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Combined Algal Biochar–Starch Bioflocculant Conditioning Reveals Distinct Responses of Extracellular Polymeric Substances in Anaerobically Digested Sludge

Yuanying Yang¹, Wenshan Guo¹, Huu Hao Ngo^{1,*} and Huiying Zhang^{2,*}

¹ Centre for Technology in Water and Wastewater, School of Civil and Environmental Engineering, University of Technology Sydney, Sydney, NSW 2007, Australia

² College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China

* Correspondence: ngohuuhaol21@gmail.com (H.H.N.); zhanghuiying_1988@163.com (H.Z.)

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Abstract: Anaerobically digested sludge (ADS) can be difficult to separate because fine particles and hydrated extracellular polymeric substances (EPS) contribute to sludge dispersion and water retention. Although particulate conditioners and bio-based flocculants have each been investigated, their combined effects on EPS distribution and sludge conditioning mechanisms are still poorly understood. This study evaluated algal biochar as an auxiliary conditioner in combination with grafted cationic starch (GCS) or etherified cationic starch (ECS), with particular attention to EPS fractions. The combined treatments achieved supernatant turbidity removals of 77.1% to 84.1%, exceeding that achieved with the corresponding starch-based flocculants alone. Although the two conditioning systems showed similar turbidity removal, they followed different zeta-potential and EPS responses. The algal biochar and ECS system shifted to less negative values and reduced soluble microbial products (SMP) and loosely bound EPS (LB-EPS). In contrast, the GCS combinations remained negatively charged and increased tightly bound EPS (TB-EPS) by 52% to 54% compared with GCS alone. These findings show that comparable supernatant clarification can be accompanied by contrasting electrokinetic and operationally extracted EPS profiles. The results highlight the value of examining EPS fractions alongside turbidity when comparing combined conditioning formulations. The implications of these profiles for sludge dewaterability and filterability remain to be established through direct performance measurements.

Keywords: algal biochar; anaerobically digested sludge; cationic starch; extracellular polymeric substances; combined conditioning

1. Introduction

Anaerobic digestion is widely used in wastewater treatment to stabilise sewage sludge and recover renewable energy in the form of methane [1]. Following digestion, the sludge still requires solid–liquid separation before further treatment or disposal [2]. Anaerobic digestion can alter particle size, soluble and colloidal organic matter, and extracellular polymeric substances (EPS), although the resulting effect on sludge dewaterability depends on the feed sludge and digestion conditions [3,4]. The release of fine particles and soluble biopolymers can impair settling, filtration and dewatering performance [5,6]. Effective conditioning of anaerobically digested sludge (ADS) is therefore important for reducing the volume and cost of subsequent sludge management.

EPS form a hydrated organic matrix associated with sludge particles and contribute to floc stability, particle interactions and water retention [7,8]. For operational analysis, EPS-related organic matter is commonly classified into soluble microbial products (SMP), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) [9,10].



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These fractions differ in their association with sludge solids and have been related to aggregation, settling and filtration behaviour [11]. In particular, proteins and polysaccharides may also contribute differently to sludge floc properties. This suggests that changes in EPS composition and distribution can provide more information than measuring total EPS alone [12]. Therefore, understanding how conditioning technologies affect individual EPS fractions is important for interpreting sludge conditioning responses.

Chemical conditioning with cationic polymers is widely used to destabilize sludge colloids through charge neutralization, adsorption and polymer bridging [13]. Among the emerging bio-based alternatives, starch-derived cationic flocculants have attracted increasing attention because starch is renewable, abundant and readily modified to obtain different charge properties and molecular structures [14,15]. Previous studies have shown that graft chain length, graft distribution, hydrophobicity and charge density affect sludge aggregation, EPS composition and distribution, and dewatering performance [16,17]. However, although polymer conditioning promotes floc formation and improves sludge filterability, the resulting sludge cake can remain highly compressible under mechanical pressure, which may restrict further water removal [18].

Particulate materials have been investigated as auxiliary conditioners or skeleton builders in combination with polymeric flocculants [18,19]. Reported materials include lignite, agricultural residues, sludge-derived biochar and mineral particles [20–23]. These materials may reinforce the filter-cake structure, reduce compressibility and maintain drainage pathways during dewatering [18,24]. Agricultural residues and sludge-derived biochar also offer opportunities to reuse waste materials. For example, Shen et al. (2023) combined an etherified cationic starch with attapulgite and examined EPS fractions, sludge flocs and dewatering performance in waste-activated sludge [25]. These studies establish a broader basis for combining cationic starch with particulate auxiliary material. However, the EPS responses of ADS conditioned with algal biochar and cationic starch flocculants prepared through different modification routes remain unclear.

