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Special Article: Algal Blooms

Microcystis Aeruginosa Needs a Microbiome in Order to Utilize Phosphorus from Organo-Phosphates

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Abstract

Microcystis aeruginosa is a toxin producing cyanobacterium responsible for dangerous Harmful Algal Blooms (HABs) in Lake Okeechobee Florida as well as worldwide. We investigated the potential utilization of organophosphates, as Dissolved Organo-phosphates (DOP), by this species to expand the knowledge of and eventually controls on nutrient sources and pollution. Axenic *M. aeruginosa* (PCC7806), which grew well on standard BG-11 media containing potassium dibasic hydrogen phosphate (K_2PO_4), was found to be unable to utilize certain organo-phosphates (D-Glucose-6-Phosphate {DG6P}, B-Glycerol-Phosphate {BGP}, Phytic Acid {PhA}). Non-axenic *M. aeruginosa* (UTEX LB2385) grew well on both standard BG-11 and BG-11 media in which the normal inorganic phosphate was substituted with DG6P or BGP but not with PhA. Heterotrophic bacteria in the non-axenic culture likely cleaved ortho-phosphate from the organophosphates while utilizing the organic portion as 'food'. The addition of alkaline bovine phosphatase to the axenic cultures did not facilitate utilization of organophosphates. Letting the axenic cultures enter the lysis (death) phase did not allow activation of intrinsic phosphatase enzymes as added orgo-phosphates did not reactivate growth. Co-culturing *M. aeruginosa* with *Anabaena flos-aquae*, known to utilize phosphatase enzymes, did not provide phosphorus for *M. aeruginosa*. Collectively, these results reconfirm the concept of a synergistic microbiome (phycosphere, 'interactome') being required for the utilization of organophosphates as a phosphorus source by *Microcystis aeruginosa*.

Keywords: Harmful algal blooms (HABs); *Microcystis aeruginosa;* Organic phosphorous; Microbiome

Introduction

The following quote is from a US-EPA Funding Opportunity (Number EPA-G2017-STAR-A1) "The occurrence of HABs" {Harmful Algal Blooms} is increasingly common in inland freshwater ecosystems. --- Yet basic questions of HAB occurrence, extent, intensity, and timing are largely unanswered." South Florida has been and still is experiencing nutrient (N,P) excesses in surface waters and sediments in Lake Okeechobee [1-5], coastal estuaries [6-9], and the Greater Everglades [10-14]. Sources include sewerage, notably septic systems (aka OSTDS, Onsite Sewerage Treatment and Disposal Systems) [15-18], agricultural operations [19-23], and a growing equestrian industry [24-27].

Drastic cyanobacterial blooms in Lake Okeechobee during the 1980s were due to the anatoxin producing diazotrophic (Nitrogen-fixing) species *Anabaena flos-aquae* and reductions in phosphorus loading in the 1990s appears to have help control that species [28,29]. However, as seen starting in 2005 and continuing to date, increasing dual nitrogen and phosphorus pollution now favors non-diazotrophic blooms of the toxic (microcystin) cyanobacterium *Microcystis aeruginosa* [30]. Cyanobacterial blooms dominate an ecosystem by blocking sunlight from photosynthetic organisms below. As a bloom senesces and dies, its organic matter is decomposed, removing available oxygen and leaving anoxic conditions leading to massive fish kills [31,32]. When the freshwater cyanobacterium *M. aeruginosa* begins to die, it releases large amounts of the hepatotoxic peptide microcystin [33] that can then leach into surrounding estuaries or marine waterways, expanding the detriment of the bloom. Microcystin-LR and its congeneric toxins are often responsible mammalian deaths such as dogs and cows [34-36]. In estuaries, this can pollute the water and decrease the success of many species that use these estuaries as safe havens for reproduction.

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M. aeruginosa growth is facilitated by eutrophic conditions in lakes, which can be excessively fueled by pollution from various anthropogenic sources [37]. Nonpoint nitrogen and phosphorus pollution is a well-known worldwide problem [38,39,70].

It is known that the mucilaginous masses of cyanobacteria that cause these cyanoHABs are not homogeneous and it has been hypothesized that the "interactome" in these globs of bacteria, or a microbiome, are facilitating the metabolic reactions needed to fuel massive blooms [41]. Since then, the idea of an active synergistic phycosphere of these microbial colonies has expanded [42,43].

