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Full Length Research Paper

Response of green alga *ULOTHRIX ZONATA* to nitrogen and humic substances

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Nitrogen and phosphorous are essential elements to aquatic biota. Different types of humic substances (HS), such as humic acid (HA) and fulvic acid (FA) have impact on the freshwater nutrients. The aim of the study was to examine how the presence of humic substances may affect nutrient availability to the growth of *ULOTHRIX ZONATA* (Weber and Mohr) Kutz. Samples were incubated on different nutrient treatments: nutrient sufficient (+NP), nitrogen deficient without HA or FA (-N) and with HA or FA (-NH/-NF) and phosphorous deficient without HA or FA (N) and with HA or FA (NH/NF). The results demonstrated that addition of HA or FA increased the production of Chlorophyll *A* and cell density as compared with the cultures exposed to nutrients only at the same concentration. *U. ZONATA* represented the highest growth efficiency (cell density 14.5 x10^7 cells/ml, determined by counting number of cells per milliliter in suspension of *ZONATA.* filamentous green alga) under N deficient with HA or FA treatment. During the experiment, whereas the N deficient treatments had the lowest cell numbers (2.2 to 2.5 x 10^7 cells /ml). It is suggested that HA and FA could be of great importance in the growth of green alga *U.*

Key words: Fulvic acid, green alga, growth efficiency, humic acid.

INTRODUCTION

Several algal species have been dominated in the phytoplankton community both fresh and marine water (Lee, 1973). Their prevalence has been related to excessive nutrients in particular nitrogen and phosphorous that is, especially Cyanobacteria. Even though nitrogen and phosphorous are essential elements for algal growth and it is generally considered that they are the main source involved in eutrophication to deteriorate water quality (Shannon and Brezonik, 1972).

In contrast, algal activity has been used in the removal of excess nutrients from aquatic ecosystem. Previous studies mentioned that many algal species can grow up in the presence of organic compounds which are the source of phosphorous in natural water (Boekel, 1991). Regarding to the work explained here, extra cellular enzymes of algal cells can utlilize phosphorous from organic compounds in phosphate-depleted media (Islam and Whitton, 1992). Nitrogen bound to humic substances (HS) may directly or indirectly stimulate algal growth especially dinoflagellates (Thurman, 1985; Doblin et al., 1999; Gagnon et al., 2005). However, there is lack of knowledge on how efficiently HS can be utilized by algal species in the presence of nitrogen in stream.

If organically bound nutrients coupled to HS can support algal growth, this process may be greatly significance in natural waters that are influenced by terrestrial run off. Conversely, the presence of HS has been shown to stimulate photosynthesis in the green alga, such as *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* by reducing toxicity of heavy metals (Prakash et al., 1973; Toledo et al., 1982). HS may also inhibit the growth of cyanobacteria, *Microcystis aeruginosa* and *Anabaena circinalis* by reducing the essential metals (Jones, 1992; Carlsson and Granéli, 1993; Doblin et al., 1999). However, the mechanism responsible for the ability of algal species to use outflow of nitrogen load in the presence of HS is insufficiently understood.

In spite of the aforementioned information, HS originating from natural sources has until now received little attention as a source of nutrients together with nitrogen (N) for periphytic algae. Periphytic green algae are dominated in many aquatic ecosystems engages

Table 1. The composition of fulvic acid and humic acid used in the experiment.

Components	C (mg)	H (mg)	N (mg)
Humic acid	35.08	3.55	0.95
Fulvic acid	49	4.33	2.89

their capability to utilize the HS as a nutrient source. These algae are referred as bioindicators because they can reflect environmental condition in stream. For example, *Ulothrix zonata* (Weber and Mohr) Kutz, and *Cladophora glomerata* (L.,) Kutz, are the dominant attached filamentous forms in the littoral zone of lake (Graham and Wilcox, 2000). These algae preferably colonize on exposed permanent substrate and are highly conspicuous especially in areas of excessive nutrient enrichment (Richter et al., 2003). Dam construction altered the natural cycle of water flow that is, nitrogen load is greater than the standard level in downstream of Nakatsugawa River in Japan.

