Chemotactic movement and zeta potential dominate *Chlamydomonas microsphaera* attachment and biocathode development

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Abstract

Microalgal cell attaching and biofilm formation are critical in the application of microalgal biocathode, which severs as one of the hopeful candidates to an original cathode in bioelectrochemical systems. Many efforts have been put in biofilm formation and bioelectrochemical systems for years, but the predominant factors shaping microalgal biocathode formation are sketchy. We launched a pair of researches to investigate microalgal attachment and biofilm formation in the presence/ absence of applied voltages using *Chlamydomonas microsphaera* as a model unicellular motile microalga. In this study, we presented how microalga attached and biofilm formed on a carbon felt surface without applied voltages and try to manifest the most important aspects in this process. Results showed that while nutrient sources did not directly regulate cell attachment onto the carbon felt, limited initial nutrient concentration nevertheless promoted cell attachment. Specifically, nutrient availability did not influence the early-stage (20 - 60 min) of microalgal cell attachment but did significantly impact cell attachment during later stages (240 - 720 min). Further analysis revealed that nutrient availability-mediated chemotactic movements and zeta potential are crucial to facilitate the initial attachment and subsequent biofilm formation of *C. microsphaera* onto the surfaces, serving as an important factor controlling microalgal surface attachment. Our results demonstrate that nutrient availability is a dominant factor controlling microalgal surface attachment and subsequent biofilm formation processes. This study provides mechanistic understanding of microalgal surface attachment and biofilm formation processes on carbon felts surfaces in the absence of applied voltages.

Keywords

Biocathode; attachment; chemotaxis; microalgae; nutrient availability

Introduction

Bioelectrochemical systems are efficient and sustainable processes for the production of valuable products, energy conversion, and bioremediation processes [1–3]. Expensive metal catalysts, such as platinum, are commonly utilized as the electrode catalyst to reduce overpotentials in conventional electrochemical systems [4–8]. Recently, the application of microalgal biocathodes have been considered as a promising alternative to replace expensive metal catalysts in bioelectrochemical systems, due to the production of pure oxygen by a photosynthetic microalgae near a cathode surface [9–11]. Efforts are now being made to employ various microalgae for biocathodes, such as *Chlorella vulgaris*, *Scenedismus obliquus*, *Scenedesmus quadricauda*, *Chlamydomonas reinhardtii*, and other mixed microalgae [12–15]. In a photosynthetic microbial desalination cell, a power density of 660 mW/m^3 could be achieved with a *Chlorella vulgaris* biocathode [16]. Similar results were also reported with another *C. vulgaris* biocathode microbial fuel cell, which gained 47% more electricity than an abiotic cathode [17].

Microalgae attachment, colonization, and biofilm formation on the electrode surface are crucial to the development of an effective microalgal biocathode. Cell attachment to biotic or abiotic surfaces and subsequent biofilm formation is ubiquitous in nature [18], where biofilms drive nearly every biogeochemical cycle in water, soil, sediment and subsurface environments [19]. Attachment enables cells to increase nutrient access and to increase resistance to environmental stress by modulating rates of gene transfer, promoting interspecies cooperation, and enabling metabolic stratification [20]. Many microalgal species have a natural tendency to attach onto surfaces forming microalgal biofilms. Generally, the process of microalgae attachment involves two steps; the initial adhesion of microalgal cells onto substratum surfaces and the following development and colonization [21]. Cell-substratum and cell-cell interactions are considered as the key factors

