Contrasting physiological responses of Chlamydomonas reinhardtii to fibrous and layered clay minerals

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Abstract

Microalgae interact with mineral particles in aqueous environment, yet how clay minerals affect physiological processes in algal cells remain unexplored. In this study, we compared the effects of palygorskite (Pal) and montmorillonite (Mt), which respectively represent fibrous and layered clay minerals, on the physiological processes of Chlamydomonas reinhardtii. It was observed that C. reinhardtii responded differently to the treatments of Pal and Mt. The Pal particles bound tightly to and even inserted cells, resulting in a significantly decrease of cell numbers from 27.35 to 21.02*107 mL-1. However, Mt was only loosely attached to the cell surface. The photosynthesis in the algal cells was greatly inhibited by Pal, with rETRmax significantly reduced from 103.80 to 56.67 μmol electrons m-2s-1 and downregulation of IF2CP, psbH and OHP1, which were key genes involved in photosynthesis. In addition, Pal reduced the quantities of proteins and polysaccharides in extracellular polymeric substances (EPS) and the P uptake by C. reinhardtii when the P level in the culture was 3.15 mg/L. However, no significant changes were found regarding the above EPS components or the amount of P in algal cells upon the addition of Mt. Together, the impacts of fibrous Pal on C. reinhardtii was more profound than those of layered Mt.

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List of abbreviations

BCA Bicinchoninic acid

C carbon

Chlamydomonas reinhardtii C. reinhardtii

Chl *a* chlorophyll *a*

Chl *b* chlorophyll *b*

DEGs differentially expressed genes

Em emission wavelength

EPS extracellular polymeric substances

Ex excitation wavelength

Fig. figure

K potassium

Mt montmorillonite

N nitrogen

P phosphorus

Pal palygorskite

PBS phosphorus buffer saline

S sulfur

TAP Tris-acetate-phosphate

Abstract:

Microalgae interact with mineral particles in aqueous environment, yet how clay minerals affect physiological processes in algal cells remain unexplored. In this study, we compared the effects of palygorskite (Pal) and montmorillonite (Mt), which respectively represent fibrous and layered clay minerals, on the physiological processes of*Chlamydomonas reinhardtii* . It was observed that *C. reinhardtii* responded differently to the treatments of Pal and Mt. The Pal particles bound tightly to and even inserted cells, resulting in a significantly decrease of cell numbers from 27.35 to 21.02*10^7 mL^{-1}. However, Mt was only loosely attached to the cell surface. The photosynthesis in the algal cells was greatly inhibited by Pal, with $rETR_{max}$significantly reduced from 103.80 to 56.67 μmol electrons $m^{-2}s^{-1}$. Several key genes involved in photosynthesis, namely IF2CP, psbH and OHP1, were also downregulated. In addition, Pal reduced the quantities of proteins and polysaccharides in extracellular polymeric substances (EPS) and the P uptake by *C. reinhardtii* when the P level in the culture was 3.15 mg/L. However, no significant changes were found regarding the above EPS components or the amount of P in algal cells upon the addition of Mt. Together, the impacts of fibrous Pal on *C. reinhardtii* was more profound than those of layered Mt.

Keywords:

clay minerals, microalgae, physiology, RNA-Seq, 3D-EEM

1. Introduction

Clay minerals commonly exist in water and sediments [1]. They closely interact with microorganisms, providing them habitats and nutrients [2], adsorbing microbial exudates and promote formation of biofilm [3]. Microalgae form foundations of the aquatic food web and directly influence global carbon and nutrient

cycling, as well as the energy flow in ecosystems [4]. Clays may influence a number of microalgal physiological processes, such as flocculation and nutrient consumption [5,6].

