Journal of Applied Phycology (2020) 32:889–899
https://doi.org/10.1007/s10811-020-02088-2

Culture study on utilization of phosphite by green microalgae

Michiyo Hashizume[1] · Masaki Yoshida[2] · Mikihide Demura[2] · Makoto M. Watanabe[2]

Received: 5 November 2019 / Revised and accepted: 21 February 2020 / Published online: 7 March 2020

Abstract

The continuous decline in phosphorus (P) resources is a serious global issue. Therefore, it is important to develop methods to recover P from waste and wastewater. Most P ores are currently used in the phosphate form in the agriculture industry and in detergents, which results in a large release of phosphates into natural aquatic environments. Much attention has been given to measuring phosphate levels and monitoring water quality, survey, and control of algal phytoplankton dynamics. However, phosphite is oxidized from hypophosphite after plating and discharged as waste, so methods to recycle and reuse phosphite should also be developed. Currently, there is no evidence of phosphite utilization by photosynthetic eukaryotes, including eukaryotic algae. Thus, except for the possible utilization by some bacteria when phosphate is unavailable, the fate of the phosphite that is discharged is mostly unknown. *Chlorella vulgaris* (NIES-2170), *Coccomyxa subellipsoidea* (NIES-2166), *Scenedesmus obliquus* (NIES-2280), and *Botryococcus braunii* (BOT-22) were cultured in phosphite medium under conditions that prevented phosphate contamination and phosphite oxidation. As a result, the number of *C. vulgaris* and *C. subellipsoidea* increased in the phosphite medium, demonstrating the availability of phosphite for the growth of these strains. In particular, the growth of *C. vulgaris* increased as the phosphite concentration increased. After being cultured for 180 days in photosynthetic conditions, phosphite utilization rates were 32–38%. In contrast, *S. obliquus* and *B. braunii* strains did not grow in the phosphite medium. In conclusion, *C. subellipsoidea* and *C. vulgaris* utilize phosphite as a P resource, which is a novel finding in photosynthetic eukaryotes. The results of this study may have important implications for the phosphorus redox cycle.

Keywords Phosphite utilization · *Chlorella vulgaris* · *Coccomyxa subellipsoidea*

Introduction

Phosphorus (P) is an essential component for all life on Earth, and its function cannot be compensated by other nutrients. The majority of phosphates in nature exist as calcium phosphate apatite, and their supply solely depends on weathering and the dissolution of this ore, which has a very low solubility. The annual production of phosphate is approximately 40 million t by P_2O_5 conversion, which comes from roughly 140 million t of phosphate rock (Steen 1998). Approximately 82% of P ores are currently used in fertilizers and another 5% for livestock feed, with the remaining used for detergents and metal processing (Edixhoven et al. 2014). Currently, there are different views on global phosphate rock reserves (Steen 1998; Van Vuuren et al. 2010). In the worst-case scenario, it is estimated that all economically mined phosphate rock could be depleted by the 2060s if there is a 3% annual increase in the consumption of phosphate fertilizer (Steen 1998). Alternatively, about 40–60% of the current resource base will be extracted by 2100 (Van Vuuren et al. 2010). Thus, it is important to develop methods to recycle and reuse phosphorus from waste and wastewater within the next 50–80 years to reduce the current consumption of phosphorus by half and avoid these worst-case scenarios (Steen 1998).

The forms of P include inorganic and organic. Inorganic P is widely used in agriculture and other industries. Inorganic P compounds include orthophosphate (PO_4-P), its deoxidized forms, such as phosphite (PO_3-P), hypophosphite (PO_2-P), phosphine (PH_3-P), and polyphosphate, which is a polymer composed of pyrophosphate and tripolyphosphate. Among inorganic P compounds, phosphates, including orthophosphate and polyphosphate, are essential nutrients for plants, algae, and microorganisms. Algae can only use phosphate

Michiyo Hashizume and Masaki Yoshida contributed equally to this work.

✉ Makoto M. Watanabe
watanabe.makoto.ga@u.tsukuba.ac.jp

[1] Canon Electronics Inc., Saitama, Japan

[2] Faculty of Life and Environmental Sciences and Algae Biomass and Energy System R&D Center (ABES), University of Tsukuba, Tsukuba, Japan

forms for their growth, and they accumulate P in their cells in the polyphosphate form. The majority of P ores are currently used in the phosphate form in the agriculture industry and in detergents. This results in a large release of phosphates into domestic and agricultural sewage and activated sludge into natural aquatic environments. Much attention has been given to measuring phosphate levels and monitoring water quality, survey, and control of algal phytoplankton dynamics. In addition, algal accumulation of phosphates has been extensively researched to evaluate their sustainable use of phosphates (De-Bashan and Bashan 2004).

Phosphite and hypophosphite components are used in several industries. Particularly, hypophosphite is used in plating, and phosphite oxidized from hypophosphite after plating is discharged as industrial waste. The phosphite that is discharged may be metabolized by some bacteria, including cyanobacteria that evolved mechanisms to acquire phosphite as a P source when phosphate is unavailable in natural habitats (Quinn et al. 2007; White and Metcalf 2007; Martinez et al. 2012; Stone and White 2012; Villarreal-Chiu et al. 2012; McGrath et al. 2013; Karl 2014; Horsman and Zechel 2017). In these bacteria, the phosphite oxidation enzyme NAD-dependent phosphite dehydrogenase (PtxD) catalyzes the oxidation of phosphite to orthophosphate and produces NADH from NAD^+ (Metcalf and Wanner 1991; Imazu et al. 1998; Metcalf and Wolfe 1998; Costas et al. 2001; White and Metcalf 2004; Yang and Metcalf 2004; Wilson and Metcalf 2005; Martinez et al. 2012). In terrestrial plants, it was suggested that soil or the foliar application of phosphite could replace phosphate as a P source in avocado (Lovatt 1990). Phosphite products are sold in US and European markets as fertilizers, fungicides, and/or defense stimulators (Leymonie 2007). However, their fertilizing effects have been questioned for the following reasons: (1) phosphite has never shown any beneficial effect on the growth of healthy plants (Thao and Yamakawa 2009; Thao et al. 2009); (2) deleterious effects of phosphite were shown in phosphate-starved plants (McDonald et al. 2001; Ratjen and Gerendas 2009; Thao and Yamakawa 2009; Thao et al. 2009; Avila et al. 2012; Berkowitz et al. 2013; Manna et al. 2015); (3) phosphite inhibited phosphate uptake in a competitive manner (Danova-Alt et al. 2008); (4) phosphite functions as a fungicide and herbicide to reduce plant diseases, resulting in positive plant responses to phosphite (McDonald et al. 2001; Danova-Alt et al. 2008; Thao et al. 2009); and (5) although phosphite is presumed to be utilized as a phosphate fertilizer by soil microorganisms (McDonald et al. 2001), the indirect provision of phosphite by microbial oxidation is not an effective means as a P nutrition source compared with phosphate fertilizer (Thao and Yamakawa 2009). Thus, there is no convincing evidence for phosphite utilization in wild plants.

The utilization of phosphate by algae is not well understood. In *Ulva lactuca*, the growth rates of both phosphate-starved and phosphate-satisfied cells were reduced in the presence of 2 mM of phosphite, and it was concluded that *U. lactuca* were unable to metabolize phosphite (Lee et al. 2005). In addition, *Chlamydomonas reinhardtii*, *Botryococcus braunii*, and *Ettlia oleoabundans* were unable to use phosphite as a P nutrition source (Loera-Quezada et al. 2015). The high concentration of phosphite did not show any deleterious effect for *C. reinhardtii* in spite of growth suppression, because *C. reinhardtii* cells survived for a long time in the presence of high phosphite levels and recovered the capacity for cell division after their transfer to an orthophosphate medium. These reports strongly support the central concept that eukaryotic microalgae cannot use phosphite and can only directly use phosphate as a sole P source.

