

Strategies adopted by *Aphanizomenon flos-aquae* in response to phosphorus deficiency and their role on growth

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Research

Keywords: *Aphanizomenon flos-aquae*, P deficiency, extracellular alkaline phosphatase, polyphosphate body, strategies for P utilization

Posted Date: March 12th, 2020

DOI: <https://doi.org/10.21203/rs.2.19948/v2>

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Version of Record: A version of this preprint was published at Environmental Sciences Europe on March 16th, 2020. See the published version at <https://doi.org/10.1186/s12302-020-00328-3>.

Abstract

Background The N₂-fixing cyanobacterium, *Aphanizomenon flos-aquae* is a globally distributed bloom causing species that degrades water quality of fresh and marine water bodies. Overcoming phosphorus (P) deficiency is one of the ecological advantages for bloom forming cyanobacteria. It remains unclear to what extent can *A. flos-aquae* alleviate P deficiency by regulating P using strategies.

Results Based on in situ observations of extracellular alkaline phosphatase (APase) in *A. flos-aquae* via enzyme-labeled fluorescence in freshwater bodies in China, Poland and Czechia, we further investigated responses of isolated *A. flos-aquae* to different P supplies (dissolved inorganic P (Pi) as +DIP, dissolved organic α -glycerophosphate and β -glycerophosphate as +DOP α and +DOP β , P-free condition as P-depleted). The significantly negative relationships between percentage of APase producing cells and soluble reactive P concentration in both fields and cultures suggested that the excretion of APase in cyanobacterium was regulated by ambient Pi supply. Suffering from P deficiency in the P-depleted treatments, *A. flos-aquae* showed the highest APase activity but a vigorous growth at the early culture stage, which might also benefit from the formation of polyphosphate body (PPB) and the decrease of cell P quota. In the +DOP treatments, the coordination of dissolved DOP mineralization and continuous prompt utilization of PPB might contribute to a maintenance but not reproduction of *A. flos-aquae* when relying on DOP, since the specific growth rate kept around 0 cells L⁻¹ day⁻¹ at the second half culture period and the highest cell density reached only 13.38% of that in +DIP treatments while photochemical efficiency was comparable during the whole experiment. Luxury uptake of phosphate as PPB in the +DIP treatments was consistent to the canonical view of polyphosphate as P storage.

Conclusion *A. flos-aquae* could achieve an instantaneous growth in response to P deficiency with the coordination of P utilization strategies, while it maintained a long-term sustainable growth but not reproduction under sole DOP supply. Persistent and active reproduction could only be achieved in high Pi supply, which implying that an effective consequence can be expected for combating the bloom of *A. flos-aquae* when controlling P supply.

Background

Filamentous cyanobacterial blooms of the diazotrophic genus *Aphanizomenon* occurred worldwide and posed deleterious effects on aquatic ecosystem [1, 2, 3, 4]. Theoretically, the nitrogen demand of the developing population of *Aphanizomenon* can be provided by nitrogen fixation in heterocysts [1, 5, 6]. Thus, the phosphorus (P) scarcity can most often lead to its growth limitation. For example, the first appearance of *Aphanizomenon* in Lake Kinneret in August 1994 was apparently elevated by relatively high total dissolved P concentrations [1]. *Aphanizomenon* behaved sensitively when exposed to changes in ambient dissolved inorganic P (Pi) so that it might be adapted to environments with higher P concentrations [7]. Orthophosphate in low concentrations led to a strong decrease in the growth of *A. issatschenkoi*, *A. aphanizomenoides* and *A. gracile* [8]. Correspondingly, bloom-forming cyanobacteria developed various strategies to overcome P deficiency. A strategy for cyanobacteria to survive in P

deficiency status was that they store P in the form of polyphosphate bodies (PPB) in cells when P is abundant, and breakdown it upon P stress [9]. Polyphosphate granules observed in cells of *Aphanizomenon* sp. from blooms in Baltic Sea suggested that the population was not subjected to P starvation [10]. Furthermore, *Aphanizomenon* had the ability to decrease cell P quota when P in their environment is scarce [7]. In addition, *Aphanizomenon* could meet its P demand by expressing extracellular alkaline phosphatase (APase), enzymes hydrolyzing bio-available phosphate from dissolved organic P (DOP) molecules [7, 11, 12, 13]. Like the *Aphanizomenon* in Lake Kinneret, whose biomass increased accompanying high enzymatic alkaline phosphatase activity (APA) in phosphate limited summer and autumn [1]. *Aphanizomenon* sp. which is one main bloom-forming filamentous diazotrophic species in Baltic Sea showed cell-surface-bound phosphatase activity as indicated by enzyme-labeled fluorescence (ELF) assays [13]. On the contrary, a combination of field and lab-cultures study showed that *A. ovalisporum* excreted APase only under severe P deficiency, it firstly enslaved another phytoplankton other than itself to produce APase [14]. In summary, APase production in the development of *Aphanizomenon* bloom at the single cell level in freshwater is rarely studied. Furthermore, it is still unclear to what extent can *A. flos-aquae* alleviate P deficiency by regulating P using strategies.

In this study, single-cell assays of APase were conducted at species level using the ELF technique in 2 Chinese lakes, 1 Czechia reservoir and 1 Polish reservoir. Furthermore, laboratory studies were performed with batch cultures, and three strains of *Aphanizomenon flos-aquae* (*A. flos-aquae* FACHB 1249, *A. flos-aquae* FACHB 1171 and *A. flos-aquae* FACHB 1260) isolated from three Chinese shallow lakes such as Lake Chaohu, Lake Taihu and Lake Dianchi were inoculated in medium with four P status with additions of Pi and two DOP species respectively, as well as without P addition. Multiple variables related to algal growth such as cell density and photosystem II quantum efficiency (F_v/F_m), P cycling such as bulk and size-fractionation APA, concentrations of soluble reactive P (SRP) and DOP in culture medium, as well as the ELF labeling and PPB containing cells of the strains were determined at different intervals. Objectives of this study were 1) to investigate whether *A. flos-aquae* could employ its own APase in acquiring P from DOP for cell growth and reproduction, and 2) to evaluate to what extent can *A. flos-aquae* abate the P limitation with various P using strategies.

Materials And Methods

Field sample collection and analysis

Field samples of surface water (0-50 cm) from Lake Chaohu, Lake Zhiyin (China), Reservoir Orлік (Czechia) and Reservoir Stefanski (Poland) were collected for chemical, biological and biochemical analysis on 05th Jun. 2018, 18th Jan. 2018, 22nd Oct. 2014 and 26th Aug. 2010 respectively (Fig. S1; Table. S2). 2 L of samples were taken and transported to the laboratory immediately. Fresh phytoplankton samples were inspected to minimize difficulties in determining species on the membrane filters. SRP concentrations were determined according to Murphy and Riley [15] after filtering with cellulose acetate membrane (0.45 μm pore diameter size). ELF method was used to quality detect APase on single-cell level. Samples obtained from field were treated using ELF[®] 97 phosphate (ELFP,

Invitrogen™) on the basis of the protocol described in Strojsova et al. [11]. 0.5 mL incubations were started by adding the ELFP solution (final concentration 27 μM) and samples were incubated at 25 °C for 2.5 hours. Each incubation was terminated by transferring the sample to a filter holder (diameter 7 mm) with a membrane filter (Millipore; 0.22 μm pore size). The filter with retained algae were placed on a microscope slide, embedded it with the anti-fading reagent Citifluor AF1 (Citifluor, London, UK), and covered with a cover slide for microscopic inspection. ELF-labelling cells were inspected by epifluorescence microscopy (Olympus BX51FL). 20-50 (depending on the cell density) images were taken randomly on the filters. The active and inactive cells with fluorescence were recorded in each image to quantify the percentages of cells with ELF labeling.

Experiment design in laboratory culture

Three strains of *A. flos-aquae* FACHB 1249 (*A. flos-aquae* 1249 for short), *A. flos-aquae* FACHB1171 (*A. flos-aquae* 1171 for short) and *A. flos-aquae* FACHB 1260 (*A. flos-aquae* 1260 for short) were attained from Freshwater Algae Culture Collection of Institute of Hydrobiology (Wuhan, China). Prior to experiments, cells were harvested in the exponential growth period by centrifugation (3000 rpm, 10 min), rinsed 3 times with P-free BG11 medium and then cultivated in P-free BG11 medium for 48 h to consume P stored in cells to a low level and equal basis [16]. After that, the culture was inoculated in BG11 [17] medium with modified P concentrations and the modifying sets were as follows:

A culture using K_2HPO_4 as the sole P source was defined as +DIP treatment. In P-depleted treatment, K_2HPO_4 was replaced by KCl. α -glycerophosphate and β -glycerophosphate alternated K_2HPO_4 with the same amount of P in the treatments of +DOP $_{\alpha}$ and +DOP $_{\beta}$. We conducted the experiments in sterile condition to avoid exotic bacterial contamination.