Previous studies have shown that clay minerals may protect the microalgal cells from heavy metal stress [7]. Some minerals can also be used as carriers of nutrients to promote the growth and reproduction of algae [8]. Moreover, clay could form aggregates with algae and prevent chlorophyll from decomposition [9]. On the other hand, clay minerals may negatively affect algae. For example, they may inhibit algae growth by reducing their mobility, suppressing nutrient acquisition, or releasing metal cations (e.g., $Al^{3+}$and Fe^{3+}) [1,10]. Moreover, minerals also absorb or reflect light and may change transparency, color and other optical properties of water. Thus, the growth and reproduction of algae may be inhibited, thereby affecting the stability of aquatic ecosystem [11,12]. As a result, clays have been used as algaecide agents. It was documented that more than 90% of *Microcystis aeruginosa* in water was removed by clays through netting and bridging with cells, leading to algal death [13].

Clay mineral' structures may influence their properties and interactions with microalgae [14]. For instance, Montmorillonite (Mt) is a layered phyllosilicate and has a large specific surface area and widely exists in aqueous environments [15]. In comparison, palygorskite (Pal) is a hydrated magnesium aluminum silicate mineral with a unique 3D structure and fibrous morphology [3,16]. However, no comparison was made on the effects of Pal and Mt on microalgal cells.

Chlamydomonas reinhardtii is a model green algae [17] and is involved in multiple element cycles between biosphere and hydrosphere, e.g., carbon (C), nitrogen (N), sulfur (S) and phosphorus (P) [18]. It has been documented that P plays a critical role in algal growth [19]. Previous studies reported that *C. reinhardtii* could accumulate large quantities of P (up to 2-4% of their cell dry weight) [20]. On the other hand, extracellular polymeric substances (EPS) in algae are enriched in proteins and polysaccharides [21]. However, how minerals affect the uptake of P and secretion of EPS has not been clarified.

The aim of this study was to compare interactions between *C. reinhardtii* and two types of clay minerals, namely Mt (layered) and Pal (fibrous). Variations of physiological parameters in the algal cells, including growth, photosynthesis, respiration, EPS induction, cell morphology and differentially expressed genes, were systematically investigated upon exposure to Mt and Pal.

2. Materials and Methods

2.1 Algal incubation and mineral treatments

C. reinhardtii (CC-125) was purchased from the Chlamydomonas Resource Center, Department of Plant and Microbial Biology, University of Minnesota, United States. It was initially cultured in Tris-Acetate-Phosphate (TAP) medium at (25 ± 2) °C (light/dark 12/12h, light intensity 2000 Lux) [22]. The algal strain was then purified by streaking on an agar plate with TAP medium and forming algal colonies. A 0.5 cm x 0.5 cm algal sample was collected from the plate, and then placed in a 100 mL liquid TAP medium prior to the mineral treatments.

Mt (STx-1) and Pal (PFl-1) were purchased from the Clay Minerals Society. For Mt, its cation exchange capacity (CEC) was 84.4 meq/100g and the specific area was 83.79 ± 0.22 m^2/g. For Pal, the CEC was 19.5 meq/100g and the specific area was 136.35 ± 0.31 m^2/g (data taken from the website of Clay Minerals Society, https://www.clays.org/sourceclays_data/).

The two minerals were added into a 100 mL TAP medium to obtain the Mt and Pal concentrations (0, 200, and 500 mg/L). For the mineral exposure, 1 mL suspension of *C. reinhardtii* (in the logarithmic phase of growth) was incubated in the above mineral-TAP composite. The mineral treatments were denoted as Mt_0, Mt_{200}, Mt_{500}, Pal_0, Pal_{200}, Pal_{500}, respectively. All the samples were cultured by shaking three times every day during four-day incubation. Additionally, in order to explore the ability of phosphorus (P) uptake by *C. reinhardtii* under mineral treatments, we set two P concentrations, 3.15 mg/L and 31.5 mg/L, respectively in the algal culture. For other analyses, the P concentration in the culture medium was 3.15 mg/L. Three replicates were prepared for each treatment.