To the best of our knowledge, there is no report on the phosphite utilization of wild photosynthetic eukaryotes, including eukaryotic microalgae. Therefore, except for the possible utilization by some bacteria when phosphate is unavailable, the fate of the phosphite that is discharged as waste is largely unknown.

Chlorella species are a major algal model in plant biochemistry and physiology (Safi et al. 2014). Their high growth potential and production of useful compounds have contributed to the fields of algae industrial and environmental technologies, including carbon capture and utilization. *C. vulgaris* is a photosynthetic and mixotrophic species and grows in a variety of environments. Many studies have demonstrated the high potential of *C. vulgaris* for the treatment of different types of wastewater, including municipal, agricultural, and industrial. As *C. vulgaris* accumulates a considerable amount of phosphorus in its cells as polyphosphate, *C. vulgaris* is expected to recycle and reuse phosphate from wastewater. However, previous studies have focused on total phosphorus and phosphate and no study has investigated the phosphite utilization of this species (Safi et al. 2014). In this study, we investigated the availability of phosphite in *C. vulgaris* and three other green algae species, such as *Coccomyxa subellipsoidea*, *Scenedesmus obliquus*, and *B. braunii*. The results of this study clearly demonstrate that *C. vulgaris* and *C. subellipsoidea* are capable of directly metabolizing phosphite. Significantly, our study is the first to detail such dynamics in photosynthetic eukaryotes.

Materials and methods

Algal strains

Axenic strains of *Chlorella vulgaris* (NIES-2170), *Coccomyxa subellipsoidea* (NIES-2166), and *Scenedesmus obliquus* (NIES-2280) obtained from Microbial Culture Collection, National Institute for Environmental Studies, and an axenic strain of *Botryococcus braunii* (BOT-22),

maintained in the Faculty of Life and Environmental Sciences and Algal Biomass and Energy System R&D Center (ABES), University of Tsukuba, were used in this study.

Preparation of medium

Chlorella vulgaris, *C. subellipsoidea*, and *S. obliquus* were cultured in P-free C medium, and *B. braunii* was cultured in AF-6 medium. The P-free C medium contained $Ca(NO_3)_2.4H_2O$ 150 mg L^{-1}, KNO_3 100 mg L^{-1}, $MgSO_4.7H_2O$ 40 mg L^{-1}, vitamin B_{12} 0.1 μg L^{-1}, biotin 0.1 μg L^{-1}, thiamine HCl 10 μg L^{-1}, PIV metal solution 3 mL L^{-1}, and tris (hydroxymethyl) aminomethane 500 mg L^{-1}. The PIV metal solution contained $Na_2EDTA.2H_2O$ 1000 mg L^{-1}, $FeCl_3.6H_2O$ 196 mg L^{-1}, $MnCl_2.4H_2O$ 36 mg L^{-1}, $ZnSO_4.7H_2O$ 22 mg L^{-1}, $CoCl_2.6H_2O$ 4 mg L^{-1}, and $Na_2MoO_4.2H_2O$ 2.5 mg L^{-1}. The pH was adjusted to 7.5. The P-free AF-6 medium contained $NaNO_3$ 280 mg L^{-1}, NH_4NO_3 44 mg L^{-1}, $MgSO_4.7H_2O$ 60 mg L^{-1}, $CaCl_2.2H_2O$ 20 mg L^{-1}, KNO_3 10 mg L^{-1}, Fe-citrate 4 mg L^{-1}, citric acid 4 mg L^{-1}, biotin 2 μg L^{-1}, thiamine HCl 10 μg L^{-1}, vitamin B_6 1 μg L^{-1}, vitamin B_{12} 1 μg L^{-1}, PIV metals 5 mL L^{-1}, and MES 400 mg L^{-1}. The pH was adjusted to 6.6. The original C medium contained 7.2 mg-phosphorus L^{-1} (Na_2-glycerophosphate $5H_20$ 50 mg L^{-1}), and the original AF-6 medium contained 3.2 mg-P L^{-1} (KH_2PO_4 10 mg L^{-1} and K_2HPO_4 5 mg L^{-1}), but the P-free C and AF-6 medium were prepared in this study. A bacteria-check medium containing 1 g yeast extract and 2 g tryptone in 1 L of C medium or AF-6 medium was used to check bacterial contamination once a week throughout the experiment. The axenicity of the culture was determined by observation under a microscope.

Stock solutions of orthophosphate ($NaH_2PO_4.2H_2O$ 36.1 g L^{-1}) with a P concentration that was 1000 times higher than the original C medium were prepared, and the pH was adjusted to 7.5. Stock solutions of phosphite ($Na_2HPO_3.5H_2O$ 50 or 500 g L^{-1}) with a P concentration that was 1000 or 10,000 times higher than the original C medium were prepared, and the pH was adjusted to 7.5. Similarly, stock solutions of orthophosphate (16.1 g L^{-1}) and phosphite (22.3 g L^{-1}) with a P concentration that was 1000 times higher than the original AF-6 medium were prepared, and the pH was adjusted to 6.6. All P-component stock solutions were aseptically filter sterilized through a 0.2 μm cellulose acetate filter sterilized by ethylene oxide (DISMIC-25CS, Advantech Japan, Co., Ltd., Japan) on a clean bench. One milliliter of orthophosphate and phosphite stock solutions (1000 times higher) was added to the P-free C medium and P-free AF-6 medium, respectively, to obtain C medium containing 7.2 mg-P L^{-1} and AF-6 medium containing 3.2 mg-P L^{-1}. Additionally, 0.05, 0.1, 0.5, 1, and 10 mL of the phosphite stock solution (10,000 times higher) were added to the P-free C medium, to generate C medium containing 3.6, 7.2, 72, and 720 mg-P L^{-1}, respectively. These media were then sterilized through a 0.2-μm cellulose acetate filter. Media containing orthophosphate were named "PO_4-P," and media containing phosphite were named "PO_3-P." Autoclave-sterilized media were named "AS," and filtered-sterilized media were named "FS."

Preparation of P-starved algae cells

Algal cells were pre-cultured in PO_4-P C or AF-6 medium until they reached the stationary phase. The pre-cultured cells were inoculated into P-free C or AF-6 medium and cultured until they reached the stationary phase, indicating the P-starved state of algal cells. These intracellular P-starved cells were washed several times with sterilized water by centrifugation to washout P from the cells. These P-starved cells were used for this study.

Measurements of growth and phosphoric acid concentrations

Growth was measured by determining the optical density at 680 nm (OD 680 nm) using a UV spectrophotometer (UV1800, Shimadzu, Japan). The concentrations of PO_4-P and PO_3-P in the media were measured using a capillary electrophoresis apparatus (G1600A, Agilent Technologies Japan, Ltd., Japan) before and after cultivation. Reagent grades of sodium orthophosphate and sodium phosphite were used as standards.

C. vulgaris NIES-2170 culture experiments

C medium was used to culture *C. vulgaris*. P-starved cells of *C. vulgaris* were grown in 500-mL Erlenmeyer flasks with 250 mL of PO_4-P and PO_3-P added C media and P-free C medium under continuous white fluorescent light (53–69 μmol photons m^{-2} s^{-1}) or heterotrophically grown in 500-mL Erlenmeyer flasks with 250 mL of 20 g L^{-1} glucose-added to PO_4-P and PO_3-P C media and P-free C medium under dark conditions. To observe the effects of PO_3-P concentrations on the growth of *C. vulgaris*, P-starved cells were grown in 500-mL Erlenmeyer flasks with 250 mL of PO_3-P C media with 3.6, 7.2, 36, 72, and 720 mg-P L^{-1}. P-free and PO_4-P C media were used as a negative control and positive control, respectively. All of these experiments were performed in triplicate at 25 °C ± 1 °C on a shaker (NR-150, Taitec Corp, Japan) at 120 rpm. During these culture experiments, the amount of evaporated water was replenished regularly by adding sterile water.