Initial cell density of *A. flos-aquae* in each culture was roughly 5×10^8 cells L^{-1} . All cultures were incubated in conditions suitable for cyanobacterial growth: constant white light intensity of 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiance, 12 h of light: 12 h of dark, and 25°C.

Subsamples were collected at different intervals over 59 days for chemical and biochemical analysis, e.g. SRP and DOP concentrations, cell density, photochemical efficiency, cell P quota, quality and quantity determination of APase, P storage detection etc.

Cell density, specific growth rate and photochemical efficiency of photosystem II

Samples for cell density estimation were preserved with Lugol's solution and counted with an Olympus BX 41 microscope (Olympus Corporation, Japan) [18]. The specific growth rate was calculated according to the following equation: $\mu = (\ln Ct_2 - \ln Ct_1) / (t_2 - t_1)$, where Ct_2 and Ct_1 are cell densities at times t_2 and t_1 , respectively [19].

F_v/F_m is one of the chlorophyll fluorescence parameters which could reflect the maximum quantum yield of the photochemistry. The value of F_v/F_m will decrease when plant was stressed by environmental

conditions so that it is often used to evaluate whether the plant is subjected to conditional inhibition [20]. It was examined by Water PAM2100 (Walz, Germany) and determined according to Ting and Owens [21].

P concentrations and cell P quota

SRP concentration was determined using the same method as field investigation. Total phosphorus (TP) and total dissolved phosphorus (DTP) concentrations were measured following digestion according to the measurement reported by Beattie et al. [22]. DOP concentration was calculated as the difference between DTP and SRP concentrations.

Aliquots of 5 mL from individual cultures were harvested onto 25 mm GF/F glass fiber filters (Waterman, UK) for the measurement of TP in samples to determine the specific total P content of cells according to Vahtera, et al. [12].

Size-fractionation of APA

APA was quantified by fluorometric assay [23]. It was determined in unfiltered sample (APA_T) and the filtrates through 3.0 μm and 0.45 μm membrane filters ($APA_{<3.0\mu\text{m}}$ and $APA_{<0.45\mu\text{m}}$ respectively). 150 μL of Tris-HCl (pH = 8.5) was added to 2.7 mL subsample, after pipetting aliquots of fluorogenic substrate (MUFp-phosphate, final concentration of 100 μM), fluorescence was measured immediately, after incubation at room temperature for 2 hours, fluorescence was read again. The contribution of APA to the algal ($APA_{>3.0\mu\text{m}}$) and bacterial ($APA_{0.45-3.0\mu\text{m}}$) fractions were calculated as follows:

$APA_{>3.0\mu\text{m}} = APA_T - APA_{<3.0\mu\text{m}}$, $APA_{0.45-3.0\mu\text{m}} = APA_{<3.0\mu\text{m}} - APA_{<0.45\mu\text{m}}$, in which $APA_{<0.45\mu\text{m}}$ represented the dissolved phosphatase activity [24].

Fluorescence measurements and microscopic inspections

In addition to quality determine algal APase by ELF method, we detected PPB in cultures of *A. flos-aquae* by DAPI (4',6-diamidino-2P-depletedhenylindole) according to the procedure of Bar-Yosef et al. [14]. 0.5 mL incubations were started by adding the DAPI solution (final concentration 15 μM) and samples were incubated at room temperature in dark for 5 min to detect PPB accumulation. Incubation termination, slide embedded protocol and inspection were the same as the stain of ELF. DAPI could bind to polyphosphate and show a yellow fluorescence at 526 nm under UV excitation [25, 26].

Statistical analysis

Pearson's correlation analysis and Linear regression were carried out to analyze the relationships between SRP concentration and ELFp, APA and ELFp. The significant differences among four treatments were determined by ANOVA analysis and the differences were considered significant at $P < 0.05$ or $P < 0.01$. SPSS statistical software (version 18.0, Chicago, IL, USA) was used for those analysis. Data were log transformed to fit normal distribution. The plots of regression were drawn by SigmaPlot 10.0.

Results

Differences in growth of *A. flos-aquae* in various P supply

The three *A. flos-aquae* strains of the four treatments yielded similar cell density increasing from around 5.0×10^8 cells L^{-1} to 1.5×10^9 cells L^{-1} at first 8 days (Fig. 1). The cells in the P-depleted and +DIP treatments were then rapidly growing until the day 20 as indicated by the gradually increasing of cell densities and positive specific growth rates (Fig. 1 A, D; Fig. 2 A, D). Moreover, the specific growth rates in the P-depleted treatments had the highest values among all the treatments in the first 20 days (Fig. 2 A). After that, specific growth rates fluctuated negatively and positively leading the peak cell densities at day 31 (4.6 , 6.6 and 6.5×10^9 cells L^{-1} in *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 respectively), followed by specific negative growth rates (Fig. 2 A) together with remarkable drop in cell densities (Fig. 1 A) till the end of the experiment. On the contrary, the growth rates kept constantly positive in the +DIP treatments (Fig. 2 D). As a result, the highest cell densities were observed at the end of the experiment (1.75 , 2.55 and 2.86×10^{10} cells L^{-1} in *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 respectively) (Fig. 1).

The specific growth rates in the +DOP_α and +DOP_β treatments were the lowest compared with other two treatments in the first 20 days, after then they fluctuated around zero till the end of the experiment (Fig. 2 B, C). Furthermore, the specific growth rates in the +DOP treatments were significantly lower than that of +DIP treatment among three culture strains ($P < 0.05$). In terms of cell density, DOP treatments gave comparable value firstly. After day 8, the lowest cell density was observed in the DOP treatments spatially. In details, the maximum cell density in the +DOP_α and +DOP_β treatments was 2.3×10^9 cells L^{-1} for the three strains, reaching only 47.16% and 50.82% for *A. flos-aquae* 1249, 28.94% and 30.43% for *A. flos-aquae* 1171, 26.84% and 29.02% for *A. flos-aquae* 1260 of that in P-depleted treatments, respectively. When compared with +DIP treatments, the maximum cell densities in the +DOP_α and +DOP_β treatments were 12.42 % and 13.38 % for *A. flos-aquae* 1249, 7.64 % and 8.04 % for *A. flos-aquae* 1171, 6.1% and 6.66% for *A. flos-aquae* 1260 of that, respectively. Noticeably, the cell densities in the +DOP_α and +DOP_β kept increased before day 11, then they stabilized ranging from 1.5×10^9 cells L^{-1} to 2.0×10^9 cells mL^{-1} (Fig. 1 B, C).

Photochemical efficiency of photosystem II in different treatments

The photochemical efficiency of photosystem II (Fv/Fm) values remained relatively consistent at 0.400 to 0.538 during the whole incubation period in the +DIP treatments (Fig. 3 D). So did the Fv/Fm values in the +DOP_α and +DOP_β treatments fluctuating from 0.251 to 0.590 (Fig. 3 B, C). In the P-depleted treatments, Fv/Fm values were comparable with that in the +DIP treatments before day 31, while it dropped sharply at the end of the experiment (0.014, 0.077 and 0.037 for *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 respectively; Fig. 3 A).

SRP and DOP concentrations in different treatments

SRP concentrations were determined in all treatments while DOP concentrations were determined only in +DOP_α and +DOP_β treatments. The P-depleted treatments always showed the lowest SRP concentrations among the treatments (Fig. 4 A). In the +DOP_α and +DOP_β treatments, the SRP concentrations rapidly peaked the value of around 3500 μg L⁻¹ at the first 15 days with the DOP concentrations decreasing to almost 0 μg L⁻¹. The SRP concentrations stabilized around 2700 to 3000 μg L⁻¹ after then (Fig. 4 B, C). In the +DIP treatments, the SRP concentrations declined gradually until day 20 and more rapidly thereafter. Moreover, they decreased under the levels of detection limit at day 46 in the strains of *A. flos-aquae* 1171 and *A. flos-aquae* 1260 (Fig. 4 D).

