**Aim:** The objective of the present study was to determine the factors that regulate the response of algal growth in wetland ecosystem by *in situ* nutrient-spiking bioassays (NSBs).

**Methodology:** The NSBs tested algal responses as a ratio of final (Chl) to initial Chl (Chl) in the control (no nutrient addition) and several treatments of phosphorus (P), nitrogen (NH$_3$-N, NO$_3$-N), and NO$_3$-N+P under various environmental conditions of inorganic suspended solids (ISS) and free-floating plants (FP).

**Results:** Experiments of *in situ* NSBs with low inorganic solids showed that the response of P treatments were significantly (p<0.05) greater than the controls and the treatments of NH$_3$-N or NO$_3$-N. Regression analysis of Chl : Chl ratios against log$_{10}$-transformed TP in the cubitainers, thus showed that *in situ* algal response in P treatments was directly determined ($R^2 = 0.965$, $F = 40.049$, $p < 0.001$) by concentrations of spiked TP. In contrast, the *in situ* NSBs with high ISS (> 15 mg l$^{-1}$) or high FfP cover (> 95%) showed that the response in treatments of N, P or P+NO$_3$-N had no significant (p>0.05) difference with the controls.

**Interpretation:** Light limitation dominated the system, thus the ratio of Chl : Chl had no relation (p>0.10) with the spiked TP. Overall, the experiments of *in situ* NSBs suggest that high P-inputs from the watershed increased algal growth, but dense FP or high inorganic solids suppressed eutrophication in the wetland.
Introduction

Wetland studies are recognized as one of hot ecological research topics for conservation of biodiversity and wildlife habitats. They have diverse hydrological and ecological functions such as flood control (Ogawa and Male, 1986; Hey and Philipp, 1995), providing natural habitat for aquatic biota (Ralph and Rogers, 2011), water purification (Gersberg et al., 1986), nutrient retention balancing terrestrial and aquatic ecosystems (Craft CB, 1997), sediment filtering capacity from the watersheds (Mc Jannet et al., 2012), as well as aesthetic and economic aspect of ecological tourism. For these reasons, water pollution and eutrophication are largely focused in the wetlands (Brix, 1994), and nutrients such as nitrogen and phosphorus have been primary chemicals regulating the wetland productivity (Elser et al., 2007), although chemicals such as carbon and silica (diatom) have frequently been proposed as important (Goldman et al., 1972; Struyf and Conley, 2008). Phosphorus in wetland ecosystems is lost from the water column by sedimentation processes and has no gas phase, so it is often considered the most important limiting nutrient, which thereby regulates eutrophication of wetlands (Reddy et al., 1995). The input-output models (Vollenweider, 1975, 1976) or empirical models (Mitsch and Reeder, 1991) show how nutrients and chlorophyll or primary production, are functionally related to external loading or input of nitrogen and phosphorus from the watersheds.

Many published studies on wetlands have pointed out that phytoplankton dominate in waterbodies with a large phosphorus input (Pan et al., 2000), low underwater light regimes (O’Farrell et al., 2007), high water temperature (Kadlec and Reddy, 2001), water column stability (Reynolds and Walzby, 1975) or low N:P ratio (Güsewell et al., 2003). Nitrogen and phosphorus have been considered as key variables for algal blooms and the magnitude of wetland eutrophication is closely related to supply ratios of these two key nutrients along with underwater light availability. In particular, nutrient loading of P or N in temperate Asian monsoon regions is controlled by frequency and intensity of monsoon rainfall or runoff during the monsoon periods.