Coccomyxa subellipsoidea NIES-2160, *S. obliquus* NIES-2280, and *B. braunii* BOT-22 culture experiments

C medium was used for *C. subellipsoidea* and *S. obliquus*, and AF-6 medium was used for *B. braunii*. P-starved cells of

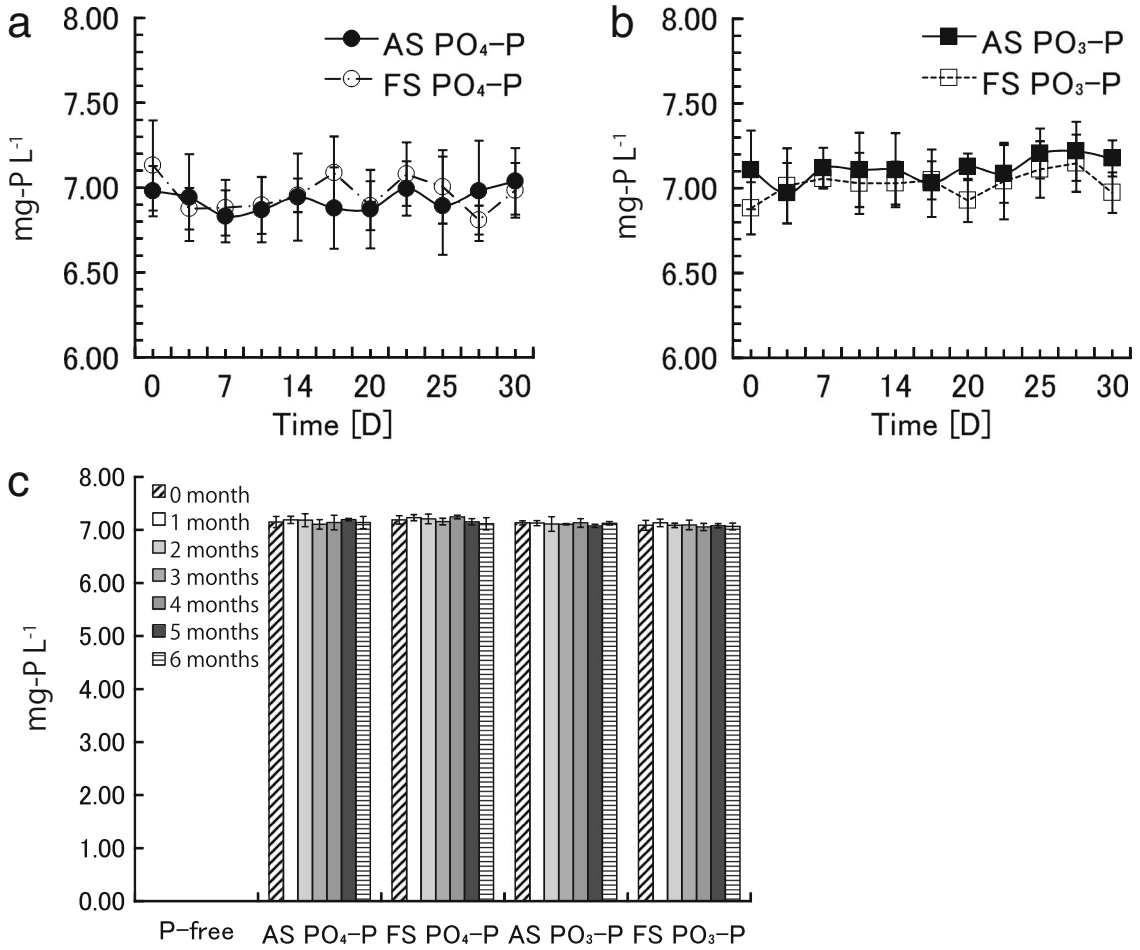

Fig. 1 Orthophosphate (PO_4-P) and phosphite (PO_3-P) stabilities over 6 months in algae-free media. The phosphorus concentration of the PO_4-P and PO_3-P media was adjusted to 7.2 mg-P L^{-1}. **a** PO_4-P concentration measured every few days for the first month. **b** PO_3-P concentrations measured every few days for the first month. **c** The PO_4-P and PO_3-P concentrations determined every month for 180 days (6 months). Data represent the mean ± standard deviation (SD) of three replicates. All bars in **c** are not statistically different from each other. AS autoclave sterilization, FS filter sterilization

C. subellipsoidea and *S. obliquus* strains were grown in 500-mL Erlenmeyer flasks with 250 mL of PO_4-P and PO_3-P C media and P-free C medium on a shaker (NR-150, Taitec Corp, Japan) at 120 rpm. P-starved cells of *B. braunii* were grown in 90-mL tubes with 50 mL of PO_4-P and PO_3-P AF-6 media and P-free AF-6 medium and aerated with sterile air containing 1% (v/v) CO_2 through a polytetrafluoroethylene filter (0.2 μm, Tokyo Roshi Kaisha, Ltd., Japan). All of these experiments were performed in triplicate at 25 °C ± 1 °C under continuous white fluorescent light (53–69 μmol photons m^{-2} s^{-1}). During these culture experiments, the amount of evaporated water was replenished regularly by adding sterile water.

Statistical analysis

Results are expressed as the mean ± standard deviation (SD) of three replicates. Significant differences were determined by an analysis of variance (ANOVA) of the measured OD values. The effects of phosphoric acid concentrations before and after cultivation were also analyzed by ANOVA. Differences were considered significant when $p < 0.05$.

Results

Stability of orthophosphate and phosphite in medium

Orthophosphate and phosphite stabilities in algae-free media were analyzed for 180 days (6 months) by determining the change in orthophosphate and phosphite concentrations every several days in the first month (Fig. 1a and b) and every month for the next 5 months (Fig. 1c). We found that orthophosphate and phosphite concentrations did not change in the algae-free media (Fig. 1c). In addition, phosphoric acid was the only form found in each medium, and the levels of both orthophosphate and phosphite did not differ between AS and FS.