Algal biomass is a renewable feedstock that can be produced rapidly without direct competition for arable land [26]. Biochars produced from algae can differ from lignocellulosic biochars in their chemical composition and ash characteristics [27,28]. These properties may affect surface charge and the availability of sites for interactions with sludge particles and EPS. This provides a rationale for examining algal biochar as an auxiliary conditioner, although its behaviour in ADS must be evaluated experimentally. Although algal biochar has been studied for water treatment and sediment remediation [29,30], its use as an auxiliary conditioner for ADS has received limited attention. This is especially true when it is combined with starch-based bioflocculants and evaluated through operational EPS fractionation. As a result, the mechanisms underlying its interaction with different starch bioflocculants are still not well understood.

Accordingly, this study investigated the combined conditioning of ADS using algal biochar together with grafted cationic starch (GCS) or etherified cationic starch (ECS). Both addition sequences were evaluated. The conditioning systems were compared by measuring pH, supernatant turbidity, zeta potential, and the protein and polysaccharide contents of SMP, LB-EPS and TB-EPS. This study examined whether comparable supernatant clarification was accompanied by different PN and PS profiles across the operationally extracted SMP, LB-EPS and TB-EPS fractions. By linking sludge clarification with operationally extracted EPS fractionation, this work provides new insight into hybrid biochar and bioflocculant conditioning.

2. Materials and Methods

2.1. Anaerobically Digested Sludge

ADS was collected from a wastewater treatment plant in Sydney, Australia. The collected sludge had measured total solids (TS) and volatile solids (VS) concentrations of 19.045 and 12.085 g L⁻¹, respectively. The sludge was stored at 4 °C for approximately 48 h before use. Before experiments, it was allowed to reach room temperature and gently homogenised.

2.2. Preparation and Characterisation of Algal Biochar

Red algal powder was obtained from Xi'an Victory Biochemical Technology Co., Ltd. The feedstock had a loss on drying of 6.81% and an ash content of 10.60%. It also met the particle-size specification requiring 98% to pass through a 100-mesh sieve. Its species identity was not specified. The powder was washed thoroughly with deionised water and dried at 105 °C to constant weight. The dried powder was pyrolysed in a tube furnace under a nitrogen atmosphere. The temperature was increased to 600 °C at a heating rate of 5 °C/min and maintained at 600 °C for 2 h. After cooling, the resulting algal biochar was washed with deionised water, dried at 105 °C and stored in a sealed container until use.

2.3. Preparation and Characterisation of Starch-Base Biofloculants

Two starch-based cationic biofloculants were prepared through graft copolymerisation and etherification. Soluble starch and the chemical reagents used for synthesis were obtained from Sigma-Aldrich. The graft-copolymerised product was designated grafted cationic starch (GCS), whereas the etherified product was designated etherified cationic starch (ECS).

For GCS preparation, 1.0 g of soluble starch was added to 50 mL of boiling deionised water and gelatinised under magnetic stirring until a clear and homogeneous dispersion was obtained. The reaction mixture was then maintained at 55 °C and purged with nitrogen for approximately 30 min to remove dissolved oxygen. Ammonium persulfate (APS, 0.10 g), corresponding to an APS-to-starch mass ratio of 1:10, was dissolved in 5 mL of deionised water and added to the reaction system. The mixture was allowed to react for 10 min before addition of the cationic monomer.

[2-(Acryloyloxy)ethyl]trimethylammonium chloride (AETAC, 80 wt.% in H₂O, Sigma-Aldrich; 6.6 mL) was diluted with 20 mL of deionised water and added gradually over approximately 3 h. After completion of AETAC addition, the graft-copolymerisation reaction was continued for a further 30 min at 55 °C. The reaction mixture was then cooled to room temperature and the product was precipitated with acetone. The precipitate was separated by centrifugation at 3000× g for 5 min and washed three times with ethanol. The recovered product was vacuum-dried at 45 °C to constant mass and ground into powder.

For ECS preparation, a 2.5 wt.% NaOH solution was prepared and preheated to 70 °C. Soluble starch was added to the alkaline solution and activated at 70 °C for 1 h. (3-Chloro-2-hydroxypropyl)trimethylammonium chloride (60 wt.% in H₂O, Sigma-Aldrich) was subsequently added dropwise, and the etherification reaction was maintained at 70 °C for 2 h.

For ECS preparation, a 2.5 wt.% NaOH solution was prepared and preheated to 70 °C. Soluble starch was added and activated at 70 °C for 1 h. (3-Chloro-2-hydroxypropyl)trimethylammonium chloride (CHPTAC, 60 wt.% in H₂O, Sigma-Aldrich) was then added dropwise, corresponding to a starch to active-CHPTAC mass ratio of 1:1.5. The etherification reaction was maintained at 70 °C for 2 h. The product was precipitated with ethanol and separated by centrifugation at 3000× g for 5 min. The precipitate was washed three times with ethanol, vacuum-dried at 45 °C to constant mass and ground into powder.

