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This review provides a current understanding of algal biological response mechanisms to NPs exposure, based on such mechanisms, implications were addressed for NPs application on mitigating algal bloom. Further, this critical review may inspire NPs application for promoting growth of beneficial algae.

Algae Response to Engineered Nanoparticles: Current Understanding, Mechanisms and Implications

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Abstract

The growing application of nanotechnology causes the release of engineered nanoparticles (NPs) into the aquatic environment. With increasing concerns on the potential effect of NPs to the aquatic organisms, investigations on NPs toxicity to algae are rising. To date, the overall algal responses to cope with NPs toxicity are still uncertain. In this review, a meta-analysis was conducted to quantitatively assess the toxicity mechanisms dominated by oxidative stress. The reactive oxygen species elevated by 90 % caused retarded algal growth by reduction (38 %) in cell density, and NPs toxicity was strongly dependent on NPs type and dose. Specifically, the mechanisms of NPs toxicity were discussed in different "omics" level. Further, we summarized the current knowledge and mechanisms of defense strategies, including formation of a protective bio-barrier and adjustment in intracellular processes (internalization, transformation and compartmentation) to decrease cellular NPs concentrations. Based on the response patterns of algae to NPs, we addressed the possibility of NPs application for algal bloom control. A systematic understanding of algal response mechanisms to NPs will help develop safe and sustainable nanotechnology in aquatic ecosystem.

1. Introduction

The special physiochemical properties impart engineered nanoparticles (NPs) with wide applications. According to the Nanotechnology Consumer Products Inventory, there are 1814 nanomaterial-containing products from 622 companies in 32 countries,

among which metals and metal oxides (Ag, TiO_2, SiO_2, ZnO etc.) comprise the largest nanomaterial composition.[1] The booming use of NPs in consumer products entails their release into surface water as items degrade and are discarded, ultimately, aquatic environments become one of the main sinks for NPs pollution.[2–4] The small size, low mass concentration, high activities and complex matrixes make it sophisticated to accurately detect aquatic NPs, the estimated concentration in surface water is in the range of ng/L to μg/L.[5] NPs in the aquatic environments could undergo physical and chemical processes such as dispersion, agglomeration/aggregation, dissolution, transformation and sedimentation,[4] which are governed by numerous factors including the properties of NPs (size, coating material and shape) and exposure conditions (pH, ionic strength and dissolved organic matter).[6–13] Even though 90 % of NPs can be removed from water column by deposition and heteroaggregation within 10 - 100 h, this limited residence time can still lead to a great deal of interactions between NPs and aquatic organisms.[14] Consumption of NPs-infected aquatic organisms is expected to cause human exposure to NPs.[4] Algae, constituting the basis for aquatic food chain and involving in the nutrient cycling of aquatic ecosystems, has been shown as a sensitive receptor with low 50 % effective concentration (EC_{50}) of NPs.[15] Over the last decade, substantial research regarding the effects of NPs to algae has risen exponentially. Most studies indicated a certain degree of NPs toxicity to algae based on the 374 articles published during 2006 - 2017.[16] Metallic NPs appear to be the most frequently studied type of NPs, their toxicities to algae are mediated by the dissolved ions and nano-properties of NPs, such as dissolution mostly accounts for the

toxicities of Ag NPs and CuO NPs,[17,18] while nano-effect contributes to the major toxicities of TiO_2 NPs and CeO_2 NPs.[19,20] NPs stress is known to induce formation of reactive oxygen species (ROS), damage on organelles, depletion of nutrients, and reduction in photosynthetic yield in algae.[21,22] In response to NPs exposure, algae employ a variety of defense strategies, such as activation of antioxidative defense system to eliminate ROS,[23,24] excretion of biomolecules to form a protective layer,[25] and intracellular processes to decrease cellular content of NPs.[26] Currently, exact mechanistic understanding in terms of algal responses to NPs remains to be elucidated, a systematic investigation with this respect can facilitate the development of safe and effective NPs-based technology.

This review attempts to clarify the toxicity mechanisms of NPs to algae with the approach of meta-analysis. Particularly, we emphasize the occurrence of extracellular polymeric substances (EPS) as a defense strategy and the regulation of intracellular processes (internalization, transformation, compartmentation) towards the intrusion of NPs. Also, suggestions will be provided on how to extrapolate laboratory findings regarding response mechanisms for mitigation of algal bloom. On this basis, the potential of NPs applications for controlling harmful algae and promoting beneficial algae will be addressed to guide future research.

2. Mechanism for NPs toxicity to algae: meta-analysis and omics-based perspective

2.1 Meta-analysis

Physical restraints and oxidative stress are responsible for NPs toxicity to algae (Fig. 1).[6] The entrapment of algal cells by large NPs aggregates not only reduces light available for photosynthesis, but also prevents uptake of nutrients.[27,28] Oxidative stress occurs via over-accumulation of intracellular ROS initiated by the exposure of NPs.[29–33] Recently, employment of quantification methods to thoroughly explain the mechanism of NPs toxicity has been a new research trend.[16] Meta-analysis is a quantitative, scientific synthesis of research results,[34] it has been applied for examing the toxicity of NPs. Wang *et al*.[35] explored the behavior of Ag NPs along the source-receptor pathway by meta-analysis, and proposed that the risk of Ag NPs to terrestrial plants and fauna is low. Meta-analysis has also been adopted to identify: the main organ to accumulate TiO_2 NPs,[36] the primary factor to the cellular toxicity of cadmium-containing quantum dots (QDs),[37] and the human cellular responses to carbon nanofibers.[38] To date, no coherent results in terms of NPs exposure and algal health status have been revealed by meta-analysis. To close this gap, this review mined the literature data and quantitatively assessed the key NPs toxicity mechanism to algae. The criteria of the selected studies and methods of the meta-analysis referred to previous studies[37,39] and are detailly described in appendix S1. Study list and dataset used for meta-analysis are shown in Table S1.

A large number of studies indicate that the oxidative stress is the dominant toxicity mechanism of NPs to algae.[40] Our meta-analysis results showed that the level of ROS significantly increased by 90 % in the presence of NPs, indicating the accumulation of excess ROS in algal cells which ultimately caused oxidative stress. NPs-induced ROS

accumulation was not significantly influenced by NPs surface modification (Q_B = 2.659, df = 1, p =0.103), but was strongly influenced by NPs type (Q_B =8.08, df = 3, p = 0.044), NPs dose (Q_B = 21.95, df = 6, p = 0.001) and algae species (Q_B = 39.06 df = 11, $p < 0.001$) (Table S2). Particularly, NPs at 200 – 500 ppm induced highest ROS level by 207 % and *Microcystis aeruginosa* is the most vulnerable algae species upon NPs exposure. In response to ROS stress, algal cells initiate immune warfare, where superoxide dismutase (SOD) and peroxidase (POD) are triggered as the major antioxidative enzymes to scavenge ROS. Correspondingly, the activities of SOD and POD increased by 231 % and 270 % based on the meta-analysis, respectively (Fig. 2). Between-study variation explained 99.8 % and 99.3 % of the observed variation in the magnitude of NPs-mediated effect on SOD and POD, respectively. The heterogeneity of NPs toxicity on algae SOD were accounted for 5 %, 71 % and 56 % by NPs type, NPs dose and algae species, respectively. Similarly, NPs toxicity on algae POD were strongly dependent on NPs type (Q_B = 18.47, df = 2, $p < 0.001$), NPs dose (Q_B = 53.32, df = 4, $p < 0.001$) and algae species (Q_B = 12.67, df = 1, $p < 0.001$) (Table S2). It is noted that catalase (CAT) is another important ROS scavenging enzyme,[41] however, due to only two data points in the meta-analysis, we cannot quantitatively estimate the NPs-induced changes in CAT. Therefore, further studies are needed to confirm whether CAT plays a role in algae to diminish ROS upon NPs exposure. Indeed, the over-accumulated ROS exceeding the scavenging capacity of antioxidative enzymes would directly damage cell membrane, which was evidenced by the 172 % increase of its end product malonaldehyde (MDA) as shown in Fig. 2.

