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Research article

Application of extracellular polymeric substances recovered from aerobic granular sludge as bio-based hydrogels for plant cultivation systems

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ABSTRACT

Wastewater treatment plants generate large amounts of excess sludge, whose management represents an environmental and economic challenge, motivating resource recovery strategies in line with European Union Directive 2024/3019. Among these, the extraction of extracellular polymeric substances (EPS) enables conversion of sludge-derived biopolymers into sustainable hydrogel materials. However, the effects of structural EPS (sEPS) on nutrient availability and plant performance remain poorly understood. This study investigated aerobic granular sludge derived sEPS as hydrogel-based substrates for in vitro seedling cultivation. Garden cress (*Lepidium sativum* L.) was grown in substrates containing 0, 1, 2, and 4 g TS_{sEPS} L^{-1} sEPS, applied as stand-alone hydrogels (sEPS-CM) or combined with Murashige and Skoog medium, with and without sucrose (MS + sEPS-CM; MS + sEPS + S-CM). Nutrient availability, Ca/N stoichiometry, and plant responses were evaluated. In MS-based systems, increasing sEPS concentrations reduced bioavailable Ca^{2+} and inorganic nitrogen, inducing a shift in Ca/N stoichiometry. Germination was not affected. In stand-alone hydrogels, the best responses were observed at 1 g TS_{sEPS} L^{-1}, with maximum shoot and root growth and vitality, whereas 4 g TS_{sEPS} L^{-1} showed inhibitory effects. Nutrient-rich systems, with increasing sEPS concentrations, reduced dry biomass, pigments, soluble sugars, and photosynthetic performance, with the strongest negative effects at 4 g TS_{sEPS} L^{-1}. The MS + sEPS + S system showed the highest performance, highlighting the role of carbon availability. Overall, sEPS act as structural matrices and regulators of nutrient availability. Moderate concentrations (~1 g TS_{sEPS} L^{-1}) are compatible with plant development, whereas high levels induce nutrient limitation, highlighting potential as bio-based substrates for sustainable cultivation systems.

1. Introduction

Wastewater treatment plants (WWTPs) generate large amounts of excess sludge as a by-product of biological treatment processes. The management and disposal of excess sludge represent a major environmental and economic challenge for the water sector, accounting for a significant share of the operational costs of wastewater treatment. In the context of circular bioeconomy strategies, with the new directive concerning the urban wastewater treatment (Directive 2024/3019, 2024) attention has been dedicated to resource recovery approaches capable of converting wastewater-derived residues into valuable secondary raw materials.

Among the most promising valorization pathways is the extraction of extracellular polymeric substances (EPS) from waste sludge. EPS are complex biopolymers mainly composed of polysaccharides, proteins, lipids, and humic-like substances, characterized by a high density of functional groups capable of interacting with water and dissolved ions (Felz et al., 2019; Seviour et al., 2019). Due to their physicochemical properties, EPS are of growing interest as sustainable bio-based materials with potential applications in environmental engineering, biotechnology, and agriculture.

Particularly relevant in this context are EPS derived from aerobic granular sludge (AGS). AGS technology has recently been established as an efficient alternative to conventional activated sludge systems for biological wastewater treatment (Pronk et al., 2015). Granular sludge consists of dense microbial aggregates characterized by high settling

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velocities and a multilayered structure that allows the simultaneous removal of organic carbon, nitrogen, and phosphorus (Campo et al., 2020). AGS waste can reach solid concentrations of approximately 11% in weight through simple draining processes, making it a valuable feedstock for recovery and valorization strategies (Campo et al., 2022; Pagliaccia et al., 2024).

In AGS systems, microorganisms produce large quantities of highly hydrated EPS that form a hydrogel-like matrix in which the microbial cells are embedded (Felz et al., 2016). Within the EPS matrix, structural extracellular polymeric substances (sEPS) represent a specific fraction capable of forming three-dimensional hydrogel networks that contribute to the mechanical stability of granular sludge (Felz et al., 2016). These polymers can be extracted using physicochemical methods, and are then subsequently valorized in a variety of applications, including textile and paper coatings (Feng et al., 2024; Lin et al., 2015), cement additives (Karakas et al., 2020) bio-flocculants (Amin Vieira da Costa et al., 2022), and metal adsorption materials (Li et al., 2017).

Beyond these industrial applications, the recovery and valorization of structural extracellular polymeric substances from wastewater-derived sludge may also offer promise in the production of functional bio-based materials. In fact, AGS-derived sEPS have recently attracted interest for their potential role as bio-based hydrogel materials in environmental and agricultural systems. Due to their high water-binding capacity, swelling properties, and the presence of functional groups such as carboxyl and hydroxyl moieties, EPS can interact with dissolved ions and nutrients in aqueous environments (Felz et al., 2016; Seviour et al., 2019). Previous studies have shown that sEPS hydrogels can absorb and release nutrients and water, suggesting possible applications in water and nutrient management for agricultural substrates (Pagliaccia et al., 2024). To date, a patented sEPS derived bioproduct (named Kaumera®) is currently tested as soil conditioner/amendment (Kaumera, 2026; Miranda et al., 2025).

In this sector, plant cultivation systems represent a potential application domain for EPS-derived hydrogel materials. Protected crop cultivation and plant propagation increasingly rely on specialized culture media that support plant germination and development under controlled conditions, particularly in soilless production systems (Gruda et al., 2013). In addition to plant tissue culture, hydrogel-based cultivation systems are increasingly used for the production of high-value crops characterized by very short production cycles, such as microgreens, where rapid germination and early seedling development determine crop quality and marketability (Di Gioia et al., 2021; Kyriacou et al., 2016; Truschi et al., 2026). In these systems, the ability of hydrogel matrices to regulate water and nutrient availability during the first days of growth is particularly relevant. In plant biotechnology and tissue culture applications, these substrates provide a controlled environment that supply essential macro- and micronutrients, carbon sources, vitamins, and growth regulators necessary for plant development (FAO, 2017). Among the most widely used formulations, the Murashige and Skoog (MS) medium remains a standard reference due to its balanced nutrient composition, particularly its high nitrogen (N) content supplied as ammonium and nitrate (Murashige and Skoog, 1962; Phillips and Garda, 2019). Nevertheless, alternative bio-based materials are of increasing interest to improve nutrient management and sustainability in controlled cultivation systems.

Conventional plant tissue culture systems generally combine a nutrient-rich medium, such as MS, with gelling agents including agar or gellan gum (Phytagel®/Gelrite®), whose primary function is to provide physical support for plant tissues rather than actively regulate nutrient availability (George et al., 2008; Phillips and Garda, 2019). In parallel, hydrogel materials based on alginate, cellulose derivatives, or synthetic polymers have been increasingly investigated for water retention and controlled nutrient delivery in agricultural systems (Guilherme et al., 2015). However, these materials are generally produced from dedicated raw materials and do not contribute to resource recovery from waste streams. In contrast, AGS-derived sEPS combine hydrogel-forming properties with a high density of ion-binding functional groups and originate from wastewater treatment residues, suggesting that they may simultaneously act as structural matrices, regulators of nutrient availability, and renewable bio-based materials within a circular bioeconomy framework. These characteristics make sEPS an attractive candidate for multifunctional cultivation substrates, although their interactions with nutrient dynamics and plant responses remain largely unexplored.

Despite these potential advantages, previous studies on EPS-derived materials have mainly focused on their physicochemical properties and environmental engineering applications, such as flocculation, adsorption and water retention. Only limited research has explored their potential use in plant cultivation systems (Miranda et al., 2025), and, to the best of the authors' knowledge, no studies have investigated their role as hydrogel-based substrates. In particular, limited information is available on how EPS influence nutrient availability and stoichiometric balances in plant growth substrates and how these chemical changes translate into plant physiological responses. Essential nutrients such as calcium (Ca) and N play crucial roles in plant metabolism, cell wall stability, and signal transduction processes (Hawkesford et al., 2011; White and Broadley, 2003). Therfore, alterations in their relative availability may therefore influence seedling development and photosynthetic performance.

The central research question addressed in this work is how sEPS-derived hydrogels influence nutrient availability and Ca/N stoichiometry in plant cultivation systems, and how these changes affect seed germination, seedling growth, and physiological performance. We hypothesized that, owing to their hydrogel-forming properties and ion-binding capacity, AGS-derived sEPS can act as a functional component of plant cultivation systems by modulating nutrient availability in a concentration-dependent manner. Specifically, we expected that moderate sEPS concentrations would be compatible with plant development, whereas higher concentrations would reduce nutrient availability through excessive ion complexation, leading to altered physiological responses. To test this hypothesis, the present study investigates the potential use of sEPS extracted from aerobic granular sludge as hydrogel-based component of plant cultivation system, using garden cress (*Lepidium sativum* L.) as a model species. Specifically, the objectives of this work were to.