The aims of this study are to examine how the presence of humic substances (humic acid (HA) and fulvic acid (FA)) may affect excessive nitrogen bioavailability and observe the interactions between humic substances and N on the growth of periphytic green algae. The periphytic green alga *U. zonata* was used as the test organism. Experiments were carried out in the laboratory under nitrogen- deficient and sufficient conditions. Humic substances added to the growth media were isolated from Suwannee River (aquatic humic substances).

MATERIALS AND METHODS

Sample preparation

Non-axenic culture sample of the periphytic green alga, *U. zonata* was used in the experiments. The culture sample was obtained from the National Institute for Environmental Studies, Japan (NIES) collection and was initially grown in WC medium (Guillard and Lorenzen, 1972). Then cultures were transferred to natural media which was prepared with filtered (0.45 m) and autoclaved river water collected from Nakatsugawa River, Japan to provide natural conditions similar to field.

U. zonata is a filamentous form and filaments of the alga were inoculated on N and P deficient and sufficient growth medium containing 0.5 to 14 m P and 0.5 to 200 m N. The inoculums of $1x10^{7}$ cells ml^{-1} (around 200 filaments ml^{-1}) was added at the beginning. The cultures were incubated 40 days at 10C ± 0.1°C under 12 h:12 h dark-light photo period. The photon flux density of light source was maintained at c. 15 E m^{-2} s^{-1} of photosynthetically available light (PAR). All experiments were run in incubator (LH-55-RD/S(CT), Japan) providing constant natural conditions. The controls were prepared in same culture medium with sufficient nutrient to compare the growth of *U. zonata* in nutrient sufficient and deficient condition without HA/FA and with HA/FA.

The experiments consisted of five series of sample sets with the following treatments, 4 controls with nutrient sufficient medium (NP); N 200 mol l^{-1} and P 14 mol l^{-1}, 4 samples with addition of K_2HPO_4, and $NaNO_3$, phosphorous sufficient medium (-N) to give final concentration 0.5 P including 200 N mol l^{-1} , 4 samples with addition of $NaNO_3$ and K_2HPO_4, nitrogen sufficient medium (+N), to give final concentration 200 N including 0.5 P mol l^{-1} and 4 samples with initial addition of HA/FA that gave HA/FA concen-tration of 4 mg/l with N deficient (-NH/NF). 4 samples with initial addition of HA/FA that gave HA/FA concentration of 4 mg/l with P deficient (NH/-NF). Cultured samples were mixed with river water.

The initial nutrient concentrations of collected river water were as follows: NO_3^- <0.01 mol l^{-1}, PO_4^{3-} 0.001 mol l^{-1}. Vitamins were added according to Schöne and Schöne (1982) in order to prevent growth limitations caused by lack of these compounds.

Humic substances characteristics and sample preparation

Elemental compositions of humic substances were measured using CHN analyser (Yanaco, CHN CORDER MT-5). Suwannee River humic substances such as humic acid (HA) and fulvic acid (FA) were obtained from the International Humic substances Society. Results are presented in Table 1.

Humic substances were dissolved in a $NaClO_4$ solution containing dilute NaOH, and FA and HA concentration adjusted to 100 mg/l in 0.01 M at pH 8.0. FA and HA solutions with concentrations of 0.01 to 10 mg/l were made by dilution of the 100 mg/l of the 100 mg/l solution (Nagao et al., 2003).

Measurements

Small portion of *U. zonata* samples in each culture on different nutrient treatments (nutrient sufficient) (+NP), nitrogen deficient without HA or FA (-N) and with HA or FA (-NH/-NF) and phosphorous deficient without HA or FA (N) and with HA or FA (NH/NF) was mounted on a glass slide. Filaments of the alga were separated carefully using needles and a microscope. Three sub samples of each were taken for measurements of optical density and Chlorophyll a. Another three subsamples of each were preserved with 2% formaldehyde solution for cell counting. According to the method described by APHA (1995), cell density was performed by counting number of cells per milliliter in suspensions of filamentous green alga *U. zonata* using a light microscope (Olympus B40). Cell counting was carried out nine occasions during the experiment. At least 50, but often than 400, filaments were counted for each treatment (depending on the abundance of the samples), giving standard deviations ±28% or ±10% respectively.