2.2 Analysis of physiological parameters

In this study, photosynthesis, respiration, P accumulation, fluorescence, intra- and extracellular proteins and polysaccharides were determined. For photosynthesis, the algal cells were measured for Fv/Fm and $rETR_{max}$ using a multiple excitation wavelength phytoplankton & photosynthesis analyzer (Phyto-PAM-II Modular Version, USA) after four days of incubation. The plate reader (SpectraMax i3X, USA) was used to measure OD_{680} in each sample and then the number of cells was calculated [23]. For respiration rate, standard gas was evenly injected into a saline bottle for 2 min, and then the culture was sealed in darkness for 1 h. Gas chromatography (GC, Agilent GC-7890B, USA) was used to analyze the concentration of CO_2 in a 5 mL bottle with a 10 mL syringe [24].

Another aliquot of the algal sample was centrifuged (5000 rpm, 5 min) and the supernatant was filtered via a 0.45 μm membrane for the P measurement by ICP-MS (Perkin Elmer NexION 2000, USA). In addition, for the fluorescence analysis of dissolved EPS from algal cells, the excitation wavelength (Ex) and emission wavelength (Em) were set to 200˜500 nm with a step size of 10 nm, the scanning speed was 6000 nm/min, with a spectral bandwidth of 3 nm (SHIMADZU RF-6000, Japan). The pellet was extracted with the equal volume of methanol. After re-suspension, the algal solution was placed in a refrigerator at 4 °C for 24 h. Then absorbance of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids were measured using the plate reader (SpectraMax i3X, USA) [25].

For measurements of intracellular and extracellular proteins and polysaccharides, the suspension of *C. reinhardtii* was filtered directly through 0.45 μm PES membrane (soluble proteins and polysaccharides (P_s)). The samples left on the filter membrane were resupsended, heated at 50 °C for 2 h and homogenized by sonication (total proteins and polysaccharides (P_t)). Then, they were filtered through PES membrane (soluble and cell-surface bound proteins and polysaccharides (P_{s+c})). The cell-surface bound proteins and polysaccharides was defined as the difference between the P_{s+c} and the P_s. The content of intracellular proteins and polysaccharides (P_i) was the contrast between the P_t and P_{s+c}. The proteins and polysaccharides contents were measured by bicinchoninic acid method (BCA) and the phenol sulfuric acid digestion method, respectively [22].

2.3 Functional groups and cell morphology

The algal sample was centrifuged and stored in a refrigerator for freeze-drying. Then, the functional groups were analyzed by ATR-IR (Nicolet iS5 FTIR, USA) [16]. The centrifuged cells were fixed with ethanol and freeze-dried with acetone. The samples were stored for SEM (Zeiss-Supra55) analysis [2]. For TEM (Tecnai G2 F20S-TWIN + AZtec X-Max 80T) observation, the samples were first fixed with glutaraldehyde and then stored at 4 °C. Finally, ultrathin (60–80 nm) was cut by a diamond knife for TEM analysis [26].

2.4 Transcriptomic analysis

Algal pellets were separated from the medium by centrifugation. Then, the pellets were washed three times (5 min each) with phosphorus buffer solution (PBS) and stored at -80 °C prior to transcriptomic analysis [23]. According to the SEM results, there was a significant difference in the cell morphology between the 200 mg/L mineral additions and the control, however upon the 500 mg/L exposure to Mt and Pal, a large number of dead cells was observed. Therefore, the transcriptome analyses were performed at the 200mg/L mineral treatments.

2.5 Data analysis

All the data were analyzed by R project (version 4.1.0) and SPSS Statistics software (IBM version 17.0) with a significance level of $p < 0.05$, as well as Origin Pro were used for graph preparation. The significant differences between the control and treatment groups were determined using a one-way analysis of variance (ANOVA, Duncan). The data in the figures was all presented as the mean value with standard deviation (n = 3).