Expression of APase and its relationship with ambient P

The active ELF signals, indicating extracellular Apase expression, in cells of *A. flos-aquae* were observed in the samples from different subtropical and temperate water bodies in Czechia, Poland and China, as well as in the studied strains (Fig. 5). Noticeably, the ambient SRP concentration was 681 μg L⁻¹ in Reservoir Stefanski in Poland, which was 13-49 times higher than that in the other three field samples (Table. S1). In addition, the ELFp of *A. flos-aquae* was negatively related to the SRP concentration in Lake Chaohu ($r=-0.1159$, $P<0.05$, Fig. 6 A). The two parameters also correlated negatively in the cultures among four treatments in the lab-culture ($r=-0.2590$, -0.2391 and -0.1694 for *A. flos-aquae* 1249, 1171 and 1260 respectively; $P < 0.01$, Fig. 6 B).

In the cultures, the ELFp exhibited significantly positive relationship with APA_{>3.0μm} for all strains ($r=0.8145$, 0.7118 , 0.834 for P-depleted, +DOP_α and +DOP_β treatments, respectively; $P<0.01$) (Fig. S2), and the APA_{>3.0μm} accounted for the largest part of APA_T (round to 50-70%) for each treatment (Fig. S3). In the P-depleted treatments, APA_{>3.0μm} together with the percentages of cells with ELF labeling (ELFp) were significantly higher than those in the other treatments for all strains ($P<0.01$) (Fig. S3, S4). In addition, the ELFp in +DOP_α and +DOP_β treatments were significantly lower than that in the P-depleted treatments (6.6% to 9.5% of that in the P-depleted treatments; $P < 0.01$) while they were significantly higher than that in +DIP treatments (17.8 to 25.6 - fold of that in the +DIP treatments; $P < 0.01$) (Fig. S4).

Cell P quota and PPB containing cells in different culture treatments

The cell P quota in the P-depleted treatments showed significant lower values compared to that in the other ones ($P < 0.01$) (Fig. 7). The PPB were observed in each culture, with the initial percentages of PPB containing cells (PPBp) being around 0.05%. PPBp in the P-depleted treatments was significantly lower than that in the other treatments ($P < 0.01$) (Fig. 8). Noticeably, PPB containing cells could be observed at first day 15 (Fig. 8 A). PPBp began to increase at first (for *A. flos-aquae* 1171, 1260) or second (for *A. flos-aquae* 1249) day and peaked at day 5. It rapidly declined to 0 at day 15 in *A. flos-aquae* 1171 and 1260. Though demonstrating the same pattern, PPBp in *A. flos-aquae* 1249 was obviously lower than that in the other two strains. In the +DIP treatments, PPBp increased from day 1 and reached maximum at day 2, and remaining constant (85-93%) until day 42. In terms of strains, the PPBp decreased in *A. flos-aquae* 1171 and *A. flos-aquae* 1260 at day 46 to 74% and 78%, respectively (Fig. 8 D). In the +DOP_α and +DOP_β

treatments, the PPBp fluctuated regularly with incubation time. In details, the PPBp reached a level (over 80%) comparable to that in the +DIP treatments at first days and decreased to 10-40% several days later and went up and down later on (Fig. 8 B, C). Furthermore, PPB numbers per cell varied greatly among the different cultures (Fig. 9). It was rich and constant in the +DIP treatments (Fig. 9 E and F) while fluctuated in +DOP_α and +DOP_β treatments (Fig. 9 A-D).

Discussion

Whether cyanobacteria alleviated P deficiency by producing APase is rarely studied, despite numerous studies have showed that it did so in phytoplankton in freshwater and ocean by evaluating the APA *in situ* and cultures [27-32]. The current study illustrated the P utilization adoption of *A. flos-aquae* in response to various P supply and its role in regulating the growth and reproduction. A vigorous growth at the early stage in the P-depleted treatment might be owing to the cooperation of P utilization strategies in *A. flos-aquae*, e.g. producing APase, increase PPB formation and decrease cell P quota. Unexpectedly, *A. flos-aquae* in the treatment of DOP gave the cell density reaching round to 13% of that in replete Pi control, even though the quantity and quality of APase were observed and the substrate monoester was sufficient. No growth rate could be determined, while the photochemical efficiency was comparable to the treatment of +DIP, indicating that *A. flos-aquae* maintained its metabolic activity by enzymatic hydrolyzing P but not reproduced when DOP was supplied as a sole source of P.

The ELF signals in cells of *A. flos-aquae* in the field and lab-culture and the inverse relationship between the percentage of active cells (or the APA contributed by algal cells) and the SRP concentration indicated that the APase excretion by *A. flos-aquae* was regulated by the ambient P concentration, which was commonly observed so that high APA was recommended as an indicator of P stress or limitation [28, 33, 34]. It is reasonable that *A. flos-aquae* produced APase in Lake Chaohu, Zhiyin and Reservoir Orlik where the SRP concentration varied between 14 to 51 μg L⁻¹. Unexpectedly, it still produced APase where SRP concentration was 13-49 times higher in Reservoir Stefanski in Poland (Table. S1). A possible explanation for these observations might be that the threshold for APase initiation may be strain-specific so as to response to local environmental conditions. Phytoplankton species would respond to P deficiency at various concentrations of SRP. For example, *Prorocentrum minimum* began to express APA at a higher SRP concentration than *Chaetoceros neogracile*, *Chlorella autotrophica*, *Isochrysis sp.* and *Tetraselmis chui*. did using the ELF method [35]. In addition, a repression of the synthesis of APase was observed over an orthophosphate concentration threshold between 0.4 and 1 μM in *Alexandrium catenella* in the France lagoon [36]. In time-course experiment, the APA was induced at orthophosphate concentration at 0.43 mM for *Alexandrium tamarense* isolated from Hiroshima Bay [37]. These results indicated that the threshold for producing APase is species specific, while our field and culture observations indicated that it might be strain-specific among *A. flos-aquae*. On the other hand, scientists also referred to APA's contribution to phytoplankton P absorption and growth in mineralizing DOP [31, 38, 39]. It was quantified that up to 89% of the total phytoplankton P uptake was represented by APA in Lake Wivenhoe of Australia [40]. In addition to algae, free living and attached bacteria were the main contributors to APA [41-43]. In

our experiments, $APA_{>3.0\mu\text{m}}$ accounted for the most part of APA_T (see Fig. S3). We couldn't exclude the contribution of the attached bacteria to $APA_{>3.0\mu\text{m}}$. But the positive relationship between ELFp and $APA_{>3.0\mu\text{m}}$ (see Fig. S2) indicated that an anomaly thriving growth of *A. flos-aquae* in the P-depleted treatments at the first 31 days might attribute to the DOP mineralization by APase secreted by *A. flos-aquae*. The origin of the DOP might be released from the algal cells. For example, DOP was excreted when phosphate concentrations were less than the sensitivity of the molybdate test for orthophosphate in freshwater algae of *Chlorella*, *Scenedesmus*, *Navicula* and *Anabaena* [44]. Therefore, the new formation of PPB in P-depleted treatments was probably from the hydrolysis of DOP released from *A. flos-aquae* as evidenced by the fluctuation of PPBp (Fig. 8 A). This pattern of PPB forming was contradicted to the canonical point of polyphosphate as a luxury P storage molecule. In accordance with our study, polyphosphate accumulation was observed in cells of *A. flos-aquae* obtained from a summer bloom in the central Baltic Sea, where only small amounts of phosphate were available in the ambient environment, which could help to compete with other microorganisms for phosphate during bloom [45]. In addition, the phytoplankton in the western North Atlantic was enriched in polyphosphate in the P-depleted subtropical Sargasso Sea compared with nutrient-replete temperate waters, proposing that the polyphosphate cycle might produce a feedback loop, which reduces the P export when it is lacking and makes contribution to bio-available P for primary production [46]. Another explanation for this transitory growth is that *A. flos-aquae* decreased the requirement of cellular P and alleviated P deficiency to some extent. The lowest cell P quota was found in this treatment (Fig. 7). Consistently, the N:P ratios and intracellular P demand in *A. flos-aquae* TR183, *Nodularia spumigena* AV1 and *Trichodesmium erythraeum* IMS101 can be remarkable plastic in response to P deficiency [12, 47]. It was, therefore, inferred that the *A. flos-aquae* utilized some strategies such as the promotion of excreting APase, elevation of PPB formation and reduction of P demand to gain maximum growth at the circumstance of P deprivation. This hypothesis was verified by the field observation in Lake Chaohu that high percentage of ELF labeling was accompanied by high biomass. Our previous work about another N_2 -fixing genus *Dolichospermum flos-aquae* also suggested that it suffered from severe P limitation at the end of the bloom [24]. An obviously lower cell P quota was observed in cyanobacteria during their bloom (unpublished data) and its underlying mechanism needs further investigation.

