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

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

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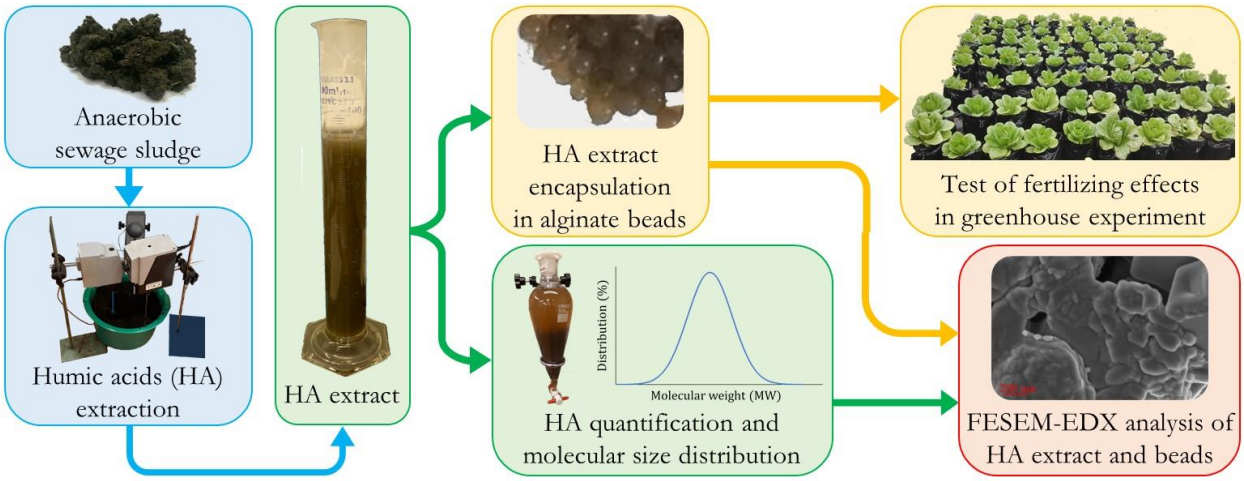
Highlights:

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

Graphical abstract: (in colors)



Abstract:

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

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

Keywords: humic acids extraction, circular economy, greenhouse experiment

Declaration of interest: none.

Glossary of abbreviations and acronyms

Abbreviation	Definition
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

1. Introduction

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

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

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

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

Different biodegradable polymers have been studied as matrixes for the encapsulation of bioactive compounds for different applications with the aim of having a controlled release of the substances in time. The most used matrixes are chitosan, collagen, gelatin and alginate [[28], [29], [30]]. In the field of agriculture, different bioactive capsules can be found for the purpose of releasing herbicides [31], fertilizers [32, 33], pesticides [34] or even complete cells that have a symbiotic effect with the plant growth [35]. Generally, the objective of having a controlled release is to reduce the amount of product that is added to the soil, which permits the operational costs to be reduced and, more 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)-β-D-mannuronic acid (M) and (1,4)-α-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 Ca^{2+} and 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 sequestrate the divalent cations yielding
114 to a disruption of the electrostatic interactions between the alginate chains and the 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 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

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

2.2. Extraction protocol

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

2.3. Chemical analysis and HA quantification

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

2.4. Molecular size distribution of HA

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

Figure 1 (in greyscale)

2.5. Encapsulation

The HS extract obtained from ASS was firstly sieved (0.8 mm) to remove residual debris. After that, micronutrients were added to the solution in proportion inspired by Epstein and Bloom [39]. Concentration of these elements were: B 0.57 g L^{-1}, Cu 0.57 g L^{-1}, Fe 7.36 g L^{-1}, Mn 4.34 g L^{-1}, Mo 0.13 g L^{-1} and Zn 1.12 g L^{-1}. After, the solution was mixed at 40°C with sodium alginate powder to a final concentration of 2.3% w/v, until the solution resulted homogeneous. Hence, the mixture was poured into a glass bottle and put under a nitrogen (N_2) pressure of 450 mbar to allow the injection in the encapsulator Buchi B-390. The encapsulator was set at a frequency of 40 Hz and a voltage of 250 V. The solution extruded from the encapsulator was drop-shaped by a nozzle with a diameter of 1 mm. Drops fell in a hardening bath of $CaCl_2$ in the range of 0.06 - 6 M where the Na/Ca exchange took place. Finally, beads were air dried. In order to measure diameters of dry and wet beads, beads were photographed, and their pictures were analyzed with the ImageJ software [40].