Lipid peroxidation can increase the cell membrane permeability,[42] which increased by 216 % based on the meta-analysis, leading to the loss of membrane selectivity, fluidity and integrity.[42] Membrane damage and change in photosynthetic activity are among the most frequent biological markers to assess the toxicity to algae after NPs exposure.[43] Given that photosynthesis is an essential activity for algal growth,[44] the chlorophyll content and photosystem II (PSII) yield can indicate their growth and health status. The meta-analysis showed that NPs exposure reduced the contents of chlorophyll a and b in photosynthetic algae by 35 % and 36 %, respectively, thereby inhibiting the PSII reaction by 18 %. Chlorophyll a was notably dependent on NPs type (QB = 25.53, df = 3, $p < 0.001$), NPs modification ($Q_B = 7.82$, df = 1, $p = 0.020$), NPs dose ($Q_B = 97.28$, df = 6, $p < 0.001$), and algae species ($Q_B = 98.72$ df = 10, $p < 0.001$). PSII was not significantly influenced by NPs type, NPs modification and algae species, but was strongly dependent on NPs dose (Table S2). Presumably, the accumulated ROS in chloroplast could reduce the content of chlorophyll via altering the lipid-protein ratio of the pigment-protein complexes.[23,27,45] NPs-mediated decrease in the chlorophyll level interrupts energy transduction in light reactions, thus impairing algal photosynthesis, leading to the retarded algal growth, and accompanying 38 % decrease in cell density by the meta-analysis. Given that phytoplankton photosynthesis in the sea contributes to almost half of the global photosynthetic activity,[46] the reduced algal photosynthesis by NPs may decrease algal productivity, which in turn suppresses the growth of higher trophic consumers in the aquatic ecosystem. Therefore, it is of high importance to further study algal biological

responses particularly defense mechanisms towards NPs to help prevent the damage and maintain algal growth.

2.2 Omics-based perspective

The NPs toxicity to algae was often evaluated by the biological endpoints (e.g., ROS, chlorophyll and cell density) as shown in meta-analysis (Fig. 2). Recently, with advanced "omics" technologies, analysis at genomic, transcriptomic, proteomic and metabolomic levels are more sensitive to reveal the toxicity mechanisms of NPs in-depth besides oxidative damage.[47,48] At genomic level, genes encoding light-harvesting proteins of photosystem (*LHCA3*,*5*,*8*, *LHCB4*,*5*, *LHCBM2*,*3*,*5*,*6*,*7*, *3HfcpB*), electron transport chain (*cox3*, *nad5*, *atpA*, *psaB*, *petF*, *psbD*), reaction center protein of PSII (*D1*), carbonic anhydrase and RuBisCo of carbon fixation (*cah2*, *rbcL*), diacylglycerol acyltransferase (*dgat*) for triacylglycerol biosynthesis, and protein of cell division (*ftsH*) were down-regulated.[45,47,49] The reduced conversion of light energy into photosynthetic electron followed by the decreased electron transport rate slowed down the synthesis of ATP and NADPH. The lower energy supply could not only inhibit the assimilation of CO_2 which finally decreased the sugar production from Calvin cycle, but also down-regulate the gene transcription of cell division.[50] Correspondingly, transcription and translation related-proteins (ribosomal proteins, protein NusG and elongation factors Ts, Tu, and G) were also down-regulated, demonstrating that the presence of NPs could inhibit protein translation and influence protein folding.[24] Proteomic analysis revealed that cytochrome b6-f complex, F1 ATP synthase alpha, phosphoribulokinase and phycobiliproteins involved in the

photosynthesis were down-regulated upon NPs exposure.[24] Lately, at metabolomic level, NPs exposure significantly inhibited algae metabolic function by reducing metabolites of carbon-fixation pathway and repressing synthesis of fatty acid, amino acid andnucleotide.[24,47,49] In general, "omics" results indicate that NPs exposure predominantly inhibits gene expressions related to photosynthesis, lipid biosynthesis and cell proliferation in algal cells. The rapid development of "omics" approach brings many studies mapping the molecular responses to several types of stresses including heat, exposure to metal and herbicide,[51,52] most of which reported overlapping results on the central metabolism such as Calvin cycle and lipid biosynthesis. Whilst understanding on the regulation of specific defense responses across different "omics" level is scarce. By now, investigation at metabolomic level enables early detection of ROS under NPs exposure. A metabolic profile of *Chlorella vulgaris* revealed that 13 metabolites (e.g., alkane, lysine and propanoic acid) contributed positively to the ROS generation in the presence of carbon nanotubes, which could be the new biomarkers for evaluating ROS levels.[53] Comprehending biomarkers and signaling pathway for regulation of algal response raises opportunities of using candidate genes to generate NPs-tolerant/intolerant algae.

In addition, "omics" analysis indicates an initiation of repair in algal cells under NPs exposure. Interestingly, among the studies selected for meta-analysis, it is noteworthy that some studies presented no effect or even stimulation of NPs on algal growth, such as increased cell density and chlorophyll b content in *Picochlorum sp*. (Table S2). *Picochlorum sp*. is high in nutritional value and can be used for mariculture.[30] The

stimulation is considered as a hormesis response at a low dose of toxicant.[54] For example, low concentrations of QDs (0.043 - 0.073 nM) or Ag NPs (0.01 - 0.1 mg/L) promoted the growth rate of *Phaeodactylum tricornutum* and *Chlorella autotrophica,* respectively.[43,54] 5 mg/L zero-valent iron NPs or Fe_2O_3 NPs not only stimulated green algae growth but also elevated the contents of lipid and polyunsaturated fatty acids,[55,56] this induction may result from the dissolved trace ions of NPs which can function as a trace element for algal growth.[55] Thereby, NPs are proposed to be alternative stimuli to improve algal biofuel production. Hormesis appears to be related to the development of several response strategies to counter the damage caused by NPs, and knowledge of response mechanisms can facilitate NPs application in algae cultures.

3. Bioavailability of NPs affected by extracellular polymeric substances

NPs bioavailability, namely the extent to which NPs are free for uptake by algae and to which they can cause an effect, is fundamental for evaluating algal responses.[13] NPs reaching algae first encounter the extracellular matrix, target algae can modify interactions at the nano-bio interface by releasing biomolecules,[57] the bioavailability of NPs is thus modified by the biogenic materials. Therefore, understanding the function of these substances is crucial for NPs risk assessment.

Natural organic matters (NOM) are known to interact with NPs and affect their fate in aquatic systems.[6,58,59] The impacts of commonly considered NOM including humic acid and fulvic acid have been well described by Wang *et al*.[2] and Ma & Lin,[60] whereas for a particular type of NOM known as extracellular polymeric substances

(EPS), their impacts on the bioaccumulation of NPs in algae remain to be elucidated. EPS excreted by algae are mainly consisted of polysaccharides and proteins.[7] The existence of EPS has been considered as a protective layer for algae against external interferences, preventing direct physical contact and reducing NPs dissolution by surface coating and modification.[7,15] The bioavailability of NPs is thus shaped by the successively or simultaneously occurring processes in the presence of EPS, which are inherently complex.

