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Effects of shading, fertilization and snail grazing on the productivity of the water fern *Azolla filiculoides* for tropical freshwater aquaculture

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Abstract:

Water ferns (*Azolla* spp.) are among the main important floating macrophytes used for feeding farmed animals such as fish, because they have high growth potential and high protein content. Nevertheless, their use as feed requires sustainable mass production, which can be difficult to maintain in field conditions. We performed a first experiment to assess the effects of shading and fertilization levels on the growth of *Azolla filiculoides* with complementary information regarding the morphology and the chemical composition of the plant cultivated under the different experimental conditions. Plants were cultivated in floating 50-L plastic drums at three fertilization levels ("no", "low" and "high") using an inorganic multi-nutrient fertilizer rich in P (NPK = 1:2:1) and maintained under full natural light or shaded by one of two different types of shading materials, transparent polyethylene sheet and 60% shade net, respectively. A second experiment was carried out to evaluate the effects of grazing by the invasive golden apple snail *Pomacea canaliculata* on *A. filiculoides* previously cultivated without addition of fertilizer (treatment "n") or in high fertilizer concentrations (treatment "high"). Fertilization levels and shading materials significantly affected the growth of *Azolla*. The highest productivity was reached using the highest fertilization level under direct sunlight. *Azolla* produced in these culture conditions was preferentially grazed by snails compared to *Azolla* cultivated without added fertilizer. Based on these findings, we make recommendations regarding the best culture conditions for *A. filiculoides* in ponds for its use as sustainable fish feed.

<u>Keywords:</u> Aquaculture, Floating macrophytes, Light, Nutrients, Productivity, Grazing

1. Introduction

Water fern species (*Azolla* spp.) are considered as the most economically important floating aquatic macrophytes in the world (Brouwer et al., 2014; Kollah et al., 2016). *Azolla* spp. are among the fastest growing plants and can reach very high growth rates via asexual reproduction with a doubling time of only 2-5 days (Wagner, 1997; Sadeghi et al., 2013). These aquatic fern species are native to Central and South America and western North America (Sadeghi et al., 2013). Nevertheless, *Azolla* spp. are nowadays widely established in freshwater ecosystems of temperate and tropical regions all over the world (Sadeghi et al., 2013), demonstrating high adaptability to various environmental conditions. This excellent adaptability is explained by specific physiological characteristics.

Due to their symbiosis with nitrogen (N)-fixing microorganisms (diazotroph cyanobacteria *Nostoc* (ex. *Anabaena*) *azollae*; Kahindi et al., 1997), *Azolla* growth is vigorous even in natural N-limited conditions (Brouwer et al., 2017). Physiologically, the *Azolla-Nostoc* complex is outstanding, because it can fix N at substantial rates - approximately the double of *Rhizobia* living in the root nodules of soybean (Hung et al., 2013; Brouwer et al., 2017). The two symbiotic organisms' light-harvesting pigments are complementary and can capture a wide range of wavelengths of light (Wagner, 1997). These specific characteristics make *Azolla* spp. very suitable for many uses in agriculture and aquaculture (Pabby et al., 2004; Sithara and Kamalaveni, 2008; Kollah et al., 2016).

Beyond its high productivity, and according to the environmental conditions, *Azolla* spp. can reach high protein contents (15-40% of its dry weight; Brouwer et al., 2018; Feedipedia, 2018; Slembrouck et al., 2018) and can therefore be a valuable alternative to costly fish meal and soybean, especially in pond aquaculture systems (Datta, 2011; Gangadhar et al., 2015; Das et al., 2018). *Azolla* may represent a valuable resource to meet the current challenge for aquaculture: improvement in production to match the growing demand for aquatic products and in a sustainable manner. Thus, experiments carried out on many herbivorous or omnivorous fish species have shown that reasonable inclusion rates (usually not exceeding 30%, Abdel-Tawwab, 2008; Datta, 2011; Das et al., 2018) of *Azolla* (fresh or dry form) in the fish diet reduce production costs without affecting growth (for review, see Mosha, 2018). However, this use requires continuous mass production, which is generally not yet common on a large scale (Brouwer et al., 2018). Although, *Azolla* species have undeniable growth capacities, many factors can affect the mass production of these macrophytes.