The optical density and chlorophyll a concentration were used to track algal growth. Chlorophyll a was measured following standard methods after filtration (GF/F), extraction with acetone, absorption measurements at 680 and 750 nm (Jeffrey and Humphrey, 1975). Optical density (OD) was measured as absorbance at 680 nm.

DNA concentration

10 to 12 mg portion of each sample on different nutrient treatments was weighed and washed several times using distilled water. After adding 500 l distilled water, samples were vortexes well around 5

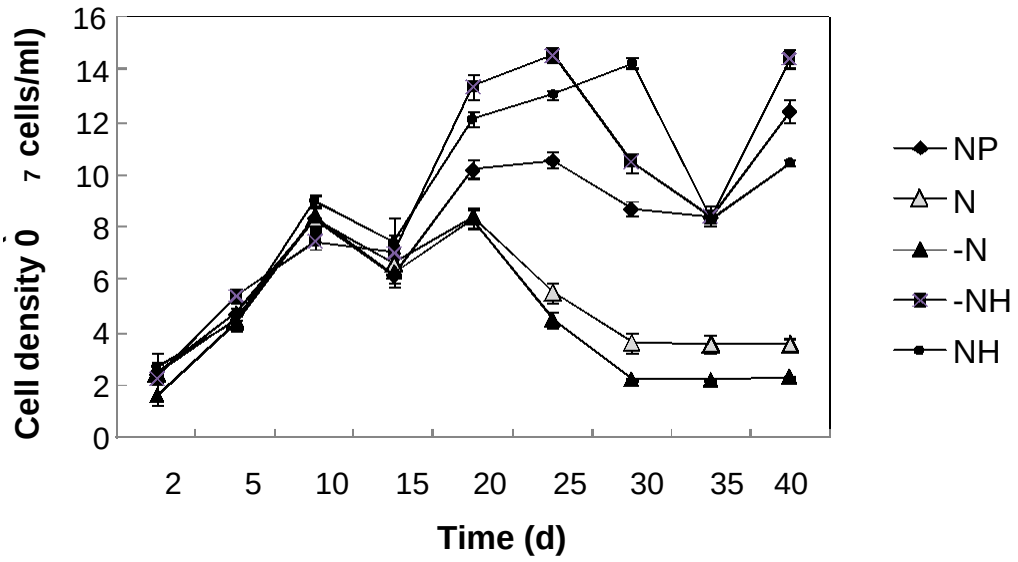

Figure 1. *Ulothrix zonata* cell densities were in different nutrient media (NP – N and P sufficient medium; -N – N deficient medium; N – N sufficient medium; NH – N sufficient medium with humic acid; -NH – N deficient medium with humic acid).

min and water was replaced with 0.5 M NaOH. Then samples were transferred to micro homogenizer vessel and well grounded samples were centrifuged at 15000 rpm for 5 min 4°C. 30 l supernatant of each sample was transferred to another fresh tube and was mixed well with 90 l of 200 mM Tris HCl (pH 8.8) (Sambrook et al., 1989). DNA concentration of each treatment was measured using spectrophotometer (Bio Spec - nano, Shimadzu, Japan).

Carbonic anhydrase activity

Carbonic anhydrase (CA, EC 4.2.1.1) activity of *U. zonata* was assayed potentiometrically using the procedure of Wilbur and Anderson (1948) . Each sample of 10 to 20 mg fresh weight (FW) on different nutrient treatments was ground in mortar, at 0 to 2°C, and then transferred to a test tube containing 3 ml of ice cold buffer (Tris, 25 mM ascorbic acid, 5 ml EDTA, pH 9.0). The reaction was initiated by addition of 1 ml of ice cold CO_2-water (bubbled with CO_2, commercial). The time required for the pH to decrease from 8.0 to 7.0 was measured. Relative enzyme activity (REA) in the test samples was calculated using the Equation (1), expressed as:

$$[(t_0/t_c)-1]g^{-1}FW, \quad (1)$$

where t_0 is the time required for pH change when non-catalyzed (buffer only) was used in place of sample and t_c the time required for pH change when catalyzed (in the presence of sample extract) was present in reactions.