2.6. Microscopy analysis of extract and beads

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

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

2.7. ATR-FTIR spectroscopy

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

2.8. Pot experiment

In order to evaluate beads effects on plant growth, a pot experiment was performed in a greenhouse located in Quillota, Valparaiso Region (Chile). Pots of 30 cm of diameter were filled with a sandy soil previously sieved at 2.5 mm. Before the transplanting of Chilean lettuce plants (*Lactuca sativa* L.), a basal dressing with a commercial NPK fertilizer for vegetables (N: 8%, P_2O_5: 15%, K_2O: 25%) and a fungicide treatment with Captan (5 g L^{-1}) were applied in each pot following manufacturer instructions. Three days after the transplanting, half of pots (9 replicates) were randomly treated with alginate-extract beads and the second half was no treated (control). The quantity of beads per-pot was of 8.7 kg ha^{-1} of HA due to a commercially recommended dosage as reported in the datasheet of Humic plus®. A drip irrigation plant was installed, and plants were irrigated every 3 days. Finally, 70 days after transplanting, plants were cut at the basis, and epigean fresh biomass was immediately weighted. Fresh biomass of root apparatus was measured after washing with water to remove residual soil particles. Finally, dry matter of the epigean and hypogean part of plants was weighted after thermal treatment (105°C, 48 hours). Furthermore, chemical characterization of soils was performed.

2.9. Statistical analysis

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

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

3. Results and discussion

3.1. Quantification and characterization of HA

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

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

As reported by Steelink [43], several techniques have been exploited to clarify the size and shape of HA, such as sedimentation, size exclusion chromatography (SEC), light scattering and many others; however, each approach has pros and cons, revealing a broad range of molecular weight values. Hence, the purpose of this study was not to investigate the theoretical molecular size distribution of HA and their chemical moieties, as described by Shin and co-workers [44] for commercial HA. Instead, the goal was to adopt an engineering approach which would permit a general characterization, and at the same time would offer a potential process to obtain a more concentrated 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 µm [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 µm and half with a size included between 1 and 0.45
257 µm.
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 K^+) and phosphorous

262 (as 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 µm membrane followed by a 0.45 µm 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 $CaCl_2$ (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 25ºC 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 ± 0.4 mm and 1.1 ± 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 µm [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 µm. 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

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

Figure 2 (print: in greyscale)

Figure 3 (print: in greyscale)

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

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

Figure 3 (in greyscale)

Figure 4 (in greyscale)

Figure 5 (in greyscale)

3.3. Pot experiment

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

It is important to underline that the enhancement of shoot biomass driven by HA addition to soil is more unusual. A work on tomato showed that only one out of nine dosages of HA applied promoted shoot biomass, but roots biomass production was promoted in all cases [69]. On the other hand, a recent meta-analysis of 89 papers on random-effects revealed that the dry weight of shoot and root increases of 22.4% and 21.6%, respectively, in response to HS application [23]. Furthermore, this 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 μm, 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,

