. *Y. lipolytica*

446 fluorescence was localised in one of the cytoplasmic organelles. RSB-CN fluoresced in all the
447 yeast (**Figure 5b**) with a varied localisation and fluorescence intensity dependent on the yeast.
448 *S. cerevisiae* fluorescence localisation was low with small points inside the cells. This can be
449 associated with some interaction between RSB-CN and molecules in the cytosol. *C. albicans*
450 evidenced a diverse distribution of RSB-CN inside the cells. However, it was possible to
451 identify particles concentrated in specific zones in the cells. In *Y. lipolytica*, RSB-CN exhibited
452 a well-localised fluorescence inside the cells demonstrating the introduction of these materials
453 in a specific organelle. SSB-CN exhibited fluorescence in all the yeast species (**Figure 5c**). In
454 *S. cerevisiae*, SSB-CN had fluorescence throughout the entire cell. In *C. albicans* and *Y.*
455 *lipolytica*, SSB-CN the fluorescence was localised in cellular compartments. The control using
456 only PDB did not generate any fluorescence either associated with the BCN or any
457 autofluorescence from the cells.

458

459 The CTCF differences among the combinations of yeast species and BCN types were analysed
460 with a two-way non-balanced ANOVA (**Annex 3, supplementary material**). As the ANOVA
461 *p-value* (<0.0001) was lower than the alpha (0.05), at least one of the 9 combinations of BCN
462 and yeast species were different. Additionally, the two main factors (yeast: <0.0001 and BCN:
463 <0.0001) and the interaction between factors (YEAS*BCN: 0.0018) were significant for the
464 model. As the interaction between the factors was significant, the interaction plots (**Figure 6**)
465 were necessary to analyse the fluorescence emitted by the yeast cells with each BCN. **Figure**
466 **6a** describes how the yeast species were influenced by each BCN. *S. cerevisiae* had the lowest
467 CTCF for all the BCN. *C. albicans* and *Y. lipolytica* had similar CTCF when grown with RSB-
468 CN and SSB-CN. In contrast, the cultures with MAB-CN had a CTCF significantly higher in
469 *Y. lipolytica* than *C. albicans*. Moreover, MAB-CN was the only BCN presenting significant
470 CTCF differences among the three yeast species CTCF. **Figure 6b** depicts the effect of each

471 BCN in the yeast species. RSB-CN exhibited the lowest CTCF in all the yeast species. *C.*
472 *albicans* had the highest CTCF with SSB-CN and was significantly different from RSB-CN
473 and MAB-CN, which had a similar CTCF. In contrast, *Y. lipolytica* and *S. cerevisiae* generated
474 the largest CTCF when mixed with MAB-CN and SSB-CN. In those BCN, the CTCF was not
475 significantly different. MAB-CN present advantages as a future discrimination probe since the
476 yeast species' CTCF varied. However, the SSB-CN exhibited the highest CTCF in all the yeast,
477 making this BCN the most appropriate for fluorescence imaging

478

479 **3.5 Heavy metal ions detection in aqueous systems**

480 The interactions between 12 heavy metal ions and the three BCN is depicted in **Figure 7**. Hg
481 (II) and Cu (II) ions quenched MAB-CN significantly, having fluorescence reduction
482 percentages of 41.5% and 27%, respectively (**Figure 7a**). Pb (II), Ni (II), Co (II) and Ag (I)
483 ions quenched MAB-CN in percentages between 10% and 15%. Mn (II), Mo (IV), Li (I), and
484 Ba (II) ions did not quench the MAB-CN fluorescence. In contrast with the other heavy metal
485 ions, Zn (II) ions increased MAB-CN fluorescence significantly (15%). RSB-CN was
486 significantly quenched by Cu (II) and Pb (II) ions with fluorescence reduction percentages of
487 39% and 29%, respectively (**Figure 7b**). Similar to MAB-CN, the second tier of quenching
488 included metal ions with fluorescence reduction percentages between 10% and 15% including
489 Ni (II), Co (II), Fe (II) Hg (II), Mn (II) and Ag (I). Cu (II) (43%) was the only heavy metal ion
490 that significantly quenched SSB-CN fluorescence. Pb (II) ions obtained the second highest
491 quenching with 15%, while the rest of the heavy metal ions achieved fluorescence reductions
492 below 10%. The significant difference between the quenching obtained by Cu (II) ions and the
493 other metal ions indicates a selectivity between SSB-CN and Cu (II) ions that was not observed
494 in the other BCN (**Figure 7c**). Similar to MAB-CN, Mo (IV), Li (I), and Ba (II) ions did not