3. Results

3.1 Photosynthesis parameters

Under the treatments of Mt_{200} and Mt_{500}, there was no significant change of Fv/Fm compared with the control group (Fig.1A). However, the value decreased significantly under Pal addition. When the alga was incubated with Pal_{200} and Pal_{500} for 72 h, the Fv/Fm data decreased from 0.6 to 0.5 (Fig. 1A).

The $rETR_{max}$ did not vary significantly upon Mt addition (Fig. 1B), although it increased at 24h and decreased afterwards. After 24h, the $rETR_{max}$under Mt_{500} reached were 128.5 μmol electrons $m^{-2}s^{-1}$ and remained higher than the control group. In contrast, Pal addition dramatically reduced the $rETR_{max}$ values from about 105 to 62.9 μmol electrons $m^{-2}s^{-1}$ at Pal_{200} and 56.7 μmol electrons $m^{-2}s^{-1}$ at Pal_{500}.

3.2 P uptake, cell number andCO_2emission rate

When P supply was 3.15 mg/L, the P uptake declined from 3.0 to 2.8 mg/L at Pal_{500} (Fig. 2A). When the P concentration in the medium was 31.5 mg/L, the P uptake increased significantly under Pal addition. However, the increase of P uptake was much lower under Mt addition (Fig. 2A).

As for the growth of *C. reinhardtii* , Mt and Pal had contrasting effects. Mt_{200} and Mt_{500} respectively increased the cell number from 27.4 to 28.6 and $29.9*10^7$ ml^{-1} (Fig. 2B). However, the number of cells significantly decreased to 26.1 and $21.0*10^7$ ml^{-1}treated by Pal_{200} and Pal_{500}, respectively

At Mt_{200}, the respiration showed no evident fluctuation. When Mt was elevated to 500 mg/L, the CO_2 emission rate slightly decreased from 35.0 to 32.3 mg C 10^{-10} cells h^{-1} (Fig. 2C). Nevertheless, the CO_2emission rate showed a persistent increasing trend from Pal_0 to Pal_{500}, reaching 46.0 mg C 10^{-10} cells h^{-1}.

3.3 Three-dimensional excitation-emission matrix fluorescence spectra

C1 was identified as tryptophan-like substances [27]. Two apparent peaks were observed in C1 (Fig. 3. A & B), i.e., a strong peak at 225/325 nm (Ex/Em) and a weak peak at 225/290 nm. Under Mt_{200} and Mt_{500}, the relative intensity of C1 declined to 31.6% and 30.2%. The addition of Pal_{200} and Pal_{500}, also led to a decrease of C1 to 39% and 36.5%, respectively (Fig. 3B). The major peaks of C2 had represented tyrosine-like substance [28], which was located at 280/340 nm (Ex/Em). The treatments of $Mt_{200}$and Mt_{500} increased the intensity of C2 to 34.5% and 31.8%, but the Pal addition did not significantly alter the level of C2. The major peaks of C3 and C4, which were located at wavelengths of 280/475 nm and 290/460 nm (Ex/Em) respectively, indicating that the EPS contained humic-like substances [29]. The intensities of these two components were lower than C1 and C2, although they also varied upon Mt and Pal additions (Fig. 3B).

3.4 Polysaccharides, proteins, chlorophyll and carotenoids

Compared to the control, the soluble and cell surface proteins had no significant change at Mt treatments (Fig. 4A). However, the content of intracellular protein increased gradually with the elevation of Mt concentration, reaching 6.0 mg 10^{-12} cell at Mt_{200} and 7.6 mg 10^{-12} cell at Mt_{500}. After Pal addition, the soluble proteins rose from 1.6 mg 10^{-12}cell to 2.0 and 3.0 mg 10^{-12} cells for Pal_{200} and Pal_{500}, respectively (Fig. 4A). However, the intracellular protein declined significantly from 5.5 to 3.7 mg 10^{-12} cells at Pal_{200} and increased slightly to 4.0 mg 10^{-12} cells at Pal_{500}.

