

Recovery of humic acids from anaerobic sewage sludge: Extraction, characterization and encapsulation in alginate beads

*Original*

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1 **Title:**

2 Recovery of humic acids from anaerobic sewage sludge: extraction, characterization and  
3 encapsulation in alginate beads

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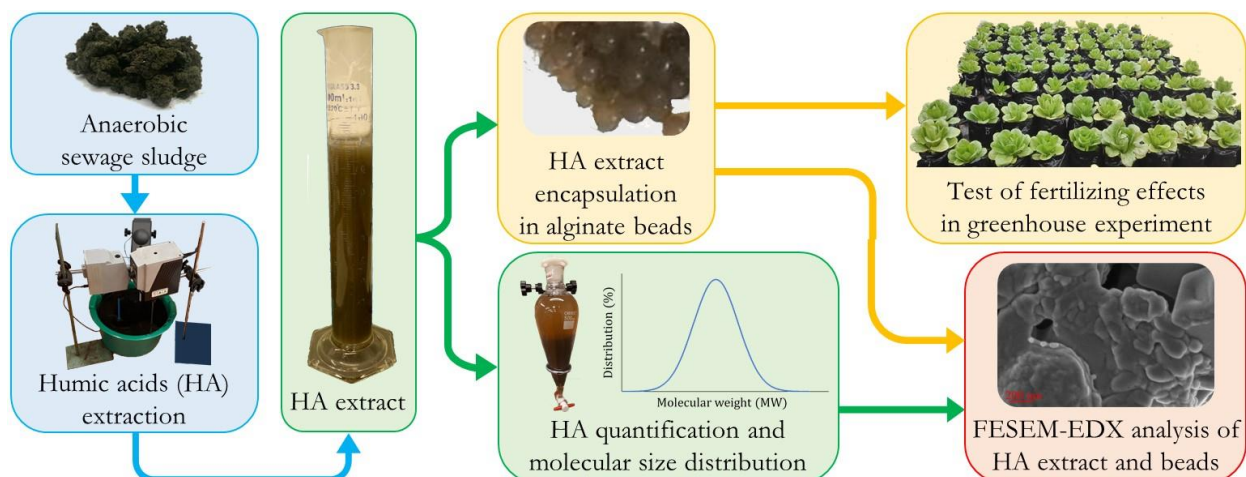
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16 **Highlights:**

- 17
- 18 • Humic acids extraction from sewage sludge anaerobically digested
  - 19 • Humic acids extracted without heavy metals
  - 20 • Study of humic acids molecular weight in the extract
  - 21 • Encapsulation of humic acids within alginate beads for controlled releasing in soil
  - 22 • Humic acids beads allows roots biomass enhancement in greenhouse experiment
- 23

24 **Graphical abstract: (in colors)**



26 **Abstract:**

27 Wastewater production is rising all over the world and one of the most difficult problems is the  
28 disposal of sewage sludge (SS). It is known that SS contains certain quantities of added-value  
29 compounds, such as humic acids (HA) which in turn have beneficial effects on soil quality and plant

30 growth. On the other hand, SS can retain many pollutants, such as heavy metals. The present work  
31 aimed to implement an HA alkaline extraction protocol from anaerobic sewage sludge (ASS).  
32 Subsequently, the HA were quantified in ASS, in HA extract and in commercial HA, used as a  
33 benchmark, which gave results of 12.53%, 26.87% and 77.87% (on dry matter basis), respectively.  
34 FESEM and EDX analyses on lyophilized HA extract confirmed that no heavy metals had passed  
35 into the extract. Afterwards, in order to allow controlled release of the HA in soils, alginate beads  
36 containing the HA extract were created. Finally, a pot experiment in a greenhouse was performed  
37 using Chilean lettuce plants (*Lactuca sativa* L.) treated with alginate-HA extract beads. At the end of  
38 the greenhouse experiments, the hypogean dry biomass of the treated plants was significantly higher  
39 than for non-treated plants. The relevance of this study relies not only on the exploitation of green  
40 chemistry principles, by converting a waste stream into a high-value product, but also on the  
41 application of an approach following a circular economy model.

42 **Keywords:** humic acids extraction, circular economy, greenhouse experiment

43 **Declaration of interest:** none.

#### 44 **Glossary of abbreviations and acronyms**

<b>Abbreviation</b>	<b>Definition</b>
ANOVA	Analysis Of Variance
ASS	Anaerobic Sewage Sludge
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy
d.m.b.	Dry matter basis
E.C.	Electrical Conductivity
EDX	Energy Dispersive X-ray
FA	Fulvic Acids
FESEM	Field Emission Scanning Electron Microscopy
HA	Humic Acids
HS	Humic Substances
NOM	Natural Organic Matter
SEC	Size Exclusion Chromatography
SS	Sewage Sludge
TOC	Total Organic Carbon
TS	Total solids
WWTP	Wastewater Treatment Plant

45

46 1. Introduction

47 Wastewater production is rising all over the world as a consequence of the increasing population  
48 and industrialization [1]. A recent study estimated that approximately 330 km<sup>3</sup> of wastewater are  
49 produced worldwide yearly [2]. For purification purposes, different treatment strategies can be  
50 carried out, producing sewage sludge (SS) as the main by-product, which is stabilized mostly via  
51 anaerobic digestion [3]. A study by the European Commission revealed that Europe produces 17 kg  
52 per capita of dry sludge per year [4]. SS is particularly rich in terms of plant nutrients, such as nitrogen  
53 and phosphorous [5] and other beneficial compounds for good plant development, such as humic  
54 substances [6]. Hence, agricultural reuse is the principal solution for SS disposal in many European  
55 countries, such as Portugal, Spain and the United Kingdom [7], as well as Chile and some other  
56 Latin American states [8]. On the other hand, SS can retain many pollutants, such as heavy metals  
57 [9]. Thus, the direct application of SS in soils can cause the accumulation of heavy metals in both  
58 the soils and plants [10]. The extraction of humic substances (HS) is a potentially interesting strategy  
59 to solve the problems associated with the presence of heavy metals and other undesirable  
60 substances in SS.

61 HS are defined as the highly transformed part of non-living natural organic matter (NOM), which is  
62 formed by organic compounds with structures that vary in their degree of complexity [11]. These  
63 substances are also defined as “the black gold of agriculture” [12] due to their beneficial effects on  
64 soil quality and plant growth, a concept present in the literature for many years [13]. HS are natural  
65 polymers with a highly heterogeneous structure and are traditionally classified as humic acids (HA),  
66 fulvic acids (FA) and humins according to their solubility. In fact, FA are soluble at all pH, HA are  
67 insoluble in acids, and humins are insoluble at all pH [14]. The molecular sizes range typically  
68 between 5 to 100 kDa for HA, and less than 10 kDa for FA [14, 15]. According to Grinhut and  
69 colleagues [16], the half-life time of HS in nature can reach thousands of years, attributable mainly  
70 to HA and humins, whose biodegradability is very slow. In sewage sludges from wastewater  
71 treatment plants, HS are present in concentrations ranging from 7.7 to 28.6%, expressed as volatile  
72 solids [17]. Typically, sludge HS are adsorbed to extracellular polymeric substances [18]. In  
73 particular, the distribution between HA and FA in sewage sludge varies between 24% and 76%,

74 depending on the characteristics of the wastewater as well as the operational conditions of the  
75 wastewater treatment plant [[19], [20], [21], [22]].