The prepared GCS and ECS were characterised by cationic charge-density measurement, Fourier-transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (¹H NMR) spectroscopy. Charge density was determined by colloid titration using poly(sodium 4-styrenesulfonate) as the anionic titrant and toluidine blue O as the indicator. The measured charge densities were approximately 2.7 mmol g⁻¹ for GCS and 1.4 mmol g⁻¹ for ECS. FTIR and ¹H NMR spectra were used to verify the chemical modifications introduced by graft copolymerisation and etherification, respectively (Fig. S1).

2.4. Conditioning Procedure

Algal biochar was applied at 2 g/L, corresponding to approximately 105.0 mg/g TS based on the initial sludge TS concentration of 19.045 g/L. GCS and ECS were applied at 20 and 30 mg/g TS, respectively, corresponding to approximately 0.381 and 0.571 g/L. The flocculant dosages were selected separately in preliminary optimisation experiments and therefore represent selected operating conditions rather than equal polymer-mass or equal cationic-charge inputs. Within each flocculant system, the same polymer dose was used for individual and sequential treatments. The experimental groups comprised an untreated ADS control, individual treatments with algal biochar, GCS or ECS, and sequential treatments in which algal biochar was added either before or after GCS or ECS.

For treatments containing one conditioning material, the material was added to the sludge and dispersed by rapid mixing at 200 rpm for 2 min. The sample was then mixed at 50 rpm for 25 min to promote floc formation and growth, followed by settling for 60 min.

Sequential conditioning comprised two successive mixing stages. After addition of the first material, the sludge was mixed at 200 rpm for 2 min and then at 50 rpm for 25 min. The second material was subsequently added, followed by a further 2 min of rapid mixing at 200 rpm and 25 min of slow mixing at 50 rpm. The conditioned sludge was then allowed to settle for 60 min. Each treatment was performed in three independently conducted conditioning runs using separately processed aliquots from the same homogenised sludge batch.

2.5. Analytical Methods

The pH of each sludge sample was measured before and after conditioning using a pH meter. The zeta potential of the whole conditioned sludge suspension was measured directly without dilution using a Zetasizer

Nano ZS (Malvern Panalytical, Malvern, UK) at 25 °C with a DTS1070 folded capillary cell. Three instrumental measurements were performed for each sample. Because the conditioned suspension contained heterogeneous sludge flocs and biochar particles, the measured zeta potential was interpreted as an operational electrokinetic indicator of the sampled suspension rather than as a uniform surface-potential value for all particles. Following settling, the upper supernatant was carefully collected, and its residual turbidity was measured using a portable turbidity meter (2100Q, Hach Company, Loveland, CO, USA).

EPS were operationally separated into SMP, LB-EPS and TB-EPS using a sequential extraction procedure adapted from Wang et al. (2009) [31]. For each treatment, 10 mL of conditioned sludge was used for each independent extraction. The sample was centrifuged at 5000 r min⁻¹ for 10 min, and approximately 8 mL of the supernatant was collected as the SMP fraction. The remaining sludge was resuspended to the original volume with 0.9% NaCl solution and centrifuged at 5000 r min⁻¹ for 10 min. Approximately 8 mL of the resulting supernatant was collected as the LB-EPS fraction. The remaining sludge was subsequently washed twice with 0.9% NaCl solution, with each washing step followed by centrifugation at 5000 r min⁻¹ for 10 min. The washing supernatants were discarded. For TB-EPS extraction, the washed sludge suspension was adjusted to 10 mL and heated in a water bath at 80 °C for 30 min. The sample was then centrifuged at 8000 r min⁻¹ for 20 min, and approximately 8 mL of the supernatant was collected as the TB-EPS fraction.

Protein concentrations in the SMP, LB-EPS and TB-EPS fractions were determined using the modified Lowry method [32]. Polysaccharide concentrations were measured using the anthrone-sulfuric acid method following Deng et al. (2014) [33]. For each operational fraction, the reported EPS concentration was calculated as the sum of the measured protein and polysaccharide concentrations.

For each fraction, recovered PN and PS masses were calculated from the measured concentrations and final extract volumes and normalised to the initial sludge VS mass, excluding the added conditioners. Total recovered PN and PS were calculated separately by summing the corresponding recovered masses across SMP, LB-EPS and TB-EPS within each independent extraction.

The same extraction procedure was applied to all treatments. Extraction recovery was not independently quantified for the sludge–biochar–starch matrix. Therefore, SMP, LB-EPS and TB-EPS are interpreted as operationally extracted fractions rather than as a complete quantitative recovery of native sludge EPS.

3. Results and Discussion

3.1. Physicochemical Responses

The untreated ADS had a pH of 7.24, whereas the conditioned samples had pH values ranging from 7.36 to 7.57 (Figure 1a). The highest pH values were observed in the combined treatments containing ECS and algal biochar. Although pH can affect EPS ionisation and conformation and thus influence sludge surface properties [34], the variation among the conditioned samples was limited in the present study.