The contents of soluble and intracellular polysaccharides decreased with the elevation of Mt, i.e., from 2.9 to 2.3 mg 10^{-12}cells and from 7.2 to 5.8 mg 10^{-12} cells, respectively (Fig. 4B). In contrast, these polysaccharides increased with the elevation of Pal. Upon Pal_{500}, the intracellular polysaccharides increased remarkably from 7.2 to 13.4 mg 10^{-12} cells. The cell surface polysaccharides contents hence showed an increasing trend under Pal addition.

The changes of chlorophyll *a* , chlorophyll *b* and carotenoids in cells showed an upward trend under Mt and Pal addition (Fig. 4C). Following the addition of Mt_{500}, chlorophyll*a* increased from 21.9 to 23.9 mg 10^{-12} cells, whereas this value increased to 28.5 mg 10^{-12} cells at Pal_{500}. In addition, upon the 500 mg/L Mt and Pal treatment, the content of chlorophyll *b* rose from 12.2 to 15.3 mg 10^{-12}cells and to 17.4 mg 10^{-12} cells,

respectively. The carotenoids content did not change significantly at Mt_{500}, but increased from 1.6 to 2.7 mg 10^{-12} cells at Pal_{500} (Fig. 4C).

3.5 Functional groups on the cell surface

The peaks of ATR-IR spectra located at 977 and 1000 cm^{-1} could be assigned to Si-O vibration (Fig. 5) [30]. The peaks at 1020 cm^{-1} represented the vibration of Si-O-Si [31], which disappeared after Mt and Pal addition (Fig. 5). Peaks at 1387 cm^{-1} and 1460 cm^{-1} were assigned to -COOH and P-O-H groups of polysaccharides, while the bands at 1534 and 1650 cm^{-1}were contributed by N-H and C=O groups in proteins [22], which declined after the addition of Mt and Pal, indicating that the content of amide group decreased [32,33]. The peak at 3548 cm^{-1} was related to the stretching vibration of -OH [34]. The weakened peak intensity (for peaks 1500-1600 and 2900-3500 cm^{-1}) were possibly due to the binding of functional groups with clay particles.

3.6 Cell morphology

SEM images showed that the algal cells only weakly interacted with 200 mg/L Mt (Fig. 6A&B). At Mt_{500}, Mt particles were more tightly adsorbed by the cells (Fig. 6C). In addition, abundant fibrous Pal particles were adhered to the cell surface at Pal_{200} (Fig. 6D&E). With the gradual elevation of Pal concentration, the density of mineral particles on the cell surface gradually increased. At Pal_{500}, the cells were almost wrapped in Pal (Fig. 6F), suggesting strong binding between the Pal and algal cells.

TEM observations showed that, Mt crystals were adsorbed onto the cells at Mt_{200} (Fig. 7A). With the increase of Mt addition, more Mt particles were observed on the cell surface (Fig. 7B). In addition, there were only scarce mineral particles around the cell wall at Pal_{200}, which appeared as separated mineral fibers (Fig. 7C). The Pal particles even inserted into the cell wall due to its fibrous shape. Compared with Mt, cell lysis could be observed with large amounts of Pal adsorbed on the cell surface at Pal_{500} (Fig. 7D).

3.7 Annotations of differentially expressed genes (DEGs)

Compared with control, differentially expressed genes (DEGs) were identified in the 200 mg/L Mt and Pal treatments. Since photosynthesis is an important physiological process in algae, we focused on those significantly different genes involved in photosynthesis (Figure. 8). IF2CP and CPLS1 genes were related to chloroplast synthesis based on the KEGG [35]. After the addition of Pal, these genes were down-regulated, which inhibited the chloroplast precursor material conversion to chloroplast. CAB4, CAB7 and CHLG genes regulated chlorophyll synthesis [36,37]. These genes were significantly upregulated compared with addition of 200 mg/L Mt. In addition, psbH and OHP1 genes related to PSII synthesis were significantly downregulated at Pal_{200} [38,39]. In addition, odhA and SAMC1 genes related to cellular respiration were upregulated at Pal_{200} [40,41]. However, the change was not evident in the gene expressions when Mt was added.