495 quench RSB-CN and SSB-CN. Whereas, Zn (II) ions increased the fluorescence emitted by
496 RSB-CN and SSB-CN, but the fluorescence rise in those BCN was 50% and 75% lower than
497 the rise in MAB-CN, respectively. The lowest heavy metal ions quenching in all the BCN was
498 obtained by Mo (IV), Li (I) and Ba (II) ions.

499

500 The metal ions with the highest quenching in each BCN were used to evaluate the correlation
501 between heavy metal ion concentration and BCN fluorescence reduction or increase (**Figure**
502 **8**). The RSB-CN and SSB-CN emission fluorescence spectra at different concentrations of Cu
503 (II) ions are shown in **Figures 8a** and **8b**. The limit of detection (LOD) for the RSB-CN and
504 Cu (II) ions and SSB-CN and Cu (II) combinations was 0.5 μM . The Stern-Volmer plot for
505 these combinations evidenced a liner correlation (**Figure 8a and 8b embedded figures**). A
506 linear Stern-Volmer plot indicates collisional quenching and can be modelled using the Stern-
507 Volmer equation: $F_0/F = 1 + K_{SV}[Q]$, where K_{SV} is the Stern-Volmer quenching constant and
508 $[Q]$ is the concentration of the quencher molecule, in this case the Cu (II) ions. The SSB-
509 CN/Cu(II) combination had a K_{SV} of $0.017 \text{ L}\cdot\mu\text{Mol}^{-1}$ while the RSB-CN/Cu(II) combination
510 had a K_{SV} of $0.0162 \text{ L}\cdot\mu\text{Mol}^{-1}$. A larger K_{SV} indicates a larger interaction between heavy metal
511 ions and the fluorophores. Therefore, the greater quenching observed in SSB-CN/Cu (II) is
512 explained by the higher SSB-CN's K_{SV} . As Hg (II) was the metal ion with the largest
513 fluorescence reduction in MAB-CN, the effect of Hg (II) ions concentration on MAB-CN
514 concentration was evaluated (**Figure 8c**). The LOD for the MAB-CN/ Hg (II) combination was
515 $0.4 \mu\text{M}$. The Stern-Volmer plot for the MAB-CN/ Hg (II) combination evidenced a nonlinear
516 behaviour with a downward curvature (**Figure 8c embedded figure**). Such curves are obtained
517 by pure collisional quenching when some of the fluorophores are less accessible than others
518 [28,29]. The non-linear downward behaviour depends of diverse variables and could not be
519 empirically modelled. Although, the fluorescence reduction percentage obtained by MAB-

520 CN/Hg (II) had a similar percentage as the RSB-CN/Cu(II) and SBB-CN/Cu(II), the difference
521 between their Stern-Volmer plots evidenced a lower interaction between the MAB-CN/Hg (II)
522 than the RSB-CN/Cu(II) and SBB-CN/Cu(II).