Lentic and lotic ecosystems in Asian regions potentially have unique trophic characteristics determined by the summer monsoon. One third or half of total annual precipitation in Korea occurs during short period summer monsoon July - August. Thus, nutrients, primary production, and light regime may largely vary by them on soon flood events, which will result in short hydraulic residence time (An and Park, 2002). Especially, large inputs of non-volatile suspended solids (NVSS) during flood can reduce the underwater light availability, and may reduce phytoplankton biomass relative to available nutrient supplies. This phenomenon is frequently observed in wetland ecosystems of Asian regions (Muzaffar and Ahmed, 2007). Under these circumstances, phytoplankton production or growth may be closely associated light availability rather than by amounts of nutrients, and phytoplankton growth is largely reduced. Empirical relation between nutrients (N, P) and chlorophyll-a or between chlorophyll-a and nutrient regime, thus, may be modified, resulting in modification of ecological functions in the wetland ecosystems.

Assessment of trophic variables such as nitrogen, phosphorus, or chlorophyll, are important in the wetland ecosystems, but more important thing is to evaluate the chlorophyll-a response to the trophic variables using an in situ cubitainer approach like nutrient-spiking bioassay. The technique of nutrient enrichment bioassays or nutrient-spiking bioassays (NSBs) is easy to conduct in fields, and is cost effective in diagnosing the nutrient response to phytoplankton growth and are widely applied for determining nutrient or light limitations in well and lake stream ecosystems (Elser et al., 2007; Pulatsu and Topcu, 2009). Various nutrient-spiking bioassays such as short-term batch culture method, radioactive nutrient or whole lake approaches employing in situ polyethylene cubitainers are used and especially in situ bioassays using enclosures or cubitainers have been commonly used for identification of key or primary nutrients most limiting in aquatic ecosystems (Elser et al., 1990).

The aim of the research was to apply a new approach of in situ nutrient stimulation experiments (NSEs) for determining phosphorus and light limitation in Woopo Wetland, and evaluate temporal variability of algal growth responses under different wetland conditions such as dominance of non-volatile suspended solids (NVSS) or free-floating plants (FP) in wetland ecosystem.

Materials and Methods

Experimental designs of nutrient-spiking bioassays: In situ nutrient stimulation experiments (NSEs) were conducted in Woopo Wetland, which is located in the southern part of South Korea and influenced by Asian monsoon. For the experiments, two different sites (W1, W2) with a dominance of non-volatile suspended solids and free-floating plants (FP, i.e., Spirodela polyrhiza), respectively were selected to determine nutrient or light limitations of algae. Surface water was mixed in a 120-L polyethylene-lined container and then dispensed into 10-L translucent polyethylene cubitainers (Naigene Company). Unfiltered surface waters were suspended at 0.5m depths and incubated 3 days in the cubitainers. Five different individual bioassays (BIO-I to BIO-V) had controls and various treatments consisting of additions of N and/or P, with two or three replicates each.

Temporal effects of bioassays in relation to water temperature, free-floating plants (FP), and inorganic solids were tested in each bioassay. First tests of BIO-I were conducted during summer season (July-August), with intense rainfall and runoffs from the watershed, and inorganic suspended solids (ISS) dominated in the water-column of wetland along with high inorganic turbidity (> 70 NTU). The bioassays of BIO-II were conducted in the fall season (November-December) with 10 - 15
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response in the controls (C) did not differ from the NO$_3$-N treatment (Fig. 1c). These conditions indicate that a partial light limitation was due to decreases of underwater light by inorganic solids (non-volatile suspended solids) in the bioassay.

In situ nutrient-spiking bioassays (NSBs) during fall season (November - early December) showed that the ratio of (Chl - Chl)/Chl in the treatments of P and NO$_3$-N+P was 0.66 and 0.59, respectively and the ratios in the control, NH$_4$-N and NO$_3$-N treatments were < 0.25. Two treatments of P and NO$_3$-N+P were significantly ($p<0.05$, ANOVA tests) greater than the control (C) or treatments enriched with nitrate-nitrogen or ammonia-nitrogen (Fig. 2a). This was due to increased light availability by low ISS (range= 3.1 - 6.3 mg l$^{-1}$; mean = 4.9 mg l$^{-1}$) and the absence of free-floating plants (FP; < 5%) in the surface, even if the mean water temperature were low (9.2 - 12.2 °C ) compared to BIO-I. (Chl - Chl)/Chl, in the treatments of P and NO$_3$-N+P of Exp-II was 0.54 and 0.49, respectively, which were significantly ($p<0.05$) greater than the control (C) or treatments enriched with nitrate-nitrogen or ammonia-nitrogen (Fig. 2b). The ratios in the treatments of P and NO$_3$-N+P were decreased in the Exp-I than in the Exp-II, and this was mainly attributed to a reduced underwater light of partial coverage of FP (< 5%). In other words, P-limitation was consistent in the postmonsoon period.