Regarding sources of phosphorus, specifically organo-phosphates, we previously found that sugarcane leaves, husks, stalks and roots contain beta-glycerol-phosphate, fructose-6-phosphate, glucose-6-phosphate as well as phosphate mono- and di-esters in addition to ortho-phosphate [21]. Therefore, the leaching of organo-phosphates into adjacent water bodies, such as Lake Okeechobee, can well be expected from sugarcane and other land plants as well [44]. One study has revealed that meadow or forest soils had between 79-92% or 13-37% organic phosphorous compounds, respectively [45]. Organo-phosphates are therefore phosphorus sources that need to be fully examined for their participation in the nutrient supplies creating harmful algal blooms.

Microcystis aeruginosa must compete with all other autotrophic and heterotrophic organisms, as well as inorganic precipitation reactions (*e.g*. $Fe^{3+} + PO_4^{3-} \rightarrow FePO_4$), for soluble reactive phosphorus (SRP, ortho-phosphate). Therefore, we undertook the current study to investigate the potential utilization of organic phosphorus by *M. aeruginosa*.

Materials and Methods

Stock culture conditions utilized light at 70 µmol phota m^{-2} s^{-1} in a 12hr light/dark cycle with BG-11 media [46,47] containing 2 mM $NaNO_3$ and 0.23 mM K_2PO_4. The nitrate concentration was reduced from the standard 17.6 mM to 2 mM to better mimic [48,49] the Redfield Ratio of 16:1 N:P [50]. Tests on the utilization of organic-phosphates were performed by substituting the 0.23 mM K_2PO_4 with an equimolar amount of D-glucose-6-phosphate {DG6P: Sigma Aldrich #G7375}, b-glycerol-phosphate {BGP: SigmaAldrich #50020}, or phytic acid {PhA: SigmaAldrich #P8810}. Bovine alkaline phosphatase (SigmaAldrich # API-RO) was utilized for testing exogenous phosphatase activity on dissolved organophosphates. Glyphosate (SigmaAldrich #45521) was also tested as a phosphorous source in place of dipotassium phosphate. All samples were cultured in 125 mL PETG flasks (ThermoFisher # 50-233-5807) rotating at 130 rpm in a gyratory water bath shaker at 26ºC. To ensure the axenic stocks and inoculates stayed axenic, all culture manipulations were performed in a Baker Sterile GARD-111 biosafety cabinet with sterile conditions. Media was sterile filtered or autoclaved, depending on the organic contents of the media. Extreme caution was taken when working with all stock and inoculates to ensure there was no contamination. Routine checks for contamination were performed using a fluorescent microscope and a light microscope, utilizing DAPI (ThermoFisher # EN62248) staining and the natural fluorescence of the cyanobacteria to evaluate contamination. All experimental trials were inoculated at a level of about $1x10^5$ cells per mL and cultured in 50 mL of media. Growth was tracked over time utilizing cell counts (cells per mL) using a Thermo Fisher Invitrogen Countess II Cell Counter. The counter was checked for accuracy and precision. A standard curve was created with an r^2 value of 0.996. All inoculates were grown into their stationary phase unless there was negative or no growth. Cell counts were performed every 3-4 days after an initial 4-day inoculation / lag phase period. Sterile procedures were ensured using the biosafety cabinet. It is estimated that a normal growth curve would take approximately 30-40 days, as cell counts will stop after reaching the end of their stationary phase (i.e., their lysis or death phase).

Axenic *Microcystis aeruginosa* stock (PCC7806) was from the Pasteur Culture Collection of Cyanobacteria (Institut Pasteur of Paris). The culture was incubated for at least 5 days to allow for adequate growth prior to being separated further into additional stock cultures. Stock solutions for these trials were kept under the same conditions as all other experimental trials.

The non-axenic strain of *M. aeruginosa* previously studied by our group. The UTEX LB2385 strain is another widely used and studied culture of *M. aeruginosa*. This strain was obtained through the University of Texas at Austin's Algal culturing center (UTEX). This species was grown in the same conditions given above and stocks were allowed to incubate and routinely refreshed.

A culture of *Anabaena sp.,* UTEX 2576, was acquired from the University of Texas at Austin's algae culturing center (UTEX) and used as a potential source of phosphatase activity. This culture strain was also grown in the same conditions as the *M. aeruginosa* strains of PCC7806 and UTEX LB 2385. The same gyratory water bath shaker with the same RPM and water temperature, as well as the 125 mL PETG flasks, were used. The *Anabaena sp.* stocks were also grown in BG-11 medium. *Anabaena sp.* cells could not be counted with the cell counter as they are filamentous. Therefore, manual microscopic cell counts were utilized to assess growth.