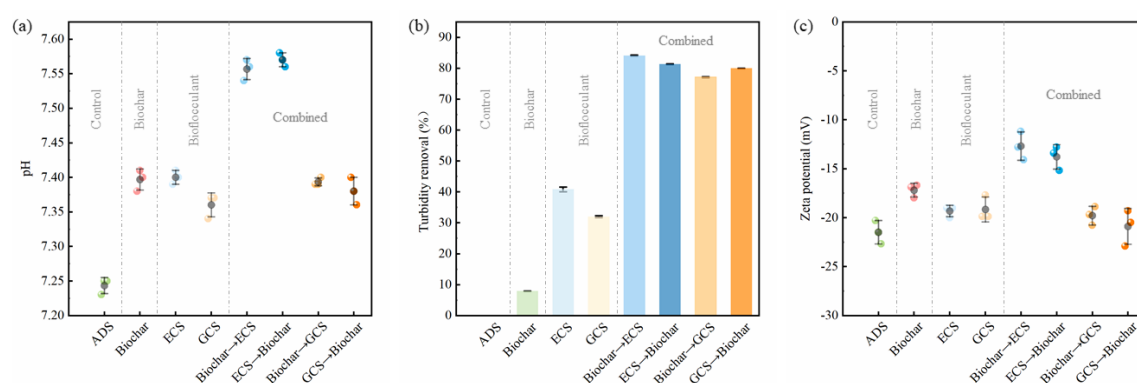


Figure 1. Physicochemical responses of anaerobically digested sludge to individual and sequential conditioning treatments. (a) pH, (b) turbidity removal and (c) zeta potential.

Algal biochar alone achieved only 7.9% turbidity removal, compared with 40.8% for ECS and 32.0% for GCS (Figure 1b). Although biochar shifted the measured zeta potential from −21.5 to −17.2 mV, this change was accompanied by limited clarification (Figure 1c). The modest shift towards a less negative zeta potential indicates a measurable electrokinetic response, although this change alone does not establish charge neutralisation as the dominant mechanism. In contrast, the combined treatments achieved removals of 77.1–84.1%. These observations

suggest that algal biochar has potential as an auxiliary conditioner alongside starch-based flocculants. Enhanced sludge conditioning has also been reported when cationic starch was combined with a particulate auxiliary material. However, the properties of the auxiliary material and the sludge matrix can affect the resulting interactions [25].

The relationship between turbidity removal and zeta potential differed between the ECS and GCS systems. Combining ECS with algal biochar shifted the zeta potential to less negative values of -12.7 and -13.8 mV for the two addition orders. This shift coincided with turbidity removal above 81%, suggesting that electrostatic interactions may have contributed to clarification. However, the present measurements cannot determine whether electrostatic destabilisation was the dominant mechanism. Charge neutralisation is a recognised mechanism of cationic starch flocculation. However, flocculation also depends on polymer structure and adsorption configuration [16].

In contrast, the combined treatments containing GCS and algal biochar achieved turbidity removal of 77.1% and 80.0% while retaining zeta potentials of -19.8 and -20.9 mV. Effective clarification in this system therefore did not require the bulk zeta potential to approach zero. Effective aggregation can occur while particles remain negatively charged when mechanisms such as polymer bridging and electrostatic charge patch interactions contribute [35,36]. Therefore, the present results may be consistent with contributions from mechanisms other than charge neutralisation. Given the heterogeneous nature of the conditioned sludge suspension, the zeta-potential measurements were used to compare overall electrokinetic responses among treatments and were not interpreted as direct evidence of a specific aggregation mechanism. Within each conditioning system, reversing the addition order changed turbidity removal by no more than 2.9 percentage points and zeta potential by no more than 1.1 mV. Under the tested doses, the physicochemical differences between the ECS and GCS systems were larger than those caused by reversing the addition order.

3.2. Concentration and Composition of SMP

The operationally defined SMP concentration of untreated ADS was 204.7 mg L^{-1} , comprising 141.2 mg L^{-1} PN and 63.5 mg L^{-1} PS (Figure 2). PN therefore accounted for approximately 69% of the measured SMP. The distinction between these components is important because the relative contributions of PN and PS vary among EPS fractions and sludge structures. Therefore, changes in total EPS concentration may not necessarily reflect similar changes in the individual components [12,37].