1. Evaluate the potential phytotoxic or stimulatory effects of sEPS when used as a standalone hydrogel substrate by assessing seed germination, early seedling growth, and physiological responses of *Lepidium sativum* L.;
2. Investigate how increasing sEPS concentrations influence nutrient availability and Ca/N stoichiometry when combined with a nutrient-rich culture medium MS;
3. Explore the relationships between hydrogel chemical properties and plant physiological traits through multivariate analysis.

By integrating chemical and physiological approaches, the objective was to provide new insights into the interactions between AGS-derived sEPS and plant cultivation systems, contributing to the evaluation of sEPS as sustainable biomaterials for controlled plant production systems.

2. Materials and methods

2.1. sEPS extraction and characterization

The AGS used as feedstock for sEPS recovery was collected from a full-scale aerobic granular sludge reactor treating real municipal wastewater in Utrecht (The Netherlands) and was previously used in a study on the agronomic valorization of sEPS (Pagliaccia et al., 2024). The extraction of sEPS was carried out according to an agriculture-oriented physicochemical method reported by Pagliaccia et al. (2024). Briefly, 200 μm-sieved AGS was kept in contact in a

K_2CO_3 aqueous solution (6.5 g L^{-1}, pH = 11.3) at a concentration of 0.06 g mL^{-1} on a wet weight basis (i.e. 60 g WW L^{-1} with 0.11 ± 0.01 g TS g WW^{-1}; WW: Wet weight, TS: Total solids) under stirred conditions (400 rpm) at a controlled temperature of 80 °C for 1 h to promote the solubilization of the EPS matrix and thus the dissolution of the granular 3D-structure. The disintegrated granules in the alkaline aqueous phase were then centrifuged (4000 g, 20 min, 4 °C). The resulting supernatant, containing the solubilized EPS, was first dialyzed in a 3500 Da dialysis bag (SnakeSkin™ Dialysis Tubing, 3.5 kDa MWCO, 35 mm, Thermo Scientific™; about 24 h of dialysis with one water replacement) against demineralized water and then further processed via acidic precipitation by adding 0.1 M HNO_3 until a pH of 2.20 ± 0.05 was achieved. The fraction of sEPS was recovered after centrifugation (4000 g, 20 min, 4 °C) in the form of acidic pellets and then re-suspended under alkaline conditions (pH = 8.5 - 9) by dosing 1 M KOH aqueous solution. Total Solids (TS) and Volatile Solids (VS) of the polymeric aqueous suspensions thus formed were measured according to standard methods (Baird et al., 2017) and their sEPS concentrations were expressed as a TS to WW ratio. This method yielded about 207 ± 9 mg VS_{sEPS}/g VS_{AGS} (Pagliaccia et al., 2024). Among the chemicals tested in the previous research (Pagliaccia et al., 2024), those applied for the present study resulted in an extraction yield closer to that reported in the literature for a well-established method assumed as a benchmark (Felz et al., 2016) while decreasing the concentrations of potentially phytotoxic elements (e.g. sodium and chlorine) in the extracted biopolymers compared to such a reference protocol. The main characterization data of the sEPS here used, including biochemical composition, elemental analysis and relevant hydrogel-forming properties, are listed in Supplementary Table 1; for the complete dataset, refer to Pagliaccia et al. (2024) with reference to extraction protocol named "Agro 1".

2.2. Preparation of the plant culture media

The sEPS aqueous suspensions obtained as described in section 2.1 were then used for the preparation of plant culture media under various operative conditions (Fig. 1). The sEPS were incorporated into gelled culture media because the objective of this study was to evaluate their potential as functional components of hydrogel-based plant cultivation systems rather than as soluble amendments. Four concentrations of sEPS (0, 1, 2, and 4 g TS_{sEPS} L^{-1}) were tested in three different media: (i) sEPS as a stand-alone plant culture medium (referred to as "sEPS-CM"), (ii) Murashige and Skoog medium supplemented with sEPS (referred to as "MS + sEPS-CM"), and (iii) MS + sEPS-CM supplemented with sucrose (8%, w/v), a standard carbon source used in plant tissue culture media (Murashige and Skoog, 1962; Phillips and Garda, 2019). The sucrose treatment was included to distinguish the effects of an external carbon source from those associated with the carbohydrate fraction naturally present in sEPS (Felz et al., 2019; Pagliaccia et al., 2024).

Culture media were prepared by adding the required amount of sEPS suspension to obtain the desired concentration on a dry weight basis (g TS_{sEPS} per L of media). Phytagel (Sigma-Aldrich; USA), was added to all culture media at a concentration of 2 g L^{-1}. Calcium chloride dihydrate (400 mg L^{-1} of $CaCl_2{\cdot}2H_2O$) was added only to sEPS-CM to provide sufficient calcium for gelling, whereas no additional calcium chloride was added to MS-based media because calcium was already present in the MS salt mixture.

The pH was adjusted to 5.8 before the final volume (80 mL) was reached, and the media were sterilized by autoclaving (120 °C, 20 min). Three biological replicates (90-mm Petri dishes containing approximately 20 mL of the medium) were prepared for each treatment and then stored at 20 °C to allow the solution to solidify overnight.

2.3. Overlay-leachate collection and quantification of bioavailable Ca, NH_4^+, and NO_3^-

After gel solidification, 10.0 mL of sterile liquid MS medium prepared at the standard (1×) concentration (without gelling agents) was added onto each gel surface (90-mm Petri dish; gel volume 20 mL). The systems were left undisturbed at 20 °C for 1 h to allow gel–solution equilibration. The overlay leachate (approximately 8-9 mL) was then collected, clarified by centrifugation (3000 × g, 5 min), and used for subsequent analyses.

The electrical conductivity (EC) of the clarified overlay leachates (prior to ultrafiltration) was measured using a calibrated conductivity meter (InLab® 751-4 mm conductivity probe, Mettler Toledo, Greifensee, Switzerland; measuring range 0.01-100 mS cm^{-1}). Measurements were performed at 25 °C with automatic temperature compensation, and EC values were expressed in mS cm^{-1}.

Clarified leachates were subjected to centrifugal ultrafiltration (3 kDa molecular-weight cut-off membranes). The permeate, operationally defined as the low-molecular-weight fraction containing freely dissolved ions and small inorganic complexes, was analyzed. Liquid MS 1× s processed under the same conditions served as a matrix-matched blank.

The permeate was divided into two aliquots. One aliquot was acidified with HNO_3 (1% v/v; trace metal grade, Carlo Erba, Rodano, Milan, Italy) and analyzed for Ca by inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 7300 DV, PerkinElmer Inc., Shelton, CT, USA). The second aliquot was kept at 4 °C and analyzed spectrophotometrically for inorganic N species. Samples were diluted as

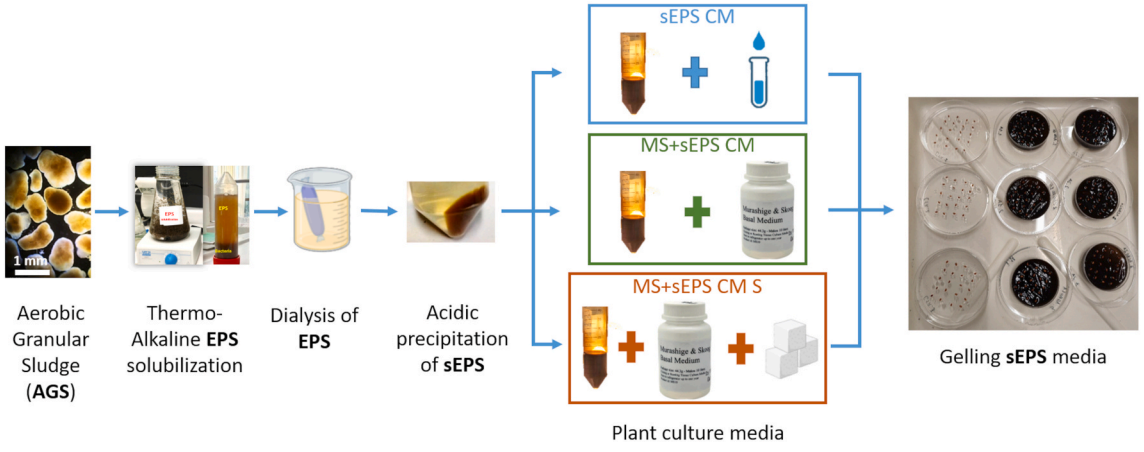

Fig. 1. Schematic workflow overview for the preparation of gelling media used in plant culture. Media formulations include from the top to the bottom: (i) stand-alone sEPS medium (sEPS-CM), (ii) a combination of Murashige and Skoog medium with sEPS (MS + sEPS-CM), and (iii) MS + sEPS-CM supplemented with sucrose (MS + sEPS-CM + S).

required (1:200 for MS-based leachates and 1:5 for sEPS-only leachates), and calibration standards were prepared in matrix-matched blanks. Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were determined using a Lambda 20 UV–Vis spectrophotometer (PerkinElmer, Waltham, MA, USA). Nitrate was quantified using the Griess method following reduction to nitrite, with absorbance measured at 540 nm (Irandoust et al., 2013), whereas ammonium was determined using the salicylate-hypochlorite (indophenol blue) method, with absorbance measured at 655 nm, according to APHA et al. (2017).