Results and Discussion

Growth of Axenic and Non-Axenic *M. Aeruginosa* on BG-11 Media

Both the axenic (Figure 1a) and non-axenic (Figure 1b) *M. aeruginosa* cultures grew well on normal BG-11 in which phosphorus is provided as a form of ortho-phosphate (PO_4^{3-} as K_2HPO_4). Each of the three separate trial data sets are plotted as the mean of three runs, equaling nine trials for both the axenic (1a) and non-axenic (1b) cultures. Cell counts and growth was stopped when the cultures were more than 10 days int the stationary phase.

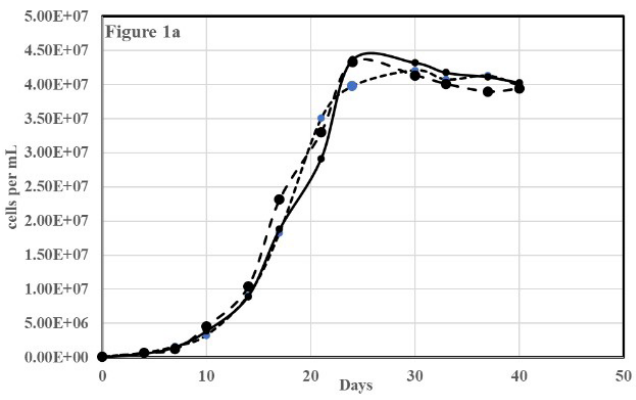

Figure 1: Growth of (1a) axenic PCC-7806 and (1b) non-axenic UTEX-LB2385 *Microcystis aeruginosa* in standard BG-11 media.

Zero (0.00E+00) on the Y-axis in these and following plots indicates the inoculation stage of ~ 1E+05 cell / mL.

Growth Trials of Axenic and Non-Axenic *M. Aeruginosa* with Organophosphate Substituted BG-11 Media

Next, we substituted the standard Dissolved Inorganic Phosphate (DIP), dipotassium phosphate, with various Dissolved Organic Phosphate (DOP) species. These are coded within the legend for Figure 2. As always, each set of trial data plotted is the mean of triplicate cultures. Each trial also included a reference run (black trendline) with standard BG-11 for both the axenic and non-axenic cultures.

The axenic *M. aeruginosa* cultures were unable to grow on any of the three DOP compounds provided as a potential phosphorus source. This indicates a lack of 'active' phosphatase enzymes in this species, or at least this clade. The very slight increase in cell density for the phytic acid and b-glycerol-6-phosphate trials on the axenic culture is attributed to the use of 'storage' phosphorus reserves [51-53]. The non-axenic culture grew equally well on D-glucose-6-phosphate and b-glycerol-phosphate but was unable to utilize the hexaphosphate compound phytic acid. The activities if coincident heterotrophic bacteria in the microbiome [42,43,54] forming the "interactome" [41] of this culture are responsible for the cleavage of phosphate from the organophosphate species.

Aside from synergistic heterotrophic bacterial activities releasing phosphate from organophosphate compounds for use by *M. aeruginosa*, it is reported that high Ultraviolet (UV) radiation can alter phosphatase activities and high Dissolved Organic

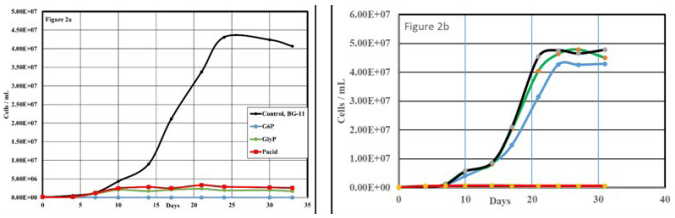

Figure 2: Axenic (2a) and non-axenic (2b) cultures of *M. aeruginosa* grown on BG-11 (black) and BG-11 phosphate substituted with D-glucose-6-phosphate (blue), β-Glycerol-phosphate (green) or phytic acid (red).