regulating microalgae attachment, including Lewis acid-base interactions, hydraulic turbulence, and hydrophobicity and roughness of substratum surfaces[22–25]. Early attachment begins with short-range Lewis acid-base interactions before the cells begin migrating towards the substratum surface induced by the electrostatic attractive force [26]. Many factors have been hypothesized as the most important for microalgae attachment, including light intensity, nutrient concentration, pH, hydraulic status, microalgae strains, and substratum properties [27]. Some studies found that appropriate surface treating would benefit for microalgae cell attachment and substantially shorten the initial attachment time [28]. Numerous types of support materials, including filter paper, membrane, polyethylene fabric silicone rubber, glass fiber, cotton, loofah sponge, etc., have been employed to grow attached microalgae and evaluate biomass productivity [29–32]. Meanwhile, operational optimization of initial nitrogen concentration and pH remarkably enhanced microalgal attachment [33]. For example, in a ceramic substrate photo-bioreactor, the optimum biomass productivity of 4.65 ± 0.42 g/m^2/d was obtained after 8-d cultivation at a light intensity of 17,000 lux and 14 mL/d BG-11 medium [34]. It was reported that the initial nutrient concentration, including nitrogen and phosphate, influences bacterial [35–37] and microalgal [38,39] cell attachment/aggregation. For instance, *Scenedesmus* LX1 was found to increase the attached life form under relatively lower nitrogen and phosphate concentrations as compared with those of high-nutrient circumstances [36]. Therefore, we applied different initial nitrate and phosphate concentrations to quantify the effects of nutrient concentration on *C. microsphaera*'s attachment and biocathode development. Though progress has been achieved in microalgal cultivated with attached methods, the formation of microalgae layer on electrode surfaces, such as carbon felt, is a complex process, and the mechanisms involved in how nutrient levels affect microalgal attachment are not fully understood [21,40,41].

To better understand the process of microalgal attachment and underlying mechanisms, we employed a model unicellular microalga, *Chlamydomonas microsphaera*, to investigate how do nutrient patterns and external voltages affect microalgal attachment onto a carbon felt surface and subsequent biofilm formation, and to identify the determinant factors. We performed nutrient-controlled laboratory experiments consisting of pure cultures of *C. microsphaera* and estimated microalgal cell numbers attached onto the carbon felt, cell velocity, EPS excretion, and zeta potential values. We further quantified nitrate and phosphate adsorption patterns onto the carbon felt under different nutrient conditions. Finally, we performed principal component analysis (PCA) to examine hypotheses about the main driving forces of *C. microsphaera* cell attachment onto the carbon felt, including cell velocity, nitrate and phosphate adsorption, EPS excretion, and zeta potentials of cells, for various nitrate or phosphate concentrations. For the paired research in the presence of voltage, related results could be found in the paired investigation [42].

2. Materials and Methods

2.1 Microalgal strain and growth conditions

C. microsphaera (FACHB 52) was selected as a model single-cell green alga for investigating microalgal attachment and biofilm formation and was acquired from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China). The microalgal inoculum was diluted at 10% ($V_{inoculation}/V_{media}$) in 500 mL Erlenmeyer flasks containing 250 mL of sterile Bristol's solution and grown in a climate chamber (BDP-250CO2, BaiDianTech, China) at 25 ± 1°C. The climate chamber was set at 2000 lux illumination intensity under a 12-12 h light-dark cycle condition. Cell growth was monitored by measuring the optical density at 680 nm using a UV/Vis spectrophotometer (Unico, Shanghai). *C. microsphaera* cells were harvested at the mid-log phase by centrifugation (5810R, Eppendorf, Hamburg, Germany) at 2000×g for 10 min, washed twice with

deionized water, and resuspended in deionized water to a finial cell density of ~ 1.0×10^6 cells/mL. Experiments were performed in triplicate if not specifically indicated otherwise. The composition of sterile Bristol's solution was (in mg/L): $NaNO_3$, 250.0; K_2HPO_4, 75.0; $MgSO_4\cdot7H_2O$, 75.0; $CaCl_2\cdot2H_2O$, 25.0; KH_2PO_4, 175.0; NaCl, 25.0; $FeCl_3\cdot6H_2O$, 5.0; H_3BO_3, 2.86; $MnCl_2\cdot4H_2O$, 1.86; $ZnSO_4\cdot7H_2O$, 0.22; $Na_2MoO_4\cdot2H_2O$, 0.39; $CuSO_4\cdot5H_2O$, 0.08; $Co(NO_3)_2\cdot6H_2O$, 0.05. The chemicals used in this study were analytical reagent grade and were acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Reactor configuration

For the nutrient adsorption experiment, bottle-type batch reactors with 100-mL working volumes sealed with a butyl rubber stopper were used in this study [42]. Two 6 mm diameter holes were milled in the stopper with two plain graphite rods inserted (6.0 mm in diameter, 16.0 cm in height). The graphite rod spacing between each other was fixed at 15 ± 2.0 mm. The cathodic graphite rod was connected to a piece of carbon felt as the substratum for microalgae attachment. Prior to experiments, the carbon felts were washed three times with deionized water, soaked in 1.0 M HCl solution followed by 1.0 M NaOH solution for 10 min to remove possible metal and biomass contaminations, and finally rinsed three times with deionized water. All reactors and carbon felts were sterilized by autoclaving at 121^{o}C for 15 min prior to beginning the experiments.