Quality control (QC) included duplicate analysis of each sample. In addition, four QC standards (distilled water blank and 0.5, 5, and 50 mg L^{-1}) were analyzed for every 20 samples to verify analytical accuracy and instrument stability.

Total available N was calculated as $NH_4^+ + NO_3^-$. Relative Ca and Relative N were expressed as the ratio between each treatment and the corresponding 0 g TS_{sEPS} L^{-1} control within the same medium type. The Ca/N ratio was calculated as Ca^{2+} divided by total available N.

2.4. Growth conditions

Common garden cress (*Lepidium sativum* L.) seeds were surface-sterilized by immersion in 10% (v/v) sodium hypochlorite for 10 min and rinsed five times with sterile distilled water. Twenty sterile seeds were sown in each prepared Petri dishes under a fume hood. The Petri dishes were incubated at 20 °C in darkness. Germination and early seedling development were assessed following the European Standard protocol EN 16086-1 and EN 16086-2. The trial was initially designed for a 72 h, but extended to 168 h to evaluate whether treatment effects persisted beyond the standard test duration.

2.5. Phytotoxicity assessment

Germination rate (%) was measured at 72 h and 168 h after sowing by counting the germinated seeds out of the total sown seeds. Root and shoot lengths (cm) were also measured at 72 h and 168 h. After 168 h, shoot and root dry biomass weights were determined after oven drying at 105 °C for 24 h. The phytotoxicity of the different sEPS concentrations was assessed using the Munoo–Liisa Vitality Index (MLV; %) (Eq. (1)):

$$MLV\ index_{treat_j} = \frac{\sum_{i=1}^{n}\left[(G.sd * Rt\ lgth)_{Rep_i.treat_j}\right]}{\left[n * \left(G.sd_{CT} * Rt\ lgth_{CT}\right)\right]} * 100 \quad (1)$$

where *G.sd* and *Rt lgth* are the number of germinated seed and root length, respectively for each replication (Rep_i) of the j-esim sEPS level ($treat_j$); n is the number of replications, and $G.sd_{CT}$ and $Rt\ lgth_{CT}$ are the average germinated seeds and the average root length of the control (0 g TS_{sEPS} L^{-1}), respectively.

According to Campos et al. (2022), MLV values > 100% indicated enhanced germination and root growth; values of 80-100%, 60-80%, 40-60% and <40% indicated non-phytotoxic, moderately phytotoxic, phytotoxic, and pronounced phytotoxic effects of the substrate, respectively.

2.6. Determination of seedling physiological parameters

At the end of the incubation period, seedling physiological parameters were measured to evaluate the effects of sEPS concentration on plant growth, carbon metabolism, and photosynthetic performance.

2.6.1. Dry matter determination

Dry matter (DM) was determined on harvesting seedlings at the end of the incubation period after drying seedlings at 65 °C in a ventilated oven until constant weight. Dried biomass was weighed using an analytical balance and expressed as dry matter per sample (mg).

2.6.2. Total soluble sugars

Total soluble sugars (TSS) were quantified using the anthrone colorimetric method according to Yemm and Willis (1954). Fresh plant tissue was homogenized and the soluble carbohydrate fraction was extracted prior to analysis. The anthrone method permits the direct quantification of total soluble carbohydrates, including reducing sugars, sucrose, and other soluble carbohydrate fractions, within a single assay. This approach was selected to avoid potential underestimation of total sugar content that may occur when sucrose and reducing sugars are quantified separately. A calibration curve was prepared using sucrose as the reference standard, and total soluble sugars were expressed as sucrose equivalents (mg g^{-1} FW). Because the anthrone assay responds to different soluble carbohydrates with slightly different sensitivities, the reported values represent estimates of total soluble sugars expressed as sucrose equivalents rather than absolute concentrations of individual carbohydrates. Absorbance was measured spectrophotometrically at 620 nm.

2.6.3. Photosynthetic pigments

Photosynthetic pigments were determined by quantifying chlorophyll *a* (Chla, mg g^{-1} FW), chlorophyll *b* (Chlb, mg g^{-1} FW), and carotenoids (Car, mg g^{-1} FW) from fresh seedling tissues. Chlorophyll extraction was performed using N,N-dimethylformamide (DMF), according to the method of Moran (1982), and absorbance was measured at 647 and 664 nm for Chla and Chlb, Respectively. Carotenoids were extracted with acetone according to Gitelson et al. (2002), and absorbance was measured at 510 nm. Pigment concentrations were calculated from spectrophotometric absorbance using the validated equations and specific extinction coefficients reported by Moran (1982) and Gitelson et al. (2002), respectively. Therefore, no calibration curves based on purified pigment standards were required.

2.6.4. Photochemical Pigment Index

To provide an integrated descriptor of pigment composition, a Photochemical Pigment Index (PPI) was calculated as the ratio between total chlorophylls and carotenoids according to the following equation:

$$PPI = (Chla + Chlb) * Car^{-1}$$

Higher PPI values indicate a greater relative abundance of photosynthetic pigments compared with carotenoids.

2.7. Statistical analysis

Statistical analyses were performed using R software (R Core Team, Vienna, Austria). Data normality was assessed using the Shapiro-Wilk test, and Box-Cox transformation was applied when necessary.

The effects of culture medium type and sEPS concentration on the physiological parameters (DM, TSS, Chla, Chlb, carotenoids, and PPI) were evaluated using a two-way analysis of variance (ANOVA). The experimental design included two fixed factors: culture medium type and sEPS concentration. When significant effects were detected, differences among treatment means were assessed using the Tukey's honestly significant difference (HSD) post hoc test at a significance level of $p < 0.05$.

For parameters measured over time (bioavailable Ca^{2+}, NH_4^+, NO_3^-, and Ca/N ratio), a two-way ANOVA was applied by considering sEPS concentration and incubation time as factors. Significant differences among treatments and time points were evaluated using the Tukey's HSD test ($p < 0.05$).

To explore the relationships between hydrogel chemical properties and plant physiological traits, a principal component analysis (PCA) was performed using standardized variables. The PCA included chemical parameters (Ca^{2+}, NH_4^+, NO_3^-, Ca/N ratio, and EC) and plant physiological variables (DM, TSS, Chla, Chlb, carotenoids, and PPI).

In addition, pairwise relationships between chemical and

physiological variables were assessed using Spearman's rank correlation coefficients. The correlation structure was visualized using a correlation matrix combined with scatterplots and variable distributions.

3. Results

3.1. Effects of sEPS on the ion availability and Ca/N stoichiometry

The two-way ANOVA revealed that both medium type and sEPS concentration significantly affected the concentrations of bioavailable ions and EC in the overlay leachates (Supplementary Table 2).

Significant main effects of both factors were observed for Ca^{2+}, NH_4^+, NO_3^-, total available N, relative Ca availability, and EC ($p < 0.001$ in most cases). Moreover, significant interaction effects between medium type and sEPS concentration were detected for all variables, indicating that the influence of sEPS on the ion availability depended strongly on the chemical composition of the surrounding medium.

Relative N availability and the Ca/N ratio were calculated only for MS-based media. In sEPS-only media (sEPS-CM), absolute N concentrations were negligible compared to those in MS-containing media and were therefore not considered suitable for calculating relative N availability or Ca/N ratios. A separate two-way ANOVA was performed on the relative N availability and the Ca/N ratio. Results confirmed that the sEPS concentration had a highly significant effect on both parameters ($p < 0.001$), whereas medium type did not significantly influence relative N availability (Supplementary Table 3).

The detailed concentrations of bioavailable Ca^{2+}, inorganic N species, total available N, and EC for each treatment are reported in Supplementary Table 4. In the sEPS-CM systems, increasing sEPS concentrations were associated with a progressive decrease in bioavailable Ca^{2+} and a simultaneous increase in inorganic N species, resulting in a gradual increase in total available N and EC. In contrast, in the MS-based media, increasing sEPS concentrations were associated with a consistent reduction in both bioavailable Ca^{2+} and inorganic N species. As a consequence, total available N and EC decreased progressively with increasing sEPS levels.