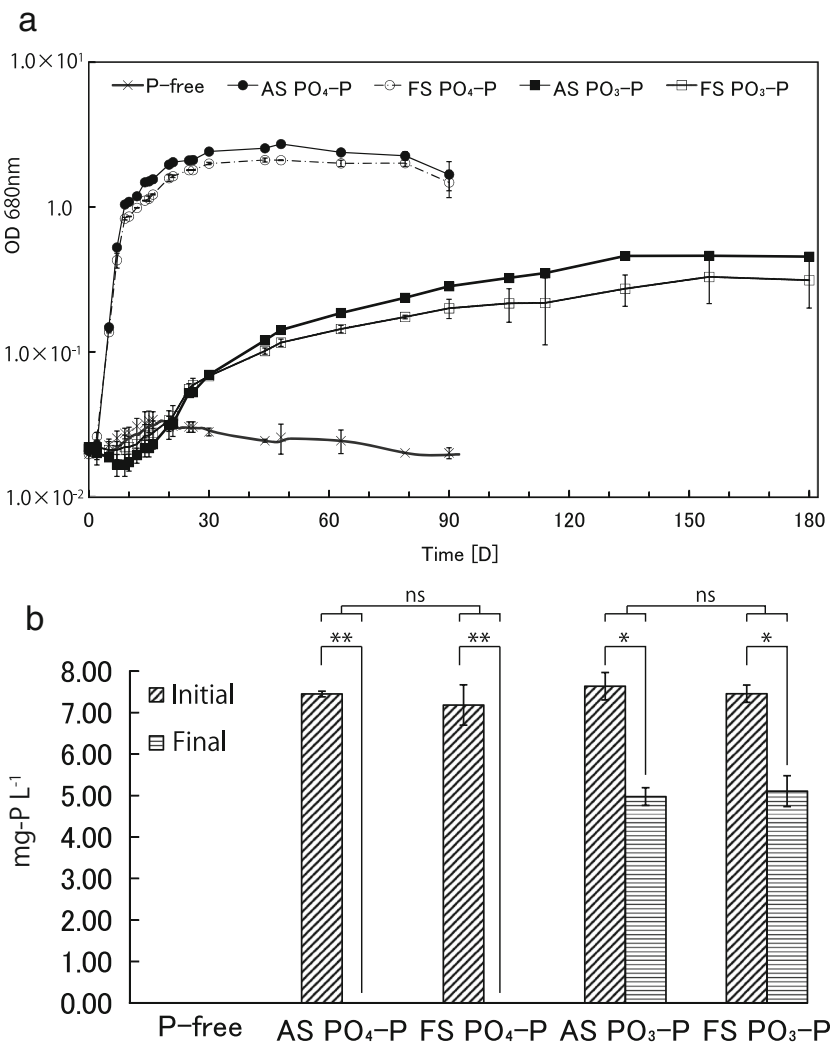

Fig. 2 Effects of sterilization methods. **a** Growth of *C. vulgaris* in P-free, PO_4-P, and PO_3-P media. The phosphorus concentration in PO_4-P and PO_3-P media was 7.2 mg-P L^{-1}. Cell growth was determined during the 90 days of culture in PO_4-P media and 180 days of culture in PO_3-P media by measuring the optical density (OD). **b** Changes in P concentration in PO_4-P and PO_3-P media before and after the cultivation of *C. vulgaris*. After 90 days of culture, 100% of PO_4-P was consumed. Data represent the mean ± SD of three replicates. Asterisks on bars indicate statistical significance, $^{**}p < 0.001$, $^{*} < 0.005$, ns not significant ($p > 0.05$). AS autoclave sterilization, FS filtered-sterilization

Growth and phosphite utilization of *C. vulgaris* NIES-2170

As mentioned above, the oxidation of phosphite to orthophosphate by the AS medium was not observed, but the possibility of oxidation occurrence was considered. Compared with autoclave sterilization, filter sterilization does not include treatments that cause the oxidation of phosphite to orthophosphate. The growth and phosphite utilization of this strain were investigated in the AS and FS media to confirm that there was no difference between the two media in terms of cultivation. In the P-free medium, the growth of *C. vulgaris* was not observed during the three-month culture period (Fig. 2a). However, this strain exhibited high growth rates and yields in the PO_4-P media and reached the early stationary phase 20 days after inoculation. The algae aliquot was filtrated 90 days prior to the death phase, and orthophosphate concentrations in the filtrates were analyzed. However, no trace of orthophosphates was detected (Fig. 2b). In the PO_3-P media, this strain showed reduced growth rates and yields than those in the PO_4-P media. Specifically, growth was observed after a lag phase of 20 days and reached an OD680 of 0.3–0.5 after 180 days. The algae aliquot was filtered at 180 days and phosphite concentrations in the filtrates were analyzed. A 32–35% reduction (hereafter called the "utilization rate") of phosphite in the media was detected (Fig. 2b). There were no significant differences in the growth and phosphate utilization rates between AS and FS media (Fig. 2a and b), and thus, FS media were used for all subsequent experiments. After 180 days of culture in the FS PO_3-P medium, the strain was inoculated again into fresh phosphite medium to confirm the sustainability of growth. Similar to our previous results, growth was observed without a lag phase (Fig. 3a), and a 38% utilization rate of phosphite was detected (Fig. 3b).

In order to completely exclude the possibility of oxidation due to oxygen species generated by photosynthesis, the growth and utilization of orthophosphate and phosphite of this strain were investigated under heterotrophic conditions for 2 months. The same results were obtained under these conditions (Fig. 3c and d). Specifically, no growth was observed in

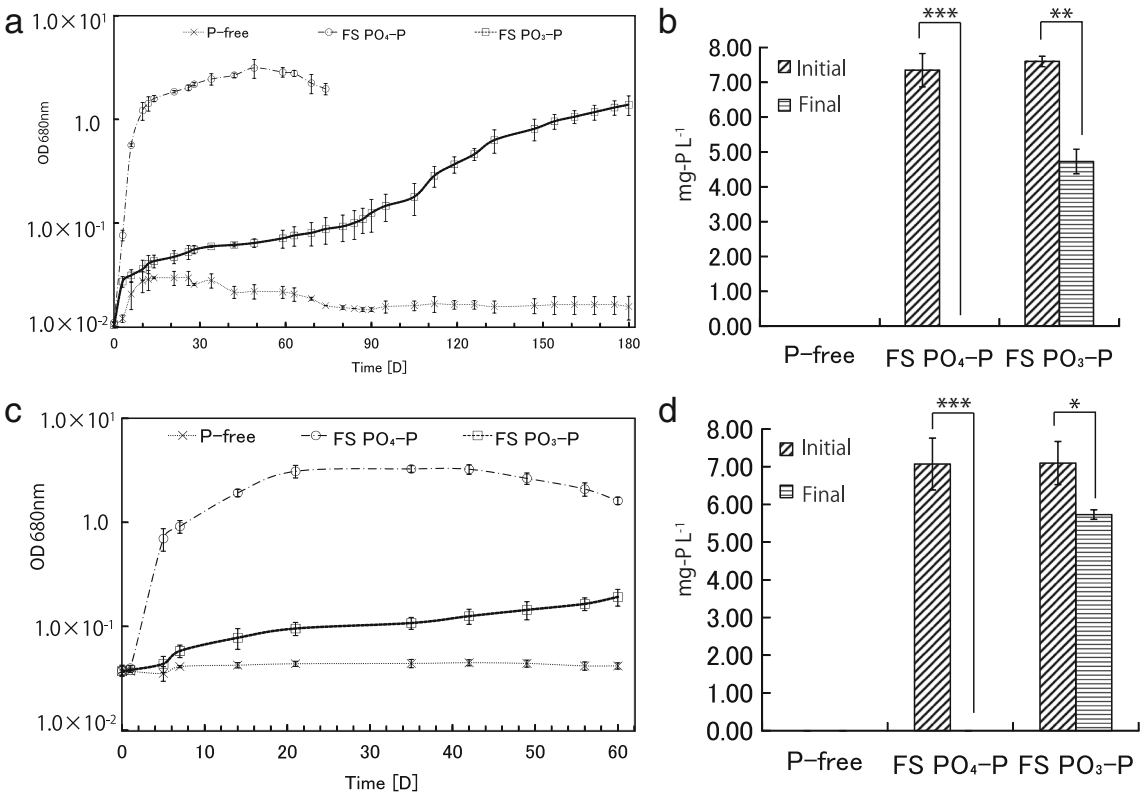

Fig. 3 *Chlorella vulgaris* in P-free, PO_4-P, and PO_3-P media. **a** Phototrophic growth after the inoculation of cells from the first cultures shown in Fig. 2. The phosphorus concentration in PO_4-P and PO_3-P media was 7.2 mg-P L^{-1}. Cell growth was determined during the 80 days of culture in PO_4-P medium and 180 days of culture in PO_3-P medium by measuring the optical density (OD) at 680 nm. **b** Changes of P concentration in PO_4-P and PO_3-P media before and after the long-term cultivation of *C. vulgaris*. After 80 days in culture, 100% of PO_4-P was consumed. **c** Heterotrophic growth of *C. vulgaris* in P-free, PO_4-P, and PO_3-P media under dark conditions. The phosphorus concentration in PO_4-P and PO_3-P media was 7.2 mg-P L^{-1}. Cell growth was determined during 60 days of culture by measuring the optical density (OD) at 680 nm. **d** Changes in P concentration in PO_4-P and PO_3-P media before and after the long-term heterotrophic cultivation of *C. vulgaris* for 60 days. After 60 days in culture, 100% of PO_4-P was consumed. Data represent the mean ± SD of three replicates. Asterisks on bars indicate statistical significance, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. FS filter sterilization

the P-free medium, whereas abundant growth and complete utilization of orthophosphate occurred in the PO_4-P medium. In addition, a lower net growth and a 19% utilization rate of phosphite was observed in the PO_3-P medium.