Statistical analysis

We performed ANOVA for the samples variables, that is, cell density, nutrient concentration, Chlorophyll a and enzyme activity, in order to search for differences between the treatments. For those variables ANOVA reveals the significant differences between the treatments. Here, we performed the univariate test (One way ANOVA) for each data variables in order to identify the differences between the treatments which were significant.

RESULTS

The periphytic green alga *U. zonata* was grown in both N deficiency and sufficiency medium with and without HA and FA. *U. zonata* was reached to steady state condition after day 15 of the experiment (Figure 1). *U. zonata* growing in the N-deficient with HA/FA and N deficient without HA/FA exhibited large variations of cell densities (Figure 1). The highest cell numbers were observed in N sufficient and N deficient medium with addition of HA/FA. On day 35, cell density of *U. zonata* in the –NH and the NP were similar and higher than in the N deficient cultures without HA/FA (Figure 1). Cell densities of *U. zonata* were significantly lower in the P deficient medium (N) than in other treatments, with the exception of NH cultures on sampling day which had cell numbers not significantly different from -N treatments (Figure 1). The nitrate concentrations decreased from 30 to 4 mol/l in the N sufficient medium (Figure 2). Statistical analysis shows that there were significant differences ($p < 0.05$) between the treatments of all variables. The differences between the treatments were significant for ($p < 0.05$) on each day for cell density of *U. zonata* as well as NO^-_3, and Chlorophyll a.

The results presented here on the influence of HA and FA on the *U. zonata* under both nutrient sufficient and deficient conditions. From Suwannee River HA and FA were added at a concentration of 4 mg/l to media containing N at concentration $p < 0.5$ to 200 m, and $p < 0.5$ to 14 m, and stimulated *U. zonata* growth and chlorophyll production.

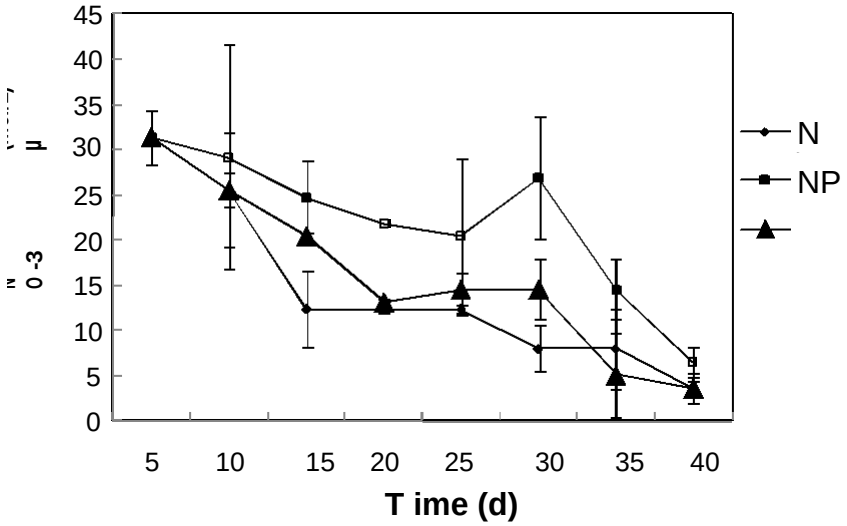

Figure 2. Changes in nutrient concentrations in different nutrient treatments media (NP – N and P sufficient medium; N – N sufficient medium; NH – N sufficient medium with humic acid).

