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Physiological Responses of *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta) Exposed to High Ammonium Effluent in a Seaweed-based Integrated Aquaculture System

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Porphyra yezoensis is known to act as a biofilter against nutrient-rich effluent in seaweed-based integrated aquaculture systems. However, few studies have examined its physiological status under such conditions. In this study, we estimated the photosynthetic activity of *P. yezoensis* by chlorophyll fluorescence of PSII ($\Delta F/F'_m$ and relative ETR_{max}) using the Diving-PAM fluorometer (Walz, Germany). In addition, bioremediation capacity, tissue nutrients, and C:N ratio of *P. yezoensis* were investigated. The ammonium concentration in seawater of seaweed tank 4 decreased from 72.1±2.2 to 33.8±0.4 μM after 24 hours. This indicates the potential role of *P. yezoensis* in removing around 43% of ammonium from the effluents. Tissue carbon contents in *P. yezoensis* were constant during the experimental period, while nitrogen contents had increased slightly by 24 hours. In comparison with the initial values, the $\Delta F/F'_m$ and $rETR_{max}$ of *P. yezoensis* had increased by about 20 and 40%, respectively, after 24 hours. This indicates that *P. yezoensis* condition improved or remained constant. These results suggest that chlorophyll fluorescence is a powerful tool in evaluating the physiological status of seaweeds in a seaweed-based integrated aquaculture system.

Key words: Ammonium removal, Chlorophyll fluorescence, *Porphyra yezoensis*, Seaweed-based integrated aquaculture, Tissue nutrient content

Introduction

Aquaculture has long been supporting human demand for fish products and is an important industry worldwide (e.g. Chopin and Yarish, 1998; Naylor et al., 2000). According to the FAO (2003), aquaculture production is growing more than 10% annually, and a production of 47 million tons of aquaculture products (mainly fish) is estimated by the year 2010 (Dar, 1999). However, excess nutrients output from fish aquaculture activities may have negative impacts on coastal and sheltered areas, such as eutrophication (Troell et al., 1999). In order to reduce or remove mass nutrients sources, Ryther et al. (1975) suggested a biofilter system based on high nutrient uptake rate and growth rate of seaweed. Recently, Chopin et al. (2001) proposed the integration of seaweed cultivation into fish aquaculture.

Previous studies have demonstrated that environmentally sound seaweed-based integrated aquaculture systems are able to successfully cultivate seaweeds using effluent from artificially fed aquaculture species (e.g. fish or shrimp) (Jiménez del Río et al., 1996; Troell et al., 1999; Hernández et al., 2002; Neori et al., 2004). For example, three red seaweed species (*Chondrus crispus*, *Gracilaria bursapastoris* and *Palmaria palmata*) have been cultivated in tanks using effluent from a local commercial turbot (*Scophthalmus maximus*) and sea bass (*Dicentrachus labrax*) farm, thus prohibiting wastewater output into the environment and ultimately reducing the risk of eutrophication (Matos et al., 2006). Troell et al. (1997) reported high growth rates of *Gracilaria*, high

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concentrations of tissue nitrogen and phosphorus and a large accumulation of agar in algae cultivated close to a fish farm. These studies indicate that seaweed-based integrated aquaculture systems are able to improve seaweed growth, as well as the quality of water sourced from aquaculture effluent. However, despite the potential applicability of seaweed as a biofilter, few studies have been conducted on physiological responses of seaweeds exposed to high nutrient levels except for their bioremediation capacity (Pinchetti et al., 1998; Figueroa et al., 2006).