To further assess whether the presence of an external carbon source influenced nutrient dynamics, MS-based media supplemented with sucrose were compared with those without sucrose (Fig. 2). This comparison was performed to evaluate whether sucrose addition could affect the availability of Ca^{2+} and inorganic nitrogen species in the hydrogel-solution system. The results indicated that the presence of sucrose did not produce significant differences in the patterns of Ca^{2+} and inorganic N availability across sEPS treatments, suggesting that nutrient dynamics were primarily driven by sEPS concentration rather than by the presence of an external carbon source. These trends resulted in a marked shift in the Ca/N balance in MS-based systems (Fig. 2). In MS-based media, the Ca/N ratio decreased progressively with increasing sEPS concentrations, from approximately 4.2-4.3% in the control treatments to values below 1.5% at 4 g TS_{sEPS} L^{-1} sEPS. Overall, increasing sEPS concentrations were associated with progressive changes in Ca-N balance within the tested cultivation systems, particularly in MS-based media.

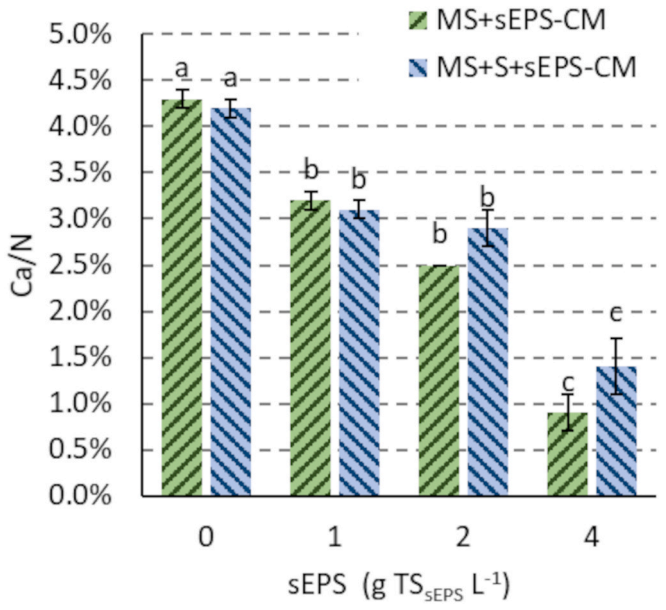

Fig. 2. Effect of increasing sEPS concentration on the Ca/N ratio in MS-based hydrogel systems. Bars represent mean ± standard error. Different letters indicate significant differences among treatments according to Tukey's HSD test ($p < 0.05$).

3.2. Effects of sEPS concentration and incubation time on Ca^{2+}, inorganic nitrogen, and Ca/N stoichiometry in MS-based hydrogel systems

This section focuses on the effects of sEPS concentration and incubation time on nutrient availability in MS-based hydrogel systems, with and without sucrose. The analysis was specifically conducted on these systems to evaluate how sEPS interacts with a nutrient-rich medium and influences the availability of Ca^{2+} and inorganic nitrogen species. The standalone sEPS system was not included in this comparison, as it does not contain a defined nutrient solution and was primarily used to assess phytotoxicity rather than nutrient dynamics.

Bioavailable Ca^{2+} concentrations in the overlay leachates declined progressively with increasing sEPS concentrations at all incubation times (Fig. 3A and B). The highest Ca^{2+} levels were consistently observed in the control treatments (0 g TS_{sEPS} L^{-1}), whereas the lowest values occurred at the highest sEPS concentration (4 g TS_{sEPS} L^{-1}). Significant differences among sEPS levels were detected within each incubation time according to the Tukey's post hoc test. In addition to the sEPS effect, Ca^{2+} availability generally decreased over time in most treatments, particularly in the absence or at low sEPS concentrations (Fig. 3). At the highest sEPS level, Ca^{2+} concentrations remained consistently low and showed limited variation during the incubation period. No significant differences were detected between MS-based media with and without sucrose.

A similar temporal pattern was observed for inorganic N species. Ammonium concentrations declined with increasing sEPS concentrations and decreased markedly over time in media with and without sucrose (Fig. 3C and D). The highest NH_4^+ concentrations were recorded at 24 h in the control treatments, whereas progressively lower values were observed at both higher sEPS levels and later incubation times. By 168 h, NH_4^+ concentrations were substantially reduced across all treatments. As observed for Ca^{2+}, no significant differences were detected between the media with and without sucrose.

NO_3^- concentrations also decreased with increasing sEPS concentrations and incubation time (Fig. 3E and F). At each time point, the control treatments exhibited the highest NO_3^- concentrations, while progressively lower values were observed with increasing sEPS levels. In both media types, NO_3^- levels generally declined from 24 h to 72 h and further decreased by 168 h, although the magnitude of this temporal decrease varied among sEPS treatments.

Since Ca and inorganic N responded differently to increasing sEPS concentrations, the Ca/N ratio changed markedly over time and across treatments (Fig. 3G and H). In both types of culture media, increasing sEPS concentrations resulted in a strong reduction of the Ca/N ratio at all incubation times. The highest Ca/N ratios were consistently observed in the control treatments, whereas the lowest values occurred at the highest sEPS concentration. In addition to the sEPS effect, the Ca/N ratio generally increased over time in most treatments, reflecting a stronger temporal decline in N availability compared with Ca. However, this temporal increase was less pronounced at the highest sEPS concentration, where Ca availability remained strongly limited throughout the

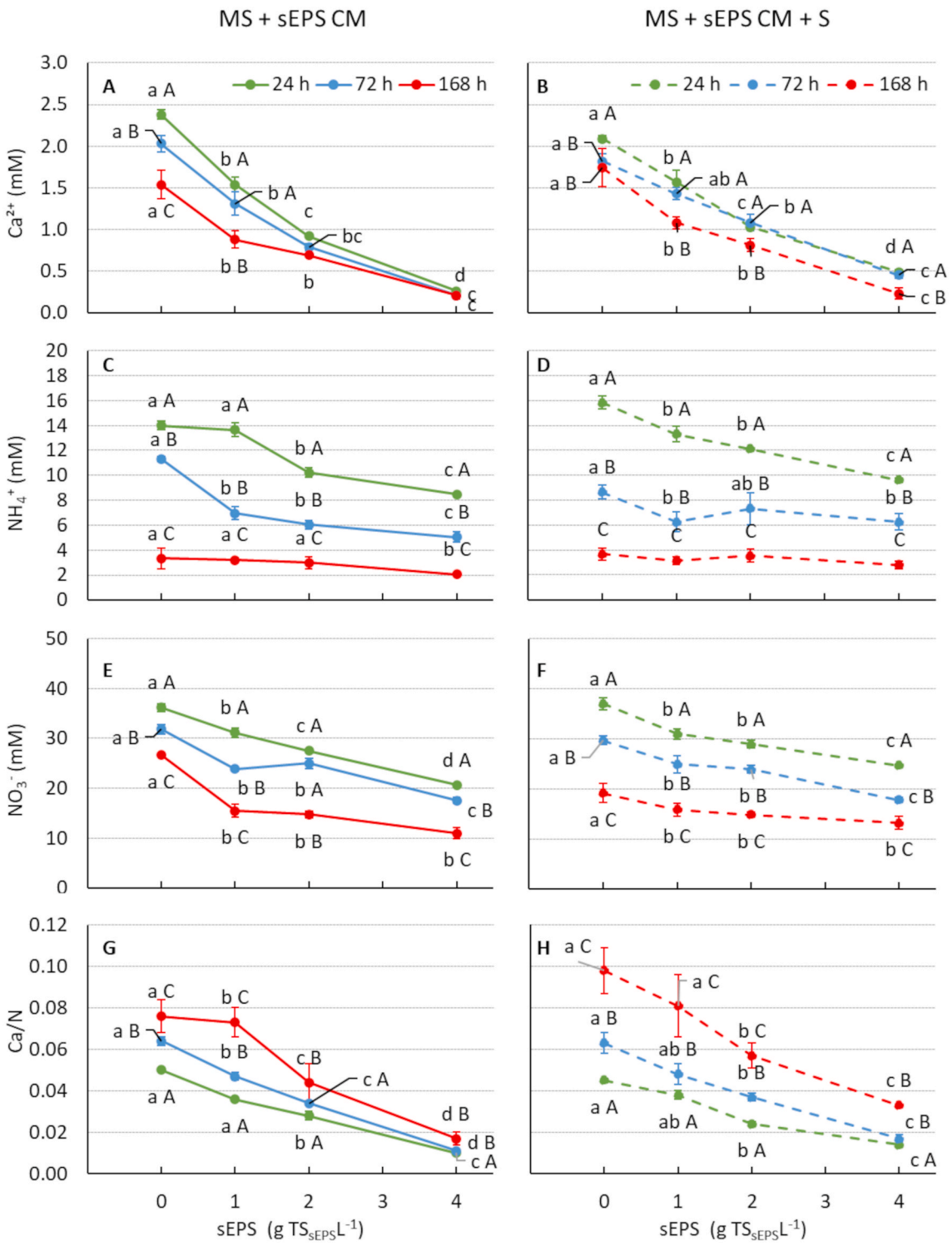

Fig. 3. Time-dependent changes in bioavailable Ca^{2+}, NH_4^+, NO_3^- concentrations and Ca/N ratio in overlay leachates of hydrogel-based culture media at increasing sEPS concentrations. Panels show MS + sEPS-CM (left column) and MS + sEPS-CM + S (right column). Measurements were performed after 24, 72, and 168 h of incubation. Points represent mean, while whiskers represent the standard errors. Different lowercase letters indicate significant differences among sEPS concentrations within the same incubation time, whereas different uppercase letters indicate significant differences among incubation times within the same sEPS level (Tukey's HSD test, $p < 0.05$).