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

Acknowledgment

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Parameter	Dry matter %		% HA (d.m.b.)	
	Mean value	Standard dev.	Mean value	Standard dev.
ASS	25.58	± 0.49	12.53	± 1.60
Extract	1.13	± 0.02	26.87	± 0.35
Commercial HA	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^{-1})	10.7	8.32	6.03	5.74
Dry matter retention	vs pure extract	-	22.24%	43.64%	46.36%
	vs GF-6 filtrate	-	-	27.52%	31.01%
	vs 45 µm filtrate	-	-	-	4.81%
Ashes (dry matter basis) (g L^{-1})		4.52	4.50	4.39	4.30
Ashes retention	vs pure extract	-	0.54%	2.80%	4.87%
	vs GF-6 filtrate	-	-	2.27%	4.35%
	vs 45 µm filtrate	-	-	-	2.13%
Humic acids (% dry matter)		26.87%	14.46%	n.a.	n.a.
	Humic acids (g L^{-1})	3.01	1.52	0	0
Humic acids retention	vs pure extract	-	49.50%	100.00%	100.00%
	vs GF-6 filtrate	-	-	100.00%	100.00%
	vs 45 µm filtrate	-	-	-	-
	TOC (g L^{-1})	3.32	2.02	0.81	0.71
TOC retention	vs pure extract	-	39.16%	75.60%	78.61%
	vs GF-6 filtrate	-	-	59.90%	64.85%
	vs 45 µm filtrate	-	-	-	12.35%

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.

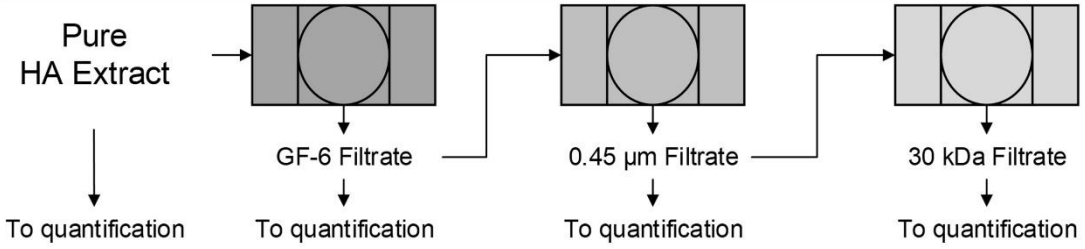
624 **Fig. 5.** ATR-FTIR spectrum of lyophilized HA extract.

625 **Fig. 6.** Mean dry epigean 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 epigean dry biomass samples and lower-case
629 letters refer to statistical analysis applied to hypogean dry biomass samples.

630

631

Commercial name	GF-6 Filter	0.45 µm	Ultracel Ultrafiltration Disc
Brand	Merck-Millipore	Membrane Solutions	Merck-Millipore
Dimension	Ø 110 mm	Ø 47 mm	Ø 25 mm
Cut-off	~ 1 µm	0.45 µm	30 kDa (6 ÷ 7.5 nm)
Material	Paper/Glass Fiber	Mixed cellulose esters (MCE)	Regenerated cellulose
Maximum flux	2.2 ml min^{-1}	0.5 ml min^{-1}	0.17 ml min^{-1}