The physiological status of seaweed is affected by water temperature, salinity, light, and nutrient concentrations (Lobban and Harrison, 1994; Floreto et al., 1996; García-Ferris et al., 1996; Kang et al., 2008). Photosynthesis is regarded as the main indicator of physiological performance. Although oxygen metabolism and ^{14}C uptake are available for measuring photosynthesis rate of seaweeds, such methods are mostly restricted to laboratory studies. Non-destructive probe of the PSII photochemical processes in the water can grow be conduted with a recently developed submersible pulse-amplitude modulated (Diving-PAM) fluorometer, which provides information on light conditioning, photosynthetic capacity and efficiency of PSII. Chlorophyll fluorescence is also used to monitor plant responses to stress (Schreiber et al., 1986). In addition to photosynthesis, excessive nitrogen sources cause changes in biochemical composition of macroalgae, which also limits their growth, leading to drastically raised levels of tissue C:N ratio (Vergara et al., 1993; Lahaye et al., 1995). For example, Pinchetti et al. (1998) reported that tissue N content of seaweed was affected by N-enrichment in tanks. C:N ratio is a powerful index of the physiological status of seaweed (Vegara et al., 1993) and is often used as an indication of seaweed health. The responses in growth and pigment concentration of macroalgae have been correlated with responses in tissue N content (Jones et al., 2002).

The selection of seaweed species as biofilters for seaweed-based integrated aquaculture systems depends on commercial value and their capacity to remove nutrients (Buschmann et al., 2001; Chopin et al., 2001). Seaweeds belonging to the *Porphyra* genus are valuable around the world, and grown extensively in Korea, Japan and China (FAO, 2003). The thin gametophyte blade of *Porphyra* is composed of only 1 or 2 cell layers with the capability for nutrient uptake (Kraemer et al., 2004). With such morphology, *Porphyra* species exhibit a high growth rate and are capable of rapid uptake and assimilation of nutrients (Chopin et al., 1999; Neori et al., 2004). Based on these characteristics, they are recommended as an attractive candidate in seaweed-based integrated aquaculture environments. In this study, we hypothesized that high ammonium levels from fish tank effluent in an integrated aquaculture system influence the biochemical composition and photosynthetic activity of tank-cultivated *P. yezoensis*. These two parameters were evaluated based on measurements of tissue nutrient content and chlorophyll a fluorescence using the Diving-PAM. Additionally, we estimated the bioremediation capacity of *P. yezoensis* as a potential biofilter of dissolved wastes from an experimental fish cultivation tank.

Materials and methods

Culture conditions and water sampling

Porphyra yezoensis, which grows from late-fall to spring, was collected from a seaweed farm located in Gamak Bay (34°35'N, 127°43'E), Korea, and was carried to the aquaculture research center (Chonnam National University) at Dolsando under humid and cool conditions. Before the experiment, the seaweed were rinsed with filtered seawater and gently scrubbed to remove sediment and epiphytes. The specimens were precultured for 4 days under continuous flow of filtered seawater pumped from the South Sea (mean nutrients concentrations 0.6 μM NH_4^+, negligible NO_3^- +NO_2^- and 0.7 μM PO_4^{3-}) at 10-12°C to 200 L aerated tanks at a density of 10 g L^{-1}. During this period the algae remained healthy.

The integrated system consisted of a fish culture tank containing 3,000 L of seawater, a sedimentation tank with sandy filter (500 L), four seaweed cultures containing a total of 800 L, and one pumping tank (100 L) for controlling water level of tanks (Fig. 1). Water was circulated by gravity from the fish tank via the sedimentation tank (500 L) to the four seaweed tanks arranged in series. Water then returned to the fish tank by an electric pump from a small tank (100 L) via the seaweed tanks. Seaweeds were stocked at a density of 10 g fresh weight L^{-1}. The seawater effluent exchange rate (2.4 vol d^{-1}) supplied approximately 70 μM of NH_4^+ from the fish tank. Irradiance ranged from 30-50 μmol photons m^{-2} s^{-1} and the water temperature was approximately 10-12°C. Water samples (n=6) were taken from the fish tank and the four seaweed tanks every 3 hours during a 24 hour period. NH_4^+ concentrations were analyzed at each sampling time using the phenol-hypochlorite method (Solorzano, 1969).