The addition of HA/FA in the presence of N <0.5 and P >14 concentrations increased the optical density and the production of chlorophyll as compared with the cultures exposed to the same concentrations at N <0.5 and P >14 without HA /FA. 4mg/l HA/FA added to media significantly support the growth of test organism under nutrient deficient condition.

DNA concentration and patterns of the CA activities

Intracellular contents of DNA of *U. zonata* cells were higher in the N deficient medium with HA than in all other cultures on day 35 (Figure 3). On day 35, no significant differences in the intracellular DNA contents were found between N and NH/NF treatments, which had higher values for these variables compared to the other treatments (–N). The N-deficient treatment without HA/FA had lower intracellular amounts of DNA than the nutrient sufficient medium (Figure 3). Although there were significant differences found under treatments of HA and FA. *U. zonata* showed higher activities under HA compared to FA.

The CA activities in the studied algae ranged between 42 and 350 REA g^{-1} FW following Equation (1) (Figure 4). In general, the CA activities did not change markedly between N sufficient and deficient medium without HA/FA ($p > 0.05$, one-way ANOVA). In contrast, the CA values measured in algae from N sufficient medium with HA/FA and N deficient medium with HA/FA showed high variability: the green algae *U. zonata* showed the highest CA activities in N deficient medium with HA/FA ($p < 0.05$; ANOVA–Turkey HSD). Significant differences were found in each sampling dates for the CA activities ($p < 0.05$).

DISCUSSION

The present study clearly demonstrated that humic substances HA and FA at concentration of 4 mg/l stimulates the growth of *U. zonata* under Nitrogen deficient and sufficient condition (Figure 1). When *U. zonata* under N deficient with HA /FA yielded finally the same cell density as the nutrient sufficient medium. Cell density of those treatments were higher than the N deficient treatments without HA/FA. Similar response was not observed with algae in freshwater by addition of HA/FA at equivalent concentration (Devo et al., 1984). In contrast, dinoflagellates responded greatly on humic acid, not fulvic acid (Prakash et al., 1973; Carlsson et al., 1993).

U. zonata species grow slowly under nitrogen deficient condition (Figure 1). The intracellular amounts of DNA in the –N cultures were lower than –NH/-NF and +NP treatments. The results presented here may be associated with the reduced cell division in the –N treatments without HA/FA addition (Figure 3). However, N: P ratios did not differ between the N deficient and nutrient sufficient treatments. The experimental results suggested that the growth efficiency of *U. zonata* under N deficient treatments with HA/FA was higher than the nutrient deficient treatments without HA/FA. *U. zonata* can successfully grow under nitrogen sufficient and deficient condition with HA/FA. Similar results indicated that dinoflagellates have potential to acquire HS as nitrogen source under nitrogen limited condition, implying that they can successfully compete with other algal species for this nitrogen source (Schnitzer, 1985; Carlsson et al., 1993). This growth stimulating effect has generally been attributed to the ability of HA/FA which can act as chelators, making elements such as iron available to algae or