76 The positive effects of HA on plant growth usually depend on many factors [23], such as the HA  
77 concentration rate, plant species and origin of the raw material used as HA source. These effects  
78 include the improvement of the physico-chemical soil properties (such as water retention and soil  
79 structure), and the increase of enzymatic activity and soil microbial diversity [24]. Moreover, Chen  
80 and Aviad [25] demonstrated that specific dosages of these substances can enhance seed  
81 germination, stimulate root initiation and lateral root development, and boost root and shoot growth.  
82 Many mechanisms are involved in plant growth and, amongst them all, the major role is mainly  
83 attributed to the HA/plant membrane interaction. Indeed, improved performance membrane  
84 transporters allow better absorption of soil nutrients [26]. A clear example is represented by  
85 phosphorus bioavailability in soils treated with HA and P-fertilizer: HA increases water-soluble  
86 phosphate, phosphorus plant uptake and plant biomass, retarding the formation of occluded  
87 phosphate [27]. Hence, HA cannot replace mineral fertilizations, but they can provide more  
88 productive cropping systems with fewer negative impacts on the environment deriving from the lower  
89 application of fertilizers. All these characteristics consent HA to be used as biostimulants in  
90 horticulture [26]. Moreover, it is worth underlining that nowadays commercial HA derive mostly from  
91 non-renewable resources, such as leonardite, coal and peat, while only in a few cases do they come  
92 from renewable sources, such as compost and vermicompost [26].

93 Different biodegradable polymers have been studied as matrixes for the encapsulation of bioactive  
94 compounds for different applications with the aim of having a controlled release of the substances  
95 in time. The most used matrixes are chitosan, collagen, gelatin and alginate [[28], [29], [30]]. In the  
96 field of agriculture, different bioactive capsules can be found for the purpose of releasing herbicides  
97 [31], fertilizers [32, 33], pesticides [34] or even complete cells that have a symbiotic effect with the  
98 plant growth [35]. Generally, the objective of having a controlled release is to reduce the amount of  
99 product that is added to the soil, which permits the operational costs to be reduced and, more  
100 importantly, ensures a constant and correct dose of each bioactive compound. As a consequence,

101 the product is not released into the environment, avoiding the environmental issues cited above.  
102 Another attractive feature of encapsulation is the reduction of bioactive compound deterioration.

103 Among the principal commercial polymers, alginate has the advantage of being a cost-effective  
104 material, which is mainly extracted from brown algae. Alginate is a linear polysaccharide composed  
105 of two monosaccharide residues, (1,4)- $\beta$ -D-mannuronic acid (M) and (1,4)- $\alpha$ -L-guluronic acid (G).  
106 The particularity of alginate is that it can form a physical hydrogel (insoluble form) in the presence of  
107 divalent cations such as  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$ , which form an ionic cross-linking between the G monomers  
108 of two adjacent polymer chains [28]. The mechanism of the release of bioactive materials  
109 encapsulated in alginate beads can be divided in two steps, the leakage of the bioactive compound  
110 and the degradation of the matrix [30]. In fact, bioactive compounds with a size smaller than the  
111 matrix pores are leached upon water irrigation, while the bigger ones are released after the  
112 degradation of the matrix. The degradation occurs through alginate solubilization due to the action  
113 of chelating compounds or extracellular enzymes. Chelators sequester the divalent cations yielding  
114 to a disruption of the electrostatic interactions between the alginate chains and the  $\text{Ca}^{2+}$  cations. On  
115 the other hand, enzymatic degradation is carried out by alginate lyases, which hydrolyze the  
116 polysaccharide bonds. Thus, the degradation of alginate varies with the number of ionic bonds  
117 between the  $\text{Ca}^{2+}$  and G monomers and will depend on the presence of microorganisms able to  
118 produce alginate lyases enzymes in the soil [36].

119 Adopting a circular economy approach, the principal aim of the present work was to evaluate the  
120 valorization of anaerobic sewage sludge from an agronomic point of view, with the purpose of  
121 improve soil quality. More in detail, a process of extraction of HA from ASS and their encapsulation  
122 in alginate beads was implemented to obtain an added-value product free from heavy metals and  
123 contaminants. The quality of the extracted HA was assessed with the size distribution analysis of the  
124 HA molecules and through electron microscopy. Finally, the effect of the HA beads on plant growth  
125 and biomass was evaluated on lettuce plants with a pot experiment under greenhouse conditions.

## 126 2. Materials and methods

### 127 2.1. Materials

128 Anaerobic sewage sludge (ASS) was sampled from a wastewater treatment plant (WWTP) in Chile.  
129 Commercial HA, extracted from leonardite, were provided by Sanagro (Chile), Sodium alginate (food  
130 grade) was purchased from Merck. All other chemicals used (KOH,  $K_4P_2O_7$ , HCl, NaOH,  $CaCl_2$ ,  
131  $H_3BO_4$ ,  $CuSO_4 \cdot H_2O$ ,  $FeSO_4 \cdot 7H_2O$ ,  $MnSO_4 \cdot H_2O$ ,  $NaMoO_4 \cdot 2H_2O$ ,  $ZnSO_4 \cdot 7H_2O$ ) were reagent-grade.

## 132 2.2. Extraction protocol

133 An extract of HA was obtained from ASS adapting the protocol of HS alkaline extraction from soil  
134 (Stevenson, 1994). The solid ASS was firstly mixed with water. Then KOH and  $K_4P_2O_7$  was added  
135 in a ratio of 1:1 and the mixture was stirred at room temperature. The soluble part of the mixture was  
136 separated from the solids to obtain the HA extract.

## 137 2.3. Chemical analysis and HA quantification

138 Methods and results of chemical analysis of ASS are reported in **Supplementary material - 1**.  
139 Quantification of HA on ASS and HA extract was performed adapting the method proposed by Lamar  
140 and co-workers [37] on the analyzed matrixes. Quantification was carried out with the same method  
141 to the commercial HA as benchmark reference.

## 142 2.4. Molecular size distribution of HA

143 Membrane filtration was exploited to study molecular size distribution of humic acids in the HA  
144 extract. To this aim, HA extract was submitted to three consecutive filtrations using membranes with  
145 a progressively smaller cut-off. The membrane filtration process was designed as reported in **Fig. 1**,  
146 using three different modules: i) filtration set-up with Buchner flask (aspired with vacuum pump) and  
147 Buchner funnel equipped with GF-6 filter paper (Merck-Millipore); ii) filtration set-up with Buchner  
148 flask (aspired with vacuum pump) and Buchner funnel equipped with 0.45  $\mu m$  membrane (Membrane  
149 Solutions); iii) MilliporeSigma™ Amicon™ Bioseparations Stirred Cell (pressurized with  $N_2$ ) equipped  
150 with Ultracel 30 kDa (pore size  $\approx 6 - 7.5$  nm [38]), ultrafiltration disc (Merck Amicon Bioseparation).  
151 Full technical details of filter membranes are provided in **Fig. 1**. After each filtration, filtrate was used  
152 partially for successive filtration, and partially for characterization of the filtrate. Characterization of  
153 the filtrate included total solid analysis, HA quantification and measurement of total organic carbon  
154 (TOC) with TOC analyzer (TOC-VWS, Shimadzu Corporation).