This strain was also observed for growth and utilization of phosphite in different concentrations of phosphite (3.6 mg-P L^{-1}, 7.2 mg-P L^{-1}, 36 mg-P L^{-1}, 72 mg-P L^{-1}, and 720 mg-P L^{-1}) for 180 days. P-free medium and PO_4-P medium (7.2 mg-P L^{-1}) were used as a negative control and positive control, respectively. As with our previous experiments, this strain did not show any growth in the P-free medium during the 180 days but showed abundant growth in the PO_4-P medium and reached an early stationary phase 20 days after inoculation. In the presence of different concentrations of phosphite, the growth of this strain increased as the phosphite concentration increased with maximum growth at 720 mg-P L^{-1}, exhibiting a growth yield at 180 days that was similar to the PO_4-P medium (Fig. 4a). In addition, the utilization rates of phosphite were 7, 39, 12, 9, and 13% in media with 3.6, 7.2, 36, 72, and 720 mg-P L^{-1}, respectively (Fig. 4b).

Growth and phosphite utilization of other green microalgae

Growth and the utilization of phosphate and phosphite of *C. subellipsoidea* (NIES-2166), *S. obliquus* (NIES-2280), and *B. braunii* (BOT-22) were investigated in FS media. *Coccomyxa subellipsoidea* showed no growth in P-free medium for 60 days, but abundant growth in PO_4-P medium, reaching an early stationary phase (> OD 2.0). The cells cultured in PO_3-P medium grew much more slowly, reaching an OD of 0.2 after 2 months (Fig. 5a) and with a 17% utilization rate of phosphite (Fig. 5b). After 60 days in culture, the strain was inoculated again into fresh phosphite medium to confirm growth sustainability. Similar growth was observed (Fig. 5c), with a 26% utilization rate of phosphite (Fig. 5d). However, *S. obliquus* and *B. braunii* cells did not grow when cultured for 2 months (56–60 days) in the PO_3-P media and P-free media, respectively. In contrast, their growth was abundant in the PO_4-P media (Fig. 6a and c). No significant phosphite reduction was observed after 2 months in culture (Fig. 6b and d).

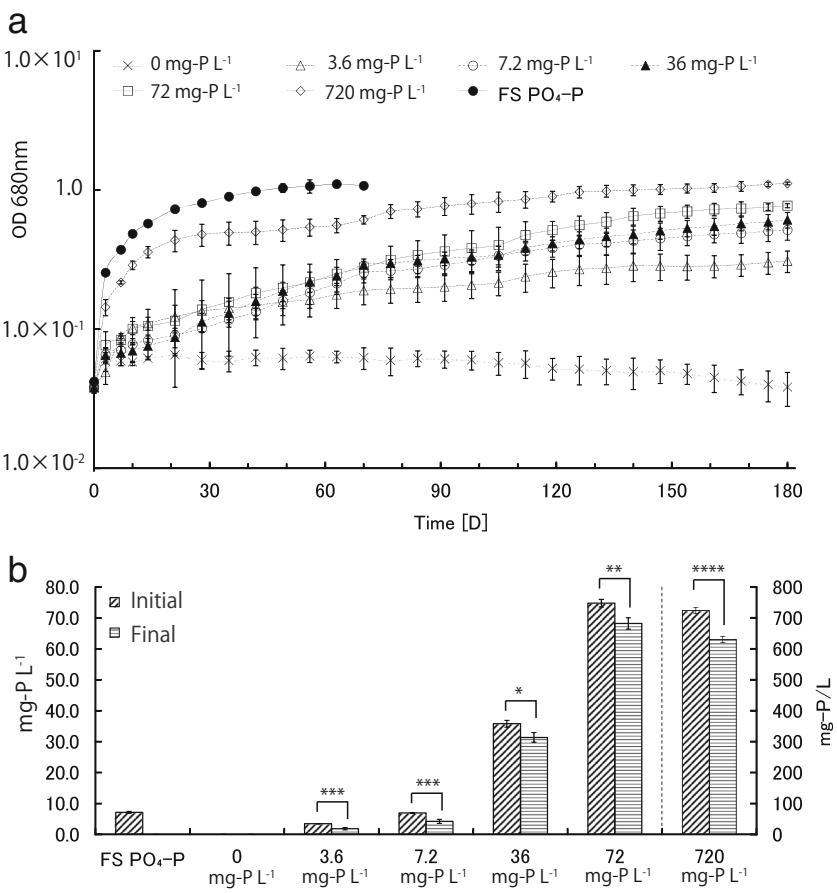

Fig. 4 *Chlorella vulgaris* at different concentrations of PO_3-P: 3.6, 7.2, 36, 72, and 720 mg-P L^{-1}. P-free medium and PO_4-P medium were used as a negative control and a positive control, respectively. The phosphorus concentration in PO_4-P medium was 7.2 mg-P L^{-1}. **a** Growth in different PO_3-P concentrations. Cell growth was determined during the 70 days of culture in PO_4-P medium and 180 days of culture in PO_3-P medium by measuring the optical density (OD) at 680 nm. **b** Changes in P concentration in PO_4-P and PO_3-P media before and after the long-term cultivation. After 70 days of culture, 100% of PO_4-P was consumed. The vertical axis shows the P concentration divided to the left for initial concentrations of 3.6–72 mg-P $L^{-1}$and right for initial concentrations of 720 mg-P L^{-1}. Data represent the mean ± SD of three replicates. Asterisks on bars indicate statistical significance, ****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$. FS filter sterilization

Discussion

Utilization of phosphite

All strains used in this study were axenic; therefore, phosphite utilization due to bacterial oxidation was eliminated. Regarding the *C. vulgaris* strain NIES-2170, we carefully looked for orthophosphate contamination from the previous culture medium and from polyphosphates accumulated in algal cells. Because P-starved cells did not grow in the P-free medium that was used as the control in all experiments (see Figs. 2a; 3a, c; 4a; 5a, c; 6a, and c), no phosphate contamination occurred in the present experiments. Since it has been reported that the conversion of phosphite to phosphate occurred in the wet oxidation of phosphite at 180 °C, under an O_2 pressure of 0.5–5 MPa, and between pH 6.05 and 7.01 (Fujita et al. 2006), we investigated whether phosphite was oxidized to orthophosphate in both AS and FS media within a 6-month period. However, the results clearly demonstrated that orthophosphate and phosphite were not oxidized in AS or FS media (Fig. 1a–c). Supported by the previous report that a large energy investment is necessary to chemically oxidize phosphite to phosphate (Liu et al. 2013), it is likely that phosphite never changed via physicochemical oxidation to orthophosphate under the normal culture conditions used in this study. In addition, when the cells were grown in P-free medium and PO_3-P and PO_4-P media under heterotrophic conditions, they showed growth and orthophosphate and phosphite utilization rates similar to those observed in photosynthetic conditions (Fig. 3c and d). These findings strongly suggested that phosphite was not oxidized to orthophosphate by oxygen species generated by photosynthesis and/or other unknown factors during the course of our study.