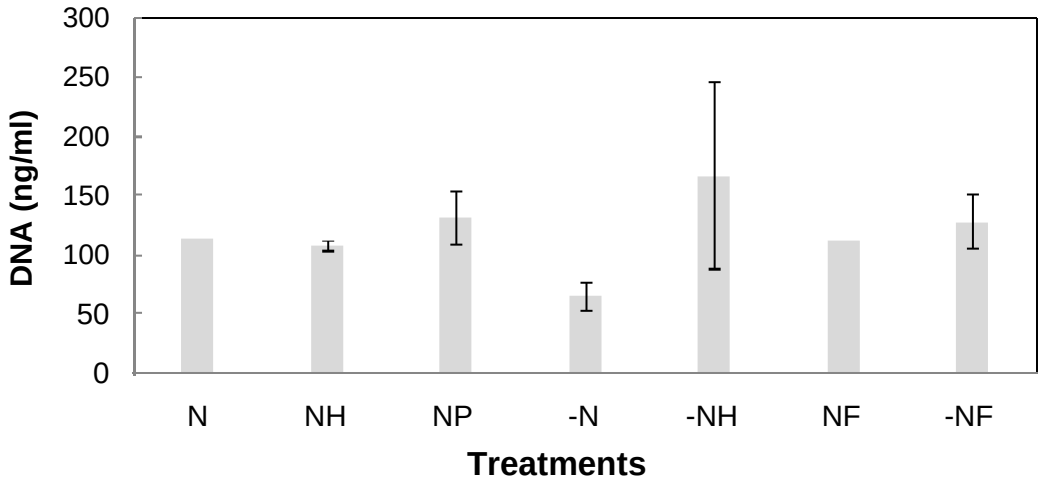

Figure 3. DNA concentration of *Ulothrix zonata* in different nutrient media (NP – N and P sufficient medium; -N – N deficient medium; N – N sufficient medium; NH – N sufficient medium with humic acid; -NH – N deficient medium with humic acid; NF – N sufficient medium with fulvic acid; -NF – N deficient medium with fulvic acid).

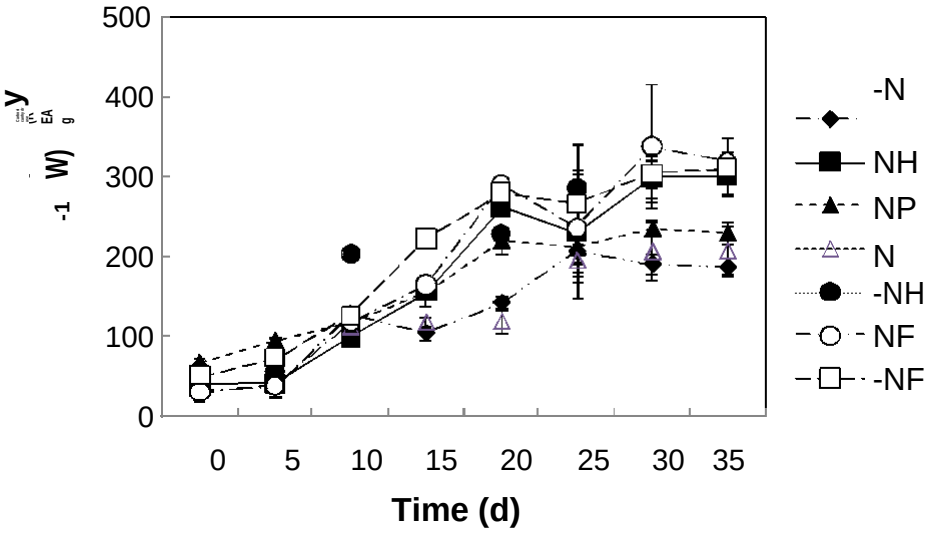

Figure 4. Carbonic anhydrase (CA, EC 4.2.1.1) activity of *Ulothrix zonata* in different nutrient media (NP – N and P sufficient medium; -N – N deficient medium; N – N sufficient medium; NH – N sufficient medium with humic acid; -NH – N deficient medium with humic acid; NF – N sufficient medium with fulvic acid; -NF – N deficient medium with fulvic acid).

binding toxic trace metals (Carlsson and Graneli, 1993; Carlsson et al., 1993).

Organic nitrogen bound to the HA and FA were mainly refractory compounds resistant to biological degradation and generally unavailable as source of N to *U. zonata*. Bacterial enzymes can hydrolyzed the organic nitrogen bound to the HS, and increase the pool of available inorganic nitrogen in the nitrogen deficient medium (Chrôst et al., 1989, 1990; Paerl and Millie, 1996). Be-cause our *U. zonata* cultures were not bacteria free. It is difficult to assume that *U. zonata* has the ability to take up organic N from the HA/FA directly. This may actually have become available to *U. zonata* through incorporation of the nitrogen into bacterial biomass and subsequent release of inorganic nitrogen. The present study indicated that *U. zonata* species has in close association with contaminant heterotrophic bacteria to stimulate biomass accumulation (Anita et al., 1991; Wetzel, 2001). It is possible to suggest a close relationship that exists between HA /FA bound nitrogen and increase in algal biomass, especially in N-depleted waters (Flynn et al., 1994).