incubation period. Taken together, these results show that nutrient availability and Ca/N stoichiometry varied as a function of both sEPS and incubation time within the tested hydrogel-based culture systems.

3.3. Plant responses to sEPS-based hydrogels

Plant responses were evaluated through a stepwise approach. Germination was first assessed across both standalone sEPS substrates and MS-based systems to identify potential inhibitory effects under different nutritional conditions. Subsequently, seedling growth and

vitality analyses were conducted specifically on the standalone sEPS system (sEPS-CM), following standard phytotoxicity assessment protocols aimed at evaluating the intrinsic effects of the material. Finally, seedling growth and physiological traits were analyzed across all culture media to assess how sEPS interacts with nutrient-rich systems and influences plant performance under different cultivation conditions.

3.3.1. sEPS effect on germination rate

The use of sEPS both as a stand-alone culture substrate (sEPS-CM) and in combination with MS nutrients did not significantly affect the germination rate of common garden cress seeds. Germination reached approximately 100% at 72 h after sowing in all treatments, and no additional germination events were observed at 168 h (Fig. 4), indicating that sEPS did not exert inhibitory effects on seed germination.

3.3.2. Seedling growth responses

In stand-alone sEPS substrates (sEPS-CM), seedling growth was influenced by the sEPS concentration. At 72 h after sowing, the longest average root length was observed at 1 g TS_{sEPS} L^{-1} sPS (1.09 ± 0.13 cm). Significantly reduced shoot length was measured in the control and in treatments with higher sEPS concentrations (Fig. 5). Shoot length followed a similar trend, with a non-significant increase at 1 g TS_{sEPS} L^{-1} level compared with the control and progressively shorter shoots at higher sEPS levels.

These differences became more pronounced after 168 h of growth (Fig. 5). Seedlings grown at 1 g TS_{sEPS} L^{-1} exhibited the longest shoots (4.86 ± 0.61 cm) and roots (3.32 ± 0.39 cm), while those grown at 4 g TS_{sEPS} L^{-1} showed significantly reduced shoot and root lengths. Intermediate responses were observed at 2 g TS_{sEPS} L^{-1}. Overall, seedling growth followed a concentration-dependent pattern, with maximum development at 1 g TS_{sEPS} L^{-1} and progressively reduced growth with increasingly higher sEPS concentrations (Fig. 5).

3.3.3. Seedling vitality response to sEPS concentration

The MLV index confirmed the sEPS concentration-dependent response observed for seedling growth (Fig. 6). At 72 h after sowing, substrates containing 1 and 2 g TS_{sEPS} L^{-1} showed higher MLV index values compared to the control, indicating a stimulatory effect on seedling development. In particular, the MLV index increased by approximately 104% and 31% in 1 and 2 g TS_{sEPS} L^{-1}, respectively, relative to the control.

These differences became more pronounced after 168 h. At this stage, the MLV index for substrates containing 1 g TS_{sEPS} L^{-1} was approximately three times higher than in the control, while that for 2 g TS_{sEPS} L^{-1} similarly showed a substantial increase in vitality compared to the control. According to the phytotoxicity classification proposed by Campos et al. (2022), these treatments can be classified as non-phytotoxic and potentially phyto-stimulatory during the early stages of seedling development.

In contrast, the highest sEPS concentration (4 g TS_{sEPS} L^{-1}) produced a clear reduction in the MLV index values at both incubation times. In this treatment, the MLV index was approximately 42% and about 56% lower than that of the control at 72 h and 168 h, respectively. This

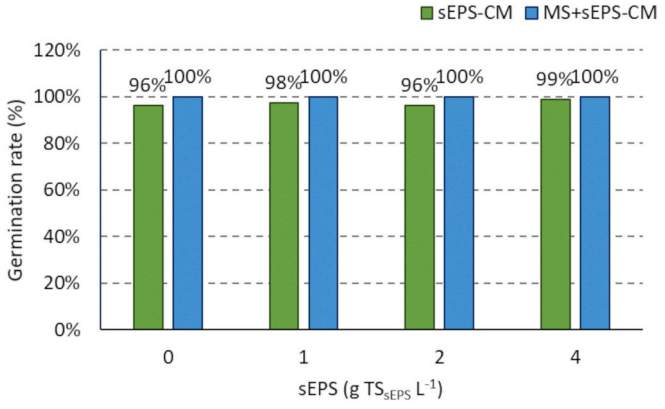

Fig. 4. Average germination rate (n = 3) of different treatments for stand-alone sEPS (sEPS-CM) and MS + sEPS combination (MS + sEPS-CM) at 72 h as a function of different concentrations of sEPS in the culture medium.

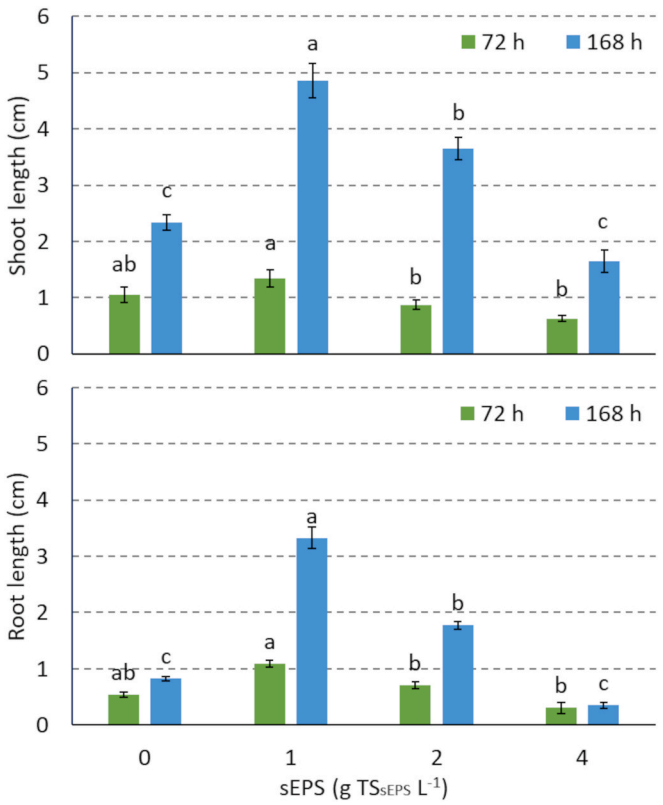

Fig. 5. Average shoot (upper panel) and root length (lower panel) of common garden cress seedlings with standard deviation (n = 3) for stand-alone sEPS (sEPS-CM) at both 72 h and 168 h as function of different levels of sEPS. Different letters indicate significant differences ($p < 0.05$) between sEPS levels according to a post-hoc Tukey test ($p < 0.05$).

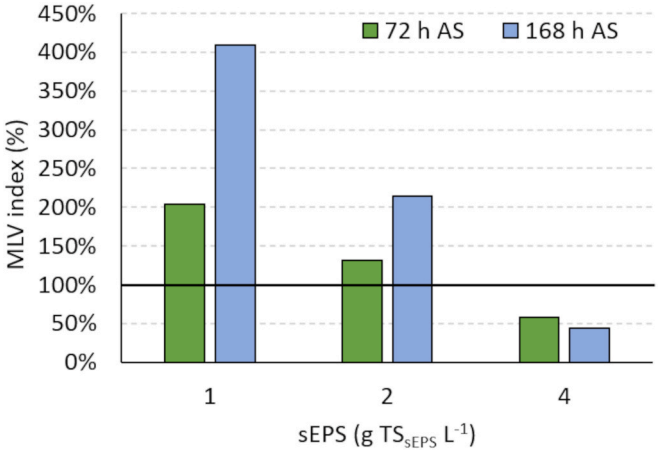

Fig. 6. Munoo-Liisa vitality index (MLV; %) for stand-alone sEPS (sEPS-CM) as function of different level of sEPS. Green histograms are referred to the MLV values at 72 h after sowing (72 h AS), while the blue ones are those at 168 h after sowing (168 h AS). The horizontal black line represents the MLV value of the control (0 g TSsEPS L^{-1}), used as the reference treatment.

indicated an inhibitory effect of high sEPS concentrations on early seedling development, consistent with a phytotoxic response under the tested conditions.