Nutrient availability is often considered as the main bottleneck in the production of *Azolla*. Phosphorus (P) is a major nutrient limiting the growth of *Azolla* spp., as in many other photoautotrophic aquatic organisms (Sadeghi et al., 2013; Temmink et al., 2018), because the N_2-fixation rates are ensured by symbionts (Cary and Weerts, 1992; Kushari and Watanabe, 1991). In experimental conditions, 2 to 10 µmol L^{-1} P are reported for the sustainable growth of *Azolla* (Subudhi and Watanabe, 1981; Kitoh and Shiomi, 1991; Temmink et al., 2018), but field observations suggest that higher concentrations are necessary (10 to 33 µmol L^{-1}, Sadeghi et al., 2013). Based on this information, P may be limiting for *Azolla* production even in fertilized ponds used for fish production, where P concentrations are usually between 1-17 µmol L^{-1} (Green and Boyd, 1995; Pengseng and Boyd, 2011; Pouil et al., 2019). In addition, temperature and light intensity are important climate variables determining *Azolla* growth rates in the field (Sadeghi et al., 2013). Although different *Azolla* species or strains have different temperature sensitivities (Uheda et al., 1999), the optimum temperature range for *Azolla* growth is about 18-26°C (Sherief and James 1994; Hasan and Chakrabarti, 2009). Furthermore, high temperatures (>35°C) are known to inhibit *Azolla* growth (Sadeghi et al., 2013). Light intensity affects *Azolla* photosynthetic activity, growth and N_2 fixation (Watanabe, 1982; Pabby et al., 2003; Sagedhi et al., 2013). Although several experimental studies showed a decrease of *Azolla* biomass under shading conditions (i.e. plants received 30 to 60% of full sunlight, Cary and Weerts, 1992; Abduh et al., 2017), slight shade (25-50% of full sunlight) can benefit *Azolla* mass production (Hasan and Chakrabarti, 2009). However, field investigations are missing to support this statement. In addition, light interacts with temperature in influencing the growth of *Azolla* species (Janes, 1998). Despite knowledge mainly gained through laboratory experiments, only few studies investigated the productivity of *Azolla* under field conditions (e.g. Brouwer et al., 2018).

The growth of *Azolla* can also be negatively affected by biological factors. Arthropods such as Lepidoptera and Diptera, or crustaceans and gastropods can affect the growth of *Azolla* by grazing (Sadeghi et al., 2013). The golden apple snail *Pomacea canaliculata* is a freshwater gastropod, native to South America that has become a serious pest in Asian agriculture and is included in the world's 100 worst invasive alien species (Lowe et al., 2000). These snails, present in aquaculture ponds, are herbivorous and can feed on floating macrophytes such as *Azolla* (Cruz et al., 2015). Golden apple snails have a strong negative effect on the biomass of all macrophyte species in Asian wetlands (Carlsson and Brönmark, 2006) but, to our knowledge, the potential effects of these snails on the production of *Azolla* in the field have not been investigated.

We aimed to promote culture of *Azolla* in fish ponds as a potential alternative source of feed for freshwater fish. Thus, the objectives of this study were to assess the combined effects of fertilization levels and shading, considered among the main parameters for plant production, on (1) productivity of *Azolla filiculoides* in a fish pond setting with complementary information on chemical composition and morphology, and (2) on the grazing of *Azolla* cultivated in two fertilizer conditions by the invasive golden apple snail *P. canaliculata*. We hypothesized that the use of shading should support mass production of *Azolla* in fish ponds by maintaining favourable light and temperature conditions while snails should cause non-negligible losses of plant production.

2. Materials and Methods

2.1. Experiment 1: Effects of shading materials and fertilization on *Azolla* production

A six-week field experiment was carried out to assess the response of *A. filiculoides* to several culture conditions (3 fertilization levels x 3 light level conditions). The experiment was performed from August to September 2018 during the dry season in an artificial pond (400 m^2, depth 0.50 m) without fish. The pond was located in a fish farm of the village of Babakan (6°28'S; 106°42'E; altitude 125 m), district Bogor, West Java, Indonesia. The experimental block design consisted of 27 plastic half drums (cut lengthwise; water volume: 50 L, water depth: 0.25 m, surface: 0.5 m^2), oriented east-west, partially immersed in the pond and maintained in place with bamboo poles. Nine of these drums were covered by a 60% shade net, nine by a transparent polyethylene sheet, and nine were exposed to direct sunlight without any cover (control condition). Light intensity, water and air temperature were recorded continuously throughout the experiment using data loggers (HOBO Pendant Temperature/Light and HOBO Water Temperature Pro v2). Data loggers were placed in three drums per light condition at 10 cm depth for water temperature and at 5 cm above the water surface for air temperature and light intensity measurements.