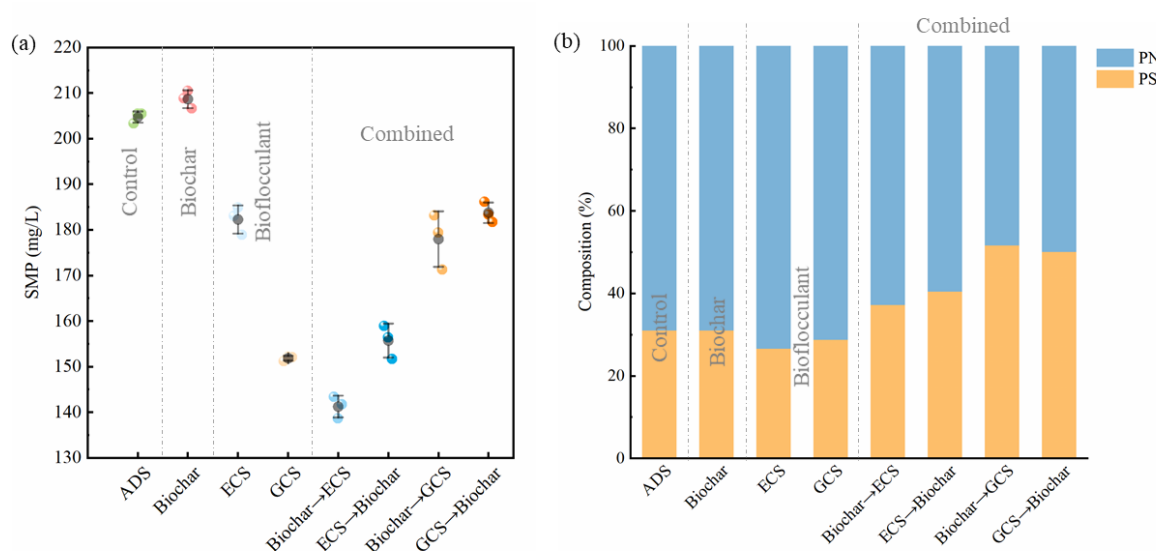


Figure 2. Concentration and composition of SMP after individual and sequential conditioning: (a) SMP concentration; (b) relative contributions of PN and PS.

Algal biochar alone resulted in an SMP concentration of 208.6 mg L^{-1} , approximately 2% higher than that of untreated ADS, while the proportions of PN and PS remained almost unchanged. This result suggests that algal biochar alone did not reduce the soluble biopolymers at the applied dosage. Therefore, adsorption of SMP by algal biochar should not be inferred from this treatment. However, conditioning systems containing biochar have been reported to alter EPS concentrations and distributions in other sludge systems [38].

ECS alone reduced the SMP concentration to 182.3 mg L^{-1} , corresponding to an 11.0% decrease compared with untreated ADS. PS decreased by 23.8%, whereas PN decreased by only 5.2%. The relative contribution of PN consequently increased from 69.0% to 73.4%. GCS alone produced a larger reduction in SMP, with the concentration decreasing to 151.8 mg L^{-1} . In this case, PN and PS decreased by 23.4% and 31.2%, respectively, while the overall composition remained dominated by PN. Therefore, the similar zeta potentials produced by ECS and GCS alone did not correspond to similar SMP responses. This indicates that bulk electrokinetic behaviour alone was insufficient to describe the changes in soluble organic components [16,39].

The combined ECS treatments produced the lowest SMP concentrations. Algal biochar followed by ECS resulted in an SMP concentration of 141.2 mg L^{-1} , whereas ECS followed by algal biochar resulted in 155.7 mg L^{-1} . These values were 31.0% and 24.0% lower than untreated ADS and 22.5% and 14.6% lower than ECS alone, respectively. The additional decrease compared with ECS alone was mainly due to PN. Its concentration decreased from 133.9 mg L^{-1} with ECS alone to 88.8 and 92.7 mg L^{-1} in the two combined treatments. In contrast, PS was slightly higher than that measured after ECS alone. The reduction in SMP should therefore be described as a preferential reduction in soluble PN rather than a uniform removal of SMP.

A different response was observed when algal biochar was combined with GCS. The SMP concentrations were 177.9 mg L^{-1} for algal biochar followed by GCS and 183.7 mg L^{-1} for the reverse order. Both values remained below that of untreated ADS by 13.1% and 10.3%, respectively. However, they were 17.2% and 21.0% higher than the value obtained with GCS alone. This increase did not result from a uniform increase in both soluble biopolymer components. PN decreased by a further 15 to 20% compared with GCS alone, whereas PS increased from 43.7 to approximately 92 mg L^{-1} . Therefore, the combined GCS systems produced a change in SMP composition that differed from the overall concentration response.

The effect of addition order was greater in the ECS system than in the GCS system. Adding algal biochar before ECS resulted in an SMP concentration 14.5 mg L^{-1} lower than that obtained with the reverse order, with most of the difference attributable to PS. In the GCS system, the two addition orders produced nearly identical PS concentrations, while their total SMP concentrations differed by only 5.8 mg L^{-1} . Previous work has shown that conditioner addition sequence can alter soluble EPS components in digested sludge, although the resulting response is likely to depend on the specific conditioning system [40].

Despite similar turbidity removal among the four combined treatments, their SMP concentrations ranged from 141.2 to 183.7 mg L^{-1} and their PS contributions ranged from 37.1% to 51.6%. Similar levels of supernatant clarification were therefore accompanied by different SMP compositions. The ECS combined treatments were characterised primarily by lower soluble PN, whereas the GCS combined treatments retained a larger PS fraction.