2.3 Nutrient adsorption at various initial concentrations

Nutrient adsorption was evaluated across a nitrite gradient (referred to as nitrite dilution series I) and a phosphate gradient (referred to as phosphate dilution series I) according to our paired study [42]. In brief, nitrate/phosphate dilutions were a series modified Bristol's solutions consisting 250 mg/L, 125 mg/L, 25 mg/L, 5 mg/L, 2.5 mg/L, or 0 mg/L $NaNO_3$/KH_2PO_4, respectively. HCl/NaOH (0.1 mol/L)

solution was used to maintain the pH value of the medium around 6.55 ± 0.01 (original pH of Bristol's solution). All dilutions were autoclaved at 121°C for 15 min before use. Nutrient adsorption experiments were conducted in 33 identical sterile batch reactors. In each reactor, 100 mL of modified sterile Bristol's solution (dilution series I) was added. One mL liquid samples were removed from each BES reactor at 0 min, 20 min, 60 min, 240 min, 480 min and 720 min for analysis. All experiments were performed at 2000 Lux illumination intensity and 25 ± 1°C without shaking in triplicate unless otherwise specified.

2.4 *C. microsphaera* attachment at various initial nutrient concentrations

C. microsphaera attachment was also evaluated across a nitrite/phosphate gradient (referred to as nitrite/phosphate dilution series II) as described for the nutrient adsorption experiment [42]. To analyze the effects of initial nitrate and phosphate concentrations on microalgae attachment, 33 sterile batch reactors were prepared. In each reactor, 40 mL of modified sterile Bristol's solution (dilution series II) was added. After 12 h, a 10 mL microalgae inoculum at a cell density of 1.0×10^6 cells/mL was transferred into each reactor, with the ultimate N/P concentration of 250 mg/L, 125 mg/L, 25 mg/L, 5 mg/L, 2.5 mg/L, or 0 mg/L. HCl/NaOH (0.1 mol/L) solution was used to maintain the pH value of the medium around 6.55 ± 0.01 (original pH of Bristol's solution). The experiment conditions were kept as 25±1°C and 2000 lux illumination intensity in a climate chamber (BDP-250CO2, BaiDianTech, China). Specifically, nitrate/phosphate dilution series II was prepared using a modified Bristol's solution where the $NaNO_3$/KH_2PO_4 concentration was set to 312.5 mg/L, 156.2 mg/L, 31.2 mg/L, 6.2 mg/L, 3.1 mg/L, or 0 mg/L. All dilutions were autoclaved for sterilization at 121°C for 15 min before use.

2.5 Microalgal cell motility

Thirty microliter aliquots of microalgal culture were removed from each reactor at 20 min, 60 min, and 720 min after incubation and transferred to a 6-well plate for cell movement tracking. An optical inverted microscope system (IX73, Olympus, Japan) equipped with a digital camera (DP73, Olympus, Japan) was employed to track cell movement via video acquisition. Real-time trajectories of microalgal cells were recorded at 12-15 frames/second for 60 seconds. Microbial cell velocity was measured frame-by-frame and was calculated at a time interval of 0.50 seconds. The average cell velocity was calculated as the mean of the complete series of trajectories [42,43]. For each sample, at least 100 randomly selected microalgae cells were used for motility analysis.

2.6 Confocal laser scanning microscopy observations

At the end of the experiment, the carbon felt substratum was removed from each reactor and fixed onto a glass slide. To visualize total cells, the sample was mixed with 100 μL 20 μmol/L Syto 63 solution (Molecular Probes, Carlsbad, CA, USA) and incubated for 30 min [44]. The stained sample was then washed twice with phosphate-buffered saline (pH 7.2) to remove excess dye and imaged using a confocal laser scanning microscope (Carl Zeiss LSM710, Germany) equipped with a 100 × oil objective. Image processing and analysis were made using ZEN blue software (version 2012, Carl Zeiss, Germany).