Overall, the MLV index results confirmed that moderate sEPS concentrations promoted early seedling development, whereas higher concentrations inhibit growth during the experimental period.

3.3.4. *Effects of sEPS on seedling growth and physiological traits*

Following the assessment of the intrinsic phytotoxicity of sEPS in standalone systems (Sections 3.3.2–3.3.3), this section evaluates seedling growth and physiological responses across all culture media. This comparison was performed to determine how sEPS interacts with nutrient-rich systems and to assess whether the effects observed in standalone substrates persist or are modified under different nutritional conditions. In particular, this analysis aims to distinguish between direct material effects and nutrient-mediated responses.

The two-way ANOVA indicated a significant interaction between medium type and sEPS concentration, demonstrating that the effect of sEPS on dry biomass was dependent on the nutritional composition of the culture medium (Supplementary Table 5).

Dry biomass measurements revealed different responses depending on whether sEPS was used as a stand-alone substrate or combined with MS nutrients (Fig. 7).

In the stand-alone sEPS substrate (sEPS-CM), seedlings produced very limited biomass and the sEPS concentration had only a minor influence on dry matter accumulation. Biomass content as dry matter remained low across treatments, with no clear dose-dependent inhibition within the tested sEPS concentration range (Fig. 7). In contrast, when sEPS was combined with MS nutrients, increasing sEPS concentrations resulted in a progressive reduction in seedling biomass (Fig. 7). In both MS + sEPS-CM and MS + sEPS + S-CM systems, dry matter decreased with increasing sEPS levels, indicating a dose-dependent inhibitory effect of sEPS on plant growth under nutrient-rich conditions. Comparisons among substrate types at the same sEPS level further showed that seedlings grown on nutrient-rich media generally accumulated more biomass than those grown in the stand-alone sEPS substrate. For most treatments, biomass followed the order MS + sEPS + S-CM > MS + sEPS-CM > sEPS-CM. Overall, these results indicated that while nutrient-rich media promoted seedling growth, increasing sEPS concentrations progressively reduced biomass accumulation, highlighting the strong influence of sEPS on plant performance in hydrogel-based culture systems. The reduction in biomass observed at higher sEPS concentrations was consistent with the decrease in Ca availability and the shift in Ca/N stoichiometry detected in the hydrogel systems (Fig. 3).

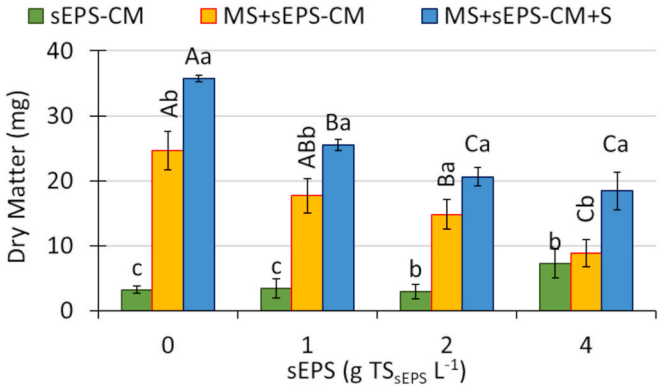

Fig. 7. Effects of increasing sEPS concentrations on seedling dry biomass under different culture media. Bars represent mean ± standard error (n = 3). Different lowercase letters indicate significant differences among sEPS concentrations within the same culture medium, whereas different uppercase letters indicate significant differences among culture media within the same sEPS level (Tukey's HSD test, $p < 0.05$).

TSS concentrations were significantly affected by both culture medium and sEPS concentration, whereas their interaction was not significant (Supplementary Table 5). Seedlings grown in the MS + sEPS-CM medium supplemented with sucrose exhibited significantly higher sugar concentrations than those grown in either MS media without sucrose or without MS media. Instead, plants grown in sEPS-CM and MS + sEPS-CM showed similar levels of TSS (Table 1). Across sEPS treatments, sugar concentrations generally decreased with increasing sEPS levels. The highest values were observed in the absence of sEPS, whereas significantly lower concentrations were recorded at the highest sEPS level. The intermediate sEPS treatments showed intermediate responses, indicating an overall decline in soluble sugar accumulation with increasing sEPS concentration.

Photosynthetic pigment concentrations were significantly influenced by sEPS concentration, whereas neither the type of culture medium nor the interaction between factors showed significant effects (Supplementary Table 5). Increasing the sEPS concentrations resulted in a marked reduction in Chla, Chlb, and carotenoid contents (Table 1). Chla concentrations remained relatively stable between 0 and 1 g TS$_{sEPS}$ L^{-1} sEPS but decreased significantly at higher sEPS levels, reaching the lowest values at 4 g TS$_{sEPS}$ L^{-1}. A similar pattern was observed for Chlb, which showed a progressive reduction with increasing sEPS concentrations. Carotenoid concentrations also decreased significantly at higher sEPS levels, although the decline was less pronounced compared with that observed for chlorophylls. The decrease in pigment concentrations observed at higher sEPS levels is consistent with the reduction in biomass accumulation and TSS content, suggesting that elevated sEPS concentrations may impair the physiological performance of the seedlings.

The PPI was significantly influenced by both culture medium and sEPS concentration, whereas their interaction was not significant (Supplementary Table 5). Seedlings grown in the MS + sEPS-CM medium supplemented with sucrose exhibited significantly higher PPI values compared with those grown in the other media types. In contrast, plants grown in the sEPS-CM and MS + sEPS-CM media showed intermediate or lower PPI values. Across sEPS treatments, PPI values showed a non-linear response to increasing sEPS concentrations. The highest values were observed at 1 g TS$_{sEPS}$ L^{-1} sEPS, whereas progressively lower values were recorded at higher sEPS levels, with the lowest PPI measured at 4 g TS$_{sEPS}$ L^{-1}. Overall, these results indicated that while moderate sEPS concentrations did not negatively affect photosynthetic performance, higher sEPS levels were associated with a decline in PPI, suggesting a reduction in the physiological efficiency of the seedlings.

3.3.5. *Multivariate relationships among hydrogel chemistry and seedling performance*

The principal component analysis (PCA) was performed to explore the relationships between hydrogel nutrient chemistry (bioavailable Ca^{2+}, inorganic nitrogen species, and Ca/N ratio) and seedling physiological traits (Supplementary Fig. 1). The first two principal components explained 78.3% of the total variance, with PC1 accounting for 66.6% and PC2 for 11.7%.

The distribution of samples along PC1 revealed a clear gradient associated with increasing sEPS concentrations. Treatments without sEPS were grouped on the negative side of PC1, whereas samples containing the highest sEPS concentration (4 g TS$_{sEPS}$ L^{-1}) were located on the positive side of the axis. Intermediate sEPS levels (1 and 2 g TS$_{sEPS}$ L^{-1}) occupied intermediate positions along this gradient. Most measured variables, including Ca, NH_4^+, NO_3^-, Ca/N ratio, EC, TSS, Chla, Chlb, carotenoids, PPI and DM, were strongly associated with the negative side of PC1. This pattern indicates that higher values of these variables were generally observed at lower sEPS concentrations, whereas increasing sEPS levels were associated with a progressive reduction in most chemical and physiological parameters. PC2 explained a smaller proportion of the variance and mainly reflected variability related to seedling physiological traits, particularly dry

Table 1
Main effects of culture medium (Medium type) and sEPS concentration on seedling dry biomass, total soluble sugars, photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids), and photosynthetic performance index (PPI). Values represent mean ± standard error (n = 3). Different uppercase letters indicate significant differences among culture media, whereas different lowercase letters indicate significant differences among sEPS levels according to the Tukey's HSD test ($p < 0.05$).