155 **Figure 1 (in greyscale)**

156 2.5. Encapsulation

157 The HS extract obtained from ASS was firstly sieved (0.8 mm) to remove residual debris. After that,  
158 micronutrients were added to the solution in proportion inspired by Epstein and Bloom [39].  
159 Concentration of these elements were: B 0.57 g L<sup>-1</sup>, Cu 0.57 g L<sup>-1</sup>, Fe 7.36 g L<sup>-1</sup>, Mn 4.34 g L<sup>-1</sup>, Mo  
160 0.13 g L<sup>-1</sup> and Zn 1.12 g L<sup>-1</sup>. After, the solution was mixed at 40°C with sodium alginate powder to a  
161 final concentration of 2.3% w/v, until the solution resulted homogeneous. Hence, the mixture was  
162 poured into a glass bottle and put under a nitrogen (N<sub>2</sub>) pressure of 450 mbar to allow the injection  
163 in the encapsulator Buchi B-390. The encapsulator was set at a frequency of 40 Hz and a voltage of  
164 250 V. The solution extruded from the encapsulator was drop-shaped by a nozzle with a diameter of  
165 1 mm. Drops fell in a hardening bath of CaCl<sub>2</sub> in the range of 0.06 - 6 M where the Na/Ca exchange  
166 took place. Finally, beads were air dried. In order to measure diameters of dry and wet beads, beads  
167 were photographed, and their pictures were analyzed with the ImageJ software [40].

168 2.6. Microscopy analysis of extract and beads

169 With the purpose of describing morphological features of HA and evaluating the elements present in  
170 the samples, the HA extract and alginate beads were investigated through Field Emission Scanning  
171 Electron Microscopy (FESEM, Zeiss MERLIN, Gemini-II column, Oberkochen, Germany) and  
172 Energy dispersive X-ray (EDX) analyses (AZTec, Oxford Instruments, Abingdon, UK). The EDX  
173 analysis was performed on a wide area (100 µm x 100 µm) in three different regions of the samples  
174 in order to have an average result of the elementary composition. Commercial HA were also  
175 analyzed in order to get qualitative information on chemical composition and as a standard of  
176 comparison. Beads without HA were created and analyzed with FESEM in order to compare  
177 structure of beads with HA and without. Previously all FESEM analysis, samples were metalized with  
178 chromium. The liquid HA extract was previously dewatered to be analyzed by the means of FESEM.  
179 Therefore, lyophilization was performed instead of classical thermal drying in order to not  
180 compromise the structure of HA. Lyophilization was performed with an IIShin FD5518 Freeze Dryer



181 with the following settings: temperature -60°C, pressure 5 mTorr, time 48 hours. As a result, a  
182 lyophilized SA extract with 82% in dry matter was obtained.

### 183 2.7. ATR-FTIR spectroscopy

184 Lyophilized HA extract was analyzed by the means of Fourier transformed infrared (FTIR)  
185 spectroscopy, which was performed in attenuated total reflectance (ATR) mode. The instrument  
186 used was a Bruker Tensor 27 spectrometer equipped with a Platinum ATR and a KBr beamsplitter.  
187 The spectra were recorded in the range 4000-400 cm<sup>-1</sup> (mid IR) with a resolution of 4 cm<sup>-1</sup>, 32 scans  
188 per sample (measurement time: 15 s) and background correction with ambient air.

### 189 2.8. Pot experiment

190 In order to evaluate beads effects on plant growth, a pot experiment was performed in a greenhouse  
191 located in Quillota, Valparaiso Region (Chile). Pots of 30 cm of diameter were filled with a sandy soil  
192 previously sieved at 2.5 mm. Before the transplanting of Chilean lettuce plants (*Lactuca sativa* L.), a  
193 basal dressing with a commercial NPK fertilizer for vegetables (N: 8%, P<sub>2</sub>O<sub>5</sub>: 15%, K<sub>2</sub>O: 25%) and a  
194 fungicide treatment with Captan (5 g L<sup>-1</sup>) were applied in each pot following manufacturer  
195 instructions. Three days after the transplanting, half of pots (9 replicates) were randomly treated with  
196 alginate-extract beads and the second half was no treated (control). The quantity of beads per-pot  
197 was of 8.7 kg ha<sup>-1</sup> of HA due to a commercially recommended dosage as reported in the datasheet  
198 of Humic plus®. A drip irrigation plant was installed, and plants were irrigated every 3 days. Finally,  
199 70 days after transplanting, plants were cut at the basis, and epigeal fresh biomass was immediately  
200 weighted. Fresh biomass of root apparatus was measured after washing with water to remove  
201 residual soil particles. Finally, dry matter of the epigeal and hypogean part of plants was weighted  
202 after thermal treatment (105°C, 48 hours). Furthermore, chemical characterization of soils was  
203 performed.

### 204 2.9. Statistical analysis

205 Data of the pot experiment were analyzed by one-way ANOVA (analysis of variance) with a Tukey's  
206 post-hoc test ( $P \leq 0.05$ ), after the assessment of the fundamental assumptions of ANOVA: the

207 normality of distributions (Shapiro-Wilk test, p-value > 0.05) and the homogeneity of the variances  
208 of the residuals (Levene's test with  $P(>F) > 0.05$ ). The statistical software R (version 3.5.1 - Feather  
209 Spray - 2018) was used for all statistical analysis.

### 210 3. Results and discussion

#### 211 3.1. Quantification and characterization of HA

212 The application of the Lamar method [37] allowed the quantification of the HA content in ASS, HA  
213 extract and commercial HA. The results are summarized in **Table 1**. The HA content in ASS was  
214  $12.53 \pm 1.60$  % on dry matter basis (d.m.b.), a value comparable to other one reported in literature  
215 (7.33%) [41]. The HA content in the extract was  $26.87 \pm 0.35$ % d.m.b., indicating that the process  
216 contributed to a more than two-fold enrichment in HA. The quantification of the HA in commercial  
217 HA powder revealed the highest content, estimated in  $77.87 \pm 1.46$ % d.m.b., in line with other  
218 commercial HA derived from leonardite (80%) [42]. Although the HA% of the extract is lower than  
219 commercial HA, it is important to underline that normally HA are extracted from non-renewable  
220 resources, such as peat, lignite and leonardite, while in this case the HA came from a waste.

221 As regards membrane separation processes, it is worth specifying that this process was designed  
222 with two aims in mind. The first was the study of the molecular size distribution of the purified HS  
223 during extraction. The second was the individuation of one or more filters to separate HA from other  
224 components of the extract, with the purpose of increasing the purity and concentration of the final  
225 product.