2.7 EPS extraction and analysis

A modified heat extraction method was used to extract EPS as described elsewhere [45]. The protein component was analyzed using a UV/Vis spectrophotometer (PerkinElmer Lambda 25) at 750 nm based on a Folin-phenol method [46]. The polysaccharide component was analyzed using a UV/VIS spectrophotometer (Unico, Shanghai) at 420 nm according to an anthrone colorimetric method [47].

2.8 Zeta potential and other chemical analysis

Zeta potentials were measured using a zeta-potential analyzer (Nano-ZS90, Malvern, UK). Total phosphorous was measured with a UV/VIS spectrophotometer (PerkinElmer Lambda 25) at 750 nm using an ascorbic acid method, and nitrate was measured with a UV/VIS spectrophotometer (PerkinElmer Lambda 25) at wavelengths of 220 nm and 275 nm using an ultraviolet spectrophotometric screening method, according to the Standard Methods [48].

2.9 Statistical Analysis

The data reported in this study were represented as mean values of three replicates and standard deviations of the mean (SD), if not specified. Polynomial fitting was applied to designate the relationship between initial nutrient concentration and nutrient adsorption with 95% confidence. One-way or two-way analysis of variance (ANOVA) was used to test the effects of nutrient concentration on microalgal cell attachment. Statistical significance was set at 95% confidence intervals and was accepted at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Linear and nonlinear correlation tests were performed with Pearson and Spearman correlations to test pairwise correlations between two factors. The general characteristics of cell attachment under different nutrient conditions were evaluated with PCA methods [42].

3. Results and discussion

3.1 *C. microsphaera* attachment under various nutrient concentrations

C. microsphaera cells attached onto the carbon felt surface in a nutrient-dependent manner (**Fig. 1**). Generally, both the initial nitrate concentration (represented by dilution rate) and the initial phosphate concentration affected the surface attachment of *C. microsphaera* on the carbon felt, where relatively limited nitrate or phosphate concentrations promoted cell attachment. The number of attached cells and attaching ratio ($Cell_A/Cell_T$, a ratio of the number of the attached cells to the

number of the total cells) onto the carbon felt at different times after inoculation are summarized and depicted in **Fig. 2.**

At the early stage of incubation (20-60 min), both nitrate and phosphate concentrations did not influence the cell attachment ($P > 0.05$, **Table S1**). For instance, during the first 20 min incubation, attached cells remained at 2.49 $\times 10^4$ cells/cm^2 in the original Bristol's solution. The number of attached cells remained around 2.33 $\times 10^4$ cells/cm^2 and 2.51$\times 10^4$ cells/cm^2 in the absence of nitrate or phosphate, respectively. Nitrate or phosphate concentrations at 2.5 to 250 mg/L did not substantially influence cell attachment, with attached cells ranging from around 2.37$\times 10^4$ cells/cm^2 and 2.67$\times 10^4$ cells/cm^2.

In the following 240-720 min, the effects of nitrate or phosphate concentrations on attached cells became significant, with P-values less than 0.01 (**Table S1**). In the original Bristol's solution (i.e., initial nitrate and phosphate concentrations of 250 mg/L), the number of attached cells (expressed as attached cell density on the carbon felt) reached 14.32$\times 10^4$ cells/cm^2 after 720 minutes of incubation, accounting for 26% of the total microalgal cells in the reactor. A moderate decrease in either the initial nitrate or phosphate concentration from 250 mg/L to 5 mg/L clearly enhanced the surface attachment of *C. microsphaera* cells onto the carbon felt, as evidenced with Pearson r values of -0.9149 and -0.9123 for nitrate and phosphate, respectively. Thereafter, further decreases in either the nitrate or phosphate concentration to 0 mg/L decreased cell attachment, with Pearson r values of 0.9992 and 0.9567 for nitrate and phosphate, respectively.

Interestingly, the maximal cell attachment occurred at intermediate nitrate and phosphate concentrations of 5 mg/L - 25 mg/L, respectively, with final cell densities and attaching ratio of ~17$\times 10^4$ cells/cm^2 and 33%, respectively. In addition, the intermediate initial nitrate or phosphate

concentration of 5 mg/L yielded a truncated cell attachment process, whereby the number of attached cells peaked at about 480 min and then stopped or even decreased.