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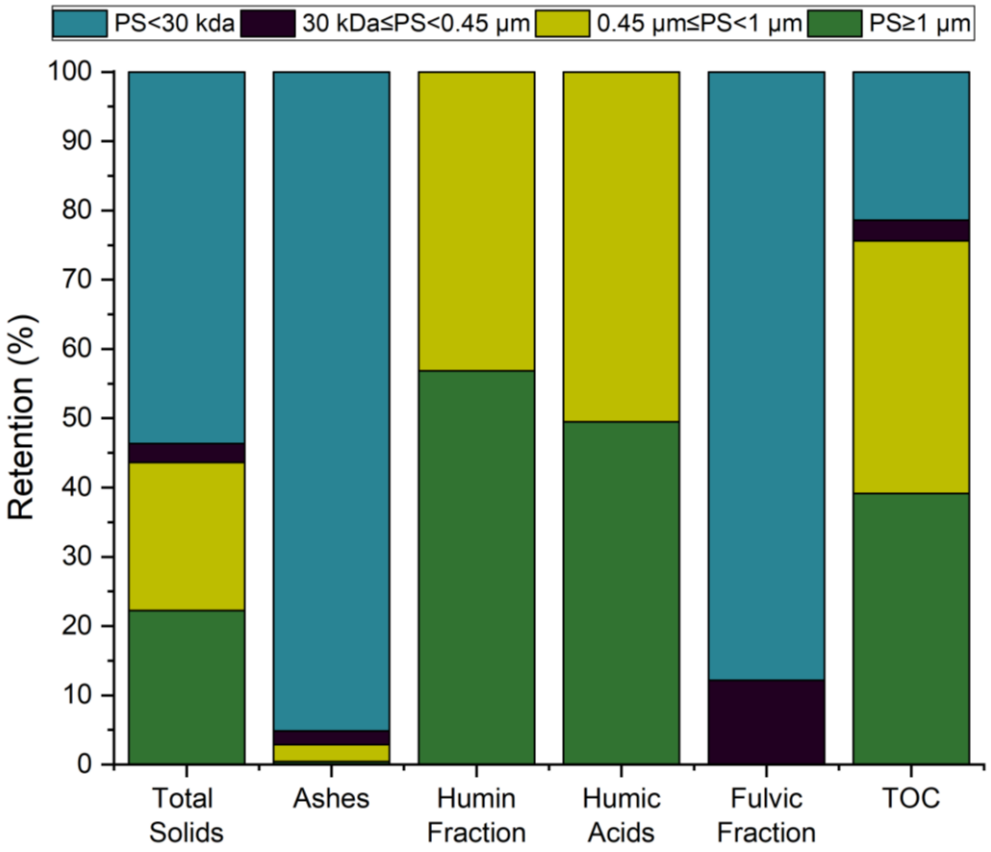
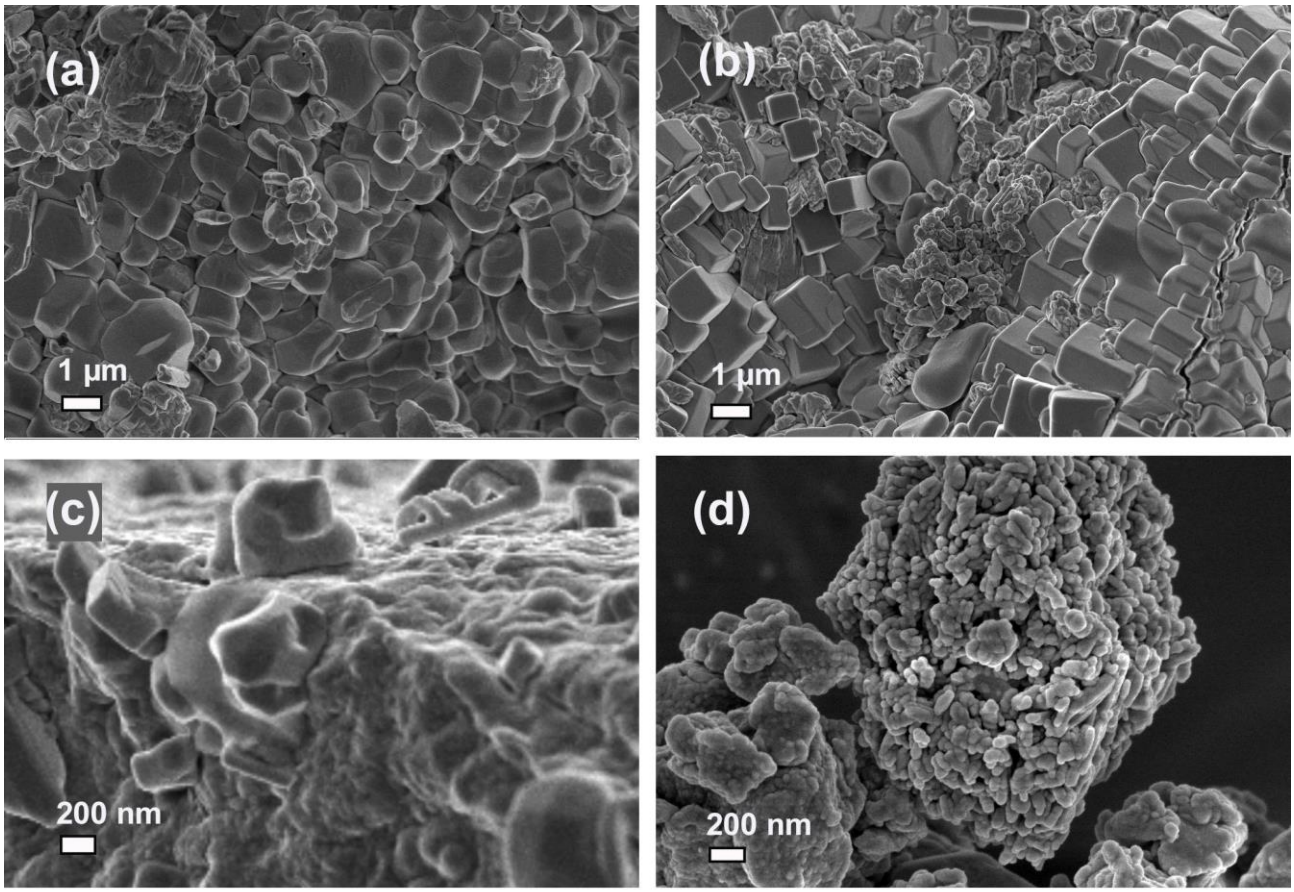