3.3. Concentration and Composition of LB-EPS and TB-EPS

The untreated ADS contained 3.84 mg g^{-1} VS of LB-EPS and 16.50 mg g^{-1} VS of TB-EPS (Figure 3). TB-EPS was more abundant than LB-EPS and had a higher proportion of PN, reaching 81.8% compared with 66.1% in LB-EPS. However, the higher concentration of TB-EPS does not necessarily mean that it has a stronger effect on sludge separation than LB-EPS. Previous studies have shown that the location and composition of EPS are important. Proteins and polysaccharides in different floc layers have different relationships with aggregation and dewaterability [11,12]. Removal and interaction energy analyses have shown that both LB-EPS and TB-EPS are involved in sludge aggregation. However, their contributions vary with the EPS layer and particle separation distance [41].

Algal biochar alone decreased LB-EPS by 33.6%, from 3.84 to 2.55 mg g^{-1} VS, whereas TB-EPS decreased by only 4.0%. The decrease in LB-EPS involved both PN and PS, although the proportional reduction was greater for PS. This difference indicates that algal biochar had a stronger effect on the more loosely associated LB-EPS fraction than on the tightly associated TB-EPS fraction [8,41]. In contrast, the SMP concentration did not decrease. The lower LB-EPS concentration therefore indicates lower concentrations of PN and PS in this fraction, rather than a corresponding decrease in soluble PN and PS.

The two individual biofloculants produced different responses in LB-EPS. ECS alone caused only a 4.5% reduction in total LB-EPS compared with untreated ADS. Its PS concentration increased slightly, while PN decreased by 11.3%. In contrast, GCS alone reduced LB-EPS by 58.4%, with decreases of 72.8% in PS and 50.9% in PN. Nevertheless, ECS alone achieved higher turbidity removal than GCS alone. Therefore, the larger decrease in LB-EPS caused by GCS did not result in greater supernatant clarification. This indicates that LB-EPS concentration alone could not explain the difference in clarification between the two flocculants.