Statistical analyses revealed that the initial nitrate or phosphate concentration did not significantly influence cell attachment at the early stage of incubation (0 - 60 mins), with the attached cell numbers being statistically indistinguishable (2.51 to 2.67 $\times 10^4$ cells/cm^2) across all concentration scenarios, as compared with that of 2.49×10^4 cells/cm^2 for the initial nitrate and phosphate concentration of 250 mg/L ($P > 0.05$ for the ANOVA test, **Table S1**). However, the impact of the initial nitrate or phosphate concentration on cell attachment gradually became significant alone with the nutrient consumption (**Table S1**).

3.2 Nutrient adsorption under various nutrient concentrations

Besides providing a substratum for cell attachment, the carbon felt might also serve as a nitrate or phosphate enrichment layer that accumulates available nitrate and phosphate to support the growth of *C. microsphaera.* The nitrate and phosphate adsorption profiles of the carbon felt support this hypothesis, where the nitrate and phosphate contents adsorbed onto the carbon felt increased rapidly within 240 min across all scenarios, followed by a further gradual accumulation throughout the remainder of the experiment (**Fig. 3A**). In addition, the adsorption ratio of nitrate or phosphate (expressed as the ratio of adsorbed to dissolved nitrate or phosphate) increased with decreasing initial nitrate and phosphate concentrations, albeit with decreased absolute adsorption amounts of nitrate or phosphate (**Fig. 3B**). For instance, the adsorption ratios of nitrate and phosphate at an initial concentration of 5 mg/L estimated at 720 min after incubation were 12.8% and 12.9%, respectively, which were nearly one order of magnitude higher than those for a higher initial nitrate and phosphate concentration of 125 mg/L. However, only a poor correlation was observed between the attached cell numbers and adsorbed nitrate or phosphate amounts for all concentration scenarios according to the

Pearson and Spearman correlation analyses (**Table S3**). This suggests the existence of alternative mechanisms (e.g., cell motility) other than nutrient sources determine cell attachment onto the carbon felt [49,50].

3.3 *C. microsphaera* motility under various nutrient concentrations

Resource acquisition is critical for microorganisms to survive in aquatic environments, microbes may interact with diffuse resources by means of a broad array of physiological and behavioral adaptations, such as chemotactic movement [51]. Chemotaxis is defined as the capacity of certain organisms to sense attractant or repellant concentration gradients and in turn move towards or away from this gradient [52–55]. For instance, cells of chemotactic strains respond to excreted signaling molecules by moving up towards the concentration gradients and forming different types of stable multicellular structures, such as cell aggregation and biofilm [45,56,57]. *C. microsphaera* cells may respond to nutrient gradients or to attractant hotspots on carbon felt via chemotactic movement. It is therefore essential to quantify microbial motility patterns at various nitrate or phosphate conditions. As shown in **Fig. 4**, cell velocity of *C. microsphaera* clearly varied with the initial nitrate and phosphate concentration. Specifically, an average cell velocity of 24.0 ± 1.1 μm/s was observed during the early incubation (20 min) for initial nitrate and phosphate concentrations of 250 mg/L, followed by a generally amplified value of up to 30.8 (SD, 1.3) μm/s at 480 min and a subsequent drop to 11.7 (SD, 0.4) μm/s at the end of the experiment. Generally, a reduction in either the initial nitrate or phosphate concentration stimulated cell velocity between 20 and 240 mins after inoculation. For example, at an initial nitrate or phosphate concentration of 5 mg/L, cell velocities at 60 min were estimated up to 45.0 (SD, 1.3) and 50.4 (SD, 0.7) μm/s, respectively. Initial nitrate and phosphate concentrations influenced cell motility significantly ($P < 0.05$ for the ANOVA test, **Table S4**), and relatively high velocities were achieved at low nitrate or phosphate concentrations, albeit

with significant fluctuations of cell velocity across the entire incubation of all nitrate and phosphate concentration scenarios (**Table S4)**. Nevertheless, the absence of either nitrate or phosphate diminished cell motility, with the velocity value remaining below 19.8 (SD, 1.0) μm/s across the entire incubation time course. Strong correlations were measured between cell velocity and attachment at initial 20 min and the following 240 min under various initial nitrate concentrations, as well as those at 20 min, 240 min and 480 min under various initial phosphate concentrations (**Table S5**).