4. Discussion

The interactions between clay minerals and microorganisms have significant environmental implications in the biogeochemical cycle. For example, microbial activities could alter mineral compositions and enrich potassium (K), leading to a significant K sink that helps to equilibrate the unbalanced K budget in the ocean [42]. In addition, the algae-mineral interactions could also be used to remediate heavy metal pollution owing to large surface area to volume ratio of algae-mineral composites [43]. However, it has not been documented as to the effects of different clay minerals on microalgal cells.

In this study, Pal greatly inhibited algal photosynthesis, whereas Mt showed mild stimulation of algal growth. In particular, due to its large surface area (136.35 $\pm$ 0.31 m^2/g) and interwoven fibrous structure (Fig. 6D), the particles of Pal were bound tightly to the cell surface and some of them even penetrated into algal cells, inducing tight and dense aggregation on the cell surface (Fig. 6E&6F, Fig. 7C&7D). Therefore, this mineral could prevent light from entering the cells, resulting in decreased photosynthesis parameters such as Fv/Fm and rETRmax (Fig. 1). Fv/Fm describes the reduction of the primary electron acceptor and may indicate the loss of function of PSII to capture enough light and interruption of photosynthetic electron transfer from

PSI to PSII [44]. The value of rETRmax can be used to evaluate the change of the capacity of the electron transport chain [45]. The decline of these two parameters indicated that photosynthesis of *C. reinhardtii* was inhibited by the particles of Pal. In order to compensate for the decline of photosynthesis, the content of chlorophyll increased (Fig. 4C) and several related genes, e.g., CAB4, CAB7 and CHLG, were upregulated (Fig. 8), which was consistent with previous literature [46-49]. In contrast to Pal, the addition of Mt did not significantly change the photosynthesis of algal cells (Fig. 1). Although Mt adsorbed on the cell surface and also prevented some light from entering the cell, energy could still move across the physical barrier from Mt on the cell surface [50]. Therefore, Mt did not negatively affect the cell photosynthesis.

The presence of Pal inhibited the P uptake by cells at P concentration of 3.15 mg/L (Fig. 2A). Lower supply of P affected the synthesis of metabolites (e.g., component of sugar phosphates) and reactions that involved the coenzyme ATP [51]. Therefore, photophosphorylation, rubisco activity, and reactions of the Calvin cycle were suppressed, which further affected the photosynthesis of algae and downregulated IF2CP, CPLS1, psbH, and OHP1 genes (Fig. 8). In addition, the metabolic reactions were not effective in supporting the cell growth under P limitation [52], finally leading to a decrease in cell numbers (Fig.2B). However, cell respiration increased even though the P was deficient (Fig. 2A). The cells were then forced to upregulated odhA and SAMC1 genes involved in cellular respiration and consume more energy to survive. Furthermore, when the P level in the culture was 31.5 mg/L, the P uptake increased to resist the negative effects [53]. In contrast, Mt did not cause much damage to cells, which did not absorb too much P to promote their growth and metabolism and to resist the adverse environment [54].

Our results showed that proteins in EPS were the key organic compounds that reacted with Pal (Fig. 4A), This finding is consistent with previous research [55]. More specifically, Mt did not lead to significant changes of EPS components while Pal reduced the contents of proteins and polysaccharides in EPS (Fig. 4A&B). In order to resist the dense aggregation of Pal on the surface of algae and prevent further interaction between cells and Pal, functional groups (such as C=O and -COOH) were declined (Fig. 5). These ligands in EPS are critical in binding clay minerals [15]. The 3D-EEM results further illustrated that tryptophan-like substance was the major substance in response to mineral treatments (Fig 3&4). This may be due to complexation between Pal and benzene ring contained in tryptophan-like substance. These responses can be explained by the self-protection mechanism of the algae [56].