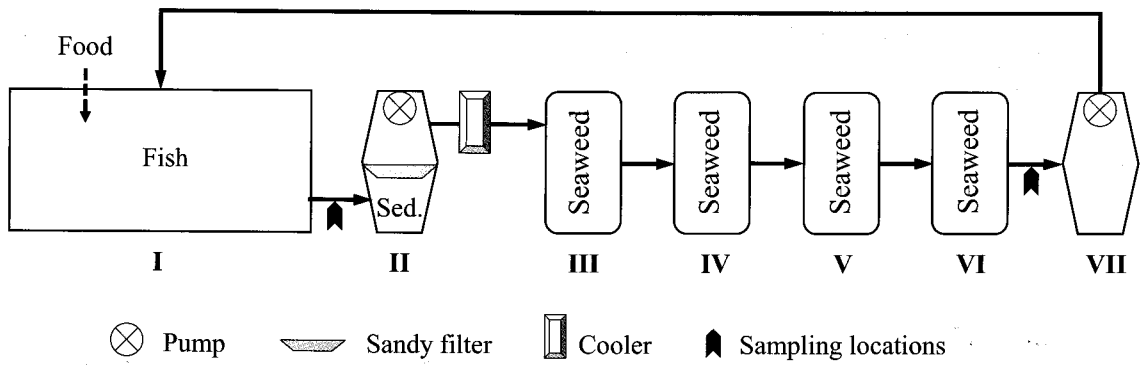

Fig. 1. Schematic diagram of land-based integrated systems. The arrows indicate the directions of water flow. There is a sandy filter before the sedimentation tank.

Chlorophyll *a* fluorescence measurements

Chlorophyll *a* fluorescence of PSII were performed using the Diving-PAM (Walz, Effeltrich, Germany) with 8 mm fiber optic. The effective quantum yield ($\Delta F/F'_m$) and rapid light curves (RLCs) of samples (n=6) from each seaweed tank were measured from the outset, and at 12 and 24 hours after initial exposure to the high nutrient concentration. Effective quantum yield ($\Delta F/F'_m$) was calculated from $(F'_m - F_t)/F'_m$, where F_t is the current steady-state fluorescence in light-adapted algae, while F'_m is the maximum fluorescence induced by a saturating white light pulse (0.8 s at approximately 1,500 mol photons m^{-2} s^{-1}; Genty et al., 1989). During the RLC measurement, samples (n=6) were exposed to 10 sec of irradiance (0-480 μmol photons m^{-2} s^{-1}) in eight incremental steps. Relative electron transfer rate (rETR) was calculated according to the following equation:

$$rETR = \Delta F/F'_m \times PAR \times 0.5 \times 0.84$$

where $\Delta F/F'_m$ is the effective quantum yield of PSII, 0.84 is the assumed absorption coefficient, 0.5 is the correction for two photosystems absorbing photons, and PPFD is the programmed level of photosynthetically active radiation (PAR, 400-700 nm) delivered by the halogen lamp (Genty et al., 1989). A standard absorption factor was used here because the best means to directly measure absorption continues to be debated among the scientific community. As such, all values are considered as relative ETR (rETR) because leaf absorptance was not directly measured (Durako et al., 2003). The RLC data were fitted to the model of Platt et al. (1980) in order to obtain values for photosynthetic efficiency (α), inhibition term (β), minimum saturating irradiance (E_k) and maximum relative electron transport rate ($rETR_{max}$).

Tissue C and N content

To determine tissue nitrogen (N) content, sample were collected prior to the experiments and again at the end of each seaweed tank. Dry weight (DW) was measured after drying the samples for 48 hours at 60°C, following which the tissues were ground using mortars and pestles. Approximately 2-3 mg of ground tissue was placed into a tin to determine N contents using a CHN elemental analyzer (Vario-EL III, Elementar Analysensysteme GmbH, Germany).

Statistical analysis

All results are presented as mean±SE. Data were tested for normality and homogeneity of variance to meet the assumptions of parametric statistics. When the assumptions were not satisfied, the data were transformed using the natural log after which they successfully met the required assumptions. Significant differences in N content of *P. yezoensis* tissues were tested using a one-way ANOVA. Differences in effective quantum yield and $rETR_{max}$ of *P. yezoensis* tissues were analysed for significance using a two-way ANOVA. Multiple post hoc comparisons between means were made using the Student-Newman-Keuls test. Statistical significance was set at the alpha <0.05 level. All statistical analyses were performed using SPSS (version 12.0).