3.4 EPS excretion of *C. microsphaera* attachment under various nutrient concentrations

Complex microbial cell surface characteristics, often linked to EPS excretion, are key factors shaping cell-substratum interactions, and thereby promoting or interrupting cell attachment patterns [54]. Results showed that the polysaccharide and protein contents of the planktonic cells gradually increased from 4.5 mg/L and 7.0 mg/L at 20 min to 6.2 mg/L and 8.4 mg/L at 480 min, respectively, with little fluctuation among different concentration scenarios, with Pearson r values of -0.3788 (nitrate) and 0.4904 (phosphate) for PN, and 0.9976 (nitrate) and 0.9974 (phosphate) for PS (**Fig. 5**). Similarly, the PS and PN contents of the attached cells were found to range from 3.3 and 8.9 μg/cm^2 to 8.9 and 12.1 μg/cm^2, respectively. It suggests that neither the nitrate nor the phosphate concentration had a significant impact on EPS excretion by *C. microsphaera* cells, whereas the PN and PS contents may have a certain effect on microalgal surface attachment.

3.5 Zeta potential under various nutrient concentrations

Zeta potential is the electrical potential at the boundary of surrounding liquid layer attached to the moving particles in the medium. The high absolute value of zeta potential generates a repulsive electrostatic force between particles, thus a more stable suspension. [23,58]. The stability of

planktonic cells depends upon the absolute high value of zeta potential, where low absolute zeta potential values (< 30 mV) of either positive or negative charge tend to promote aggregation or attachment [59]. Zeta potential is affected not only by the properties of particles, but also the nature of the solution, such as pH, ionic strength, and temperature. In this study, similar patterns of zeta potential changes were observed under various nitrate or phosphate concentrations. For example, the zeta potential of the *C. microsphaera* cells was found to gradually increase from about -20 mV to -15 mV when the initial nitrate or phosphate concentration was elevated from 0 mg/L to 25 mg/L, and then slowly dropped down to around -16 mV when the initial nitrate or phosphate was further increased to 250 mg/L. (**Fig. 6**). Noticeably, all absolute values of zeta potential at various nitrate or phosphate dilution rates were smaller than 30 mV, indicating the limiting effect on cell attachment.

3.6 PCA analysis of *C. microsphaera* attachment under various nutrient concentrations

To figure out the main driving forces on cell attachment under various nitrate or phosphate concentrations, a PCA analysis was performed, with results illustrated in **Fig. 7**. The most likely contributing factors determining cell attachment are the PS and PN contents, which accounted for approximately 69.61% of the total variance in cell attachment among all scenarios, followed by the zeta potential value (**Fig. 7**). Cell motility and nutrient adsorption have the lowest association. For both nitrate and phosphate concentration scenarios, samples collected at 60 min were clearly distinguishable from those at 20 min and 480 min, mainly owing to the PS and PN contents rather than the zeta potential, nitrate adsorption, or cell motility. For a certain sampling time, scenarios of different nitrate or phosphate concentrations were well clustered and grouped with respect to the zeta potential, cell motility, and nutrient adsorption. These results indicate that, for a simple time series, the zeta potential and cell motility are likely key determinants driving cell attachment of *C. microsphaera*, which were directly regulated by nutrient availability in the reactors (**Table S6-S7**).

4. Conclusions

Nutrient availability significantly shaped microalgal surface attaching and subsequent biofilm formation onto the carbon felt surfaces. In response to nutrient concentration, microalgal cells changed their motility accordingly via chemotaxis which played an important role for surface attachment. Meanwhile, zeta potential was found fluctuating with changing nutrient concentration, and contributed actively to trigger cell attaching processes. In summary, microalgal chemotaxis and the ambient zeta potential conspire to determine the processes of microalgal surface attachment and subsequent biofilm formation within nutrient-limiting environments. Based on these experimental and statistical assessments, it advanced our understanding of microalgal surface attachment and biofilm formation processes in the absence of applied voltages, which is hopeful for the widely application of microalgal biofilms in either the microalgal production or wastewater treatment processes.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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