Unlike the pattern of PPB forming in the P-depleted treatments, it seemed following the classical view as a luxury P storage molecule in the +DIP treatments. The types of PPB may explain this variation. Polyphosphate can be classified as acid-soluble polyphosphate (ASP) and acid-insoluble polyphosphate (AISP). ASP is considered to be used for metabolism and production of DNA and protein, it can also act as a function of short-term P pool. In contrast, AISP appears to be a form of P storage that could be utilized by the cell for growth when the P concentration is limiting in external environment [48, 49], which enables cell divisions in the absence of any exogenous phosphate [50, 51]. Additionally, this polyphosphate was only utilized by microalgae when phosphate concentration of the waste-water reached a growth limiting level [49]. The three strains of *A. flos-aquae* in the +DIP treatments remained active growth after the lag phase and gave the highest cell density with the PPBp as well as number of PPB per cell in constant levels (Fig. 8 D; Fig. 9 E, F). *A. flos-aquae* appeared to use the storage P to

achieve active growth in relation to the positive growth rate after day 46 when the SRP concentrations were decreased into the levels of that in the P-depleted treatments in the strains *A. flos-aquae* 1171 and *A. flos-aquae* 1260, where the PPBp declined then (Fig. 8 D). The main component of the PPB in the +DIP treatments might be AISP which was used upon P deficient environment.

It is expected that APase might overcome P deficiency together with amply supply of the substrate. The use of glycerophosphate and other DOP gave quite similar growth rates to that obtained with inorganic ones [52-54]. In contrast to the above results, the lowest cell densities in the glycerophosphate treatments were detected, with their growth even not as good as that in the P-depleted treatments with P-free condition. The APA in +DOP_α and +DOP_β treatments were significantly lower than that in the P-depleted treatment. Obviously, APA was not regulated by substrate DOP, which was also observed in the culture of *Microcystis aeruginosa* [32]. Low activity in the +DOP treatments could be resulted from feedback inhibition of APA by the phosphate produced in the glycerophosphate hydrolysis [54]. For example, low levels of external phosphate were sufficient to induce repression of APA in *Gelidium latifolium* [55]. Despite the repression of APA in +DOP_α and +DOP_β treatments, as well as the significantly lower exhibition of ELFp compared with the values in the P-depleted treatments, ELFp were much higher than that in +DIP treatments (round 18-to 26-fold), irrespective of the strains studied (Fig. S4). Consistently, the *Coccomyxa* isolated from Lakes Plesne and Hromnice in Czechia increased in cell-specific phosphatase activity measured combining the ELF assay with image analysis at low P concentration and the cultures treated with DOP (β-glycerol phosphate), which generated remarkably higher activity than those supplied with the same Pi concentrations [56]. Therefore, APase in *A. flos-aquae* could perform its function of hydrolyzing organic P, as illustrated by significant and positive relationship between the ELFp and APA_{>3.0μm} (Fig. S2). Furthermore, the SRP concentrations in the +DOP_α and +DOP_β treatments appeared increasing before day 15 with the expense of the DOP concentrations (Fig. 4 B, C), which was an additional evidence that the DOP had been decomposed by the enzyme. On the other hand, it was noted that three strains of *A. flos-aquae* in +DOP_α and +DOP_β treatments showed fluctuations in both PPBp (Fig. 8 B, C) and number of PPB per cell (Fig. 9 A-D) along the culture period. The PPB in the +DOP treatments might mainly be the ASP, which was rapidly utilized after approximate three days by microorganisms in waste-water [49]. Noticeably, no growth rate was determined and the photochemical efficiency in terms of *Fv/Fm* values was comparable during the whole experiment in the treatments of +DIP and +DOP. Shortly, the effective DOP mineralization and continuous processes of production and consumption of PPB in the +DOP treatments supported an active and sustainable growth but not reproduction for *A. flos-aquae* in +DOP treatments. Consistent to our observation, the growth rate of *A. flos-aquae* TR183 isolated from Baltic Sea in P-deplete treatment was a little higher than that in glycerol phosphate culture and it yielded lower biomass in glycerol phosphate culture when the Pi in the medium was not depleted than that in Pi-replete condition [12]. This suggested that *A. flos-aquae* is a species that grows better in high Pi pulse supply. The coordination of DOP mineralization and prompt utilization of PPB in *A. flos-aquae* might contribute to this maintaining growth, which was in accordance with the statement raised by Strojsova et al. [57] that the ability to decompose organic polymers and particles with

extracellular phosphatases was beneficial for longer persistence of given population in a Pi-scarce circumstance.

Conclusion

Our findings provided evidence that P utilization strategies in *A. flos-aquae*, including producing APase, increase PPB formation and decrease cell P quota, played an important role in response to P stress. DOP was an important alternative P source for longer maintenance in filamentous cyanobacteria under Pi deficiency. Persistent and active reproduction could only be achieved in high pulse Pi supply, implying that an effective [consequence](#) can be expected for combating the bloom of *A. flos-aquae* when controlling P supply.

Declarations

Availability of data and materials

The data sets supporting the conclusions of this article are included within the article and its Supplementary file.

Abbreviations

P: phosphorus

Pi: inorganic P

PPB: polyphosphate body

APase: alkaline phosphatase

SRP: soluble reactive P

DOP: dissolved organic P

APA: alkaline phosphatase activity

ELF: enzyme-labeled fluorescence

F_v/F_m: photosystem II quantum efficiency

A. flos-aquae 1249: *Aphanizomenon flos-aquae* FACHB 1249

A. flos-aquae 1171: *Aphanizomenon flos-aquae* FACHB 1171

A. flos-aquae 1260: *Aphanizomenon flos-aquae* FACHB 1260

ELFp: percentages of cells with ELF labeling

PPBp: percentages of cells with PPB staining

Funding

This work was supported by grants from the National Key Research and Development Program of China (2016YFE0202100), Natural Science Foundation of China (91951119, 41573110, 41273089), the State Key Laboratory of Freshwater Ecology and Biotechnology (2019FBZ01), Sino-Czechia cooperation financed by Chinese Academy of Sciences and Sino-Poland cooperation financed by Ministry of Science and Technology of the People's Republic of China.

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Contributions

Y. Zhou, C. Song, X. Cao, A. Sevcu and Tomasz Jurczak conceived and initiated the project. X. Cao, I. Dolinova, A. Sevcu, T. Jurczak, P. Frankiewicz, A. Wojtal-Frankiewicz and Q. Deng took field samples and chemical analysis. X. Cao and X. Chen designed the culture experiments. X. Chen and L. Wan performed the experiment and analysis. X. Chen, Y. Zhou, X. Cao, and C. Song analyzed the data and composed the manuscript. All authors analyzed the results and edited the manuscript.