523

524 As the Zn (II) ions produced a fluorescence increase, the influence of the Zn (II) ions
525 concentration was evaluated using the MAB-CN/Zn (II) combination. This combination was
526 selected because it achieved the highest fluorescence increase. The Zn (II) ions did not increase
527 the MAB-CN fluorescence at concentrations below 5 μM (**Figure 8d**). At 5 μM , the
528 fluorescence increased until 1000 μM . However, the fluorescence increase from 500 μM to
529 1000 μM was less than 15% of the total fluorescence rise. The limit of detection for this ion
530 was 9 μM with a range of detection between 10 and 1000 μM . As the MAB-CN fluorescence
531 increased, the Stern-Volmer plots could not be used. Therefore, the fluorescence increase
532 percentage (%) (**Equation 2**) was calculated to describe the interaction between MAB-CN and
533 Zn (II) ions (**Figure 8d embedded image**). In the concentration range between 5 and 1000 μM ,
534 the MAB-CN fluorescence and Zn (II) ions were correlated with a logarithmic equation ($Y =$
535 $7.0187\ln(x) - 12.773$, $R^2 = 0.9814$). As the model is an empirical approach, it was not possible
536 to correlate the constants with measurable properties from the Zn (II) ions or MAB-CN.

537

538 **4 DISCUSSION**

539 This is the first article showing the versatility of chemical depolymerisation and solvent
540 extraction (NanoRefinery) for producing biochar-derived carbonaceous nanomaterials from
541 different feedstocks (rice straw, sorghum straw and microalgae) and different thermal
542 conversion processes. These carbonaceous nanomaterials had different optical and chemical

543 properties, evidencing the importance of the original biochar feedstock and the production
544 process in the resulting materials. The effect of the thermal conversion process conditions, such
545 as reactor type, heating rate, final temperature, residence time, catalyst presence, oxygen
546 concentration etc. are significant variables that can affect the type of carbonaceous
547 nanomaterials produced. In this case, MAB and RSB were obtained with batch pyrolysis
548 whereas SSB was obtained with fluidized bed pyrolysis. However, it was not possible to
549 identify specific properties associated with the initial processing conditions. Further studies are
550 necessary to understand the details of the interaction between process conditions and feedstock
551 for the combined production of bioenergy and carbonaceous nanomaterials.

552

553 Biochar from bioenergy production used as a raw material for the production of nanomaterials has
554 the advantages of utilising a high variety of wastes, being coupled with bioenergy production, and
555 generating a diversity of carbonaceous nanomaterials with different properties. These differences
556 can be tuned to develop new types of renewable nanomaterials and novel application such as
557 the treatment of polluted water or bioimaging. BCN exhibited different heights and lateral
558 dimensions, and different chemical groups in their structure. In all cases, the materials had a
559 high negative zeta potential that can be associated with the ability to interact with heavy metal
560 ions, which generally have positive charge. Further research needs to be focused on the
561 modification of BCN, BCN applications and the development of other types of nanomaterials.

562

563 Microalgae, rice straw and sorghum straw have been utilised for the production of other
564 carbonaceous nanomaterials. Microalgae carbon dots were obtained from eutrophic algal
565 bloom (EAB-Cdots) and microwave thermolysis [30]. Rice straw has been employed for the
566 production of carbon dots [14] and a combination of silica and carbon dots materials [27].

567 Whereas, sorghum straw has been used for producing Cdots as a tool for detecting chromium
568 (Cr^{3+}) ions in aqueous media [31]. In contrast with SSB-CN, sorghum straw carbon dots detected
569 Cr^{3+} ions via fluorescence enhancement instead of quenching.

570

571 In this work, BCN biocompatibility experiments demonstrated that SSB-CN were the most
572 biocompatible material as none of the yeast species, in any of the conditions evaluated, exhibited a
573 modification in their growing curves. This result is comparable with other carbonaceous
574 nanomaterials that did not demonstrate a toxic effect on yeast [32]. *Y. lipolytica* was the most
575 compatible yeast species as only RSB-CN concentrations of 500 ppm and 1000 ppm at alkaline
576 pH were able to inhibit these yeast. *S. cerevisiae* and *C. albicans* were affected by RSB-CN
577 and MAB-CN at alkaline pH and neutral pH. In all BCN, acidic pH was associated with yeast
578 resistance to higher concentrations of carbonaceous nanomaterials. This is principally associated
579 with yeast's physiological conditions where acidic pH is the most favourable condition for growing
580 this type of microorganisms. At all pH, MAB-CN was the only nanomaterial able to inhibit the
581 growth of *S. cerevisiae* and *C. albicans* using concentrations of 1000 ppm. This result opens
582 the door to a possible application of MAB-CN as an antifungal. The concentrations that achieved
583 inhibitory effect by MAB-CN are below the concentrations that achieved antifungal effect in *Pichia*
584 *pastoris* using citric acid-derived carbon dots ($25 \text{ mg mL}^{-1} = 25000 \text{ ppm}$) [33] and close to the
585 concentrations of Vitamin C derived-Carbon dots ($300 \mu\text{g mL}^{-1} = 300 \text{ ppm}$) with antifungal effect in
586 *Rhizoctonia solani* and *Pyricularia grisea* [34]. At neutral pH, the MAB-CN inhibitory effect can
587 be achieved with a lower concentration (500 ppm) evidencing the potential of this carbonaceous
588 nanomaterial as an antimicrobial. Future work will focus on the evaluation of BCN as antimicrobial
589 agents and the mechanisms associated with the antimicrobial effect.