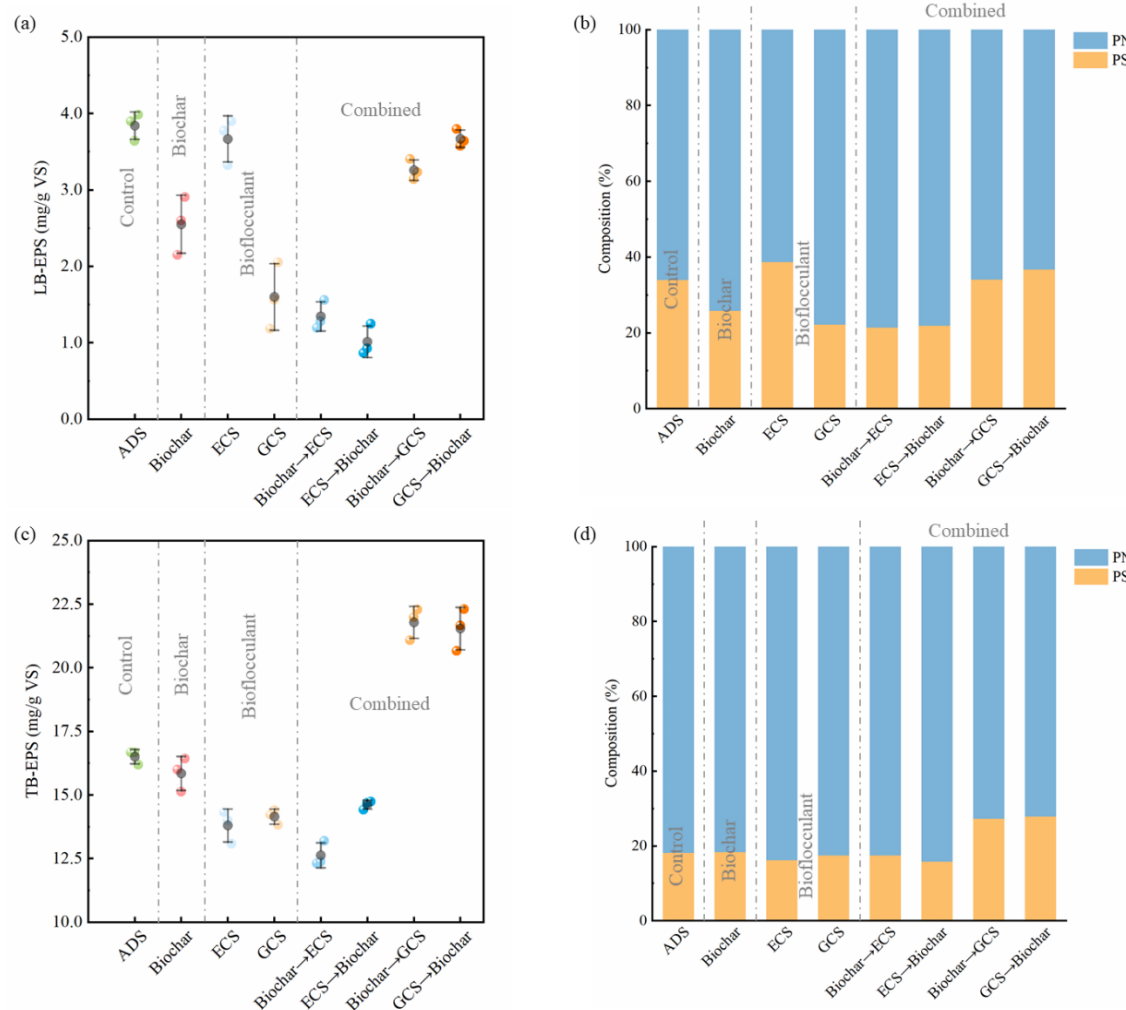


Figure 3. Concentration and composition of PN and PS in LB-EPS and TB-EPS following individual and sequential conditioning: (a) LB-EPS concentration; (b) LB-EPS composition; (c) TB-EPS concentration; (d) TB-EPS composition.

Sequential conditioning with algal biochar and ECS produced the largest decreases in LB-EPS. Algal biochar followed by ECS reduced LB-EPS to $1.34 \text{ mg g}^{-1} \text{ VS}$, while ECS followed by algal biochar reduced it to $1.01 \text{ mg g}^{-1} \text{ VS}$. These values were 65.0% and 73.7% lower than untreated ADS and 63.3% and 72.4% lower than ECS alone, respectively. Both PN and PS decreased, showing that the response was not caused by a change in only one component. The TB-EPS response in the ECS system differed from that of LB-EPS. ECS alone reduced TB-EPS by 16.4% compared with untreated ADS. Adding algal biochar before ECS resulted in the lowest TB-EPS concentration of $12.63 \text{ mg g}^{-1} \text{ VS}$. When ECS was added first, TB-EPS was $14.62 \text{ mg g}^{-1} \text{ VS}$. This represented an 11.4% decrease compared with untreated ADS, but a 5.9% increase compared with ECS alone. The two addition orders therefore had a more evident effect on TB-EPS than on turbidity removal. This agrees with previous evidence that the sequence of conditioner addition can alter EPS composition in digested sludge [40]. However, the present data do not establish whether this difference resulted from changes in organic matter association or extraction within the flocs.

In contrast to the combined ECS treatments, the combined GCS treatments increased the LB-EPS concentration. Adding algal biochar before GCS increased LB-EPS from $1.60 \text{ mg g}^{-1} \text{ VS}$ with GCS alone to $3.26 \text{ mg g}^{-1} \text{ VS}$. The reverse order increased it to $3.67 \text{ mg g}^{-1} \text{ VS}$. These concentrations were approximately twice that obtained with GCS alone and were close to the untreated ADS value. Both PN and PS contributed to this increase. Nevertheless, the combined treatments achieved higher turbidity removal than GCS alone. This shows that improved clarification did not coincide with a reduction in LB-EPS in this system. TB-EPS also increased in both combined GCS treatments, reaching 21.78 and $21.54 \text{ mg g}^{-1} \text{ VS}$. These values represented increases of 54.0% and 52.3% compared with GCS alone and approximately 31% compared with untreated ADS. PN and PS both increased by approximately 3.6 to $4.1 \text{ mg g}^{-1} \text{ VS}$ compared with GCS alone. They therefore contributed similarly

to the increase in total TB-EPS. Because the PS concentration after GCS treatment alone was much lower than the corresponding PN concentration, PS showed a greater percentage increase.

The higher recovered TB-EPS does not necessarily indicate an increase in native sludge EPS. It may reflect stronger association of biopolymers with flocs or greater release during extraction. Adsorption or retention of starch-derived material may also contribute to the measured amounts. These possibilities cannot be distinguished by the present measurements. The increase therefore cannot be interpreted as evidence of improved floc strength or dewaterability.

Overall, the combined ECS treatments had lower LB-EPS and TB-EPS concentrations than the combined GCS treatments. Within the GCS systems, the proportional increase in PS compared with GCS alone was particularly evident in the TB-EPS fraction. These contrasting EPS profiles were observed even though all four combined treatments achieved similar turbidity removal. Thus, similar clarification was achieved by combined systems with different extractable EPS responses.

3.4. Overall Comparison and Limitations

This section brings together the clarification, zeta-potential and EPS results to compare the combined conditioning systems at the selected doses. The combined ECS and GCS systems produced comparable supernatant clarification but showed different electrokinetic and operational EPS responses. Compared with the GCS combinations, the ECS combinations had less negative zeta potentials and lower SMP, LB-EPS and TB-EPS concentrations. The GCS combinations retained strongly negative zeta potentials, while their LB-EPS and TB-EPS concentrations were higher than those obtained with GCS alone. Therefore, comparable turbidity removal was not associated with a common pattern of EPS response.