226 As reported by Steelink [43], several techniques have been exploited to clarify the size and shape of  
227 HA, such as sedimentation, size exclusion chromatography (SEC), light scattering and many others;  
228 however, each approach has pros and cons, revealing a broad range of molecular weight values.  
229 Hence, the purpose of this study was not to investigate the theoretical molecular size distribution of  
230 HA and their chemical moieties, as described by Shin and co-workers [44] for commercial HA.  
231 Instead, the goal was to adopt an engineering approach which would permit a general  
232 characterization, and at the same time would offer a potential process to obtain a more concentrated  
233 product. For this reason, commercial membranes were used to design the filtration process. The

234 results of the membrane filtrations are reported in **Table 2**, which shows the concentrations of total  
235 solids (TS), ashes, HA and TOC in the HA extract and the three permeates.

236 Considering that the HA extraction protocol did not reach extremely alkaline pH (9.5 – 10) to avoid  
237 potential deterioration of HS, humins might still persist in the final extract (insoluble at pH>13 [45].  
238 Furthermore, it must be taken into account that, even when extraction conditions are kept more  
239 stringent, part of the non-humic organic material (e.g. cell material components) is solubilized and is  
240 still present in the extract [45]. In the present study, these contributes were included in the quote of  
241 humins and fulvic fraction (also known as “acid soluble fraction”), which is formed by FA and non-  
242 humic compounds [46]. In this study, TOC of the final extract is to be considered as consisting of  
243 HA, fluvic fraction and humin fraction.

244 The content of HA, fluvic fraction and humin fraction in the different steps of the process where  
245 estimated from their mean elemental composition, with a C content of 55% for HA [47], of 50 % for  
246 fulvic fraction [48], and a 56 % for humins [49].

247 In parallel, **Fig. 2** shows the distribution of the different components of the extract in function of the  
248 different membrane pore size. On one hand, the first and second filtration process retained more  
249 than a 40 % of TS and TOC, while HA and humin fraction were fully retained. Concerning humins,  
250 they were retained more by the first filter than the second one, according to their higher molecular  
251 weights [50, 51]. Different is the case of HA, where researchers commonly agree that their molecular  
252 weights ranges between 2 and 1300 kDa [52], which means that the estimated hydrodynamic radius  
253 of HA particles varies between 2 [53] and 110 nm [54]. However, the literature has demonstrated  
254 that HA may form aggregates with a mean particle diameter of 0.5  $\mu\text{m}$  [11]. Thus, the results obtained  
255 with the membrane experiments suggested that HA molecules present in the extract formed  
256 aggregates of different sizes, half bigger than 1  $\mu\text{m}$  and half with a size included between 1 and 0.45  
257  $\mu\text{m}$ .

258 On the other hand, fluvic fraction and ashes were not affected by the filtration process and were  
259 present almost in its whole in the third permeate, which corresponded to the fraction with particle  
260 size smaller than 30 kDa. This indicated that they were mainly made of small inorganic molecules.  
261 In the case of ashes, they were likely made of elements such as potassium (as  $\text{K}^+$ ) and phosphorous

262 (as  $\text{PO}_4^{3-}$ ) already present in ASS and further added during the extraction process, whose presence  
263 was confirmed by EDX analysis (see section 3.2).

264 Within the perspective to obtain an added-value product, the membrane processes yielded to an  
265 increase in the concentration of HA, moving from a 26.87% w/w d.m.b. in the extract to a calculated  
266 value of 64% d.m.b summing the contributes of the first two filtration steps, which permitted to  
267 accomplish successfully the full retention of HA. Therefore, the filtration in two steps, performed with  
268 a 1  $\mu\text{m}$  membrane followed by a 0.45  $\mu\text{m}$  one, may be a feasible solution for the HA concentration.  
269 In this context, the result of the present work indicated that the concentration and the increase in  
270 purification degree of HAs may be achieved with a microfiltration process, with smaller energy costs  
271 than the ultrafiltration process proposed previously by Li and co-workers [55].

272

273 **Table 1 (in greyscale)**

274 **Table 2 (in greyscale)**

275 **Figure 2 (in colors)**

276 3.2. Encapsulation of HA extract in calcium alginate beads

277 The HA extract was encapsulated to obtain a solid product with a slow release of the active  
278 components over time. Different concentrations of  $\text{CaCl}_2$  (0.06 M – 6 M) were studied with the scope  
279 of reducing the presence of Ca and Cl in the beads. However, concentrations below 0.6 M did not  
280 provide a solid formation of beads and therefore 0.6 M was chosen to harden the beads for pot  
281 experiments. The beads were dried with the scope of increasing their lifetime by reducing the  
282 possibility of microbial degradation, since they were mainly composed of organic matter [56]. After  
283 one year of storage at 25°C in dry conditions, no visual damage neither degradation has been  
284 observed. In addition, HA are known to be resistant to biodegradation, thus, the rate limiting step of  
285 release process can be reasonably attributed to the degradation of the alginate matrix [30]. The  
286 mean diameter of wet and dry beads was  $2.4 \pm 0.4$  mm and  $1.1 \pm 0.1$  mm, respectively (from picture  
287 analysis with ImageJ software). Considering that all the HA extract used during encapsulation was

288 successfully entrapped by beads, the content of HA in the dry beads represented the 6.09%,  
289 according to the mass balance calculation.

290 Data from the literature report that Ca-alginate beads show macro-porous in the order of 10  $\mu\text{m}$  [57]  
291 and mesoporous in the range of 8 -14 nm [58]. This complex structure has been successfully used  
292 for the entrapment of complete cells on one hand, but on the other it has revealed enzymes leaching  
293 [59]. Similarly to enzymes, HA can be washed out from the Ca-alginate matrix because of their  
294 molecule size, which ranges between 2 and 1300 kDa [52]. However, as demonstrated by the  
295 membrane process, the HA present in the extract formed aggregates bigger than 0.45  $\mu\text{m}$ . This  
296 result indicated that the aggregates were retained by the Ca-alginate matrix and that they would be  
297 released upon washing steps, (comparable to the effect of the irrigation process).

298 The FESEM images (**Fig. 3**) show the morphology of the surface of the beads, which was made of  
299 micrometer sized aggregates. Pure alginate structure is visible in **Fig. 3a**. The cluster of smaller  
300 globular particles that protrude from the entrapment made by the calcium alginate reticular structure,  
301 shown in **Fig. 3b**, confirmed the correct encapsulation of the HA extract within the calcium alginate  
302 matrix. A similar cluster-like structure is observed with the commercial HA (**Fig. 3d**). More compacted  
303 aggregates are observed from the lyophilized HA extract, which were due to the water elimination  
304 treatment (**Fig. 3c**).

305 The EDX analysis shown in **Fig. 4** confirmed that the HA extraction process yielded to a product free  
306 of heavy metals. This was an interesting result, considering that the chemical analysis of the ASS  
307 demonstrated the presence of As, Ni, Cd, Cr, Hg and Pb (**Supplementary material – 1**). Carbon,  
308 nitrogen, and oxygen elements were not included in the EDX analysis because of the high errors  
309 associated with their low atomic weight. It is worth noting that the high standard deviation of the EDX  
310 results obtained with the beads is in line with the FESEM images, which demonstrated the low  
311 homogeneity of the samples, showing regions with the presence of crystals and regions that are  
312 clearer (**Supplementary material - 2**). The higher amount of K and P in the extract was attributed  
313 to the use of a solution containing potassium hydroxide and pyrophosphate during the extraction  
314 process. As expected, the content of these two components was considerably lower in the beads

315 due to the dilution of the extract in the alginate solution during beads preparation. In contrast, there  
316 was a substantial increase of sodium, chlorine and calcium in the beads with respect to the extract,  
317 which was in line with FESEM images showing crystals of NaCl on the beads surface  
318 (**Supplementary material - 2**). Those elements came from the sodium alginate and calcium chloride  
319 solution used to harden the beads. However, further washing steps to reduce the amount of the  
320 contaminants were not conducted in order to avoid the premature leaching of the HA molecules from  
321 the beads.