590

591 This article proved that yeast species had a differential uptake and localisation of BCN. The
592 differential uptake was identified by the differences in the fluorescence emitted by the BCN
593 inside the yeast species. Differential uptake of carbonaceous nanomaterials (Cdots and CN)
594 has been previously demonstrated in human and bacterial cells. In human cells, these
595 differences were employed to differentiate between healthy and cancerous cells. Whereas in
596 bacterial cells, it was utilised to differentiate between live and dead cells [35] as well as gram
597 positive and gram negative bacteria [36]. In yeast species, to our knowledge, this is the first
598 research reporting the differential uptake of carbonaceous nanomaterials. As evidenced by the
599 confocal images (**Figure 5**), the BCN localisation inside the yeast cells also varied with some
600 yeast localising these compounds in cellular organelles (*C. albicans* and *Y. lipolytica*) while
601 others distributed them in the whole cell (*S. cerevisiae*). Additionally, these results showed the
602 effective internalisation of BCN into the yeast's cytosols and organelles, indicating the possible
603 use of BCN as nano-carriers for drug delivery or for imaging specific organelles. The
604 differences, in localisation and uptake, reported in this article are the initial steps for developing
605 fast microbial identification methods based on the combination of BCN and the different
606 interactions between microbial species and the BCN.

607

608 BCN interact with various heavy metal ions. The different quenching levels and dynamics
609 registered by each heavy metal ion/BCN combination can be correlated with the chemical,
610 electronic and vibrational characteristics of each material [37]. SSB-CN had the most
611 selective quenching as it only had high quenching with Cu (II) ions. Whereas, MAB-CN was
612 selective for Zn (II) detection as it was the only heavy metal ion producing a fluorescence
613 enhancement. Selectivity is a common property in other types of carbonaceous nanomaterials
614 such as Lotus root-derived carbon dots, chocolate derived Cdots and pigeon feathers Cdots,
615 which were selective to Hg (II), Pb (II) and Fe(III), respectively [38-40]. Compared with

616 these materials SSB-CN had similar limits of detection and a slightly wider range of
617 detection. The high selectivity evidenced by these materials make them the most promising
618 BCN for developing a sensing method to detect Cu (II) and Zn (II) in aqueous systems. BCN-
619 CN can be used as a heavy metal ion detection probe. However, other strategies are necessary
620 to improve the selectivity in detection of heavy metal ions using these materials. Some of
621 these strategies include the addition of phosphorous or nitrogen groups, introduction of a
622 secondary set of materials, and the use of multivariate statistics and additional sets of
623 measurements [13]. BCN structure is rich in C–O, C=O and C-OH linkages, these functional
624 groups with unshared electron pairs are responsible for forming coordination bonds with
625 heavy metal ions and producing the fluorescence reduction. The fluorescence increase
626 observed in all the BCN with some heavy metal ions is a significant result as the increased
627 fluorescence by the interaction with CNs has only been reported in Cdots synthesised from
628 rice using a microwave assisted method [41]. The chemical interaction between Zn (II) and
629 other carbonaceous compounds for enhancing the fluorescence is associated with linkages to
630 nitrogen groups (amide and amine) and carbon oxygen linkages with free electron pairs
631 (C=O) [42]. The presence of some nitrogen groups was evidenced in the FT-IR spectra.
632 However, the most significant signals come from carbon linkages with free electron pairs. As
633 the nitrogen groups were lower than the C=O groups, it is possible that the fluorescence
634 enhancement followed similar interactions as other fluorescent compounds such as
635 fluorescein, coumarin and rhodamine [43-45]. In these compounds, the fluorescence
636 enhancing interactions have a reduced participation of nitrogen compared with the C=O
637 linkages. Future work will focus on evaluating the combination of BCN and heavy metal ions
638 with multivariate analysis for improving their selectivity, the evaluation of matrices for easy
639 and portable detection of heavy metal ions, and the evaluation of BCN as probes for the
640 detection of biomarkers.