The use of these low-cost and locally available shading materials resulted in a gradient of shading between the different experimental conditions. Thus, *Azolla* cultivated under the shade net and the transparent polyethylene sheet received 57% and 10% less light respectively compared to the plants exposed to direct sunlight (Table 1). The nine drums of each condition were filled with water from the pond (N: 33.5 ± 15.0 µmol L^{-1}, P: 5.8 ± 3.6 µmol L^{-1}, K: 31.7 ± 30.4 µmol L^{-1}). Commercial aqueous solutions of inorganic multi-nutrient fertilizer (Rosasol®-P hydroponic fertilizer with an NPK ratio of 1:2:1, see Table S1 for composition) was used as nutrients source. Since P is the most limiting nutrient for *Azolla* growth, fertilizer was added to obtain concentrations of 2 µmol L^{-1} of P (i.e., 3.2 and 1.5 µmol L^{-1} of N and K respectively; treatment “low”) and 10 µmol L^{-1} of P (i.e., 16 and 7.5 µmol L^{-1} of N and K respectively; treatment “high”). The third series of drums was maintained without fertilizer and served as the control (treatment “no”). Each condition was triplicated.

Before starting the experiment, *A. filiculoides* maintained in an aquaculture pond was acclimated to experimental conditions by inoculating 50 g fresh weight (FW) into the 50 L drums (i.e. 100 g FW m^{-2} and surface cover of ~20%). Plants were acclimated for 11 days without harvesting to minimize P history effects (Temmink et al., 2018). During the experiment, water in drums was changed 6 times per week. *Azolla* biomass was transferred in a net and drum walls were cleaned during the water change. Fertilizer addition to the fresh pond water was according to treatment, as described above. In order to check nutrient concentrations, two times per week, water was sampled 30 min after the fertilizer addition (300 mL per drum) and the operation was renewed the next day (i.e. 24 hours later) just before to change again the water. This procedure allowed to keep nutrient concentrations in water as constant as possible (at least 70% of N, P and K remained in the water after 24 h whatever the experimental condition). Before adding fertilizer, water measurements were performed in each drum (08:00-10:00 AM, two times per week) to determine pH, dissolved oxygen (DO, mg L^{-1}), temperature (T°C), and total dissolved solid matter (TDS, mg L^{-1}) using a multi-parameter probe (HI 9829 Hanna).

Total plant biomass in each drum was determined at the beginning and at the end of the 11-d acclimation period and then one time per week during the 6-week experiment. Samples were rinsed, carefully drained and weighed (± 0.1g). After FW determination, a 50 g sample (100 g FW m^{-2}) of *A. filiculoides* was returned into its original drum to avoid too high surface densities. Productivity (expressed as g FW m^{-2} d^{-1}) was calculated as the total amount of *Azolla* harvested in each drum minus the initial seeding (50 g FW per drum). When productivity was negative (i.e. mortality of *Azolla*), plants from different stocks maintained under appropriate fertilizer and shading conditions were used to re-inoculate the drums. Samples from each drum (each 10 g FW) were carefully weighted as described above and were taken to determine dry weight (DW). The remaining samples were stored at -18°C for further analyses.

2.2. Experiment 2: Quantification of *Azolla* ingestion by snails

The grazing of the invasive golden apple snail *P. canaliculata* on *Azolla* cultivated outdoors was assessed at two extreme nutrient concentrations (treatments “no” and “high”). The experiment was carried out indoors (light: <2 µmol PAR m^{-2} s^{-1}) to prevent autogenic changes *Azolla* biomass (i.e. changes in biomass caused by endogenous factors and not snail grazing). The experimental set-up consisted of thirteen plastic buckets (6 L, 34 cm diameter) covered with a 60% shade net to prevent snails from escaping. Twelve buckets were used to quantify the ingestion of *Azolla* (from the treatments “no” and “high”) by snails (n = 6 per condition) and one basin served as the control, without snails (control). Water temperatures were recorded continuously throughout the experiment using a data logger (HOBO Water Temperature Pro v2) placed at the bottom of the bucket used as the control.

Twelve snails (FW: 20.1 ± 3.2 g, shell length: 3.8 ± 0.2 mm, shell width: 2.9 ± 0.2 mm) were randomly placed in the buckets (one snail per bucket) and acclimated for three days to the experimental conditions. Buckets were filled with water from the pond (see Section 1.2.1). During the acclimation period, each snail was fed with 10 g of fresh *Azolla* from treatments “no” and “high”. At the end of the acclimation period, 20 g of fresh *Azolla* coming from treatments “no” or “high” was added to each bucket (n = 6 per treatment). After 24 h, the remaining *Azolla* (i.e. non-ingested) was carefully drained, weighed and dried. The water was changed at the same time. Measurements were repeated for four consecutive days using the same procedure. At the end of the experiment, dry weight (DW) and the flesh-to-total FW ratio of each snail was determined. Thus, daily *Azolla* ingestion was calculated as the difference between quantity of *Azolla* added to the bucket and the quantity remaining (on a DW basis) and expressed as grams (DW) of *Azolla* eaten per gram of snail flesh (DW) per day.