The nutrient-spiking bioassays (NSBs) tests during the winter season showed that two treatments of P and NO$_3$-N+P were significantly ($p<0.05$, ANOVA tests) greater than the control (C) or treatments enriched with nitrate-nitrogen or ammonia-nitrogen (Fig. 2c) and the magnitude of productivity response to P addition was statistically greater than the ratios in the P-treatment of BIO-I and BIO-II (Fig. 1 and Fig 2). The greater responses in the NSBs were due to high water transparency (low ISS of < 2 mg l$^{-1}$) and no free-floating plants (FP), resulting in greater algal productivity. These outcome suggests that in the Asian monsoon regions phytoplankton growth is greater in the winter than summer season. Our seasonal pattern in the nutrient stimulation bioassays is opposite to those in the water bodies of North America and Europe where the algal response is greater in summer than in the winter. Our reduced algal response during summer season in the NSBs was a results of largely increased inorganic turbidity (high NVSS) and high coverage of FP, in spite of increased phosphorus in the ambient water. Also, up-stream monsoon runoff during the summer increased P-input in the system and this P was mainly composed of particulate phosphorus (PP), which is not available for the algal growth. In fact, mean ratio of PP:TP in the summer monsoon was 0.82, but was 0.11 in the winter season, resulting in decreases of particulate fractions of >7-fold in the winter.

In situ tests during spring season also indicated a P-limitation on the algal productivity response and nitrogen treatments enriched with ammonia-N and nitrate-N did not differ from the control (C). The ratio of (Chl - Chl)/Chl, in the treatments of P and NO$_3$-N+P was maximized during the spring of growing season (Fig. 2d), thus the magnitude of productivity response to phosphorus addition was greatest. This was due to increased water temperatures (15.3 - 20.0 °C) along with light availability by solar energy. The nutrient-spiking bioassays was opposite to those in the water bodies of North America and Europe where the algal response is greater in winter than in the summer season. Our seasonal pattern in the nutrient stimulation bioassays is opposite to those in the water bodies of North America and Europe where the algal response is greater in winter than summer season. Our seasonal pattern in the nutrient stimulation bioassays is opposite to those in the water bodies of North America and Europe where the algal response is greater in winter than summer season. Our seasonal pattern in the nutrient stimulation bioassays is opposite to those in the water bodies of North America and Europe where the algal response is greater in winter than summer season.

Table 1: In situ Nutrient Spiking Bioassays (NSBs), based on the ratios of Chl/Chl in the controls (C) and various treatments of ammonia-nitrogen (NH$_4$-N), nitrate-nitrogen (NO$_3$-N), phosphorus (P), and nitrate-nitrogen + phosphorus (NO$_3$-N+P). Analysis of variance (ANOVA) tests were based on significant statistical differences of $p<0.05$. The marks of † and * indicate the NSBs’ of 2003 and 2002, respectively (T = all treatments)