Most algae are unable to compete with bacteria in the uptake of HS because HS is considered as a nutrient

source for bacteria (Tranvik, 1988). Still, many aquatic systems, bacterial production is correlated with algal primary production, which suggested that alga derive carbon (C) is important for bacterial growth (Fenchel and Blackburn, 1979; Cole et al., 1988; Moran and Hodson, 1990, 1994). Notwithstanding, dinoflagellate biomass increased several fold when humic acid compounds extracted from rivers, were available with nitrate; there was an evidence of both bacterial degradation of humic substances and algal growth sustained by humic-bound nitrogen (Larson and Hagstrom, 1979; Geller, 1983; Graneli et al., 1985; Vadstein et al., 1989). The increase in cell growth in treatments, coinciding with the increase in bacterial numbers in the treatments, is also an indication that this was due to release of C from the growing *U. zonata*.

U. zonata cannot directly get dissolved organic compounds of HA/FA as a source of phosphorous in P deficient condition like some Cyanobacteria (Hübel and Hübel, 1980). But *U. zonata* grown in P deficient treatment with HA/FA addition did not become P limited. Phosphorous is usually not the main limiting macro-nutrient for algal growth in aquatic ecosystem (Heckey and Kilham, 1988).

In this experiment, the significant increase in cell growth of *U. zonata* in -NH/-NF, and NH/NF treatments, compared with –N and N treatments, P sufficient and N deficient treatments also indicated that the *U. zonata* had access to a nitrogen source, probably humic bound nitrogen. The increase yield of *U. zonata* in the NH/NF treatments may also be related to the supply of micronutrients bound in the HA/FA (Sun et al., 2005). Furthermore, the results indicated that *U. zonata* can grow well N sufficient condition with NH/NF treatments. Some authors have shown that humic substances may effect on primary production and Chl a concentration in aquatic algae depend on the photosynthetic activity (Jackson and Heckey, 1980; Hecky and Kilham, 1988; Moroney et al., 2001). The biochemical process or phenomena behind the *U. zonata* is not known well in the present study. However, the Carbonic anhydrase is one of the key enzymes of photosynthetic metabolism of algae and activity of Carbon metabolism was detected to determine the growth of *U. zonata* under the treatments aforementioned.

The results reveal the strong variation of CA activities and DNA concentration among nutrient sufficient and deficient media of *U. zonata*. In the experiment cells were grown in the N deficient medium without HA/FA coincides with a low CA activities and DNA concentration compared to other treatments. The difference between the maximum and minimum activities of CA was around six fold in nutrient sufficient and deficient media. *U. zonata* reflects different physiological activities as a result of the nutrient condition that is, the most variability of CA activities due to different photosynthetic potential of *U. zonata* among different nutrient treatments. The enzyme activity increased in the treatments -NH/-NF has proven to be a potentially important source of N for *U. zonata*. The concordance of the CA activities and photosynthetic potential has not been studied yet.

The results demonstrated that algae can modify the CA activity in response to environmental changes (nutrient media with HA or FA) in short term during the daily cycle, but that these changes (as a drop in CA activity at midday) are not followed by major changes in the photosynthetic rates (Figueroa and Vinegla, 2001).

Conclusion

Our main objective was to evaluate the stimulatory potential of HA/FA from Suwannee River on the growth of *U. zonata* under excess nitrogen condition and has demonstrated that HS stimulate biomass production in cultures of the *U. zonata*. The results indicated that HA/FA may contribute to the growth of *U. zonata* in the presence of excess nitrogen by terrestrial run off. *U. zonata* cannot meet their nitrogen requirement through biological fixation under N deplete condition, like some cyanobacteria. HA/FA may be an important source for the growth of *U. zonata* under N deficient conditions with sufficient P. Therefore, those aquatic humic substances could be of great importance in the growth of periphytic algae, especially in freshwater environment. Finally, this study concluded that HA and FA, are biologically important components in natural waters.

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