Main factor	Level	Dry Matter (mg)	Total soluble sugars (mg g^{-1} FW)	Chlorophyll a (mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	PPI
Medium type	**sEPS CM**	4.183 (0.901)	9.076 (0.491) B	1.039 (0.089)	0.414 (0.036)	0.257 (0.013)	5.758 (0.401) b
	MS + sEPS CM	16.492 (2.067)	9.929 (0.762) B	1.056 (0.104)	0.401 (0.026)	0.265 (0.016)	5.419 (0.273) ab
	MS + sEPS CM + S	25.067 (2.1)	17.688 (1.345) A	1.165 (0.075)	0.425 (0.042)	0.250 (0.012)	6.419 (0.409) a
sEPS	**0**	21.178 (4.622)	14.453 (0.365) a	1.293 (0.082) a	0.535 (0.025) a	0.291 (0.007) a	6.391 (0.448) ab
	1	15.544 (3.226)	12.525 (0.539) b	1.321 (0.045) a	0.482 (0.023) a	0.296 (0.013) a	6.859 (0.374) a
	2	12.778 (2.632)	11.513 (1.136) ab	0.984 (0.047) b	0.365 (0.014) b	0.251 (0.013) b	5.462 (0.272) bc
	4	11.489 (2.16)	9.24 (0.545) b	0.747 (0.04) c	0.273 (0.016) c	0.22 (0.018) c	4.75 (0.277) c

biomass and PPI, which showed stronger loadings along this axis. Overall, the PCA highlighted a clear structuring of treatments according to sEPS concentration, indicating that increasing sEPS levels were associated with a coordinated decline in nutrient availability and seedling physiological performance.

The correlation matrix further supported these relationships (Supplementary Fig. 2). Strong positive correlations were observed among Ca, NH_4^+, NO_3^-, and Ca/N ratio, indicating that these variables represent a common gradient of nutrient availability. In contrast, EC showed weaker correlations with individual nutrients, suggesting that it reflects overall ionic strength rather than specific nutrient concentrations. Dry biomass was positively correlated with most nutrient-related variables, indicating that seedling growth was closely associated with nutrient availability in the hydrogel system. Strong positive correlations were also detected among photosynthetic pigments, particularly between Chla and Chlb, reflecting their shared role in the photosynthetic apparatus. In addition, photosynthetic pigments were positively associated with dry biomass, total soluble sugars, and the photosynthetic performance index (PPI), suggesting that improved photosynthetic capacity was linked to enhanced carbon accumulation and plant growth. Overall, the correlation analysis revealed a coordinated relationship among nutrient availability, photosynthetic traits, and plant growth, supporting the patterns observed in the PCA.

4. Discussion

4.1. Effects of sEPS on nutrient availability and Ca–N stoichiometry

The results showed that sEPS effects strongly depended on both sEPS concentration and cultivation system. In the stand-alone sEPS hydrogel, the most favorable responses were observed at 1 g TS_{sEPS} L^{-1}, where shoot length, root length, and vitality index were highest, indicating that moderate sEPS levels support early seedling development in the absence of additional nutrients. In contrast, the highest concentration (4 g TS_{sEPS} L^{-1}) consistently reduced growth and vitality, indicating impaired plant performance.

In nutrient-rich systems, although germination was unaffected, increasing sEPS concentrations reduced nutrient availability, particularly Ca^{2+} and inorganic nitrogen, and were associated with lower biomass accumulation, pigment content, and soluble sugars. Among the tested media, MS + sEPS + sucrose supported the highest biomass accumulation, sugar concentrations, and PPI values, whereas the strongest negative effects were observed at 4 g TS_{sEPS} L^{-1}. Overall, moderate sEPS levels were compatible with plant development, whereas high concentrations were detrimental, mainly due to nutrient limitation rather than direct phytotoxicity.

These findings indicate that AGS-derived sEPS act not only as structural hydrogels but also as active regulators of nutrient availability through their ion-binding properties (Felz et al., 2019; Seviour et al., 2019), highlighting their potential as bio-based materials for controlled plant cultivation systems within a circular resource recovery framework.

The distinction between stand-alone and MS-based systems is important for interpretating the results. The development and testing of stand-alone sEPS substrates allowed the assessment of the intrinsic phytotoxicity and stimulatory effects of sEPS, whereas MS-based systems allowed the evaluation of interactions between sEPS, nutrient availability, and plant physiological responses. Because these systems also differed in calcium concentration, ionic strength, salt composition, and hydrogel constituents, the observed responses reflect the combined effects of sEPS and the surrounding matrix. Future experiments using matched formulations differing only in sEPS concentration are needed to isolate the specific contribution of sEPS.

One of the most evident effects observed in this study was the strong influence of sEPS concentration on nutrient availability within the hydrogel-nutrient solution system, particularly in MS-based media. The

most favorable conditions were observed at low or zero sEPS levels, whereas the highest concentration (4 g TS_{sEPS} L^{-1}) produced the strongest reductions in bioavailable Ca^{2+} and inorganic nitrogen. This effect was particularly evident in nutrient-rich systems (i.e. MS + sEPS and MS + sEPS + S), where increasing sEPS concentrations progressively decreased nutrient availability and Ca/N ratio.

This reduction can be attributed to the well-known ion-binding capacity of EPS, which contain functional groups, such as carboxyl, hydroxyl, and phosphate moieties, able to interact with Ca^{2+} and NO_3^-/NH_4^+ ions, potentially forming stable complexes (Felz et al., 2016; Pagliaccia et al., 2024). As a result, increasing sEPS concentrations likely reduced the fraction of freely available nutrients, altering the equilibrium between the hydrogel matrix and the solution phase.

Unlike conventional gelling agents used in plant tissue culture, such as agar or gellan gum, whose primary role is to provide mechanical support while remaining largely inert with respect to nutrient availability (George et al., 2008; Phillips and Garda, 2019), AGS-derived sEPS actively modify the chemical environment of the cultivation system through their ion-binding properties. Compared with alginate- or synthetic polymer-based hydrogels developed for water retention or controlled nutrient release (Guilherme et al., 2015), sEPS additionally offer the advantage of being recovered from wastewater treatment residues, supporting circular bioeconomy strategies.

The strong ion-binding capacity observed in this study suggests potential applications beyond in vitro cultivation. By exploiting their affinity for cations, sEPS could serve as carriers for essential nutrients, reducing nutrient losses and promoting their gradual release to plants, similarly to other hydrogel-based nutrient delivery systems (Guilherme et al., 2015). Future studies should investigate whether chemical or physicochemical modification of the sEPS matrix can tailor nutrient retention and release kinetics for specific agronomic applications.

Temporal analysis showed that bioavailable Ca^{2+} and inorganic nitrogen decreased over the incubation period, particularly at higher sEPS concentrations. Although these trends are consistent with the ion-binding properties of EPS, other processes, including diffusion within the hydrogel matrix, interactions between Ca^{2+} and the gelling components, plant nutrient uptake, and other physicochemical processes may also have contributed to the observed temporal changes. Consequently, their relative contributions could not be distinguished. As Ca and inorganic N did not decrease proportionally, the Ca/N ratio varied substantially across treatments, with the strongest imbalance observed at 4 g TSsEPS L^{-1}.

Overall, these results indicate that high sEPS concentrations induce nutrient limitation rather than direct phytotoxic effects. Such stoichiometric imbalances may have important physiological implications, as calcium plays a key role in cell wall stabilization, membrane integrity, and signaling (White and Broadley, 2003). While EPS-ion interactions are well documented, their impact on Ca-N stoichiometry in hydrogel-based plant culture systems remains largely unexplored, thus highlighting the novelty and relevance of this study.

4.2. Plant responses to sEPS-based hydrogels

Despite these pronounced chemical changes, seed germination was not affected by sEPS concentration in any tested system. Germination reached approximately 100% across all treatments, indicating that even the highest concentration (4 g TS_{sEPS} L^{-1}) did not inhibit early seed development. Similar results have been reported for EPS-based materials, which did not reduce germination even at high concentrations (Patil et al., 2011). These findings would confirm that sEPS are not directly phytotoxic and are compatible with early plant development.

In contrast, seedling growth showed a clear sEPS concentration-dependent response. The most favorable responses were observed at 1 g TS_{sEPS} L^{-1}, with the highest shoot and root length and vitality index, whereas 4 g TS_{sEPS} L^{-1} produced the strongest growth inhibition. This suggests that, in the absence of external nutrients, moderate sEPS levels may enhance development, likely through improved water retention and gradual nutrient release. EPS-based materials are known to stabilize the seed microenvironment and reduce desiccation stress, supporting early growth (Bhattacharjee et al., 2020).