3.1 Formation of EPS in response to NPs exposure

Excretion of EPS by algae may provide a feedback response to NPs exposure. Increased production and composition changes of EPS have been shown upon NPs exposure.[6,61–63] In our previous work, it was visualized that the EPS layer located outside of *Chlorella pyrenoidosa* was thickened by nearly 4-fold in response to CuO NPs exposure (Fig. 3A&B).[15] Under SiO_2 NPs treatment, EPS release was significantly induced by 400 - 1000 % in *Odontella mobiliensis*, *Thalassiosira pseudonana*, *Dunaliella tertiolecta*, *Phaeodactylum tricornutum*, and *Skeletonema grethae*.[25] Whereas under same exposure conditions, TiO_2 NPs did not stimulate EPS excretion in *O. mobiliensis and D. tertiolecta*.[25] Furthermore, exposure to Cu^{2+} at concentrations simulating that dissolved from CuO NPs resulted in a thinner EPS layer than that of CuO NPs exposure.[154] However, Taylor *et al*.[62] indicated that both Ag^{+} and Ag NPs contributed similarly to EPS production. Studies on the mechanisms for EPS release are scarce, and the roles of NPs compositions as well as their dissolved ions remain to be identified. One study proposed that intracellular Ca^{2+}

levels control the production of EPS, correspondingly, SiO_2 NPs stimulated Ca^{2+} level significantly by 50 - 300 % while TiO_2 NPs did not change Ca^{2+} concentrations.[14] In isolated rat heart cells Alvarez *et al*.[64] found that SiO_2 NPs regulated Ca^{2+} channel by altering lipid microdomains. Yet, the information on how NPs modify the signaling pathway in algal cells is still lacking, and there is no direct evidence on how NPs-mediated signaling pathway alters the release of EPS in algae cells. At a same NPs concentration, marine algae can produce higher amount of EPS than freshwater algae.[65] In addition to the genetic variation of algae species, the exposure conditions such as nutrient status may also play crucial role in mediating EPS release. Marine phytoplankton are less deficient in nitrogen than freshwater ones,[66] increased production of EPS by *T. pseudonana* has been observed in nitrogen-enriched culture.[67]

EPS composition is a sensitive endpoint for NPs toxicity. It was indicated that the energy used to promote growth and replication could be rerouted to alter EPS composition.[62] Ag NPs exposure reduced EPS dry weight in *C. reinhardtii* significantly by shifting high compositions of high molecular weight (HMW) EPS compounds to low molecular weight (LMW) ones.[62] The function of LMW EPS has not been reported yet, while HMW EPS can facilitate flocculation of NPs,[6] the decrease in HMW EPS implies the enhanced stabilization of NPs suspensions. This stabilizing capacity has been shown in several studies,[68–71] EPS excreted from *D. tertiolecta* could slacken the sedimentation of TiO_2 NPs,[72] and EPS excreted by marine algae *D. tertiolecta* have higher stabilizing potential than that of freshwater

algae *C. reinhardtii*.[70] The high ionic strength and pH of marine water[73] are likely to re-arrange EPS structure, it has been shown that low pH reduced the number of binding sites for metal ions and high ionic strength changed the secondary protein structure of bacterial EPS.[74,75] Algal EPS are divided into cell surface-bound EPS (B-EPS) and culture medium-solubilized EPS (S-EPS), S-EPS can increase the homo-aggregation and sedimentation of NPs, while B-EPS can associate NPs on algal cell surface and limit NPs internalization.[76] Noticeably, the stabilized suspensions of NPs may thus have a longer residence time in aquatic system,[6,8] the B-EPS-attached NPs are expected to travel with algae and finally enter into the food chain, causing potential ecological hazard,[7] hence, the functions of EPS at a wider context should be further investigated. Unexpectedly, rather than stabilization, the presence of EPS facilitated dissolution of CuO NPs.[69] Also, EPS extracted from *D. tertiolecta*, *Phaeocystis globose*, *T. pseudonana* and *Amphora sp*. triggered degradation of CdSe QDs which led to release of Cd^{2+}, and the degradation rate was positively correlated to EPS protein content.[71] Generally, the high glycoprotein level in the B-EPS leads to a higher total protein level than that of S-EPS,[71] indicating a high potential of B-EPS to induce NPs degradation, which is inconsistent to their function of forming a barrier against NPs adsorption. Therefore, the proteins particularly responsible for NPs degradation should be identified. Specifically, the "omics" approach can be applied to further understand how algae regulate EPS production and composition in response to NPs during different cellular processes. With this knowledge available, there is a great potential to enhance the defense system of beneficial algae by accelerating EPS

formation and improve the control efficiency of algal bloom by decreasing EPS production in harmful algae.

3.2 Regulating mechanisms of EPS for the availability of NPs

EPS alter the surface adsorption of NPs on algae by electrostatic interactions and chemical bonding, which depend on the surface charge of both EPS and NPs, and hydrophobicity of NPs.[70] Polysaccharide-rich EPS excreted from algae are anionic colloidal biopolymers,[6] which decrease algal adsorption of negatively charged NPs such as Ag NPs.[77] EPS can even reverse the surface charge of NPs and completely decrease their bioavailability. For example, negative-charged EPS could effectively coat positively charged TiO_2 NPs and significantly shift their surface charge from positive to negative.[70] Thus, the presence of EPS would prevent algae from direct contact with NPs by inhibiting the attachment of NPs to algal surface. On the other hand, chemical bonding strengthens the association between EPS and NPs. Spectrum of Fourier transform infrared (FTIR) revealed that the abundant functional groups of EPS such as amide, hydroxyl, thiolic and aromatic carboxylic groups contribute to the binding to metallic NPs.[7,61,70] Amino acids in EPS enriched with these complexation groups are shown to bind with CuO and Fe_3O_4 NPs effectively.[78] Also, TiO_2 NPs prone to attach exclusively on the proteins of EPS by *D. tertiolecta* to form a protein corona for stabilizing NPs.[72] Besides, in opposite to the aforementioned stimulated degradation of NPs by EPS, the hydroxyl groups and hemiacetal ends on the polysaccharide-rich EPS facilitated the reduction of Ag^+ to form Ag NPs,[68,79] implying a decrease in the toxicity resulted from the dissolved ions. A thorough

evaluation of NPs re-formation or dissolution can be scrutinized by the interactions between EPS and NPs of various compositions. Overall, the electrostatic repulsion maintains NPs dispersion in aqueous medium and the chemical binding reinforces retention of NPs within the B-EPS, both of which inhibit internalization of NPs.

Limited studies have addressed the internalization of NPs with regard to EPS. The thickened EPS layer attached to *C. pyrenoidosa* had minimized the interaction of CuO NPs with algal cells.[15] By atomic force microscopy (AFM), Ag NPs-EPS binding has been visualized in which Ag NPs were trapped by the fibrillar network of EPS secreted from *Cylindrotheca closterium*.[63] The EPS-Ag NPs complex was too stable and hard to enter into the cells, while removal of EPS facilitated the internalization of Ag NPs.[7] Therefore, the existence of EPS shapes a protective bio-barrier outside algal cells, which reduces NPs internalizing.