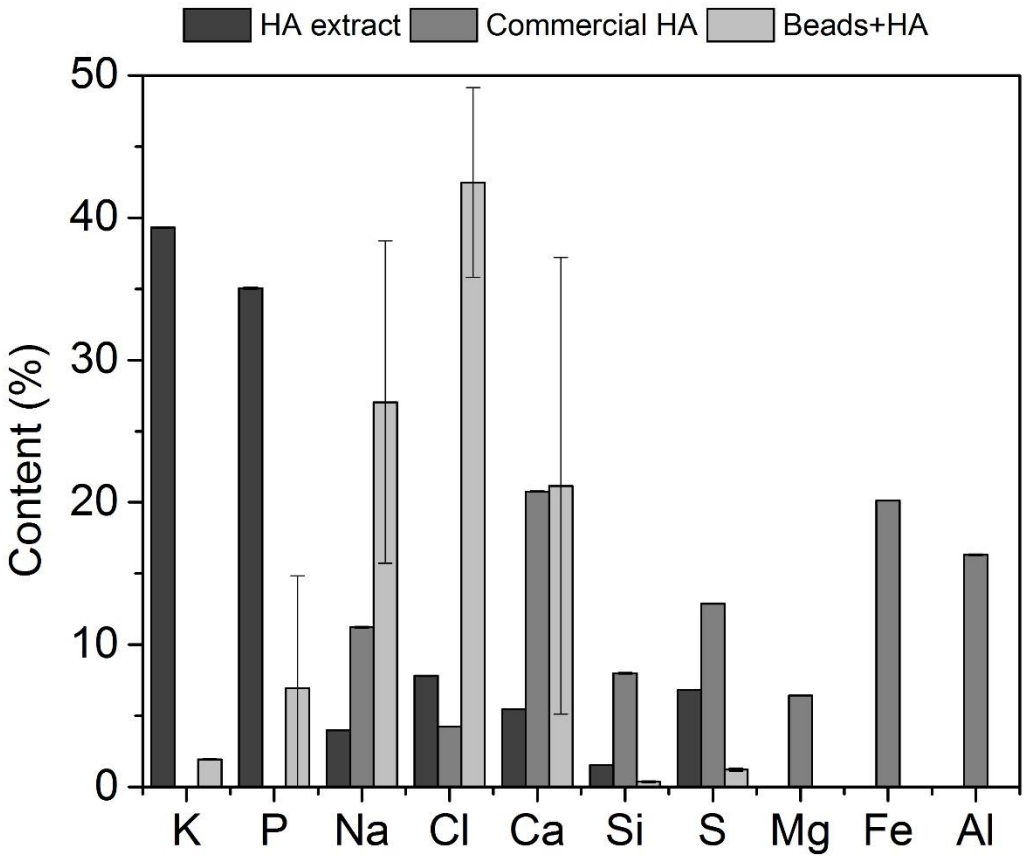
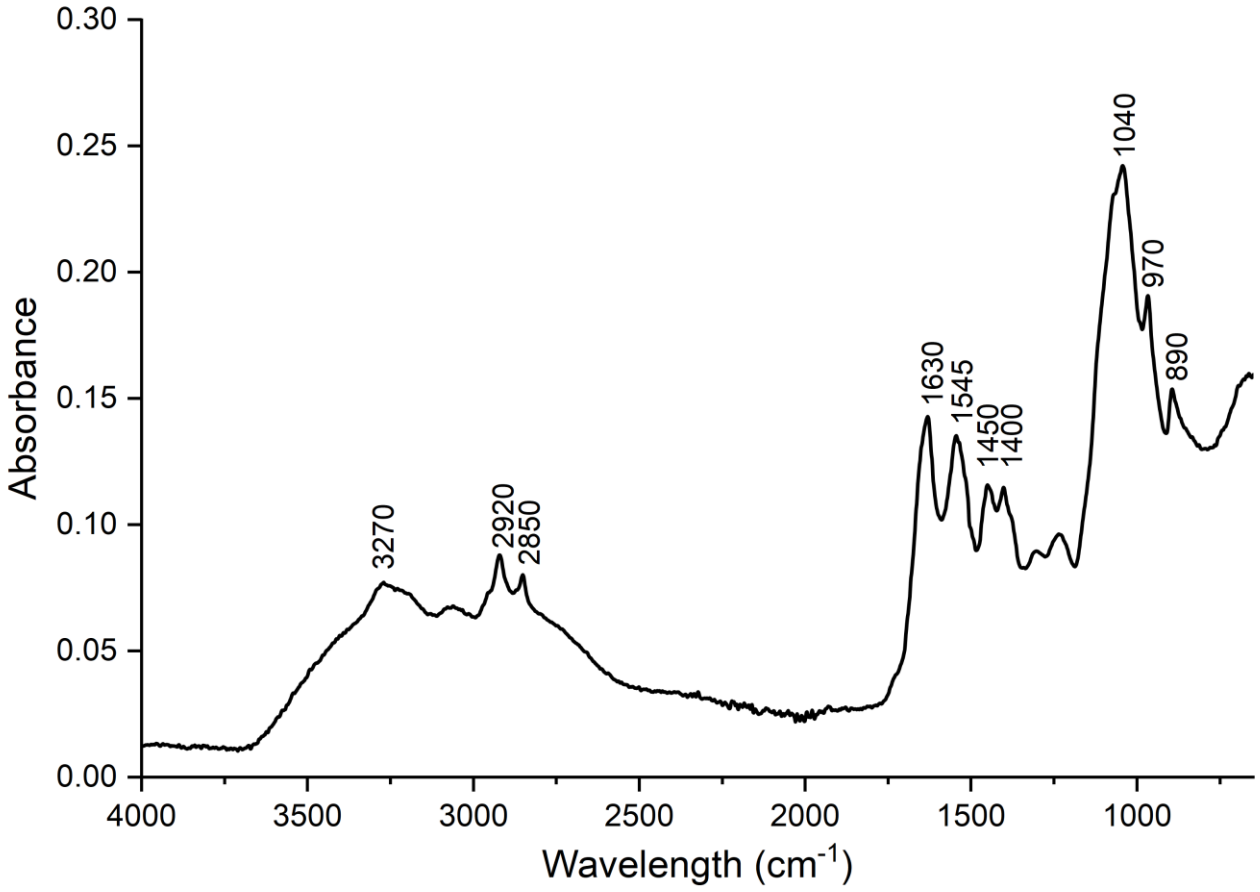