Acknowledgements

Not applicable.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

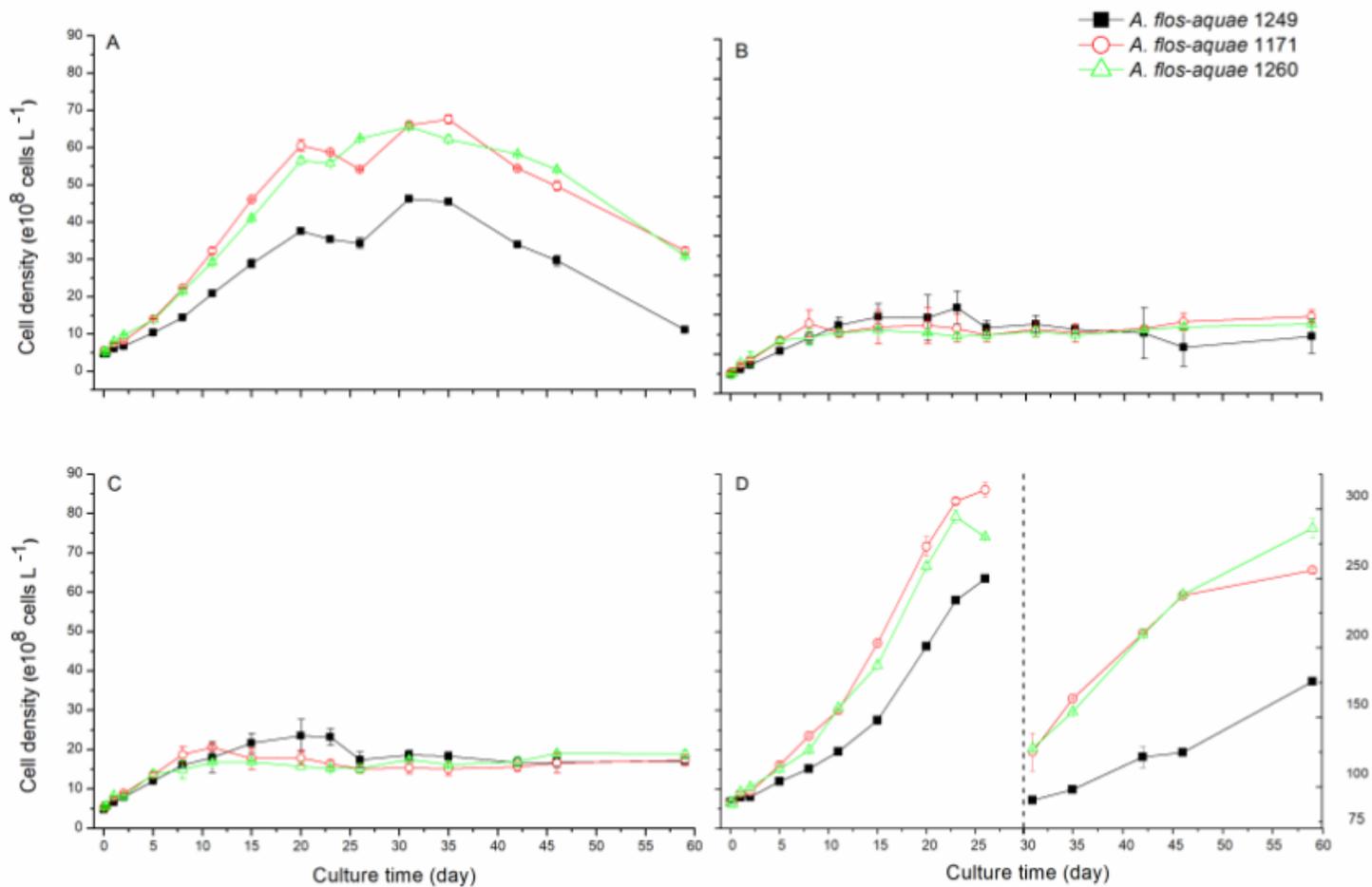


Figure 1

Time series of cell density of *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 in P-depleted (A), +DOP α (B), +DOP β (C) and +DIP treatments (D). Different ordinates scale for second half period in plot D. Error bars represent standard deviation of triplicate samples.

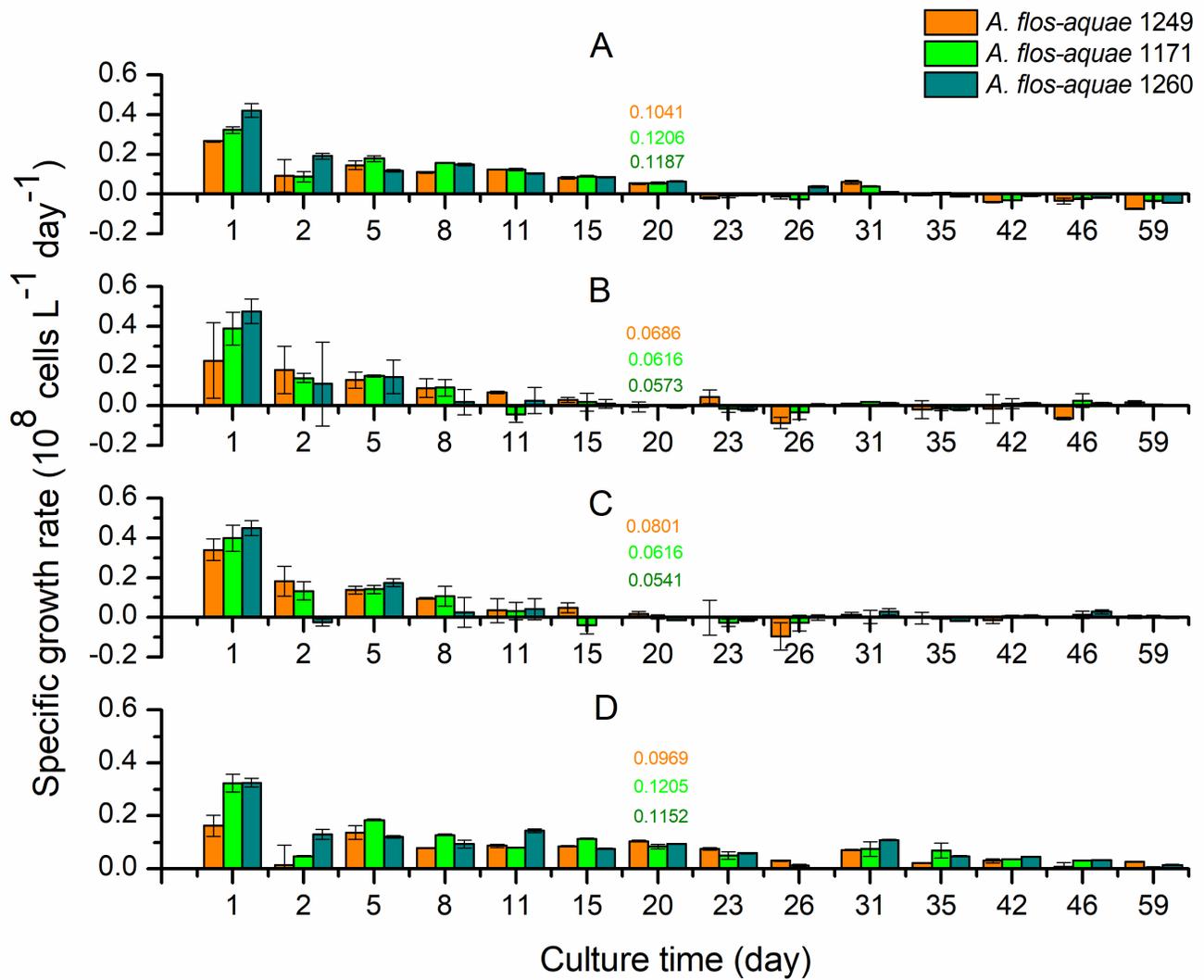


Figure 2

The specific growth rate of *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 in P-depleted (A), +DOP α (B), +DOP β (C) and +DIP treatments (D). Error bars represent standard deviation of triplicate samples. The values above day 20 mean the specific growth rate during first 20 days of *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260.