4. Internalization of NPs by algae

Even in the presence of EPS, NPs could still penetrate and subsequently accumulate intracellularly.[15] Internalization of NPs is a rather complex process, which involves interaction with cell walls and membranes. Cell wall free mutants have been used to demonstrate the inhibitive role of cell wall and the transport routes for internalization.[80] After crossing the cell wall, NPs encounter the second barrier-cell membrane. It was indicated that limited amount of Ag NPs could pass through the membrane of *C. reinhardtii* and the accumulation rate was lower than that of Ag^{+}.[81] To accurately assess the toxicity and fate of NPs, it is necessary to understand the cellular responses during internalization.[22]

4.1 Cell wall composition and ultrastructure

Studying the composition and ultrastructure of algal cell walls is crucial for understanding how they function during NPs exposure. Algal cell walls consisting of polysaccharides and glycoprotein matrix form a defense against their environments.[82] The composition of cell walls determines their reactivity with NPs. Cell walls composed of proteins, polysaccharides and uranic acid have high adhesive properties to NPs. Glycoproteins located on the cell wall of *C. reinhardtii* represent important binding sites for mannose-coated Au NPs.[83] Additionally, the presence of functional groups on the cell wall forms a negatively charged surface which enhances the electrostatic attractions to the positively-charged NPs.[84] Cell wall surface architecture such as ridges could facilitate the permeation by increasing surface contact.[84] Diatoms characterized by a siliceous skeleton represent a special type of algae.[8] Pletikapić *et al*.[63] observed that Ag NPs penetrated the frustule (cell wall of diatom) of *Cylindrotheca fusiformis* and *C. closterium* through the valve region built of silica NPs without disintegration. Besides, nano-to micrometer-scale size of pores are distributed homogeneously on the surface of frustule,[85] an opening of 100 - 160 nm wide and 30 nm deep on the valve region was visualized by AFM.[63] Hence, the penetration of NPs through the cell wall depends on the cell wall architecture, and cell wall pores have been assigned as an imperative penetration site for NPs.

Generally, inherent pores on the algal cell wall have an average diameter of 5 - 20 nm, making the cell wall semipermeable and functions as a sieve. NPs with size smaller than that of cell wall pores are suggested to enter the apertures directly,[8] such

as TiO_2 NPs of 20 - 30 nm are expected to pass through the cell wall of *Nitzschia closterium*.[86] NPs of large size are not able to enter the algal cells. In *C. reinhardtii*, uptake of uncoated CeO_2 NPs is rather unlikely due to the average large size of 159 nm.[80] However, Taylor *et al*.[47] found that CeO_2 NPs coated with PVP has a stable small size of 4 - 5 nm, which allows the penetration across the algal cell walls. NPs exposure might in turn enlarge inherent pores and induce formation of new large pores, ultimately promoting the internalization of NPs.[8,87] Previous studies attributed the enlargement of cell wall pores to the NPs damage on the cell walls,[18,27,88] such as the impairment caused by the sharp edges of reduced graphene oxide[23] and the puncture resulted from insertion of oxidized multi-walled carbon nanotubes (o-MWCNTs) tips.[89] Conversely, the number of cell wall pores decreased in higher plants due to the blockage by NPs, which in turn inhibited NPs penetration and transport of water.[90,91] Considering the similarities shared between cell walls of algae and plant,[92] pore numbers might decrease in algal cells subjected to NPs as well. With this respect, more studies are needed to evaluate the changes in algal cell wall pores after NPs exposure, and it is necessary to clarify whether the enlargement of pores is persistent.

4.2 Cell wall loosening or strengthening

Cell wall thickness is another important factor to determine the internalization of NPs. Compared to mature cells, NPs entry into newly formed algal cells are easier due to their thinner cell walls.[8,93] The small size and extremely large surface area of NPs enable the strong interaction with algal cell wall which alters the cell wall thickness. It

was demonstrated that the cell wall of *Pithophora oedogonia* was thinned by Ag NPs exposure.[94] Taking the advantages of NPs to loosen algal cell walls, Ag NPs have been used for cell wall lysis to release biomolecules during biofuel production by algae.[95] Similarly, cell wall loosening has been shown in *Arabidopsis thaliana* exposed to iron NPs (nZVI), which was attributed to the degradation of pectin polysaccharides by the OH radicals formed under iron NPs exposure.[96] Metal ions such as Cd^{2+} and Zn^{2+} are reported to decrease biosynthesis of tomato cell wall pectin by down-expressing genes for enzymes to produce pectin.[97] Decrease in pectin level leads to the failure in matrix-polysaccharide-connection and deteriorates the loading bearing capacity, which ultimately loosens cell wall.[96] With the loosening of cell walls, more NPs particularly of large size are facile to penetrate into the cell and generate potential impact. The regulating mechanisms for algal cell wall thickness have not been reported yet. It is known that plant cell wall is regulated by two groups of cell wall peroxidase (CW POD), one group loosens cell wall by generating ROS to break polysaccharides bonds, another regulates ROS level through lignin polymerization to stiffen cell wall.[98] Recently, a thickened cell wall was observed in *C. vulgaris* exposed to nanocolloids (a mixture of particles with diameters less than 100 nm), which could be attributed to the up-regulated cellulose and chitin levels.[99] The strengthened cell wall against intrusion of NPs could be considered as a self-defense strategy, which inspires the utilization of NPs such as the complex and heterogeneous nanocolloid for strengthening the barrier against toxicant at algal cellular level. Compared to plants, less attention has been paid to algal cell walls.

Even though algal cell walls are less complex than that of higher plants, the composition differs significantly among algal genera, for example, *Chlorella spp*. has two-layered cell walls consisting of sporopollenin, mannose and chitin-like polysaccharides, while *Haematococcus spp*. has three-layered cell walls composed of algaenan, mannose and cellulose.[93] Based on the fact that CW POD plays an essential role in balancing plant cell wall loosening and strengthening, the assumption for the involvement of CW POD for the rigid cell wall of algae is enlightened. Future studies could focus on the regulation of cell wall and investigate the impact of NPs on CW POD to provide new insights for algal cellular response to NPs.

4.3 Transport of NPs via cell membrane

Following traverse across the cell wall, NPs meet and interact with plasma membrane composed of phospholipid bilayer. NPs exposure can damage the structure of membrane bilayer and even trigger membrane perforation by excess ROS accumulation, physicochemical disruption or enzyme activities. Oxidative stress-induced membrane damage (lipid peroxidation) has been widely discussed and addressed in section 2 of this review. The oxidation of polyunsaturated fatty acids could induce the development of membrane pores.[100] In addition, Zhao *et al*.[101] observed that reduced graphene oxide and multi-layer graphene nanosheets could extract phospholipids from membrane of *C. pyrenoidosa*, allowing direct penetration into cells. Similarly, the phospholipids in mammalian cells were extracted drastically in several directions by the strong dispersion interactions between graphene and lipids, and the molecular dynamics for the membrane perforation was due to the depletion of

phospholipids in the confined area.[102] Enzymes involving in the metabolism of phospholipid can be activated upon NPs exposure. For example, the activities of esterases were induced in *C. reinhardtii* exposed to Cr_2O_3 NPs, leading to the degradation of phospholipid.[32] Correspondingly, a recent metabolic profile showed that Ag NPs exposure down-regulated the metabolic pathway of phospholipids in *M. aeruginosa*, resulting in reduction of phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine which are components for membrane integrity and stability.[103]