In nutrient-rich systems (MS + sEPS and MS + sEPS + S), increasing sEPS concentrations reduced dry biomass accumulation, consistent with the progressive decrease in Ca^{2+} and inorganic nitrogen availability. This finding supports the hypothesis that growth inhibition resulted primarily from nutrient limitation rather than direct phytotoxicity. Accordingly, the phytotoxicity classification presented here refers exclusively to seed germination and early seedling development under the experimental conditions adopted in this study and should not be extrapolated to complete crop cycles without additional validation. A dose-dependent effect was also recently reported by Miranda et al. (2025), who investigated the use of AGS-derived EPS as soil amendments and showed that the ability of EPS to improve plant growth, plant nutritional status, and soil enzymatic activity strongly depended on both the EPS source and the applied dosage. The concentration-dependent response was further confirmed by the physiological parameters. Increasing sEPS concentrations reduced chlorophylls, carotenoids, and total soluble sugars, with the strongest effects at 4 g TSsEPS L^{-1}, indicating impaired photosynthetic performance and carbon metabolism.

In contrast, the photosynthetic performance index (PPI) showed a non-linear response, with the highest values at intermediate sEPS concentrations (1 g TS_{sEPS} L^{-1}), particularly in nutrient-rich systems, indicating that moderate sEPS levels can sustain physiological efficiency without severely limiting nutrient availability. The presence of sucrose (i.e. MS + sEPS + sucrose) further enhanced PPI, confirming the importance of an external carbon source for plant growth in vitro, as previously reported in the literature (Tokuhara et al., 2023). These findings demonstrate that sEPS concentration influences photosynthetic performance and carbon metabolism, with optimal responses at moderate levels and strong inhibition at high concentrations due to nutrient limitation.

4.3. Linking hydrogel chemistry to plant physiological responses

Multivariate analysis provided further insight into the relationships between hydrogel chemistry and plant physiological responses. Principal component analysis revealed a clear gradient associated with increasing sEPS concentrations. The most favorable conditions (0–1 g TS_{sEPS} L^{-1}) were positioned on the negative side of PC1 and characterized by higher nutrient availability, biomass, and photosynthetic pigment, and sugar contents. In contrast, higher sEPS levels (particularly 4 g TS_{sEPS} L^{-1}) were grouped on the positive side of PC1 and associated with reduced physiological and chemical parameters, indicating a coordinated decline in nutrient availability and plant performance.

Correlation analysis supported these patterns, showing strong positive relationships among nutrient-related variables, photosynthetic pigments, soluble sugars, and biomass accumulation. In particular, Ca, NH_4^+, NO_3^-, and the Ca/N ratio were closely correlated, reflecting a common gradient of nutrient availability. Photosynthetic pigments were also positively associated with biomass production, soluble sugars, and PPI, highlighting the coupling between photosynthesis, carbon assimilation, and plant growth.

Overall, these results demonstrate that nutrient availability is the main driver of seedling physiological performance in sEPS-based hydrogel systems. While EPS–ion interactions are well known, this study shows how they translate into coordinated changes in plant physiological traits, linking hydrogel chemistry to plant performance within a unified mechanistic framework. This supports the interpretation of sEPS as dynamic regulators of plant growth conditions rather than passive hydrogel matrices.

4.4. Environmental implications and future perspectives

From an applicative perspective, the present results indicate that AGS-derived sEPS have potential for use as functional components of hydrogel-based plant cultivation systems, provided that their concentration is carefully optimized. Moderate sEPS levels supported early seedling development under the experimental conditions adopted in this study by improving substrate properties and maintaining nutrient availability, whereas excessive concentrations reduced nutrient bioavailability and impair growth. These findings support the valorization of wastewater treatment sludge-derived biopolymers as functional materials for sustainable plant cultivation within a circular bioeconomy framework.

The present results indicate that sEPS-based hydrogels influence nutrient availability and seedling physiological performance through changes in Ca-N stoichiometry. At intermediate concentrations, these materials represent promising candidates for future evaluation in hydrogel-based cultivation systems and hydroponic propagation platforms. In this context, AGS-derived sEPS may potentially fall within the European regulatory framework for fertilizing products under Regulation (EU) 2019/1009, particularly under Component Material Category (CMC) 9 "*Polymers other than nutrient polymers*" (Pagliaccia et al., 2024), provided that biodegradability and plant safety requirements are met. The absence of negative effects on seed germination supports their potential compliance with plant safety requirements for early phytotoxicity endpoints.

Another aspect that deserves consideration is the potential occurrence of emerging contaminants, such as antibiotics and microplastics, in wastewater-derived sEPS. These contaminants may accumulate in sewage sludge, be co-recovered during sEPS extraction, and affect the quality and safety of the resulting biomaterial (Michael et al., 2013; Sun et al., 2019). Recent evidence also indicates that antibiotics and microplastics can influence the properties and stability of aerobic granular sludge and its EPS matrix (Kedves et al., 2026). Future research should therefore evaluate the occurrence of these contaminants in recovered sEPS, their potential effects on plant performance and environmental safety, and establish appropriate quality control procedures to support safe agricultural applications. Process efficiency could also be improved by increasing EPS production and exploiting the polymeric matrix as a carrier for valuable compounds, for example through controlled microbial stress responses that stimulate EPS secretion and enhance the recovery of nutrients or other beneficial components (Kedves et al., 2025).

The present study was designed to evaluate the effects of sEPS during seed germination and early seedling establishment following the European Standard protocols EN 16086-1 and EN 16086-2 for phytotoxicity assessment of growing media and substrates. The experiment was extended to 168 h to verify the persistence of treatment effects. This timeframe is also relevant for short-cycle cultivation systems, such as microgreens, where harvest generally occurs within the first week after germination (Kyriacou et al., 2016). Consequently, the present findings provide evidence on the compatibility of sEPS with early plant development, whereas their suitability for longer cultivation cycles requires further investigation.

Future research should evaluate long-term agronomic performance of sEPS-based substrates beyond the early seedling stage, including complete crop cycles, biomass accumulation, root system development, nutrient uptake, and potential chronic phytotoxic effects under both soil and hydroponic cultivation conditions. Additional studies should also assess nutrient dynamics in different soil types, interactions with soil microbial communities, and the technical, economic, and environmental sustainability of sEPS production and application. Although the technical feasibility of extracting structural EPS from aerobic granular sludge has already been demonstrated at full scale through the Kaumera® process (Verschoor et al., 2023), future research should focus on improving energy efficiency, reducing chemical consumption, and integrating sEPS recovery into AGS biorefinery concepts coupled with the recovery of valuable resources such as methane and phosphorus (Bahgat et al., 2024). Ultimately, the sustainability of sEPS-based materials should be assessed across the entire production chain-from AGS to the final product-including extraction, downstream processing, material yield, and formulation. Comprehensive techno-economic analyses and comparative Life Cycle Assessments (LCA) against conventional gelling agents will therefore be essential to quantify their environmental benefits, verify their large-scale feasibility, and support their implementation within circular bioeconomy strategies.

5. Conclusions

This study investigated the potential application of structural extracellular polymeric substances (sEPS) recovered from aerobic granular sludge as hydrogel-based substrates for in vitro seedling cultivation. The results demonstrated that sEPS did not exert direct phytotoxic effects on the germination of common garden cress seeds, either as stand-alone substrates or combined with MS medium. Increasing sEPS concentrations progressively altered nutrient availability, particularly bioavailable Ca^{2+} and inorganic nitrogen, leading to shifts in Ca/N stoichiometry and seedling physiological performance. The most favorable responses were observed at 1 g TS_{sEPS} L^{-1}, which promoted seedling development in stand-alone hydrogels, suggesting phyto-stimulatory effects potentially related to improved water retention and gradual nutrient release. In contrast, the highest concentration tested (4 g TS_{sEPS} L^{-1}) reduced shoot and root growth, biomass accumulation, photosynthetic pigments, soluble sugars, and photosynthetic performance, indicating nutrient limitation effects associated with excessive ion complexation. Overall, the findings demonstrate that AGS-derived sEPS can act as bio-based hydrogel matrices capable of regulating nutrient availability and influencing plant development. At 1 g TS_{sEPS} L^{-1}, sEPS-based hydrogels represent promising substrates for hydrogel-based cultivation systems or hydroponic propagation platforms. From a circular bioeconomy perspective, recovering sEPS from wastewater sludge may provide a sustainable strategy to reduce sludge disposal while generating value-added biomaterials for plant cultivation. Further studies should evaluate long-term agronomic performance and large-scale applicability under realistic cultivation conditions.

CRediT authorship contribution statement

Antonio Pescatore: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Benedetta Pagliaccia:** Writing – review & editing, Supervision, Resources, Data curation, Conceptualization. **Tommaso Lotti:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Claudio Lubello:** Supervision, Project administration, Funding acquisition. **Riccardo Campo:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Simone Orlandini:** Supervision, Project administration, Funding acquisition. **Marco Napoli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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granular sludge used in the experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2026.130817.

Data availability

Data will be made available on request.

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