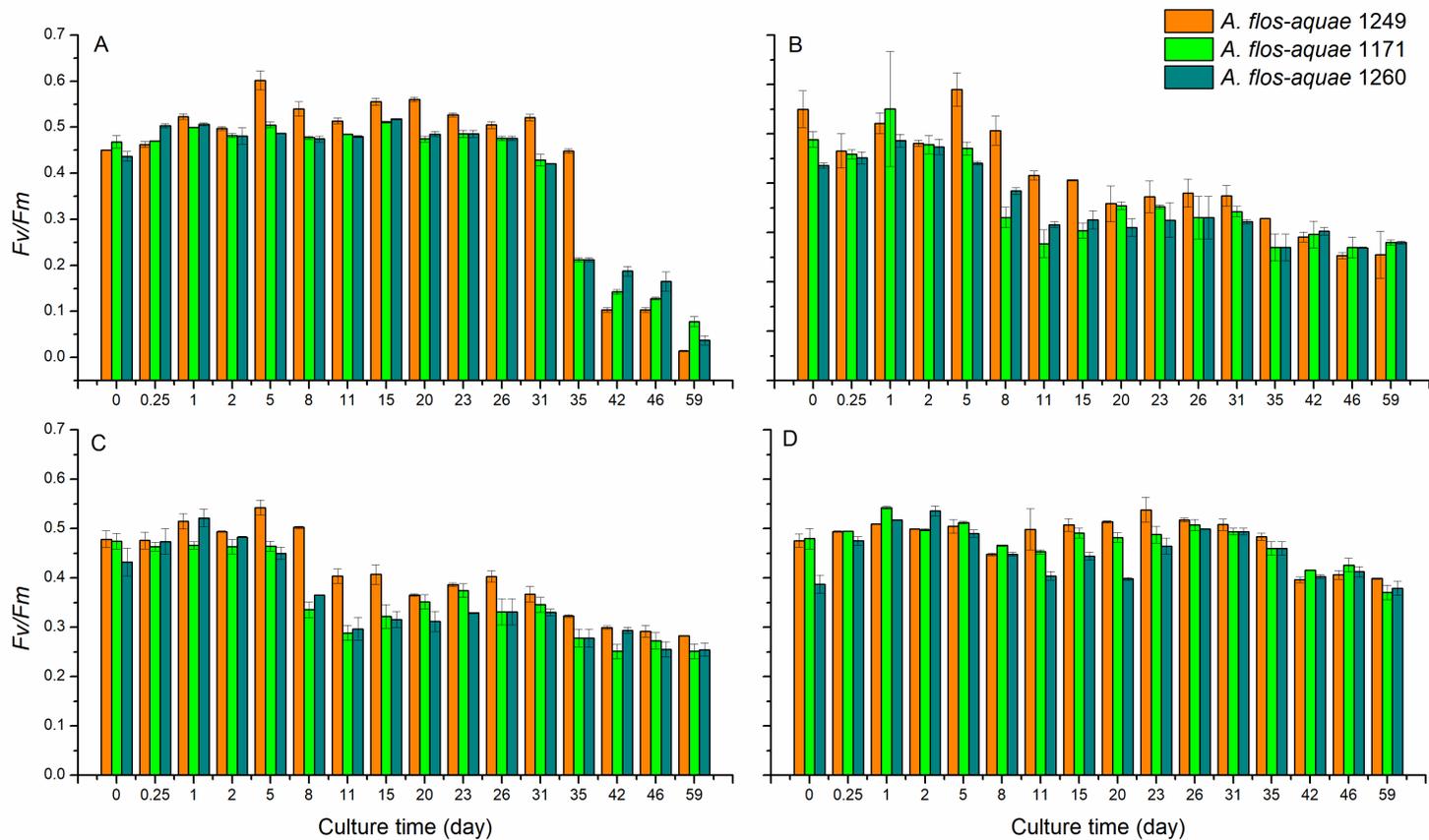


Figure 3

Photochemical efficiency of photosystem II (Fv/Fm) of *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 in P-depleted (A), +DOP α (B), +DOP β (C) and +DIP treatments (D). Error bars represent standard deviation of triplicate samples.

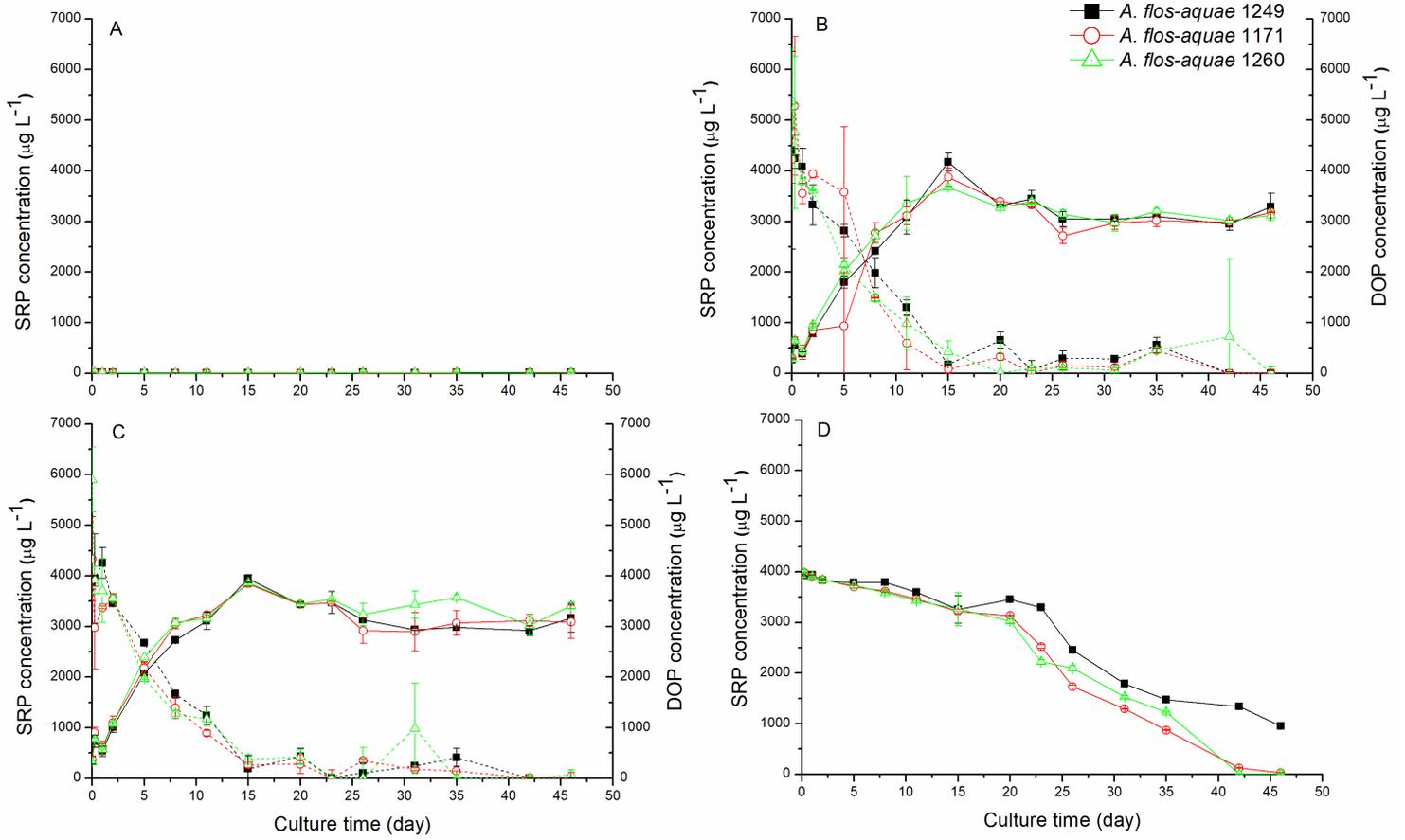


Figure 4

The P concentrations of *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 in P-depleted (A), +DOP α (B), +DOP β (C) and +DIP treatments (D). The full and dash lines represent SRP and DOP concentrations respectively, the solid square, circle and triangle represent the strains of *A. flos-aquae* 1249, 1171 and 1260 respectively. Error bars represent standard deviation of triplicate samples.