Endocytic process has been suggested as another passage for NPs to enter the cell membrane. Treatment with endocytosis inhibitors demonstrated that CuO NPs was internalized in *C. pyrenoidosa* via an energy-dependent and clathrin-mediated endocytosis process.[15] Endocytosis of o-MWCNTs through plasmalemma invagination has been observed by Zhang *et al*.[89], meanwhile, o-MWCNTs are proposed to passively penetrate the membrane like "nanoneedles". Also, Miao *et al*.[104] suggested that Ag NPs were internalized via endocytosis as the cell membrane of *Ochromonas Danica* maintained integrated. The unchanged membrane structure implies another possible mechanism that NPs could be incorporated by the transport carrier proteins or ion channels.[8] It has been shown that Ce^{3+} and Ag^{+} dissolved from NPs were internalized via Ca^{2+} and copper transporters or sodium channels.[80,105] Still, evidence on the transporters or ion channels for pristine NPs is lacking. A recent study found that phosphoinositide of membrane, associated with activation of transporters and ion channels, was up-regulated in *M. aeruginosa* exposed to Ag

NPs,[103] implying a potential interaction between NPs and transporters/ion channels. Nevertheless, it has been argued that intact NPs are too large to pass via ion transporters.[106] Interestingly, carborane-capped Au NPs having a ligand shell full of voids for Na^+ and K^+, can partition over the phospholipid bilayer and act as artificial alkali-ion transporters across biological membranes.[107] To clarify the role of ion channels for NPs internalizing, patch clamp technique could be applied to directly measure ion channel activity, which reveals how ion channels regulate cellular secretion and contraction.[108] However, application of this technique is challenging, such as extremely fragile membrane patches which are vulnerable to higher voltages, incorporation of external material to the lipid bilayer has been proposed to solve this problem.[109] Recently, an automated high-throughout-screening patch clamp device has been recommended for ion channel screens.[110] Additionally, analysis for gene expression of transporters located on algal cell membrane will demonstrate whether transporters involve in NPs transmembrane process.

The conventional quantification of NPs by averaging that measured with the entire cell population might be overestimated since cells cannot take up NPs equally under realistic conditions.[111] Recently, with the development of single cell inductively coupled plasma mass spectrometry (SC-ICP-MS), internalization of NPs has been observed on an individual cellular level. Quantification by SC-ICP-MS revealed that around only 40 % algal cells contained Au NPs after 77 h exposure at a cell: NPs ratio of 1:3.[111] SC-ICP-MS allows the precise quantification of NPs-containing cell numbers and measurement of NPs concentrations down to the attogram (ag) per cell

level,[111] thus facilitating better evaluation on the potential hazard of NPs and exploration of mechanisms under well-defined conditions. Since algae are abundant in the aquatic environment and are vulnerable to anthropogenic pollutants, the single cell-based nanometrology could contribute to develop an algal biosensor for monitoring the dynamic distribution of NPs. The improved monitoring will enhance the evaluation of ecological risks in aquatic system.

5 Post-internalization processes of NPs in algal cells

Several studies have focused on the transformation of NPs in aqueous medium,[112–114] such as the redox transformation of Ce (IV) NPs to mixed Ce (III, IV) NPs at high ionic strength exposure media.[115] Noticing the cellular internalization of NPs in several algae, the subsequent modification, localization, and corresponding toxic consequences of NPs have been studied for understanding the ultimate algal response to the risks of NPs.

5.1 Intracellular transformation of NPs

With current advanced techniques, observations on the biotransformation of NPs inside algal cells are emerging. Reduction appears to be a main process for transformation of metallic NPs. CuO NPs were reduced to Cu_2O NPs in *C. pyrenoidosa*, and intracellular reductase has been assumed to catalyze this reaction, during which ferredoxin could be the electron donator.[15] In recent years, the *in situ* detection by synchrotron X-ray absorption spectroscopy has been used to investigate the chemical speciation and their proportions. With the assistance of X-ray absorption

near edge structure (XANES), it has been shown that in *C. reinhardtii* exposed to Ag NPs for 24 h, 62 % of the internalized Ag NPs was present as Ag^0 while the rest complexed to glutathione and transformed to Ag_2O; after exposure for 72 h Ag_2O disappeared whereas Ag-glutathione complex and Ag_2S became the major speciation.[26]

NPs Transformation in algae including reduction and sulfidation are mainly stimulated by the intracellular reducing substances (e.g., ascorbic acids, sugars and phenols) or macromolecules (e.g., ferric chelate reductase, nitrate reductase and dehydrogenase).[9,116] The mechanisms involved for intracellular transformation are either a direct transformation of internalized NPs,[15] or a dissolution-sulfidation -reduction process including precipitation of released ions with sulfide followed by a reductive transformation. In *C. reinhardtii*, release of Ag^+ from Ag NPs took place in the periplasmic space, and ions were taken up inside the cytoplasm and underwent sulfidation to form Ag_2S,[26] in the case of CuO NPs, CuS was further reduced to Cu_2O NPs.[15] Reduction and complexation are known to attenuate the toxicity of metal ions,[117,118] implying that algae might initiate these processes as an adaptive mechanism to counter NPs toxicity. The transformed NPs products could have diameters smaller than that of parent NPs, such as Cu_2O (2 - 5 nm) and YPO_4 (12 - 30 nm),[15,119] the toxicity or biological benefits of these transformed products at nano-scale and their transport within the trophic chain are not well known, thus more attention should be paid on this topic.

5.2 Compartmentation of NPs

Some NPs such as Au NPs maintain their nano-form after being internalized.[115] Thus, algae could directly deposit NPs in distinct subcellular compartments to mitigate the toxicity of NPs without transformation. It is reported that a high fraction of TiO_2 NPs was bound to the cell-wall of *N. closterium*,[86] and carboxyl single-walled carbon nanotubes (C-SWCNT) were retained either outside or inside the plasma membrane of *C. vulgaris*.[53] In addition, Ag NPs were present exclusively in the periplasmic space of *C. reinhardtii* after internalization.[26] Retention of NPs in the cell wall/membrane or the periplasmic space implies the algal responses to restrain the distribution of NPs into cytoplasm, thus preventing their damage on the organelles. Once internalized in the cytoplasm, NPs would be confined in certain cell compartments such as vacuoles. CeO_2 NPs were observed within the intracellular vesicles of *C. reinhardtii*,[47] indicating the likelihood of NPs-loaded vesicles on their way to the vacuole.[120] Vacuoles are common storage sites for both NPs and their released ions.[6,121] For instance, a noticeable amount of Ag NPs and Ag^+ from Ag NPs have been shown to compart into vacuoles of *C. reinhardtii* and *O. Danica*.[26,104] Cu_2O NPs transformed from CuO NPs were also sequestered in the vacuole of *C. pyrenoidosa*.[15] Storage in the vacuole may isolate the excessive NPs and their transformed products from cytoplasm, performing as an endpoint for detoxification mechanism to tackle NPs toxicity by reducing their cytosolic concentrations. Vacuolar sequestration could also limit the exocytosis, a post-exposure study manifested no loss of NPs in the single-cell based measurement.[111] The persistence of NPs in algal cells suggested their longer residence time within algae and increased

opportunity to be passing on to the next trophic level. In addition, vacuoles are commonly considered as inert spaces for deposition of NPs,[122] nevertheless, dissolution of NPs may occur in vacuoles due to the reduced pH environment.[3] Given that the dissolved ions may be more toxic than parent NPs,[123] the potential secondary hazard of NPs transformed products from broken vacuole of dead algae should be addressed in future work.