The dietary preference of snails (FW: 18.0 ± 2.3 g, shell length: 3.8 ± 0.1 mm, shell width: 3.0 ± 0.2 mm) for one of the two *Azolla* growing conditions (treatments "no" and "high") was also tested through a multiple-choice experiment. A floating plastic separation was placed in three basins each containing one snail and 10 g each of *Azolla* coming from the treatments "no" and "high" were introduced on either side of the plastic separation. As for the previous experiment, another bucket served as the control without snail. After 72 h, the remaining *Azolla* on each side was carefully drained and weighed, and the percentage of ingestion of fresh *Azolla* was calculated.

2.3. Sample analysis

Total Kjeldahl nitrogen (TKN) in water was measured following the Indonesian National Standard (SNI) 06-6989.52-2005 using Macro-Kjeldahl apparatus coupled with titration Total N was then determined as the sum of TKN, NO_3^--N and NO_2^--N. Phosphorus (P) in water was determined based on the procedures described in the SNI 06-6989.31-2005 adapted from APHA 4500-PE (APHA, 2005). The chemical composition analyses were performed after the 11-d acclimation period (n = 1 per condition). Proximate analyses in *Azolla* samples were performed following Cunniff (1999). Moisture was determined by weight loss upon drying at 105°C for 24 h. Crude protein was determined using the standard Kjeldahl procedure; lipid content after acid hydrolysis was determined using the Weibull-Stoldt method; crude ash was determined on residue after heating at 550°C for 4-5 h in a muffle furnace. Crude fibre was determined as follows: macrophytes were extracted with 1.25% H_2SO_4 and 1.25% NaOH, dried, weighed, incinerated and reweighed. Gross energy content was calculated using energy value coefficients of 9 kcal for crude fat and 4 kcal for crude protein and carbohydrates. For total P and K determination, plant samples were weighed and digested in nitric acid for 20 min at 190°C. Measurements were then performed using an inductively-coupled plasma optical emission spectrometer. Chlorophyll content of *Azolla* was measured after acetone extraction by spectrophotometry. The absorbance was measured at wavelength of 663 nm and 645 nm. Calculation of chlorophyll levels was carried out based on Arnon's equation (Arnon, 1949).

2.4. Data treatment and statistical analysis

Prior to analysis, temperature data recorded in lux were converted to µmol PAR m^{-2} s^{-1} using x54 as conversion factor (Thimijan and Heins, 1982). Because of non-normal distribution of the data, statistical comparisons were done for the environmental parameters (air and water) monitored under the different shading conditions using the non-parametric Kruskal-Wallis and Siegel and Castellan tests (Siegel and Castellan, 1988).

A linear mixed model (LMM) was used to analyse differences in *Azolla* productivity at the nine experimental conditions (3 shading conditions x 3 fertilization levels). Shading materials and fertilizer conditions (with the interaction term) were considered as fixed effects in the model. The individual plastic drums in which *Azolla* grew were considered as random effects. Assumptions of normality and homoscedasticity were checked on residuals of the model. Contrast analysis was then performed as the mean separation procedure with a Bonferroni-corrected p-value (α of 0.05/36 = 0.0014).

Analysis of covariance (ANCOVA) was then used to test for effects of light and other environmental covariables (air and water temperatures) on the productivity of *Azolla* cultivated at the three fertilization levels ("no", "low" and "high").

Quantities of ingested *Azolla* (from treatments “no” and “high”) by snails (expressed as g g^{-1} DW d^{-1}) and feed preferences (i.e. % of ingestion) were statistically compared using the non-parametric Mann-Whitney U test because of the non-normal distribution of the data. The level of significance for ANCOVA and Mann-Whitney U test was set at $\alpha = 0.05$. All statistics were performed using R freeware version 3.3 (R Development Core Team, 2016). Unless otherwise stated, values shown are means ± SD.

3. Results

3.1. Response of *A. filiculoides* to culture conditions

In our experiment, depending on the weather and the shading conditions (Table 1), we observed air and water temperatures ranging from 23 to 40°C and light intensity reached peaks of 278 µmol m^{-2} s^{-1} (Fig. 2).