<table>
<thead>
<tr>
<th>Period</th>
<th>Control (C)</th>
<th>NH$_4$-N treatment</th>
<th>NO$_3$-N treatment</th>
<th>P treatment</th>
<th>NO$_3$-N+P treatment</th>
<th>Mean response (T/C ratios)</th>
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<tbody>
<tr>
<td>Jul 03t</td>
<td>1.134</td>
<td>1.214</td>
<td>1.113</td>
<td>1.733</td>
<td>1.655</td>
<td>1.260</td>
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<td></td>
<td></td>
<td>ANOVA Tests: P = NO$_3$-N+P &gt; NH$_4$-N &gt; NO$_3$-N = C</td>
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<tr>
<td>Jul 15t</td>
<td>0.906</td>
<td>0.765</td>
<td>0.848</td>
<td>1.054</td>
<td>1.053</td>
<td>1.026</td>
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<td></td>
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<td>ANOVA Tests: P = NO$_3$-N+P &gt; NH$_4$-N = NO$_3$-N = C</td>
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<tr>
<td>Aug 03t</td>
<td>1.063</td>
<td>1.133</td>
<td>1.077</td>
<td>1.267</td>
<td>1.243</td>
<td>1.134</td>
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<td>Nov 10t</td>
<td>1.026</td>
<td>1.092</td>
<td>1.017</td>
<td>1.658</td>
<td>1.593</td>
<td>1.306</td>
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<tr>
<td>Dec 03t</td>
<td>1.022</td>
<td>1.125</td>
<td>0.957</td>
<td>1.537</td>
<td>1.489</td>
<td>1.250</td>
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<td>Feb 16t</td>
<td>1.150</td>
<td>1.174</td>
<td>0.818</td>
<td>2.000</td>
<td>1.909</td>
<td>1.283</td>
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<td>ANOVA Tests: P = NO$_3$-N+P &gt; NH$_4$-N &gt; NO$_3$-N = C</td>
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<td>May 15t</td>
<td>1.063</td>
<td>1.206</td>
<td>1.167</td>
<td>2.278</td>
<td>2.235</td>
<td>1.619</td>
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<tr>
<td>Sep 12t</td>
<td>0.750</td>
<td>0.786</td>
<td>0.667</td>
<td>0.846</td>
<td>0.833</td>
<td>1.044</td>
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<td>ANOVA Tests: P = NO$_3$-N+P = NH$_4$-N = NO$_3$-N = C</td>
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low ISS (range = 2.2 - 3.8 mg l\(^{-1}\), mean = 2.8 mg l\(^{-1}\)) and the absence of free-floating plants (FP) in the surface. In contrast, the ratios of (Chl - Chl)/Chl in the control and all treatments were negative (range: -0.17 - -0.33; Fig. 2e) during the early summer when the flood did not occur and the FP covered the surface up to 100%. During the experiments of NSBs, mean ratio of DP:TP in the ambient water was 0.92 and mean concentration of NVSS was 2.8 mg l\(^{-1}\), indicating a high availability of P and low inorganic turbidity. But, FP blocked the underwater penetration during this period. Thus, the responses of algal productivity to the nutrient additions of N or P did not occur in the early summer season. In situ tests during the early summer, suggest that light limitation was evident for the phytoplankton growth.