Therefore, our results clearly demonstrated that the *C. vulgaris* strain NIES-2170 was able to directly use phosphite as a P nutrient source. This resulted in increased growth as the concentration of phosphite increased with the highest utilization rate of 39% in 7.2 mg-P medium.

We also found that the *C. subellipsoidea* strain NIES-2166 and *C. vulgaris* strain NIES-2170 were able to use phosphite as a P nutrient source. In contrast, *S. obliquus* (NIES-2280) and *B. braunii* (BOT-22) did not exhibit growth and phosphite utilization in the PO_3-P medium, whereas their growth and orthophosphate utilization were abundant in the PO_4-P medium. Thus, *S. obliquus* and *B. braunii* were not able to use phosphite as a P nutrient source. Taken together, *C. vulgaris* strain NIES-2170 and *C. subellipsoidea* strain NIES-2166 can utilize phosphite as a P resource. To the best of our knowledge, this is the first study demonstrating phosphite utilization in eukaryotic algae and the first convincing report in photosynthetic eukaryotes.

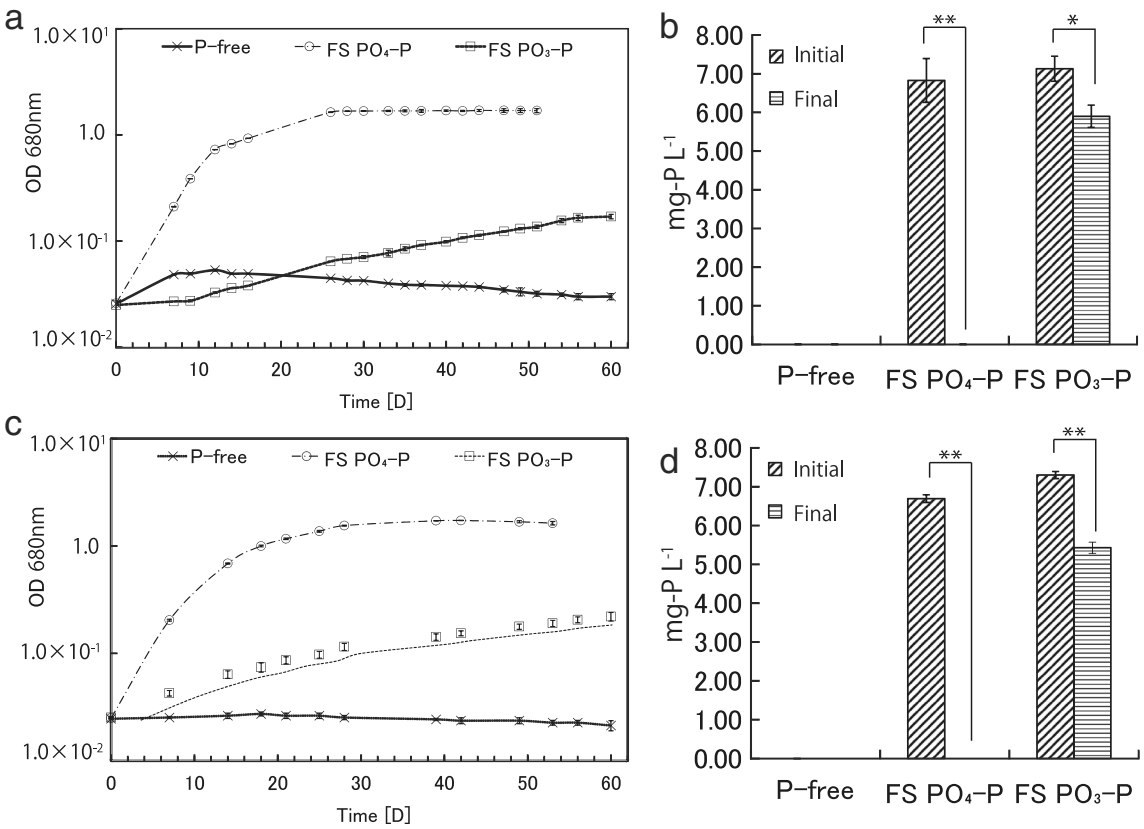

Fig. 5 *Coccomyxa subellipsoidea* in P-free, PO_4-P, and PO_3-P media. The phosphorus concentration in PO_4-P and PO_3-P media was 7.2 mg-P L^{-1}. Cell growth was determined during the 54 days in PO_4-P medium and 60 days in PO_3-P medium by measuring the optical density (OD) at 680 nm. **a** The first culture experiments using P-starved cells. **b** Changes in P concentration in the first cultures. **c** The second culture experiments using stationary phase cells from the first cultures. Data represent the mean ± SD. FS filter sterilization. **d** Change in P concentration in the second culture. Both **b** and **d** show changes in P concentration before and after the long-term cultivation of *C. subellipsoidea* for 54 days in PO_4-P medium and 60 days in PO_3-P medium. After 80 days in culture, 100% of PO_4-P was consumed. Data represent the mean ± SD of three replicates. Asterisks on bars indicate statistical significance, $^{**}p < 0.001$, $^{*}p < 0.01$. FS filter sterilization

PtxD, found in some bacteria, uses phosphite as a P source to catalyze the oxidation of phosphite to orthophosphate and reduce NAD+. Transgenic *Arabidopsis*, tobacco, and green alga *C. reinhardtii* lines expressing the *ptxD* gene from *Pseudomonas stutzeri* WM88 can use phosphite to achieve similar growth to those under orthophosphate conditions (López-Arredondo and Herrere-Estrella 2012; López-Arredondo and Herrera-Estrella 2013; Loera-Quezada et al. 2016). To identify PtxD-related sequences within the genome and transcriptome of *C. subellipsoidea* presented by Blanc et al. (2012), a BLAST search was conducted using the queries of bacterial PtxD sequences along with functional evidence (Martinez et al. 2012). As a result, several candidate sequences were suggested with identities of 29.4–50.6% (genome) and 30.2–54.2% (transcriptome). However, all candidate sequences had strong homology with eukaryotic D-3-phosphoglycerate dehydrogenase. PtxD-like enzymes were not found in *C. subellipsoidea.* It would be reasonable to presume some mechanism of intracellular phosphite oxidation in the *C. subellipsoidea* strain NIES-2166.

Future prospects

It has been suggested that P from $(Fe,Ni)_3P$ (schreibersite), which significantly influenced the geochemistry of P on the early Earth, reacts with water to form reduced P compounds, such as phosphite, hypophosphite, and phosphine. These reduced P forms may have been used as sources of P by early life on Earth (Pasek 2008). The existence of PtxD provides direct evidence that the utilization of reduced forms is a mechanism of P utilization in the present ecosystem. *Prochlorococcus*, a marine cyanobacterium, is a numerically dominant primary producer in the oligotrophic ocean that is able to use phosphite as a sole P source (Martinez et al. 2012). It was suggested that the phosphite utilization of this cyanobacterial strain is mediated by a phosphite dehydrogenase encoded by a *ptxD* gene cluster similar to that of other phosphite-utilizing heterotrophic bacteria. Phosphite utilization genes are present in diverse marine microbes, and their abundance is higher in species who live in low-P waters. In addition, Bisson et al. (2017) determined the phosphite- and hypophosphate-binding proteins from phosphite and hypophosphite ATP-binding cassette transporters of