Pal particles can penetrate the cell walls based on TEM results (Fig. 7C&D). With the increase of Pal concentration, the cell membrane could be damaged and the intracellular proteins flowed out and the soluble proteins contents increased (Fig. 4A). The DNA might also be leaked into the bacterial cytoplasm due to Pal stress [57]. To resist this stress, the cells may switch intracellular carbon flux from lipids to polysaccharides [23]. Therefore, intracellular polysaccharides contents were significantly increased (Fig. 4B). When Pal concentration was 200 ml/L, the cells could survive but at the concentration of 500 mg/L, almost all the cells died (Fig. 2B). Therefore, the damage of the whole cells, together with the impacted functions discussed above, finally led to the reduction of activities in *C. reinhardtii* by fibrous clay minerals. Compared with Pal, the content of protein and polysaccharide did not change significantly after Mt addition (Fig. 4A&B), suggesting that Mt did not need to activate its self-protection mechanism.

5. Conclusions

In this study, significant differences were found in the effects of Mt and Pal on the morphology and physiology of *C. reinhardtii* . Layered Mt attached to the cell surface and could provide positive effects for algal growth. In contrast, fibrous Pal wrapped around the cell surface and significantly inhibited the algal photosynthesis. Moreover, some Pal could even inserted the cells, leading to damage of cell membrane and eventually cell death. Further research is needed to explore interactions between various microalgae and clay minerals and their influences on the biogeochemical cycles of elements in aqueous environments.

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Declaration of Interest Statement

The authors declare no competing interests.

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Figure Legends

Fig. 1. The effects of the Montmorillonite (Mt) and Palygorskite (Pal) on (A) the maximum quantum yields of photosystem II reaction center and (B) electron transport rate. The vertical bar represents the standard deviation of triplicate samples.

Fig. 2. A: P uptake of *Chlamydomonas reinhardtii* cultured at 3.15 mg/L and 31.5 mg/L P concentrations. B: The number of counts (per 10^7 cells) of *C. reinhardtii* after four days incubation. *P<0.05. C: CO_2 emission (per 10^{10} cells) of*C. reinhardtii* . The error bars represent the standard deviation based on three parallel experiments. *P<0.05; NS, not significant.

Fig. 3. Three-dimensional excitation-emission matrix fluorescence spectra of the supernatant under different treatments. A: the four fluorescent components (C1-C4) identified by PARAFAC analysis; B: relative abundances of fluorescent components under different treatments.

Fig. 4. The content of proteins (A), polysaccharides (B), and photosynthetic pigments (C) after four days of incubation.

Fig. 5. ATR-IR spectra of *C. reinhardtii* cells and two minerals. 200Mt, 500Mt, 200Pal, and 500Pal: spectra of the algal cells incubated with 200, 500 mg/L Mt/Pal. Mt and Pal: spectra of two minerals. 0: spectra of the algal cells.

Fig. 6. A and D: SEM images of Mt (A) and Pal (D). B, C, E, and F: SEM imaging on the algae cells under 200 (B&E), 500 (C&F) Mt/Pal.

Fig. 7. TEM images of *C. reinhardtii* cells incubated with 200 (A), 500 (B) mg/L Mt and 200 (C), 500 (D) mg/L Pal of the medium in all the treatments.

Fig. 8. Main metabolic pathway network related to differentially expressed proteins (DEPs). The color bar indicates the up-down adjustment of DEPs (C for CK; P for Pal (200 mg/L); M for Mt (200 mg/L)). IF2CP, CPLS1: genes related to the transformation of precursor substances into chloroplasts; CAB4, CAB7 and CHLG: genes related to chlorophyll synthesis; SAMC1 and odhA: genes related to cellular respiration; psbH and OHP1: genes related to PhotosystemII synthesis.

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