Our experiments NSBs indicated that in situ responses of NSBs, expressed as Chl/Chl, in the treatments of P were always greater than the values of NO\(_3\)-N+P treatments, except for the season covered completely by free-floating plants (FP), even though the ratios of P-treatment did not statistically (p < 0.05) differ from the NO\(_3\)-N+P treatments (Table 1). These results suggest that P limited the phytoplankton growth, but additions of nitrate-N suppressed the phytoplankton growth in this wetland ecosystem. The influences of nitrate-N spiking on the phytoplankton growth, however, were minor because of no differences (p < 0.05) between the controls and NO\(_3\)-N treatments. Such possibility of growth inhibitions by nitrate-N are supported by the findings of Blomqvist et al., (1994) who reported that algal response in nitrate-N treatments was significantly (p < 0.1) less than the control in other nutrient enrichment bioassays. A mechanism of the suppression may be explained by nitrate assimilation efficiency in cyanobacteria, which dominated the phytoplankton community in the system. Experimental approaches in chemostats have demonstrated that this type of algae preferred ammonium over nitrate as a nitrogen source and had low-nitrate uptake rate in the high nitrate-rich environment (Blomqvist et al. 1994). In fact, concentrations of nitrate-N were > 1.10 mg l\(^{-1}\) in the ambient water of our wetland ecosystem regardless of the seasons and locations. Under this circumstance, therefore, nitrogenase activity of cyanobacteria might have inhibited the algal growth (Leonardson and Ripl 1980; Blomqvist et al. 1994), resulting in a decrease in algal growth. In the meantime, there were no significant differences (p > 0.05, ANOVA tests) among the controls and all treatments of P, NO\(_3\)-N, NO\(_3\)-N+P, and NH\(_4\)-N under the circumstance of 100% coverage of free-floating plants (FP) in the surface water. Elser et al. (1980) pointed out that alteration of environmental conditions, especially incubating phytoplankton at favorable light intensities, may artificially increase the magnitude of algal response to nutrient limitation. A variation in light condition, however, was maximized during the incubation period; a photic depth was nearly zero due to the surface cover of FP, even if non-volatile suspended solids were low (mean = 2.4 mg l\(^{-1}\); range: 1.8 - 2.9 mg l\(^{-1}\)) in the season. Also, the treatments of NO\(_3\)-N showed significantly (p < 0.05) greater or same algal response than the NH\(_4\)-N treatments (Table 1), indicating that ammonia-nitrogen influence the algal growth more than the nitrate-N, even though P-limitation is evident. These outcomes imply that ammonia-N might have stimulated the algal response weakly.
Fig. 2: Algal productivity response as an equation of \((Chl - Chl)/Chl\) in the nutrient-spiking experiments (NSEs). Experimental designs of NSEs are as follows; low ISS of < 5 mg l\(^{-1}\) without the free-floating plants (FP) at autumn (a), partial growth of FP with low ISS of < 5 mg l\(^{-1}\) at early winter (b), no FP on the surface water with low ISS of < 2 mg l\(^{-1}\) at late winter (c), growth starting of FP at early spring, (d) and 100% cover of the surface by FP with low ISS (< 3 mg l\(^{-1}\)) during summer (e).
The relation of nutrient-spiking to control ($C_r$) in the response of Chl ($R_{ch}$) also supported the phosphorus-limitation in the system (Fig. 3). Treatments of P and NO$_3$-N+P in the NSEs showed significant increases of Chl ($R^2 = 0.626, p < 0.05; R^2 = 0.561, p < 0.05$) compared to the control, and the response of Chl in the P and NO$_3$-N+P treatments increased up to 2.27 and 2.23, respectively (Fig. 3). In contrast, there was no significant relations ($p = 0.065$) between $R_{ch}$ of NO$_2$-N and $C_r$, and the all response values of Chl in the NH$_4$-N was $< 1.5$ (Fig. 3), indicating that ammonia or nitrate-nitrogen addition did not increased the algal growth.

Overall, in situ experiments of no floods or no free-floating plants (FP) showed that in 5 of total 8 NSBs, additions of phosphorus alone or with nitrate-N caused a significant increase of algal growth relative to the controls (Fig. 4). The regression analysis of CHL:CHL ratios against Log$_{10}$-transformed ambient TP in the cubitainers showed that in situ algal response in P treatments was directly determined ($R^2 = 0.965, F = 40.049, p < 0.01$) when the density of FP was low on the surface and non-volatile suspended solids were $< 5.0$ mg l$^{-1}$; Ratios of Chl$_{F}$: Chl$_{C}$ = -3.1260 + 2.06 Log$_{10}$ (TP). In contrast, the ratios of Chl$_{F}$: Chl$_{C}$ had no relations with ambient TP in the
cubitainers (Fig. 4b) when the system was completely covered by F.P and non-volatile suspended solids were > 15.0 mg l⁻¹. Under the circumstances, no nutrient limitations of P or N occurred in this system and only light limitation was key factor regulating the phytoplankton growth.

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