Results

Ammonium concentrations

NH_4^+ concentrations within the system were around 70 μM in all tanks. After culturing *P. yezoensis*, NH_4^+ concentration of the fish tank and seaweed tank 4, showed distinct decline of approximately 23 μM over the first 3 hours (Fig. 2). Following this, NH_4^+ concentration showed small fluctuations for 15 hours. The NH_4^+ concentrations of of seaweed tank 4 remained at a lower level than

those from the fish tank during the experimental period. After 24 hours, NH_4^+ concentration in the *P. yezoensis* tanks decreased from 72.1±2.2 to 40.7±0.3 (Fig. 2).

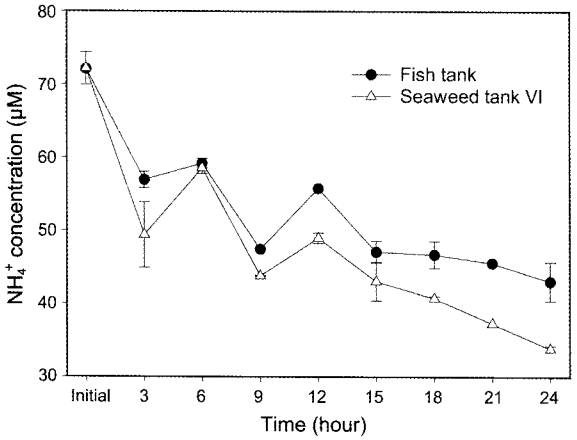

Fig. 2. Change in effluent NH_4^+ concentration in the effluents from the seaweed-based integrated aquaculture system over 24 hours. Data are expressed as mean ± SE (n=6).

Tissue N content

Porphyra yezoensis exposed to high ammonium effluent showed significant increase of tissue N content and decrease of C:N ratio compared to the initial tissue N content (Fig. 3). However, there was no any difference in tissue N content and C:N ratio among tanks (Fig. 3). Before the experiment, initial tissue N content was 5.7±0.2%. After 24 hours, tissue N contents in four tanks increased more than 10% compared to the initial value. Mean tissue N content in four tanks was 6.3±0.1%. Lower C:N ratio was observed in algae exposed to high ammonium levels after 24 hours. The initial value of C:N ratio was 7.2 while the mean C:N ratio in four tanks was 6.6 after 24 hours.

Photosynthetic activity

Effective quantum yield ($\Delta F/F'_m$) of *P. yezoensis* in the four tanks was significantly affected by effluent during the experimental period and increased with time (Fig. 4). However, there were no differences in $\Delta F/F'_m$ of *P. yezoensis* among tanks (p=0.869). The initial $\Delta F/F'_m$ of *P. yezoensis* was 0.535±0.013. After 12 hours, the $\Delta F/F'_m$ of *P. yezoensis* in all tanks was increased markedly to an average of 0.620. At the end of experiment, $\Delta F/F'_m$ of *P. yezoensis* in tank 1, 2, 3 and 4 were 0.639±0.015, 0.646±0.031, 0.644±0.007 and 0.637±0.015, respectively.