322 **Figure 2 (print: in greyscale)**

323 **Figure 3 (print: in greyscale)**

324 Infrared spectroscopy analysis of the lyophilized HA extract confirmed the presence of chemical  
325 moieties peculiar of HS, as shown by **Fig. 5**, displaying ATR-FTIR absorption spectra with bands  
326 typical of HS. The sample spectra presented a principal band around  $3270\text{ cm}^{-1}$ , corresponding to  
327 the H-bonded O-H stretching of carboxylic acids, phenols, and alcohols and it was followed by a  
328 doublet at  $2920\text{-}2850\text{ cm}^{-1}$  due to the C-H stretching of aliphatic structures [[60], [61], [62], [63]].  
329 The peak around  $1630\text{ cm}^{-1}$  was ascribable to the C=O stretching of carboxylic and ketonic groups,  
330 and to the aromatic C=C stretching [61, 63], followed by a peak around  $1545\text{ cm}^{-1}$  due to the C=N  
331 stretching of amides [60, 61].

332 Peaks at  $1450$  and  $1400\text{ cm}^{-1}$  were characteristic of the bending of aliphatic C-H and [60, 63] and  
333 of the O-H bending of carboxylic acid [64]. The large peak around  $1040\text{ cm}^{-1}$  corresponded to the C-  
334 O stretching of alcohols and aliphatic ethers [60], but it might be also assigned to the presence of  
335 Si-O silicate impurities as confirmed by EDX analysis [62, 63]. At lower wavelengths ( $900\text{-}600\text{ cm}^{-1}$ )  
336 the HA extract spectrum showed several peaks that could be reasonably attributed to the C=C  
337 bending [64]. ATR-FTIR spectra were recorded also on HA-alginate beads (data not shown), but the  
338 signal of alginate was too much intense, hindering the proper characterization of HA.

339 **Figure 3 (in greyscale)**

340 **Figure 4 (in greyscale)**

341 **Figure 5 (in greyscale)**

342 3.3. Pot experiment

343 The results of the final dry biomasses of lettuce grown in the greenhouse experiment are represented  
344 in **Fig. 6**. As regards the epigeal dry biomass of the lettuce plants, the addition of HA beads did not  
345 provide significant differences with the untreated control. FESEM and EDX analyses (**Fig. 3** and **Fig.**  
346 **4**) showed NaCl presence in beads which probably contributed to the soil electrical conductivity  
347 (E.C.) of treated samples ( $0.46 \text{ dS m}^{-1}$ ) higher than control ( $0.26 \text{ dS m}^{-1}$ ) (**Supplementary material**  
348 **- 3**). Nevertheless, E.C. did not reach potentially dangerous levels for plants ( $>2 \text{ dS m}^{-1}$ ) [65], and it  
349 likely did not affect biomass production. Many studies about the effects on shoot biomass have been  
350 conducted by adding HA to soils and the results are discordant. In some cases, no difference was  
351 reported, in others ones shoot biomass production was enhanced [26]. For studies dealing with  
352 lettuce, no differences between treated and untreated samples were reported despite the high  
353 amounts of HA applied (until  $300 \text{ kg ha}^{-1}$ ) [66]. On the contrary, in another work lettuce plants had a  
354 statistically higher growth when compared to the untreated control at a dosage of  $2000 \text{ mg of HA kg}^{-1}$   
355 of soil [67]. In the present work the estimated dosage of HA used was  $8.7 \text{ kg ha}^{-1}$ , corresponding  
356 to approximately  $2.7 \text{ mg of HA kg}^{-1}$  of soil, a dosage 740-fold lower than that reported in the work of  
357 Tüfenkçi and colleagues [67]. On the other hand, in the present study the hypogean dry biomass of  
358 plants grown in the presence of the HA beads was significantly higher (+63%) than the negative  
359 control. Hence, this result confirmed the stimulation effect of HA on root growth, already widely  
360 documented in the literature [25, 26]. Moreover, this result supports the work of Young and Chen,  
361 who demonstrated root biomass enhancement by HA in lettuce [68].

362 It is important to underline that the enhancement of shoot biomass driven by HA addition to soil is  
363 more unusual. A work on tomato showed that only one out of nine dosages of HA applied promoted  
364 shoot biomass, but roots biomass production was promoted in all cases [69]. On the other hand, a  
365 recent meta-analysis of 89 papers on random-effects revealed that the dry weight of shoot and root  
366 increases of 22.4% and 21.6%, respectively, in response to HS application [23]. Furthermore, this  
367 study elucidated which are the significant factors likely enhancing shoots and roots growth using HS.

368 The type of HS (origin and chemical moieties) was the most important parameter in affecting both  
369 shoots and roots biomasses increase. After that, the HS application rate resulted the most important  
370 parameter influencing shoot growth promotion, followed by stressing conditions and plant type. In  
371 the present experiment, the not significant increase of shoot biomass could be due to some of these  
372 parameters but, as explained before, the low application rate was likely the most conceivable cause.  
373 As regards root growth promotion, the above-mentioned study revealed that, after the HS type, the  
374 growth media and plant species were the factors that mostly affected roots enhancement. Application  
375 rate, application location and stress did not affect roots growth. In this way, our experiment  
376 demonstrated that the HA extracted and applied to the plants were adequate to increase the biomass  
377 of lettuce roots.

#### 378 **Figure 6 (in greyscale)**

#### 379 4. Conclusion

380 Sewage sludge is a waste with a high recycling potential considering its appreciable content in  
381 valuable compounds, but the simultaneous presence of toxic elements strongly limits its reuse.  
382 Hence, an effective approach is the consideration of sewage sludge as a “raw material”, candidate  
383 to the selective “mining” of added value and profitable compounds. This strategy is in line with the  
384 purposes of the present work, which dealt with the extraction, quantification, characterization, and  
385 agronomic testing of HA recovered from ASS. The protocol used allowed not only to obtain a HA  
386 extract with a HA concentration (26.87%), on dry matter basis, doubled than the starting material  
387 (ASS, 12.53%), but also to get rid of heavy metals. These positive results could be even more  
388 improved with future research, optimizing the protocol for higher HA recovery. Membrane filtration,  
389 electron microscopy and infrared spectroscopy provided insight into peculiarities of HA extract,  
390 revealing features on isolated HA comparable to those reported in the literature for what concerns  
391 molecular sizes, morphology, and chemical moieties. The sequential filtration process permitted  
392 determining that the extracted HA formed aggregates of size greater than 0.45  $\mu\text{m}$ , making them  
393 suitable for encapsulation in alginate to obtain a slow release in soil. Despite the adequate  
394 encapsulation of HA, NaCl was present in the capsules, but without affecting lettuce growth. In fact,



395 the HA-alginate beads induced beneficial effects on root apparatus growth of lettuce grown on a  
396 poor and sandy soil (+63% over untreated control). With the purpose of further deepening these  
397 issues, future perspectives should include the testing of the effectiveness of other crosslinking  
398 agents, to reduce NaCl content in beads, and the application of the slow-release HA product on  
399 different plant species. In conclusion, the encouraging results of this study suggest that HA extraction  
400 from ASS is a promising strategy for the sustainable production of commercial HS of tomorrow.  
401 Indeed, a slow-release bio-stimulant containing HA derived from a waste was achieved, successfully  
402 fulfilling the circular economy principles.