6 Implications in mitigation of algal bloom

To date, most research on the interaction between algae and NPs have been carried out under controlled laboratory conditions. The NPs toxicity to algae (Fig.1) and series of algal responses (Fig. 3C) including formation of EPS bio-barrier and regulation of NPs intracellular processes are all laboratory findings. Being aware the importance of extrapolating laboratory results for application in the field, this section will discuss the potential of NPs application in algal bloom control as an example.

Occurrence of algal bloom leads to the death of living organisms by the hypoxic aquatic condition and cyanotoxin poisoning, thus disrupts the balance formed by all aquatic lives, which ultimately threatens the aquatic ecosystems.[124] So far, effective method for managing algal bloom has not been well developed yet,[125] with increasing knowledge of algal responses to NPs, recent studies proposed the application of NPs for algal bloom control. As shown in Fig. 4, the following mechanisms are involved in mitigating algal bloom by NPs. (1) NPs may act as toxicant to algae. As shown in section 2, NPs exposure generally inhibits algal growth and finally shrinks cell density, indicating the potential of NPs to be used as an algicide. Recent findings

demonstrated the effectiveness of NPs to control algal bloom at laboratory scale. Inhibition of the most dominant algal bloom cyanobacteria (*Microcystis* and *Oscillatoria*) reached 78 - 97 % after 5 - 10 d treatment with 0.05 - 5 mg/L of Ag NPs or Co NPs.[126,127] (2) Reduction of nutrients necessary for algal growth. Enrichment of nitrogen and phosphorous is the main cause for expansion of algal bloom, *Microcystis* and *Oscillatoria* are inclined to grow and accumulate at nutrient enriched impoundment area.[127] NPs accumulation on algal surface could inhibit uptake of nutrients by algae.[32] Also, nanomaterials can compete for nutrient by their strong adsorption.[112] The presence of graphene-family nanomaterial could significantly adsorb N and P in the exposure medium to decrease the nutrients free for *C. pyrenoidosa* growth.[101] Given that algal bloom stems from eutrophication,[128] restricting the availability of key nutrients by prophylactic application of NPs could prevent algal bloom from point sources. The amount of available nutrients could also be decreased inside algal cells, transformation of NPs with phosphate has been shown to reduce the phosphorus source.[129] Despite of phosphorus and nitrogen, vitamin B_1 and B_{12} are important for the occurrence of algal blooms as well.[130] Future studies could investigate the influence of NPs on the dynamics of vitamin B for regulating algal bloom. (3) Sedimentation. The buoyant characteristic of cyanobacteria contributes to the occurrence of algal bloom at the water surface. Inspired by the diatoms which use siliceous cell wall as ballast, SiO_2 NPs were incorporated to cyanobacteria cells via polydiallyldimethylammonium chloride and subsequently sank to the water bottom, leaving the water clear.[125] The incorporation of SiO_2 NPs can

significantly inhibit cell proliferation and growth of cyanobacteria, meanwhile maintaining the O_2 saturation concentration of water bodies. The field test showed that the natural pond was cleared within only 4 h treatment with 75 mg/L SiO_2 NPs, and the treatment was still effective even after 20 d without disturbing other organisms.[125] This bio-inspired strategy opens a new potential pathway for controlling algal bloom in marine environment with efficient self-purification function; whereas the fate and treatment of sedimented algae in freshwater, as well as their impact on other organisms living at the bottom area remains to be explored and evaluated. Meanwhile, the material cost (\$ 0.6 per m^3 water)[125] of this strategy should be considered for NPs application in large area of marine water. (4) Allelopathic control of algal bloom. Studies have reported the use of allelopathic substances such as phenolics, tannin, and ethyl acetate from aquatic macrophytes, rice straw and terrestrial plant *Compositae* to control cyanobacteria *Microcystis*.[131–133] So far, research on the contribution of NPs to the allelopathic control of algal bloom is lacking. One study evidenced that 2 mg/L Cu NPs synthesized by the red algae extracts reduced the biomass of the cyanobacteria *Lyngbya* by 85 %, the inhibition was attributed to the allelopathic compounds from red algae extracts,[134] however, the interaction between NPs and allelopathic chemicals such as polyphenols, pyrogallic acid and gallic acid[134] is still unclear. It has been shown that production of allelopathic substances can be enhanced under abiotic stress conditions.[134] With this respect, it is expected that the presence of NPs could induce the production of allelopathic substances thus promoting the efficiency of algal bloom control.

Monitoring algal species and toxins are important for water management. Reliable standardized analytical methods for algal toxins are lacking.[135] Very recently, a NPs-based biosensor with high sensitivity was proposed as an innovative *in situ* detection method for algal toxin. It was suggested that nanomaterial could facilitate the specific binding between toxin and biorecognition molecule. For example, Au NPs and Ag NPs were associated with antibody and conjugate to form a NPs complex for detecting the most toxic cyanotoxin-microcystin-LR.[136] Based on this successful demonstration, future development of this mobile biosensor could be a simultaneous detection of multi-toxins. In addition to the cyanotoxins, ROS is another key factor for the algal bloom-induced mortality of organisms.[137] Recently, NPs-based enzymes have attracted special attention. NPs, such as Fe_3O_4 NPs, graphene oxide and QDs with catalytical activity can act as POD and SOD,[138] implying the potential of nano-enzymes to be applied to scavenge the ROS produced by the harmful algae, thus reducing the hazard and protecting organisms from algal bloom.

The application of NPs might be superior to other methods from the perspective of efficiency, still, aspects regarding its compatibility to different types of algal bloom need to be considered. To date, many studies on the management of algal bloom have focused on the elimination of cyanobacteria, while major algal phyla that produce blooms also includes chlorophytes, dinoflagellates, and diatoms,[139] and the responses to NPs might differ among species. It was suggested that green algae have thicker cell wall than cyanobacteria, indicating less permeability for NPs,[126] as a result, less effectiveness for treating this type of algal bloom with NPs would be expected. To

cope with this problem, knowledge on the regulation of cell wall thickness as aforementioned would be helpful for increasing the compatibility of NPs to manage different kinds of algal blooms. Surprisingly, one study reported that exposure to Au and $Cu(OH)_2$ NPs intensified algal bloom occurrence under nutrient enrichment condition, this unwanted stimulation was attributed to the increased N and dissolved organic carbon (DOC) concentrations from algal death upon exposure, thus generating appropriate conditions for the cyclical algal blooms.[140] Undoubtedly, the accordingly biological risk assessment of NPs application in mitigating algal bloom should be conducted carefully in the future.