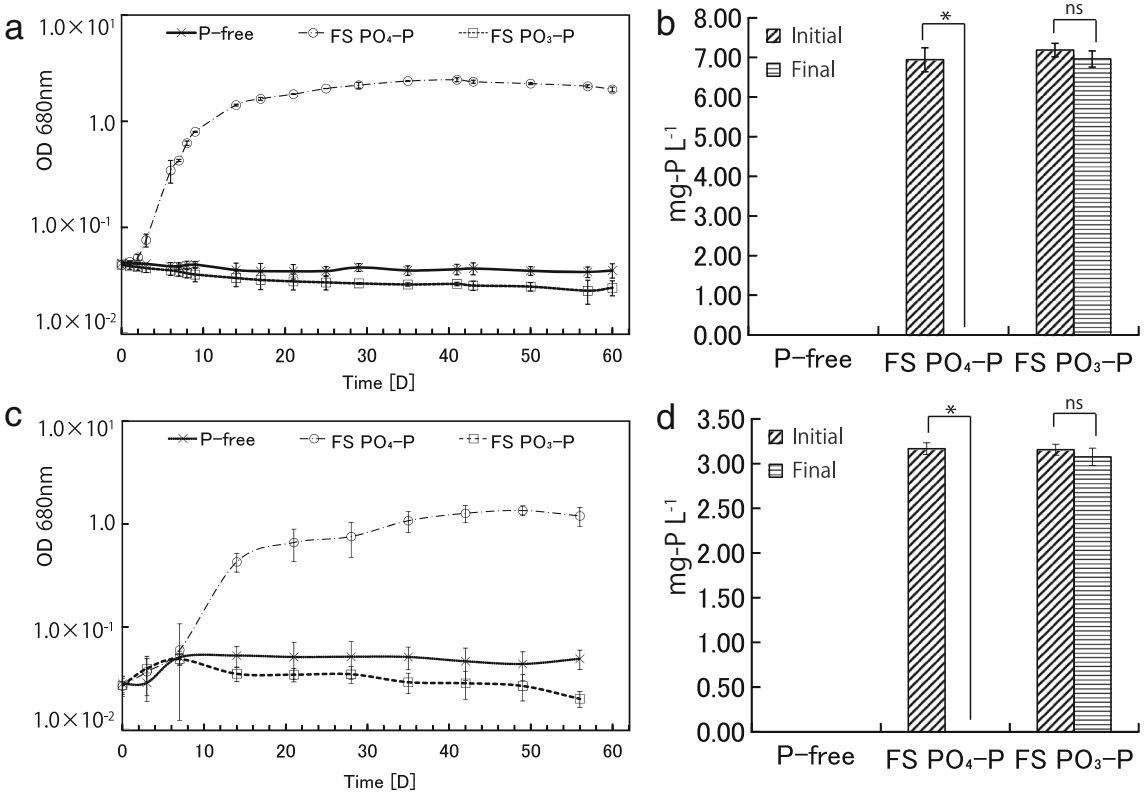

Fig. 6 *Scenedesmus obliquus* and *Botryococcus braunii* in P-free, PO_4-P, and PO_3-P media. The phosphorus concentration in PO_4-P and PO_3-P media was 7.2 mg-P L^{-1}. **a** Growth of *S. obliquus*. Cell growth was determined during the 60 days of culture by measuring the optical density (OD) at 680 nm. **b** Changes in P concentration in PO_4-P and PO_3-P media before and after the long-term cultivation of *S. obliquus* for 60 days. After 60 days in culture, 100% of PO_4-P was consumed. **c** Growth of *B. braunii* in P-free, PO_4-P, and PO_3-P media. Phosphorus concentration in PO_4-P and PO_3-P media was 3.2 mg-P L^{-1}. Cell growth was determined during the 56 days in culture by measuring the optical density (OD) at 680 nm. Data represent the mean ± SD of three replicates. FS filter sterilization. **d** Changes in P concentration in PO_4-P and PO_3-P media before and after the long-term cultivation of *B. braunii* for 56 days. After 6 days of culture, 100% of PO_4-P was consumed. Data represent the mean ± SD. Asterisk on bars indicates statistical significance, $*p < 0.001$, ns not significant ($p > 0.05$). FS filter sterilization

important globally abundant marine and terrestrial microorganisms. These two types of proteins are the initial encounters with phosphite or hypophosphite for assimilating these potentially important P species.

The present study demonstrated for the first time that the green algae *C. vulgaris* and *C. subellipsoidea* utilize phosphite as a P resource for their growth, though the intracellular phosphite oxidation mechanism is not yet clear. These species commonly inhabit lakes, reservoirs, and ponds. As previously mentioned, hypophosphite is currently used as a reducing agent in electroless nickel plating in the automotive, mechanical, electric, electronic, and semiconductor industries. The resulting liquid waste contains mainly inorganic phosphite (the oxidized by-product) at a level of approximately 1840 t-P, which was estimated in 2005 in Japan (Matsubae et al. 2015). In these conditions where phosphate is unavailable, the phosphite that is discharged as waste could be metabolized by these algae as well as phosphite-utilizing bacteria.

In the present study, the phosphite utilization rates of *C. vulgaris* and *C. subellipsoidea* were much lower than those of orthophosphate. It is possible that several algal species can use phosphite more efficiently as a P nutrient source given the vast genetic diversity of microalgae (Guiry 2012; Larkum et al. 2012). Identifying the ability of eukaryotic algae as well as heterotrophic bacteria and cyanobacteria, to use phosphite as a P source would increase the awareness that the utilization of the reduced P form by bacteria and microalgae is an important part of the global phosphorus redox cycle, similar to the geochemistry of P on the early Earth. In this context, a future study involving the analysis of the *C. subellipsoidea* NIES-2166 and the *C. vulgaris* NIES-2170 genomes is needed to determine phosphite dehydrogenase-related genes that are specific to these strains.

Conclusions

Here we report that *C. subellipsoidea* and *C. vulgaris* can utilize phosphite as a P resource, which is a novel finding in eukaryotic algae. In contrast, the other two chlorophyte algae,

S. obliquus and *B. braunii* strains, could not grow in the phosphite medium. This finding could have important implications for the evolution and distribution of the phosphorus redox system among photosynthetic eukaryotes. Furthermore, the knowledge of phosphite utilization by eukaryotic algae will lead to a better understanding of the P cycle in aquatic environments.

Acknowledgments We would like to thank the laboratory members of ABES at the University of Tsukuba for their support and valuable comments during the experiments and writing of this paper. We also thank Mr. H. Tomita for his analytical assistance. We would especially like to express our sincerest gratitude to our colleagues at Canon Electronics Inc. for their continuous support.

Author contributions This work was carried out in collaboration between all authors. MH, MY, and MMW prepared the manuscript. MH and MD designed and executed the cultivation experiments. MY contributed to the statistical data analyses and composing figures. MMW was the project leader and was responsible for the project plan, experimental design, data analyses, and writing the manuscript. All authors read and approved the final version of this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

Avila FW, Faquin V, Ramos VSJ, Pinheiro GL, Marques DJ, Da Silva Lobato AK et al (2012) Effects of phosphite supply in a weathered tropical soil on biomass yield, phosphorus status and nutrient concentrations in common bean. J Food Agric Environ 10:312–317

Berkowitz O, Jost R, Kollehn DO, Fenska R, Finnegan PM, O'Brien PA, Hardy GESJ, Lambers H (2013) Acclimation responses of *Arabidopsis thaliana* to sustained phosphite treatments. J Exp Bot 64:1731–1743

Bisson C, Adams NBP, Stevenson B, Amanda A, Brindley AA, Polyviou D, Bibby TS, Baker PJ, Hunter CN, Hitchcock A (2017) The molecular basis of phosphite and hypophosphite recognition by ABC-transporters. Nat Com 8:1–13

Blanc G, Agarkova I, Grimwood J, Kuo A, Brueggemen A, Dunigan DD et al (2012) The genome of the polar eukaryotic microalga *Coccomyxa subellipsoidea* reveals traits of cold adaptation. Genome Biol 13:R39