403

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411

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Parameter	Dry matter %		% HA (d.m.b.)	
	Mean value	Standard dev.	Mean value	Standard dev.
<b>ASS</b>	25.58	± 0.49	12.53	± 1.60
<b>Extract</b>	1.13	± 0.02	26.87	± 0.35
<b>Commercial HA</b>	83.95	± 0.08	77.87	± 1.46

607

608 **Table 1.** Dry matter mean percentages and humic acids mean percentages measured in anaerobic  
609 sewage sludge (ASS), extract of SSAD (Extract) and commercial humic acids (Commercial HA).  
610 d.m.b.: dry matter basis.

	Pure HA extract	GF-6 permeate	0.45 µm permeate	30 kDa permeate
Dry matter (%)	1.13%	0.88%	0.64%	0.61%
Total solids (g L <sup>-1</sup> )	10.7	8.32	6.03	5.74
Dry matter retention	vs pure extract	-	22.24%	43.64%
	vs GF-6 filtrate	-	-	27.52%
	vs 45 µm filtrate	-	-	-
Ashes (dry matter basis) (g L <sup>-1</sup> )	4.52	4.50	4.39	4.30
Ashes retention	vs pure extract	-	0.54%	2.80%
	vs GF-6 filtrate	-	-	2.27%
	vs 45 µm filtrate	-	-	-
Humic acids (% dry matter)	26.87%	14.46%	n.a.	n.a.
Humic acids (g L <sup>-1</sup> )	3.01	1.52	0	0
Humic acids retention	vs pure extract	-	49.50%	100.00%
	vs GF-6 filtrate	-	-	100.00%
	vs 45 µm filtrate	-	-	-
TOC (g L <sup>-1</sup> )	3.32	2.02	0.81	0.71
TOC retention	vs pure extract	-	39.16%	75.60%
	vs GF-6 filtrate	-	-	59.90%
	vs 45 µm filtrate	-	-	-

611

612 **Table 2.** Characterization of HA extract and filtrates from the three different filtration processes. Per  
613 each parameter, percentages of retention are reported at each filtration step.

614

615 **Figures**

616 **Fig. 1.** Scheme of the membrane process exploited for study of molecular size of humic acids in the  
617 extract based on membrane cut-off. Technical details are reported for each membrane.

618 **Fig. 2.** Retention percentages of total solids, ashes, humins, humic acids, fulvic fraction and total  
619 organic carbon along the different filtration steps.

620 **Fig. 3.** FESEM images of the empty (a) and with HA extract (b) calcium alginate beads and of the  
621 lyophilized HA extract (c) and commercial HA powder (d).

622 **Fig. 4.** Elementary composition by EDX analysis of the elements present in the samples of HA  
623 extract, in commercial HA and in calcium alginate beads with HA.

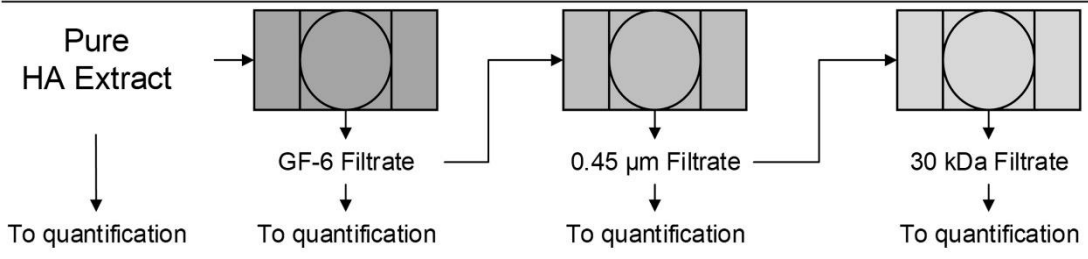
624 **Fig. 5.** ATR-FTIR spectrum of lyophilized HA extract.

625 **Fig. 6.** Mean dry epigeal and hypogean biomasses of *Lactuca sativa* L. grown on sandy soil with  
626 beads treatment and without beads treatment. Different letters indicate differences between  
627 treatments that are significant at  $P < 0.05$  (Tukey HSD). Error bars represent standard error. Upper-  
628 case letters refer to statistical analysis applied to epigeal dry biomass samples and lower-case  
629 letters refer to statistical analysis applied to hypogean dry biomass samples.

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Commercial name	GF-6 Filter	0.45 $\mu\text{m}$	Ultracel Ultrafiltration Disc
<b>Brand</b>	Merck-Millipore	Membrane Solutions	Merck-Millipore
<b>Dimension</b>	$\varnothing$ 110 mm	$\varnothing$ 47 mm	$\varnothing$ 25 mm
<b>Cut-off</b>	$\sim$ 1 $\mu\text{m}$	0.45 $\mu\text{m}$	30 kDa (6 $\div$ 7.5 nm)
<b>Material</b>	Paper/Glass Fiber	Mixed cellulose esters (MCE)	Regenerated cellulose
<b>Maximum flux</b>	2.2 ml min <sup>-1</sup>	0.5 ml min <sup>-1</sup>	0.17 ml min <sup>-1</sup>