The changes of relative electron transport rate

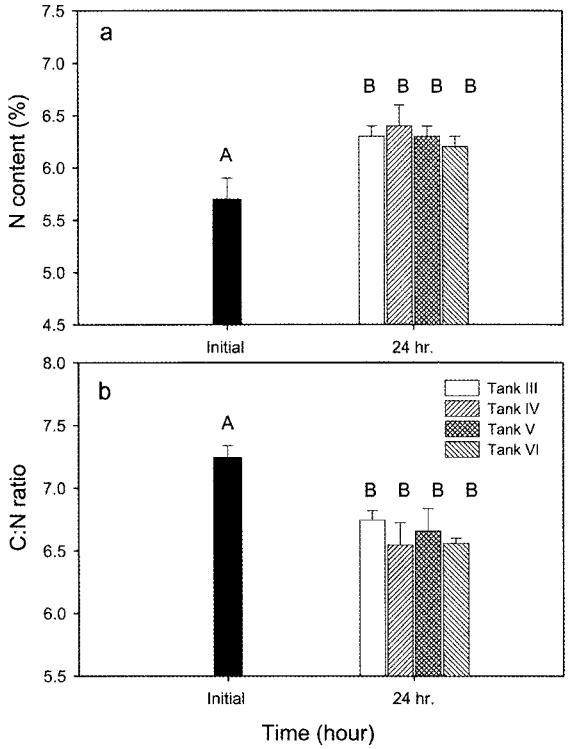

Fig. 3. The N content (a) and C:N ratio (b) of *Porphyra yezoensis* tissue before and after 24 hours of cultivation under fish tank effluent. Different letters indicate significant group at means found with SNK tests ($p<0.05$). Data are expressed as mean ± SE (n=6).

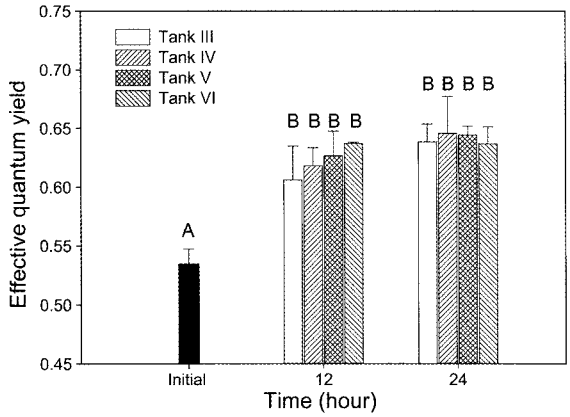

Fig. 4. The change in effective quantum yield cultivated using fish tank effluents in four seaweed tanks after 12 and 24 hours. Different letters indicate significant group at means found with SNK tests ($p< 0.05$). Data are expressed as mean ± SE (n=6).

(rETR) of *P. yezoensis* were similar to those of $\Delta F/F'_m$ of *P. yezoensis* (Fig. 5; Fig. 6). Relative ETR of *P. yezoensis* in the four tanks gradually increased during the experiment periods and reached its peak at

24 hours (Fig. 5). Their $rETR_{max}$ value after 24 hours was higher by 1.4-1.6 fold than initial $rETR_{max}$ value (10.3±0.4). After 24 hours, the $rETR_{max}$ of *P. yezoensis* in tank 1, 2, 3 and 4 were 14.3±0.7, 13.6± 0.5, 15.1±1.1 and 14.9±0.4, respectively (Fig. 6).

Discussion

Numerous species of seaweed have been assessed as a biofilters in removing or reducing mass nutrient sources from the seawater effluent in an integrated aquaculture systems. The NH_4^+ filtration efficiency of three macroaglae (*Ulva rotundata*, *Enteromorpha intestinalis* and *Gracilaria gracilis*) were more than 80% (Hernández et al., 2002). Jones et al. (2001) reported that *Gracilaria edulis* reduced more than 95% of the ammonium concentration in two hours from an integrated shrimp effluent treatment. *Porphyra* species, which have high commercial value, are also good candidates for reducing nitrogen loading from fish farm effluent (Carmona et al., 2006). He et al. (2008) found that in the absence of *Porphyra* species NH_4^+ concentration was between 43 and 61 μM, while in its presence it averaged only 20.5 μM, indicating that the N removal efficiency of the tested species ranged from 50 to 94% of incoming NH_4^+ from nearshore eutrophic coastal areas. Similarly, Carmona et al. (2006) reported that *Porphyra* species showed a strong N removal capability, with 96% of NH_4^+ being removed during a 3-4 day trial period from an initial concentration of 150 μM. In this study, our results showed that in a controlled experiment *P. yezoensis* had the potential to remove around 45% of NH_4^+ received from a fish tank within 24 hours. The NH_4^+ removal efficiency of *P. yezoensis* in this study was similar to those recorded in the literature (He et al., 2008). Therefore, our results demonstrate that *P. yezoensis* is an excellent candidate for use in seaweed-based integrated aquaculture systems.