641

642 **5 CONCLUSIONS**

643 This work demonstrated the significant effects of initial biochar feedstock and production
644 process on the final physicochemical properties as well as biocompatibility, bio-imaging, and
645 heavy metal sensing applications of BCN. The three types of BCN exhibited different optical
646 and chemical characteristics. However, the SSB-CN and RSB-CN were more similar than
647 MAB-CN. The biocompatibility between yeast species and BCN depended of the BCN type,
648 pH and BCN concentration. SSB-CN did not produce a negative effect to the yeast species at
649 any of the conditions evaluated. RSB-CN had a negative effect at alkaline pHs, In contrast,
650 MAB-CN inhibited the growth of *S. cerevisiae* and *C. albicans* at all the tested pHs and
651 concentrations above 500 ppm and evidenced its possible use as an antifungal agent. All the
652 BCN were suitable as a bioimaging probe for yeast bioimaging and had different
653 fluorescence intensity and the localisation depending of the yeast cells. The intensity of the
654 signals and lack of toxicity of SSB-CN suggest this nanomaterial as the most suitable for
655 bioimaging applications. On the other hand, an initial investigation of BCN as heavy metal
656 ions sensors demonstrated the possible use of SSB-CN and MAB-CN as transducers for the
657 detection of Cu (II) and Zn (II) ions, respectively. Cu (II) selectively quenched SSB-CN
658 (LOD 0.4 μ M) and Zn (II) enhanced MAB-CN fluorescence (LOD 9 μ M). This research is
659 the first steps to understand the differences between BCN and further utilise them to develop
660 novel and sustainable methods for cell bioimaging and chemical compounds detection.

661

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669

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FIGURES AND TABLES LIST

Table 1. Yeast species growth inhibition using different types of BCN at different concentration and pHs.

Figure 1. Depolymerised and non-depolymerised FT-IR spectra of MAB, RSB and SSB

Figure 2. Characterisation of MAB-CN a) AFM images b) Particles height distribution c) AFM image section analysis d) Emission and excitation spectra at different pH e) FT-IR spectra

Figure 3. Characterisation of SSB-CN a) AFM images b) Particles height distribution c) AFM image section analysis d) Emission and excitation spectra at different pH e) FT-IR spectra

Figure 4. Characterisation of RSB-CN a) AFM images b) Particles height distribution c) AFM image section analysis d) Emission and excitation spectra at different pH e) FT-IR spectra

Figure 5. Confocal microscope images of *S. cerevisiae*, *C. albicans* and *Y. lipolytica* with 250 ppm of BCN. a) MAB-CN, b) RSB-CN c) SSB-CN

Figure 6. Interaction plot for the normalised CTCF a) BCN b) Yeast species

Figure 7. BCN fluorescence reduction percentage using 50 μM of 12 different heavy metal ions. a) MAB-CN, b) RSB-CN c) SSB-CN

Figure 8. Fluorescence emission spectra of BCN in the presence of different concentrations of heavy metal ions. The embedded image corresponds to the Stern-Volmer plot for the respective BCN and heavy metal ion combination (a, b, c) and the fluorescence increment % (d). a) RSB-CN/Cu (II) b) SSB-CN/Cu (II) c) MAB-CN/ Hg (II) d) MAB-CN/Zn (II).