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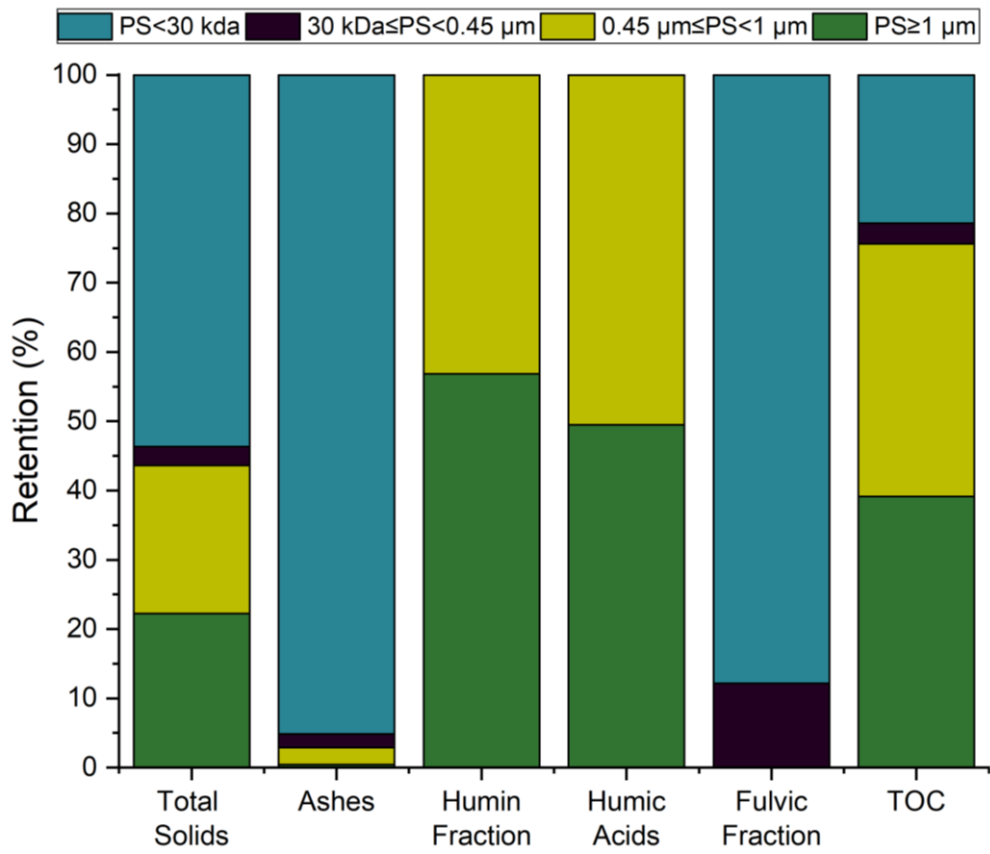
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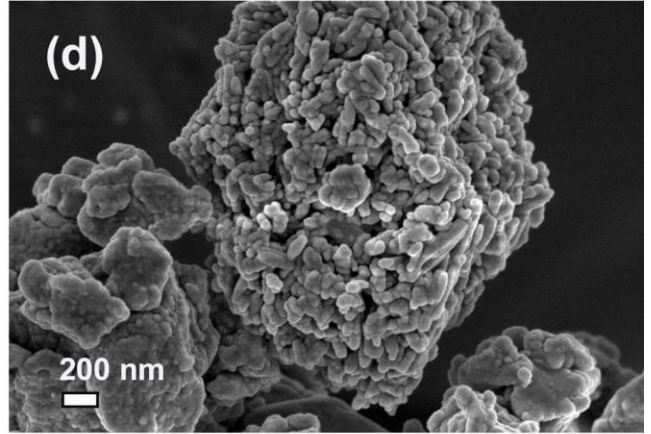
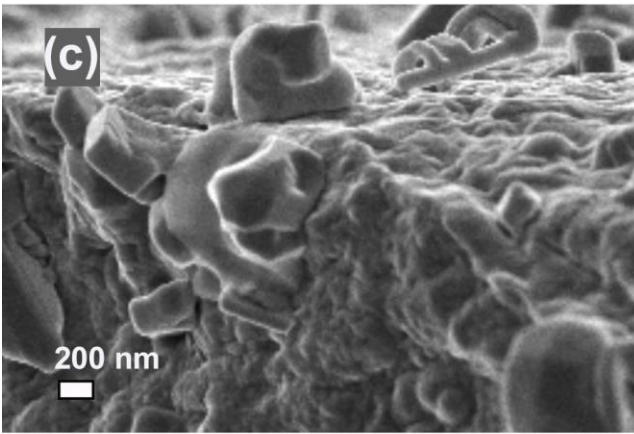
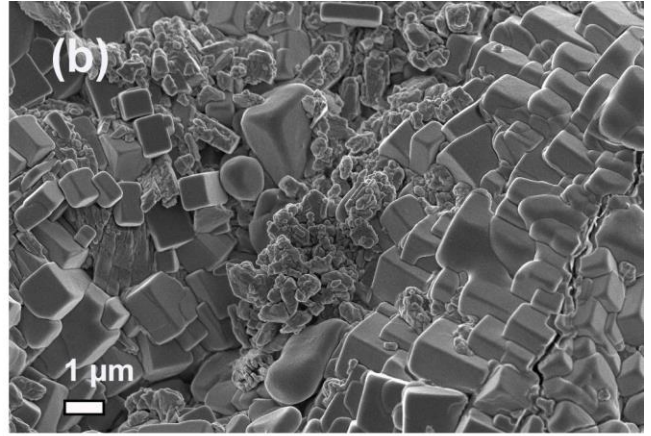
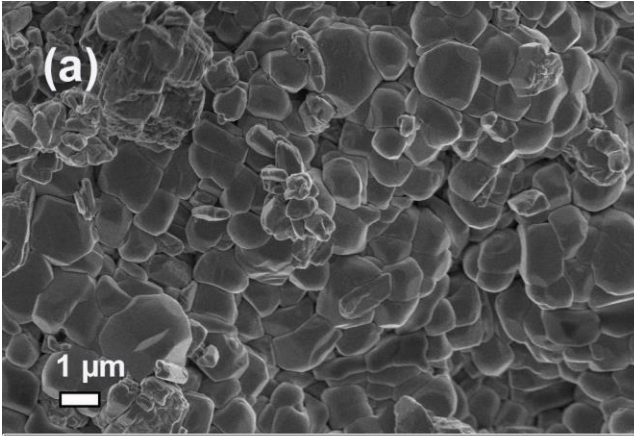