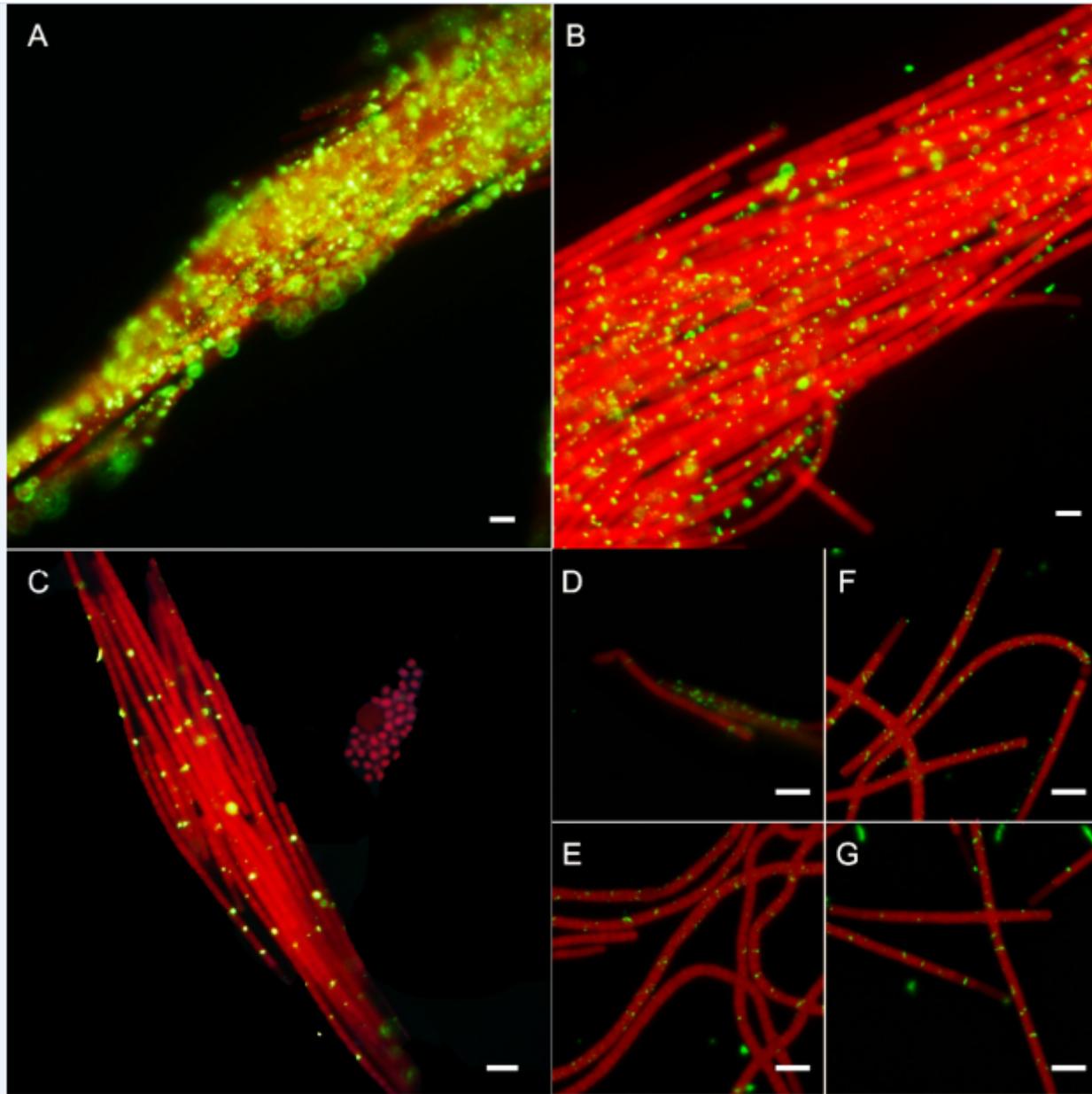


Figure 5

A. *flos-aquae* treated with ELF reagent for extracellular alkaline phosphatase (APase) detection. Green ELF fluorescence associated with *A. flos-aquae* obtained from Reservoir Orлік in Czechia (A) and Reservoir Stefanski in Poland (B), shallow lakes of Lake Chaohu (C) and Lake Zhiyin (D), as well as the culture strains of *A. flos-aquae* 1249 (E), *A. flos-aquae* 1171 (F) and *A. flos-aquae* 1260 (G). Scale bars indicate 10 μm .

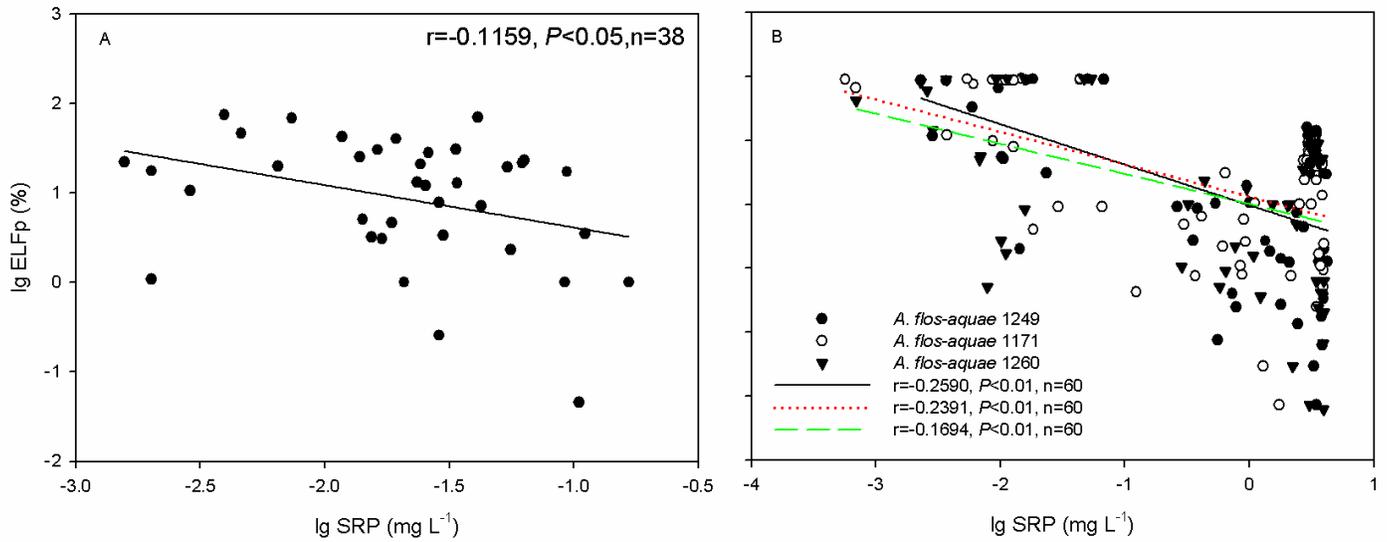


Figure 6

Relationships between the percentages of cells with ELF labeling (ELFp) in *A. flos-aquae* and the SRP concentrations in Lake Chaohu (A) and in lab-culture (B).

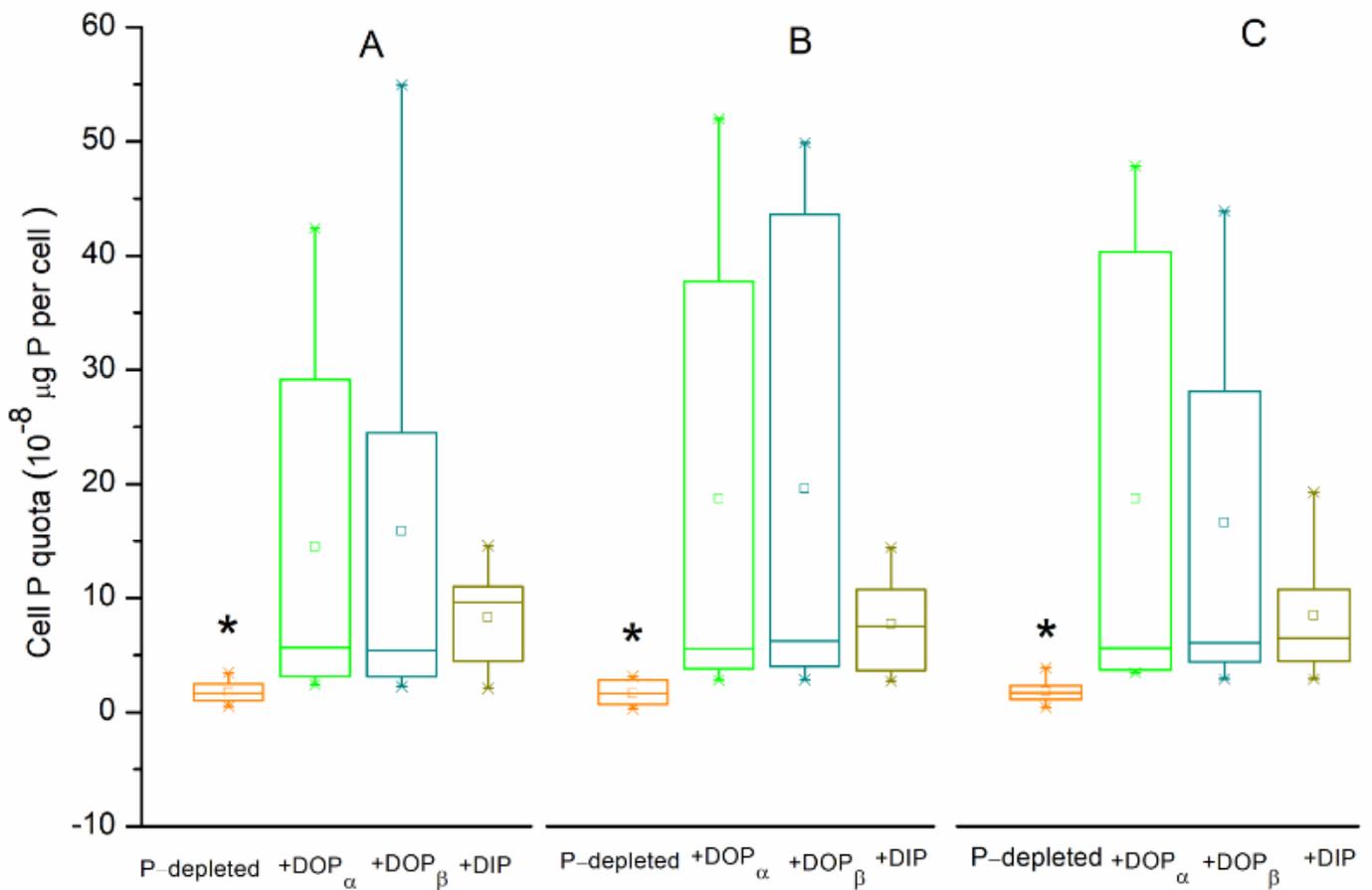


Figure 7

Box plots of temporal variations in the cell P quota in *A. flos-aquae* 1249 (A), *A. flos-aquae* 1171 (B) and *A. flos-aquae* 1260 (C) in P-depleted, +DOP $_{\alpha}$, +DOP $_{\beta}$ and +DIP treatments. The × from the two sides of the column are the minimum and maximum of ELFP, the full lines represent 25% percentiles of the variables, median values and 75% percentiles of the variables. The small squares within the column are their averages. Asterisks (*) indicate significant difference ($P < 0.01$) among treatments.