Reversing the addition order changed turbidity removal by no more than 2.9 percentage points and zeta potential by no more than 1.1 mV. However, a more noticeable effect of addition order was observed for TB-EPS in the ECS system. At the selected doses, differences between the ECS and GCS formulations were generally more evident than those between addition orders. Because ECS and GCS were applied at different doses, these between-formulation differences cannot be attributed solely to polymer structure. The comparison therefore reflects the responses of the two formulations under their respective selected operating conditions rather than an equal-dose structure–performance comparison.

Summing the three collected fractions on a consistent initial sludge VS basis revealed contrasting PN and PS recovery profiles (Table S1). Untreated ADS yielded total recovered PN and PS of 27.72 and 9.56 mg/g VS, respectively. The combined ECS treatments yielded lower recovered PN, ranging from 18.83 to 20.77 mg/g VS, and lower recovered PS, ranging from 6.83 to 7.74 mg/g VS. In contrast, the combined GCS treatments yielded lower recovered PN of 25.12–25.46 mg/g VS but higher recovered PS of 14.64–14.95 mg/g VS.

Sequential treatments involved two mixing stages, whereas individual treatments involved one. Consequently, comparisons between combined and individual treatments also reflect differences in mixing duration and exposure history. These comparisons therefore cannot by themselves isolate or demonstrate a synergistic interaction between the conditioning materials.

The recovered totals include only PN and PS measured in the three operationally extracted fractions. The discarded washing liquids and residual solids were not analysed, so a complete mass balance could not be established. Extraction efficiency and material derived from the added flocculants may also influence the measured amounts. The observed differences therefore demonstrate contrasting recovery profiles across the operational fractions but do not confirm redistribution of native sludge EPS.

The present results demonstrate distinct electrokinetic and operational EPS responses among the conditioning systems, although the dominant aggregation mechanism cannot be resolved from the current measurements alone. Direct dewatering and filtration measurements are also required to determine whether the observed profiles translate into differences in subsequent solid–liquid separation performance. Further work should combine systematic biochar characterisation, including surface chemistry, pore structure and morphology, with floc-structure analysis, adsorption tests and dewatering measurements to establish property–performance relationships and distinguish the contributions of electrostatic interactions, adsorption and particle-assisted aggregation.

The sludge used in this study came from a single wastewater treatment plant, and differences in sludge properties may influence conditioner performance. Further studies using sludge from other sources and digestion conditions are therefore needed to assess the broader applicability of the observed responses. The environmental performance of this conditioning approach was also not assessed. Biochar production requires heating and washing, while flocculant synthesis involves chemical inputs. These inputs, together with the effects of the added

materials on downstream sludge disposal or reuse, should be evaluated before broader environmental benefits are claimed.

4. Conclusions

This study extends particulate-assisted sludge conditioning to algal biochar combined with two cationic starch formulations prepared through graft copolymerisation and etherification. Combined algal biochar–starch conditioning achieved supernatant turbidity removals of 77.1–84.1%, compared with 40.8% for ECS alone and 32.0% for GCS alone. Despite comparable clarification, the ECS combinations had less negative zeta potentials and lower operational EPS concentrations than the GCS combinations. Relative to untreated ADS, the ECS combinations yielded lower total recovered PN and PS, whereas the GCS combinations yielded lower PN but higher PS. Besides, addition order had a limited effect on supernatant clarification. These findings demonstrate the importance of considering EPS fractions together with clarification when evaluating combined conditioning formulations. The findings are limited to supernatant clarification and operational EPS responses under the tested conditions. Their implications for sludge dewaterability and filterability require direct evaluation.

Supplementary Materials

The additional data and information can be downloaded at: <https://media.sciltp.com/articles/others/2610121650012692/MFE-26080152-SI.pdf>. Figure S1: Structural characterisation of the starch-based bioflocculants. (a) FTIR spectra of soluble starch, ECS and GCS. (b) ¹H NMR spectra of ECS and GCS. Table S1: Total recovered protein and polysaccharide across the operationally extracted SMP, LB-EPS and TB-EPS fractions.

Author Contributions

Y.Y.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing—original draft. W.G.: Data curation, Formal analysis, Supervision, Methodology, Validation, Writing—review & editing. H.H.N.: Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing—review & editing. H.Z.: Funding acquisition, Formal analysis, Investigation, Resources, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The datasets generated and/or analyzed during the current study are not publicly available because they contain unpublished data that form part of an ongoing research project but are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no competing financial or non-financial interests. Given their roles as Co-Editor-in-Chief and Associate Editor of this journal, respectively, Huu Hao Ngo and Huiying Zhang had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

Use of AI and AI-Assisted Technologies

During the preparation of this work, the authors used ChatGPT (OpenAI) to assist with English language editing and improving readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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