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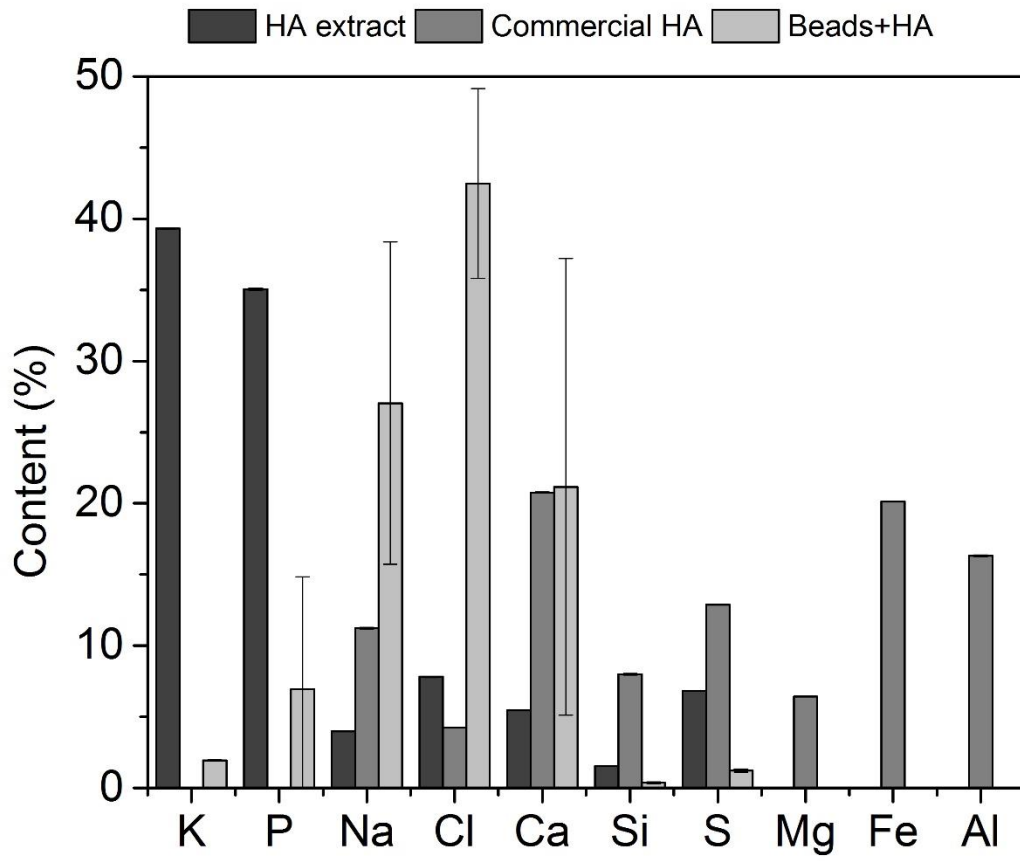
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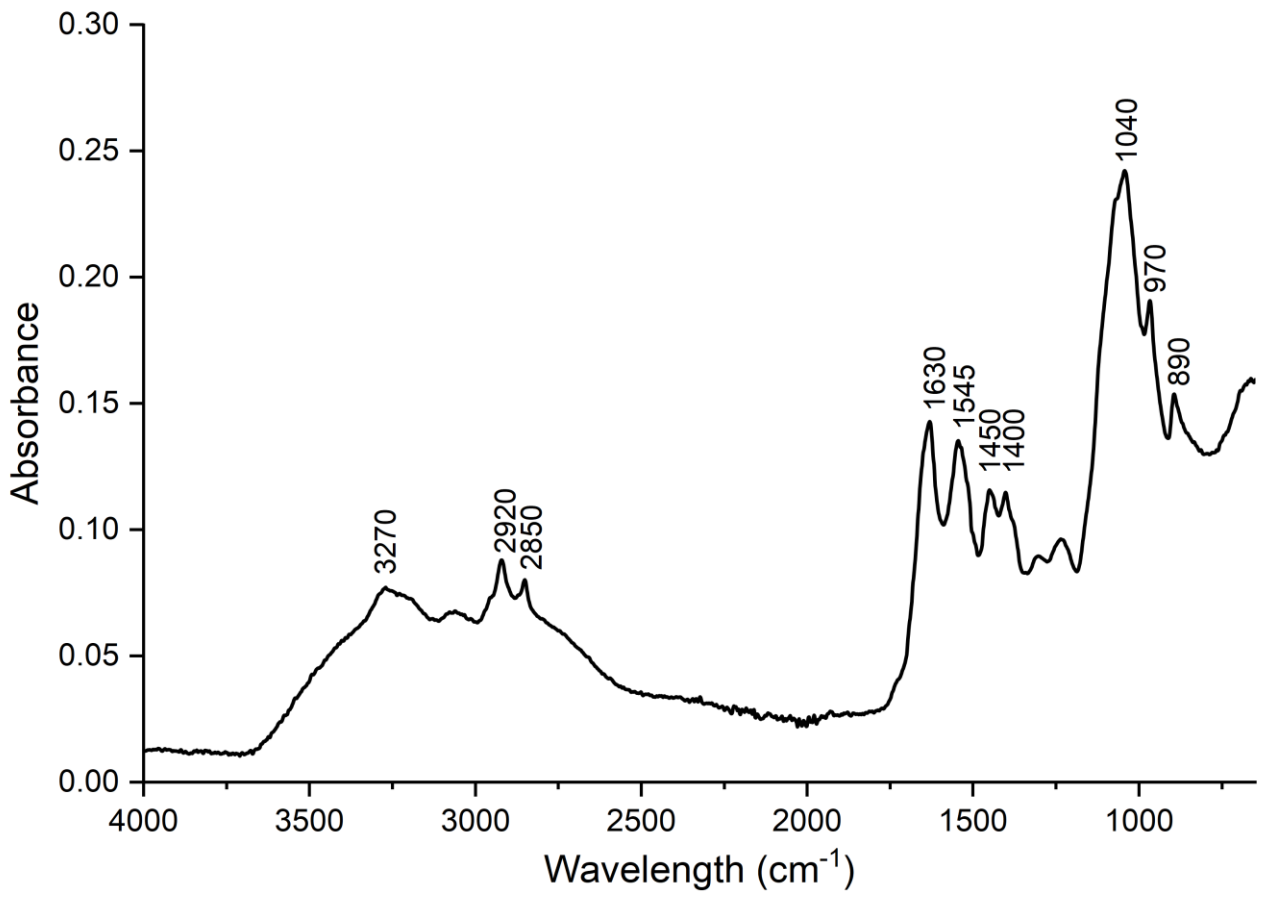
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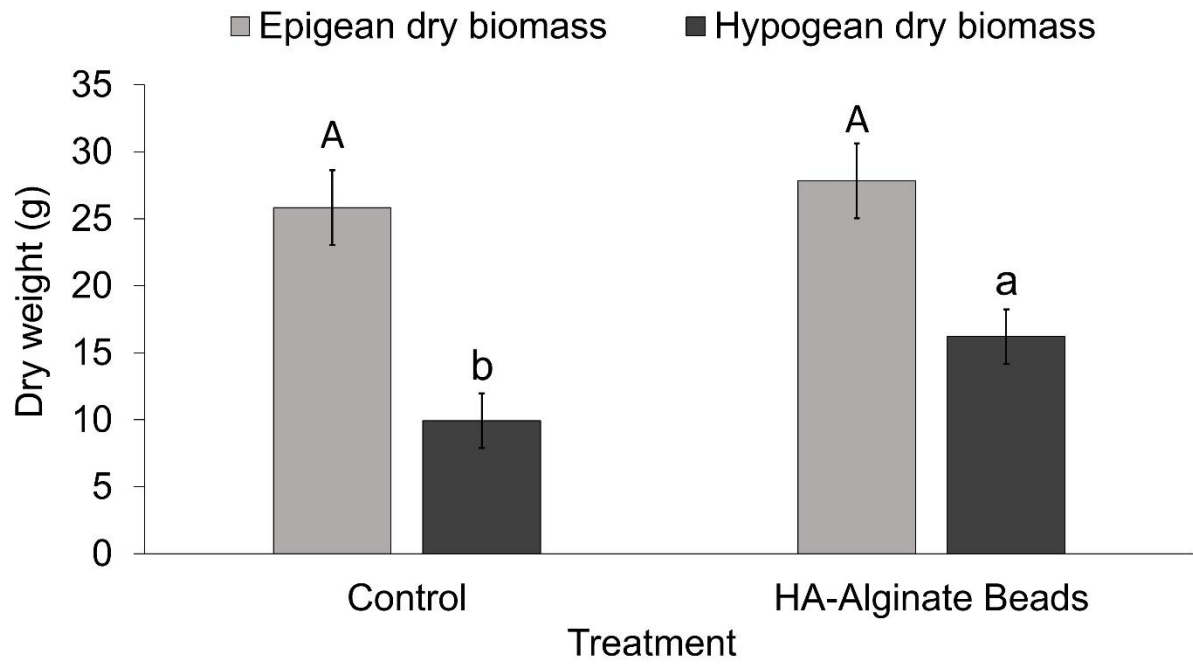
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