The genus *Porphyra* has been shown to exhibit high tissue N level corresponding to seawater increases of N concentrations (Chopin et al., 1999). Carmona et al. (2006) found that the N content in *Porphyra* tissues apparently increased in response to increasing NH_4^+ availability, though this may not account for all of the increase in tissue N. In this study, the N content of *P. yezoensis* tissues was

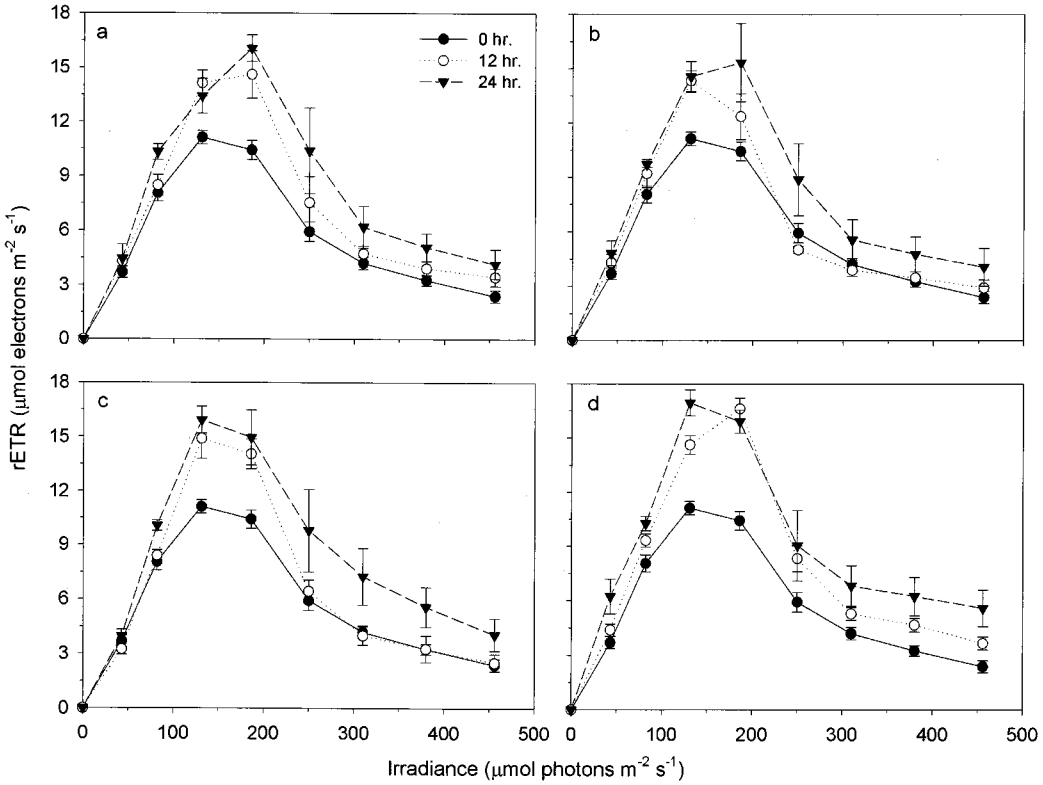

Fig. 5. The rapid light curve of relative electron transport rate (rETR) versus irradiance measured in *Porphyra yezoensis* cultivated using fish tank effluents in four seaweed tanks (a - tank III; b - tank IV; c - tank V; d - tank VI) during 24 hours. Data are expressed as mean ± SE (n=6).

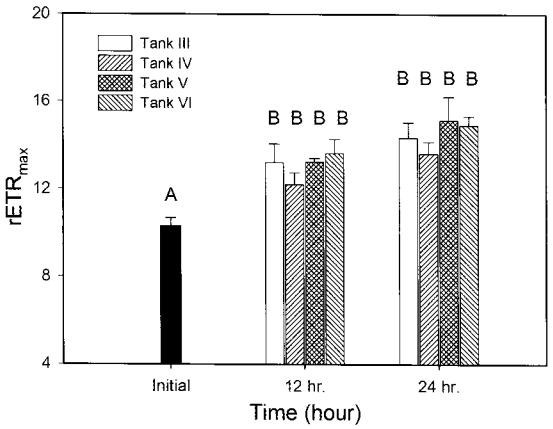

Fig. 6. The maximal relative electron transport rate ($rETR_{max}$) after 12 and 24 hours of *Porphyra yezoensis* cultivation under fish tank effluent. Different letters indicate significant group at means found with SNK tests ($p<0.05$). Data are expressed as mean±SE (n=6).