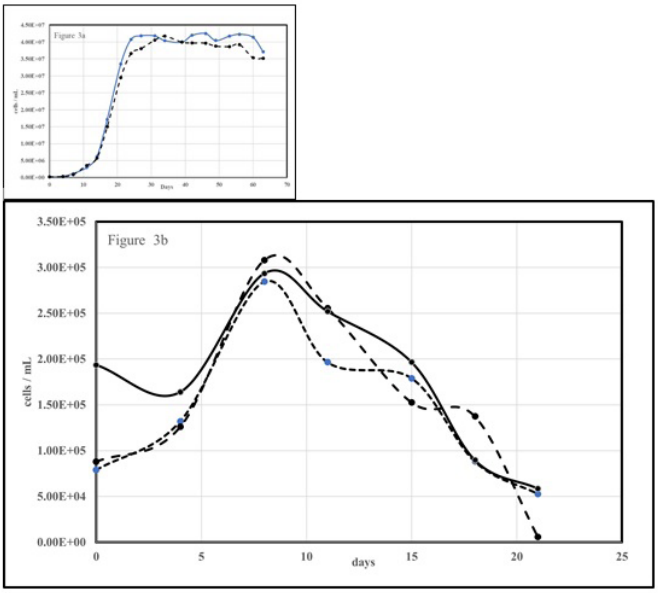

Figure 3: (3a) Axenic M. aeruginosa grown with 10% P for induction of P stress (3b) P-stressed cells incubated with B-Glycerol-6-Phosphate substituted (BGP) BG-11.

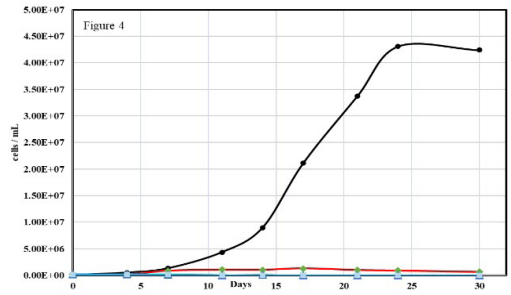

Figure 4: Axenic *Microcystis aeruginosa* grown on standard BG-11(reference, black trendline) and BG-11 media with dipotassium phosphate being substituted with an equimolar amount of b-glycerol-6-phosphate (green trendline), glucose-6-phosphate (blue trendline), or phytic acid (red trendline).

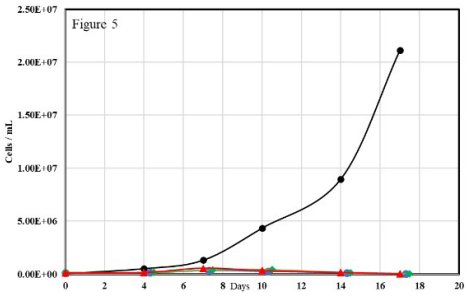

Figure 5: Axenic *Microcystis aeruginosa* co-cultured with *Anabaena flos-aquae* on standard BG-11 (reference, black trendline) and BG-11 media with dipotassium phosphate being substituted with an equimolar amount of b-glycerol-6-phosphate (green trendline), glucose-6-phosphate (blue trendline), or phytic acid (red trendline).

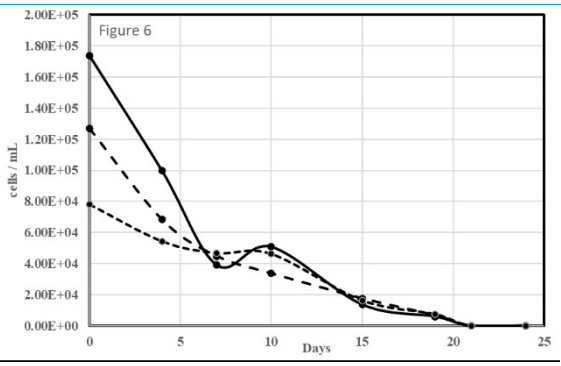

Figure 6: *Microcystis aeruginosa* growth trials of in BG-11 media having inorganic phosphate (K_2PO_4) substituted with an equimolar amount (0.23mM) of glyphosate. Trendlines for means of triplicate runs of three different starting cell concentrations.

Matter (DOM) can act as an antioxidant decreasing that effect [55]. This point should be remembered when dealing with the native 'interactomes' in waters such as Lake Okeechobee which is high in DOM. That is, the phosphatase activities of the coincident heterotrophic bacteria would likely be little affected in high DOM containing waters.