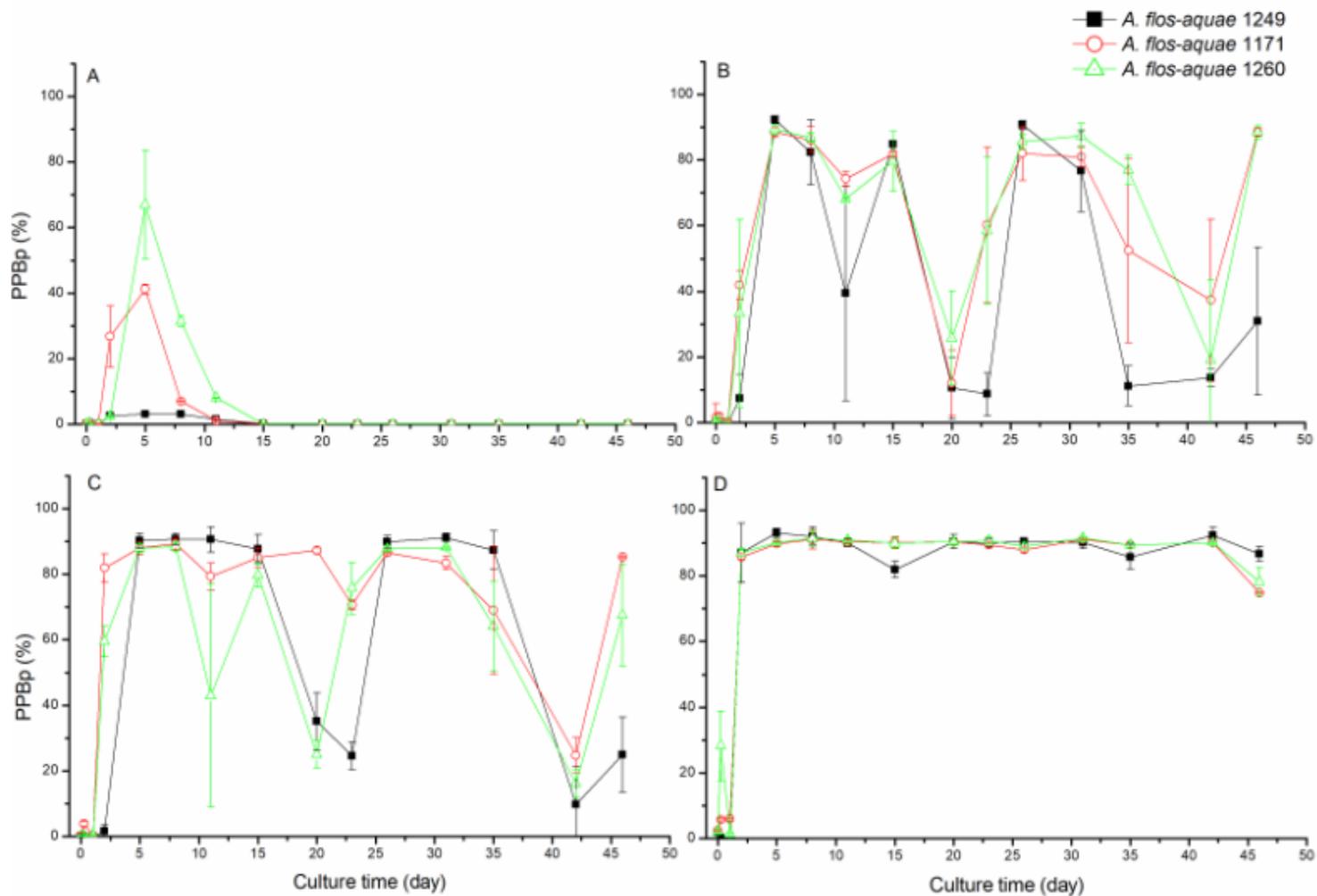


Figure 8

The percentages of PPB containing cells (PPBp) in *A. flos-aquae* 1249, *A. flos-aquae* 1171 and *A. flos-aquae* 1260 in P-depleted (A), +DOP α (B), +DOP β (C) and +DIP treatments (D). Error bars represent standard deviation of triplicate samples.

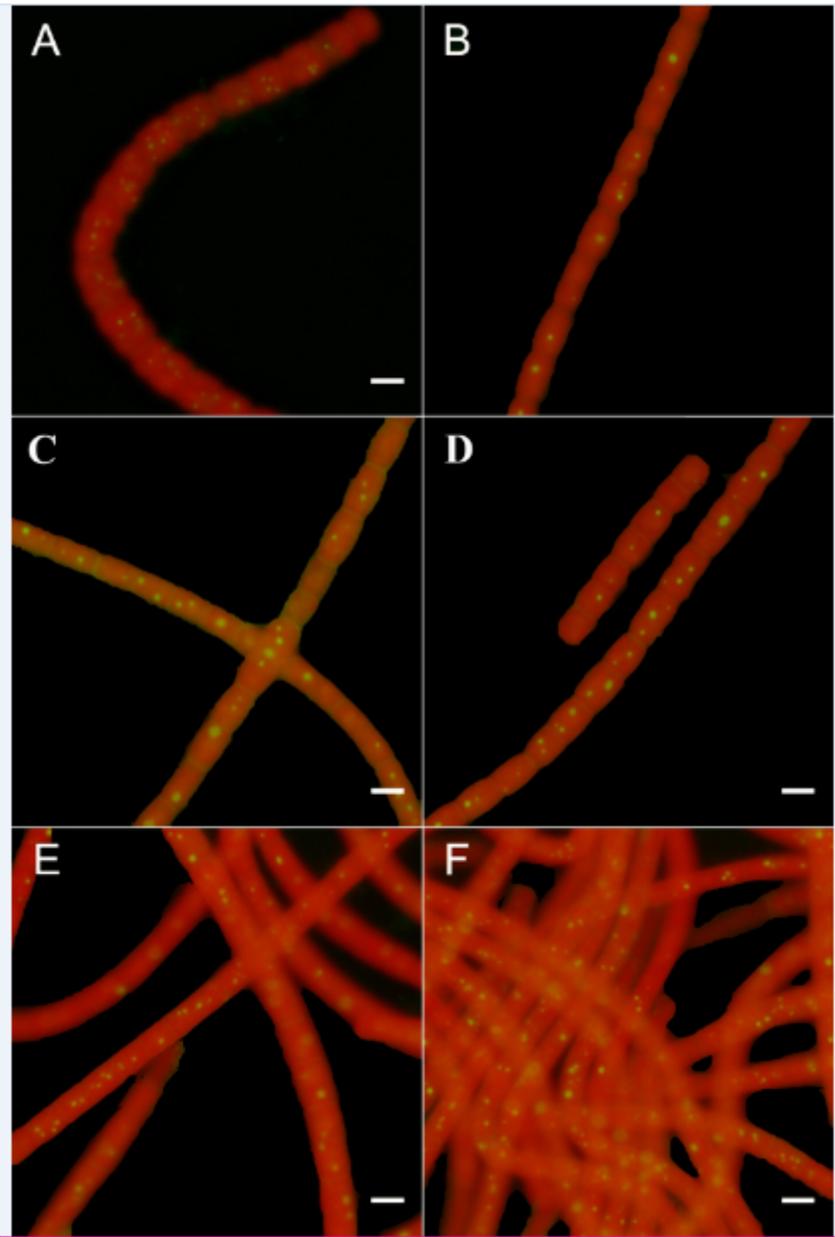


Figure 9

Polyphosphate body (PPB) detection in *A. flos-aquae* in fluorescence microscope (only shown the images of *A. flos-aquae* 1171). Chlorophyll fluorescence is red and green-yellow fluorescence marks DAPI-stained PPB. Number of PPB within cells varied in the +DOP α (A, B), and +DOP β treatments (C, D), it maintained constant in the +DIP treatment (E, F). Scale bars indicate 10 μ m.

Supplementary Files

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- [SupplementaryFilerevised.doc](#)