affected by N availability in water column and significantly ($p=0.03$) increased compared to the initial value. However, the C:N ratio of *P. yezoensis* tissues was significantly ($p<0.01$) lower after 24 hours than the initial value. The decline of C:N ratio was caused by the increase of tissue N content, as tissue carbon (C) content was relatively constant (average 35.5%) throughout the experimental period. The C:N ratio showed low values when N was abundant and increased when N supply was limited (Pinchetti et al., 1998). The capacity of *P. yezoensis* to take up, accumulate and store N during the experiment may account for these observations (Neori et al., 1991). Additionally, red algae are able to store N in red pigment and phycoerythrin and can remobilize the pigment nitrogen to support growth in the case of N deficiency (Harrsion and Hurd, 2001). Our results demonstrated that N stored in the tissue of *P. yezoensis* accounted for approximately 6% of the dry weight. This finding is similar to that of Chopin et al. (1999), where tissue nitrogen of a *Porphyra* species exceeded by 6% near salmon net pens. Therefore, the ability of *P. yezoensis* to store large amounts of nitrogen in their tissues may provide an efficient way of removing nutrients from eutrophic seawater (He et al., 2008).

It is difficult to evaluate the physiological status of seaweeds exposed to high NH_4^+ concentration in seaweed-based integrated aquaculture systems. However, PAM fluorometry has been suggested as a non-destructive technique for such purpose (Cabello-Pasini et al., 2000). The effective quantum yield ($\Delta F/F'_m$) of PS II has been found to be clearly a more sensitive indicator of photosynthetic stress than potential quantum yield (Ralph, 2000; Macinnis-Ng and Ralph, 2003). Figueroa et al. (2006) showed that the use of chlorophyll fluorescence was a powerful way to rapidly detect the health status in integrated cultivation of red macroalgae using fishpond effluents. In recent years, photosynthetic studies have emphasized that in addition to $\Delta F/F'_m$, $rETR_{max}$ is also closely related to level of photosynthetic activity (Ralph and Gademann, 2005). In this study, $\Delta F/F'_m$ and $rETR_{max}$ of *P. yezoensis* in all tanks estimated using the Diving-PAM increased significantly with time ($p<0.001$ and $p<0.001$, respectively). Both $\Delta F/F'_m$ and $rETR_{max}$ were higher at 24 hours than initially and after 12 hours, even though there were no significant difference (SNK test; $p=0.206$) in $\Delta F/F'_m$ between the measurements at 12 and 24 hours (Fig. 5). Pinchetti et al. (1998) reported that the abundance and depletion of N sources in a culture medium causes important cellular response in algae. For example, *Porphyra* species have been shown to exhibit higher pigment N contents under high N levels (Korbee et al., 2005). In red macroalgal studies, N-enrichment resulted in high levels of chlorophyll and phycobiliproteins (Figueroa et al., 2006). In addition, Peckol and Rivers (1995) found that maximum photosynthetic rate (P_{max}) and growth rate was increased under N enrichment in *Cladophora vagabunda* and *Gracilaria tikvahiae*. N enrichment was also found to lead to increases in P_{max} and $rETR_{max}$ (Longstaff et al., 2002). Together, these results indicated that the health of *P. yezoensis* estimated by $\Delta F/F'_m$ and $rETR_{max}$ was improved or remained unchanged during our relatively short experimental period of 24 hours (Fig. 4; Fig. 6). Finally, we can confirm that photosynthetic activity estimated using the Diving-PAM approach is a powerful tool for evaluating the physiological status of seaweed and that further research will improve the management of seaweed-based integrated aquaculture systems.

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