PS<30 kda
30 kDa≤PS<0.45 µm
0.45 µm≤PS<1 µm
PS≥1 µm
Retention (%)
100
90
80
70
60
50
40
30
20
10
0
Total Solids
Ashes
Humin Fraction
Humic Acids
Fulvic Fraction
TOC

647

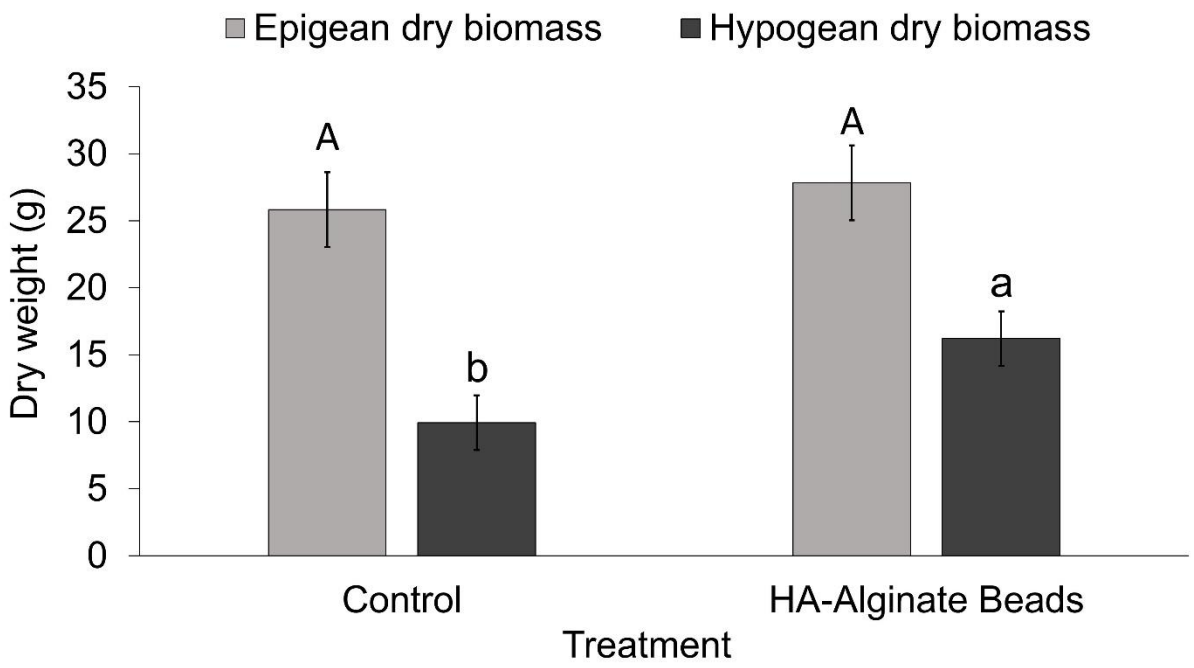

(a)
1 μm
(b)
1 μm
(c)
200 nm
(d)
200 nm


HA extract
Commercial HA
Beads+HA
Content (%)
0
10
20
30
40
50
K
P
Na
Cl
Ca
Si
S
Mg
Fe
Al


Absorbance
Wavelength (cm-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
4000
3500
3000
2500
2000
1500
1000
3270
2920
2850
1630
1545
1450
1400
1040
970
890

670

671


Epigean dry biomass
Hypogean dry biomass
Dry weight (g)
35
30
25
20
15
10
5
0
A
b
A
a
Control
HA-Alginate Beads
Treatment

672

673

CRediT authorship contribution statement

Giulio Cristina: Conceptualization, Data curation, Investigation, Software, Writing - original draft, Validation.

Enrico Camelin: Conceptualization, Data curation, Investigation, Visualization, Writing - original draft.

Carminna Ottone: Data curation, Formal analysis, Methodology, Project administration, Writing - original draft.

Silvia Fraterrigo Garofalo: Investigation, Data curation, Writing – review & editing.

Lorena Jorquera: Methodology, Resources, Writing - review & editing.

Mónica Castro: Resources, Supervision.

Debora Fino: Supervision.

Maria Cristina Schiappacasse: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision.

Tonia Tommasi: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.