7 Outlook

To date, many studies indicate oxidative stress as the main toxicity mechanism of NPs to algae, NPs toxicity is strongly dependent on NPs type and dose. The in-depth understanding of algal stress responses ultimately determines the outcome of exposure to NPs. Current knowledge indicate that algae employ several response strategies to encounter the intrusion of NPs. Future work could concentrate on how to regulate these responses for improving practical application such as algal bloom control by NPs. Most algal photosynthetic products are released back into the ocean as EPS, the signaling pathway for EPS production should be further investigated. Comprehending the interaction between signals and NPs enables the blockage of EPS formation to weaken the defense line, which consequently enhances the efficiency of algal bloom control. Notably, most NPs toxicity studies were conducted at supra-environmental levels of NPs up to 1000 mg/L.[54,141] However, environmentally relevant

concentrations of NPs such as CeO_2 NPs at 0.029, 0.144 and 0.72 mg/L did not cause any alterations in *C. reinhardtii*.[47] Generally, it can be concluded that NPs at high concentrations inhibit algal growth, while low concentrations of NPs stimulate algal performance. As one of the six "Key Enabling Technologies", nanotechnology contributes to the sustainable competitiveness in agricultural system.[142] NPs application in promoting beneficial algae growth via enhancing resistance towards stress by the strengthened EPS protective layer and cell wall, and NPs stimulation in the production of economically favorable algal products, pave a new avenue for future research.

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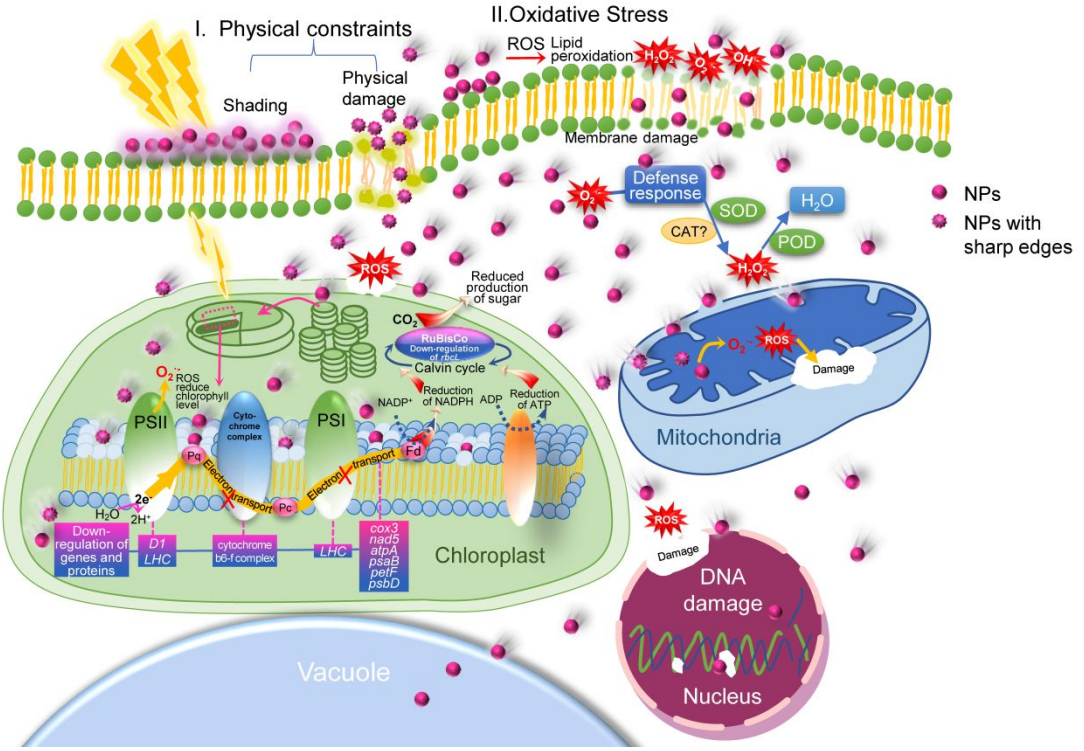

Figure 1 Mechanisms of NPs toxicity to algal cell membrane and organelles. Physical constraints and oxidative stress contribute to the toxicity of NPs. NPs exposure induces ROS production, formation of ROS results in membrane lipid peroxidation and activation of antioxidative enzymes (SOD and POD). Over-accumulation of ROS leads to impairment of algal photosynthesis as well as damage on the mitochondrial membrane and DNA. At "omics" level, NPs exposure suppresses genes encoding reaction center protein of PSII (*D1*), light-harvesting proteins of photosystem (*LHC*), electron transport chain (*cox3, nad5, atpA, psaB, petF, psbD)* and RuBisCo of carbon fixation (*rbcL*), besides, proteins (e.g., cytochrome b6-f complex) involved in the photosynthesis are down-regulated upon exposure. The lowered synthesis of NADPH and ATP thus inhibits the assimilation of CO_2 followed by decrease in sugar production in Calvin cycle. Abbreviations: Pq: plastoquinone; Pc: plastocyanin; Fd: ferrodoxin; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase.

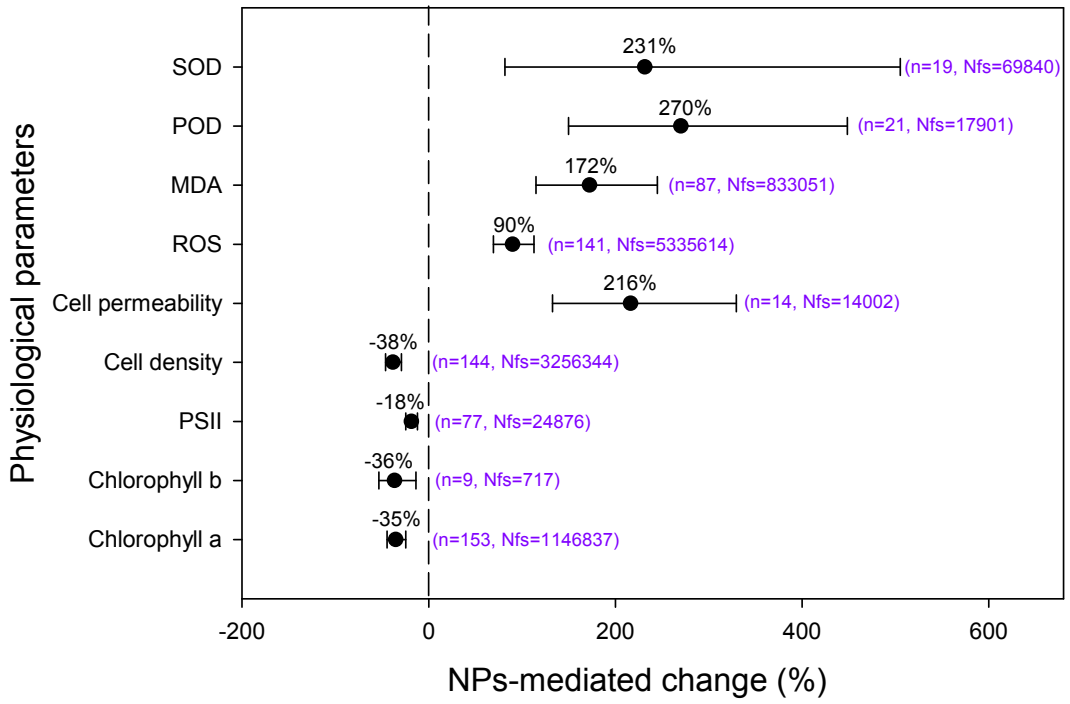

Figure 2 The mean magnitude of NPs-mediated changes in algae cell physiological parameters. Error bars denote 95 % bias-corrected confidence intervals (CIs). Sample sizes are shown beside error bars. The individual effect is significant if the 95 % CI does not include zero, fail-safe numbers (Nfs) were calculated, using Rosenberg's weighted method ($\alpha = 0.05$), Nfs indicates the number of studies reporting zero effect size that need to be added to the meta-analysis to render the observed effect non-significantly different from zero.