Costas AMG, White AK, Metcalf WW (2001) Purification and characterization of a novel phosphorus-oxidizing enzyme from *Pseudomonas stutzeri* WM88. J Biol Chem 276:17429–17436

Danova-Alt R, Dijikema C, De Waard P, Kock M (2008) Transport and compartmentation of phosphite in higher plant cells – kinetic and ^{31}P nuclear magnetic resonance studies. Plant Cell Environ 31: 1510–1521

De-Bashan LE, Bashan Y (2004) Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997-2003). Water Res 38:4222–4246

Edixhoven JD, Gupta J, Savenije HHG (2014) Recent revisions of phosphate rock reserves and resources: a critique. Earth Syst Dynam 5: 491–507

Fujita T, Kawaguchi Y, Fukuta T, Matsuda H, Kojima Y, Yagishita K (2006) Effect of pH on conversion of hypophosphite and phosphite to phosphate by wet oxidation. J Surf Finish Soc Jpn 57:368–372 (in Japanese with English abstract)

Guiry MD (2012) How many species of algae are there? J Phycol 48: 1057–1063

Horsman GP, Zechel DL (2017) Phosphonate biochemistry. Chem Rev 117:5704–5783

Imazu K, Tanaka S, Kuroda A, Anbe Y, Kato J, Ohtake H (1998) Enhanced utilization of phosphonate and phosphite by *Klebsiella aerogenes*. Appl Environ Microbiol 64:3754–3758

Karl DM (2014) Microbially mediated transformations of phosphorus in the sea: new views of an old cycle. Annu Rev Mar Sci 6:279–337

Larkum AWD, Ian L, Ross IL, Kruse O, Hankamer B (2012) Selection, breeding and engineering of microalgae for bioenergy and biofuel production. Trends Biotechnol 30:198–205

Lee TM, Tsai PF, Shyu YT, Sheu F (2005) The effect of phosphite on phosphate starvation responses of *Ulva lactuca* (Ulvales, Chlorophyta). J Phycol 41:975–982

Leymonie JP (2007) Phosphites and phosphates: when distributors and growers alike could get confused. Courtesy of New Ag International http://www.spectrumanalytic.com/support/library/pdf/Phosphites_and_Phosphates_When_distributors_and_growers_alike_could_get_confused.pdf (Accessed 17 Nov, 2016)

Liu P, Li C, Liang X, Xu L, Lu G, Ji F (2013) Advanced oxidation of hypophosphite and phosphite using a UV/H_2O_2 process. Environ Technol 34:2231–2239

Loera-Quezada MM, Leyva-Gonzalez MA, Lopez-Arrendono D, Herrera-Estrella L (2015) Phosphite cannot be used as a phosphorus source but is non-toxic for microalgae. Plant Sci 231:124–130

Loera-Quezada MM, Leyva-González MA, Velázquez-Juárez G, Sanchez-Calderón L, Nascimento MD, López-Arredondo D, Herrera-Estrella L (2016) A novel genetic engineering platform for the effective management of biological contaminants for the production of microalgae. Plant Biotechnol J 14:2066–2076

López-Arredondo DL, Herrera-Estrella L (2013) A novel dominant selectable system for the selection of transgenic plants under in vitro and greenhouse conditions based on phosphite metabolism. Plant Biotechnol J 11:516–525

López-Arredondo DL, Herrere-Estrella L (2012) Engineering phosphorus metabolism in plants to produce a dual fertilization and weed control system. Nat Biotechnol 30:889–893

Lovatt CJ (1990) A definitive test to determine whether phosphite fertilization can replace phosphate fertilization to supply P in the metabolism of 'Hass' on 'Duke 7'. Calif Avocado Soc Yearbook 74:61–64

Manna M, Islam T, Kaul T, Reddy CS, Fartyal D, James D, Reddy MK (2015) A comparative study of effects of increasing concentrations of phosphate and phosphite on rice seedlings. Acta Physiol Plant 37: 1–10

Martinez A, Osbune MS, Sharma AK, Delong EF, Chisholm SW (2012) Phosphite utilization by the marine picocyanobacterium *Prochlorococcus* MIT9301. Environ Microbiol 16:1363–1377

Matsubae K, Webeck E, Nansai K, Nakajima K, Tanaka M, Nagasaka T (2015) Hidden phosphorus flows related with non-agriculture industrial activities: a focus on steelmaking and metal surface treatment. Resour Conserv Recycl 105:360–367

McDonald AE, Grant BR, Plaxton XC (2001) Phosphite (phosphorous acid): its relevance in the environment and agriculture and influence on plant phosphate starvation response. J Plant Nutr 24:1505–1519

McGrath JW, Chin JP, Quinn JP (2013) Organophosphonates revealed: new insights into the microbial metabolism of ancient molecules. Nat Rev Microbiol 11:412–419

Metcalf WW, Wanner BL (1991) Involvement of the *Escherichia coli phn* (*psiD*) gene cluster in assimilation of phosphorous in the form of phosphonates, phosphite, P_i esters, and P_i. J Bacteriol 173:587–600

Metcalf WW, Wolfe RS (1998) Molecular genetic analysis of phosphite and hypophosphite oxidation by *Pseudomonas stutzeri* WM88. J Bacteriol 180:5547–5558

Pasek MA (2008) Rethinking early earth phosphorus geochemistry. Proc Natl Acad Sci U S A 105:853–858

Quinn JP, Kulakova AN, Cooley NA, McGrath JW (2007) New ways to break an old bond: the bacterial carbon-phosphorus hydrolases and their role in biogeochemical phosphorus cycling. Environ Microbiol 9:2392–2400

Ratjen AM, Gerendas J (2009) A critical assessment of the suitability of phosphite as a source of phosphorus. J Plant Nutr Soil Sci 172:821–828

Safi C, Zebib B, Merah O, Pontalier PY, Vaca-Garcia C (2014) Morphology, composition, production, processing and applications of *Chlorella vulgaris*: a review. Renew Sust Energ Rev 35:265–278

Steen I (1998) Phosphorus availability in the 21st century: management of a non-renewable resource. Phosphorus Potassium 217:25–31

Stone BL, White AK (2012) Most probable number quantification of hypophosphite and phosphite oxidizing bacteria in natural aquatic and terrestrial environments. Arch Microbiol 194:223–228

Thao HTB, Yamakawa T (2009) Phosphite (phosphorous acid): fungicide, fertilizer or bio-stimulator? Soil Sci Plant Nutr 55:228–234

Thao HTB, Yamakawa T, Shibata K (2009) Effect of phosphite-phosphate interaction on growth and quality of hydroponic lettuce (*Lactuca sativa*). J Plant Nutr Soil Sci 172:378–384

Van Vuuren DP, Bouwman AF, Beusen AHW (2010) Phosphorus demand for the 1970–2100 period: a scenario analysis of resource depletion. Glob Environ Change 20:428–439

Villarreal-Chiu JF, Quinn JP, McGrath JW (2012) The genes and enzymes of phosphonate metabolism by bacteria, and their distribution in the marine environment. Front Microbiol 3:1–13

White AK, Metcalf WW (2004) The *htx* and *ptx* operons of *Pseudomonas stutzeri* WM88 are new members of the Pho Regulon. J Bacteriol 186:5876–5882

White AK, Metcalf WW (2007) Microbial metabolism of reduced phosphorus compounds. Annu Rev Microbiol 61:379–400

Wilson MM, Metcalf WW (2005) Genetic diversity and horizontal transfer of genes involved in oxidation of reduced phosphorus compounds by *Alcaligenes faecalis* WM2072. Appl Environ Microbiol 71:290–296

Yang K, Metcalf WW (2004) A new activity for an old enzyme: *Escherichia coli* bacterial alkaline phosphatase is a phosphite-dependent hydrogenase. Proc Natl Acad Sci U S A 101:7919–7924