Growth Trials of Stationary-lysis Phase Axenic *M. Aeruginosa* on Organophosphate Substituted BG-11 Media

Phosphate stress in *M. aeruginosa* may induce phosphatase activities [55,56]. We grew the axenic culture (PCC7806) on BG-11 with limited (10% normal; 0.023 mM [23 mM] K_2PO_4) phosphate (Figure 3a). We then took the stressed *M. aeruginosa* in the beginning of the death or lysis stage (~62) days and inoculated it into BG-11 media that had the DIP (K_2PO_4) replaced with B-Glycerol-6-Phosphate (BGP) or glucose-6-phosphate (G6P). The three runs with the BGP substituted BG-11 is shown in Figure 3b. A very small increase (appx. doubling) in cell density occurred within the first week, likely due to legacy (storage)

P within the inoculum. After that cell death was very fast, again indicating that axenic *M. aeruginosa* cannot obtain phosphate from the cleavage of organophosphates without a synergistic microbiome. An identical trial (not shown) was performed with G6P substituted BG-11 and the results were essentially identical.

Growth Trials of Axenic *M. Aeruginosa* in Organophosphate Substituted BG-11 Media in the Presence of Alkaline Phosphatase

It is noted here that certain subspecies of *Microcystis* (e.g. have been shown to possess alkaline phosphatase enzymes [58] and/or activity [59,61]. However, as we have shown (Figure 2 above), *M. aeruginosa* (PCC-7806) lacks Alkaline Phosphatase Activity (APA). This and the following section probe potential extracellular APA.

Bovine alkaline phosphatase was added as18, 36 or 73 mL of 1500 U stock to BG-11 media that had the DIP (K_2PO_4) in BG-11 substituted with D-glucose-6-phosphate, b-Glycerol-phosphate or phytic acid. The results of these culture trials are shown in figure 4. It is noted here that all trendlines are present in this figure but overlap to the point that they, especially the blue and green trendlines, are not apparent. The presence of extracellular alkaline phosphatase without an organism's participation did not result if phosphate cleavage from these three organophosphates. Again, it is the activity of the microbiome [42,43], also called interactome [41], that is required to provide DIP from DOP for use by *M. aeruginosa*.

Growth Trials of Axenic *M. Aeruginosa* Co-Cultured with *Anabaena Flos-aquae*

Microcystis aeruginosa and *Anabaena flos-aquae* often coexist in Lake Okeechobee [62] and *A. flos-aquae* is known to utilize phosphatase enzymes [58,60]. We co-cultured these two species to determine if axenic *M. aeruginosa* could obtain DIP from the activities of *A. flos-aquae.*

The data in Figure 5 reveals that, even though *A. flos-aquae* can obtain its own inorganic P from the DOP compounds b-glycerol-6-phosphate and glucose-6-phosphate, as seen by microscopic evaluation of its growth in the co-culture, it does not supply DIP for uptake by *M. aeruginosa*.

Growth Trials of Axenic *M. Aeruginosa* Substituting Glyphosate for Inorganic Phosphorus

Glyphosate (N-phosphonomethyl-glycine), the active ingredient in Roundup® is a herbicide in widespread use [63] and degrades rapidly in the environment [63,64]. Its high use in agriculture [63], notably around Lake Okeechobee [65], and reports that it can serve as a phosphorus source for certain phytoplankton [66,67] prompted us to test it with axenic *M. aeruginosa*. Figure 6 contains the results of three separate trials using glyphosate as the sole P source. Immediately apparent is that glyphosate did not aid growth but rather led to the rapid death of all *M. aeruginosa* cells. Glyphosate in natural Lake Erie waters was shown lead to a decrease in M. aeruginosa abundance [68]. However, it is also known that glyphosate degradation by bacteria, fungi and light can lead to increases in dissolved inorganic phosphorus which aids the growth of phytoplankton in P depleted environments [68-70].

Conclusions

Axenic *Microcystis aeruginosa* (PCC7806), grew well on standard BG-11 media containing potassium dibasic hydrogen phosphate (K_2PO_4), and was found to be unable to utilize certain organo-phosphates (D-Glucose-6-Phosphate (DG6P), B-Glycerol-Phosphate {BGP}, Phytic Acid {PhA}). *M. aeruginosa* was found to not be able to use glyphosate directly as a phosphorus source. Non-axenic *M. aeruginosa* grew well on both standard BG-11 media and BG-11 media with DG6P and BGP as the sole phosphorus source but was unable to utilize phytic acid. It is apparent that heterotrophic bacteria in the non-axenic cultures were responsible for cleaving phosphate from the organophosphates. It is likely that the heterotrophic bacteria utilize organophosphates for phosphate as well as organic matter as food. Therefore, once the phosphate requirements are met for the heterotrophs, additional phosphate cleaved from the organophosphates is released into the media for use by other organisms such as *M. aeruginosa*. This synergistic action whereby the heterotrophs release phosphate for use by *M. aeruginosa* substantiates the concept of a synergistic microbiome or "interactome" [41].

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