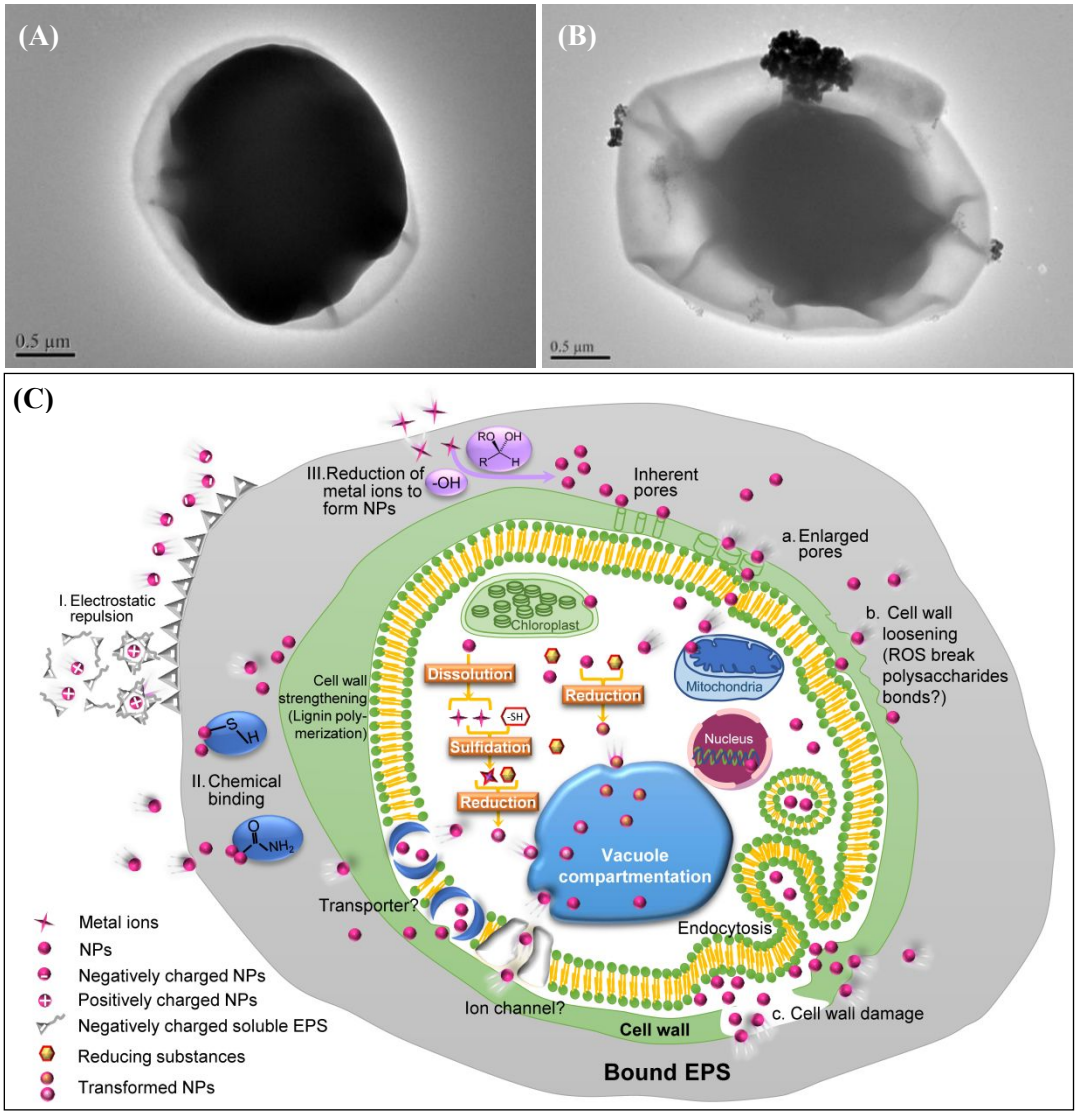

Figure 3 Transmission electron microscopy images of *Chlorella pyrenoidosa* (A) without NPs exposure and (B) with CuO NPs (reproduced from ref. 15 with permission from [Taylor & Francis], copyright [2019]). EPS layer (light gray) was thickened by nearly 4-fold upon NPs exposure. (C) Fate of NPs in contact with algal cell biological responses (formation of EPS, internalization, transformation and compartmentation). Formation of algal EPS in response to NPs prevents surface adsorption via electrostatic repulsion (I), and chemical binding (II) strengthens NPs retention in the EPS layer. Hydroxyl groups and hemiacetal ends on the EPS could promote NPs formation from metal ions with higher toxicity (III). NPs which penetrate the EPS could cross the cell wall via enlarged pores (a), loosening (b) or damaged cell wall (c), while cell wall strengthening inhibits NPs internalizing. NPs further enter the cell membrane through endocytosis, the roles of ion channel and

transporter are not yet known. Internalized NPs are shown to be transformed by direct or indirect reduction processes, NPs and transformed products would be subsequently compartmented into vacuole to decrease cytosolic concentrations.

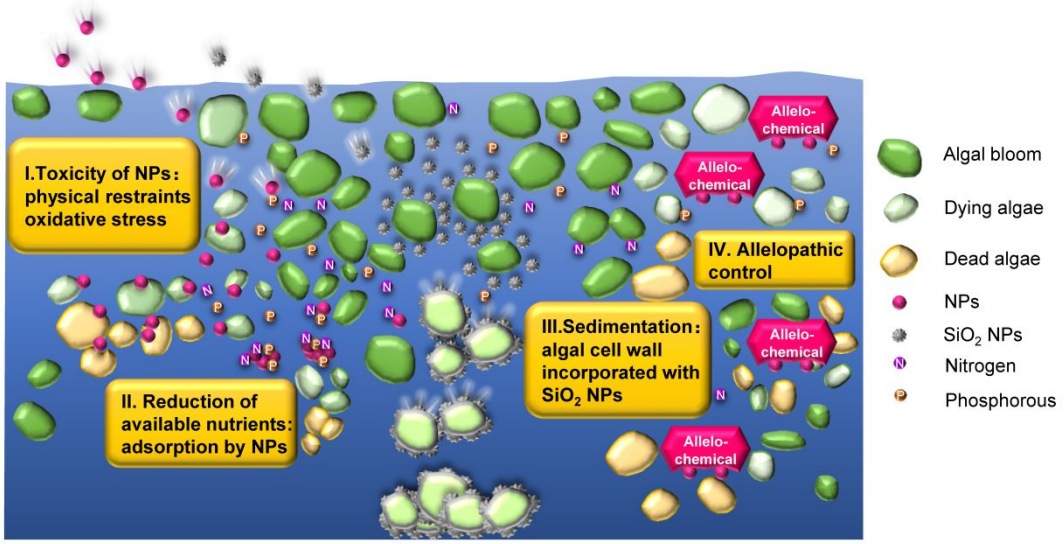

Figure 4 Mechanisms involved in mitigation of algal bloom by NPs. (I) NPs could act as fungicide to inhibit algal growth by physical restraints and oxidative stress; (II) Absorption of nutrients by NPs reduces the nitrogen and phosphorous available for algae survival; (III) Algae incorporated with SiO_2 NPs are prone to depositing to the bottom of water bodies, thus leaving the water clear; (IV) NPs function as carriers for allelochemical to control algal bloom.

Table of Contents Entry

The key algal response mechanisms to cope with NPs toxicity and implications for algal bloom control by NPs