Fertilization levels and shading materials affected the growth of *Azolla* (Fig. 1). Overall, in the absence of added fertilizer, the productivity of *Azolla* was limited to 0.15 ± 0.73 g DW m^{-2} d^{-1} (n = 54) over the experiment and *Azolla* cultures collapsed in 43% of measurements performed (23 out of a total of 54). Conversely, the productivity of *Azolla* cultivated at a higher fertilizer level (treatment “high”) reached 3.80 ± 1.44 g DW m^{-2} d^{-1} (n = 54) with doubling times of <1 d in some cases and no observed production collapse (Fig. 1).

Our LMM model indicated that both shading conditions ($p < 0.001$) and fertilizer levels ($p = 0.001$) affected the growth of *Azolla*. Significant interactions were found between the shading conditions and the fertilization treatment indicating that the effects of shading conditions are dependent of the fertilization level. Indeed, for the two lowest fertilizer addition treatments (“no” and “low”), productivity of *Azolla* was similar between the three shading conditions (t-ratio = 4.065-0.87, p = 0.016-0.992). Nevertheless, for the treatment “high”, productivity of

Azolla was significantly lower (t-ratio = 6.521, p = 0.001) under the shade net (in average -39%) and compared to the other shading conditions.

ANCOVA performed for the three fertilization levels (“no”, “low” and “high”) revealed that light intensity significantly affected *Azolla* productivity cultivated in the “high” fertilization condition (F = 16.805, p = 0.001) while there were positive significant effects of increasing air temperatures on productivity in the “low” and “high” fertilization conditions (F = 4.904, p = 0.044 and F = 13.422, p = 0.003 respectively) (Fig. 2).

We observed that morphology of *A. filiculoides* changed in response to different experimental treatments. Visual aspect indicated that plants from the treatment “no” (no fertilizer added) were smaller and more fragile than those from the “low” and “high” fertilizer treatments (lower breaking strength of the fronds). Furthermore, control plants turned brownish, whereas plants from treatments “low” and “high” stayed green (mg kg^{-1} FW of chlorophyll respectively, n = 3, Table 2). A slight difference (χ^2 = 6.057, p = 0.045) was observed in the chlorophyll concentrations between the treatments “no” and “low” (343 ± 50 and 383 ± 23 mg kg^{-1} FW of chlorophyll respectively, n = 3) and the treatment “high” (450 ± 17 mg kg^{-1} FW of chlorophyll, n = 3). Although the number of *Azolla* samples analysed was limited by the low productivity in the treatment “no” (n = 1 per condition), the chemical composition analyses indicated that, after the acclimation period, P and K concentrations increased with fertilizer concentrations from 121 to 331 and 616 to 1113 mg kg^{-1} FW respectively, Table 2.

3.2. Impacts of the golden apple snail on *Azolla* production

During the experiments, no biomass change was observed in *Azolla* from both the treatment “no” and the treatment “high” maintained without snail (i.e. control condition). The consumption of *Azolla* by the golden apple snail *P. canaliculata* reached 0.06 ± 0.04 g g^{-1} DW d^{-1} and 0.17 ± 0.07 g DW g^{-1} d^{-1} when snails (n = 6 snails x 4 d) were fed on *Azolla* from the treatment “no” and the treatment “high” respectively (U = 536, $p < 0.001$, Fig. 3). These results were confirmed by estimation of the dietary preference of snails between *Azolla* from the treatments “no” and “high”. When snails (n = 3) were able to choose between *Azolla* from the two fertilizer conditions, they slightly preferred (U = 9, p = 0.045) *Azolla* coming from the treatment “high” (79%, 80% and 88% of consumption after 72 h) to *Azolla* from treatment “no” (16%, 21% and 30% of consumption after 72 h).

4. Discussion

Some authors found that *Azolla* is able to grow in shade conditions (50% sunlight) and partial shade may favour its growth in field conditions (see for review: Hasan and Chakrabarti, 2009) contrasting with other experimental studies. Abduh et al. (2017) highlighted that *Azolla* growth gradually decreased under shading conditions (up to 50% sunlight) while Cary and Weerts (1992) found that biomass yields of plants receiving 30% of greenhouse sunlight were less than one-third those of plants receiving no shading. Nevertheless, comparisons between studies are complex. Indeed, shading materials used are likely to affect not only the intensity but also the spectrum of the light received. Although we did not measure light spectra, another study performed on a large number of shading materials, revealed that the photon irradiance is reduced at wavelengths from 260 to 500 nm (-42% for UVB: 280-315 nm, -30% for UVA: 315-400 nm and -13% for blue: 400-500 nm) by polyethylene sheets (Kotilainen et al., 2018). The same authors found that the light spectrum under 50-70% shade nets remained similar to ambient sunlight spectrum. In this field experiment, we experimentally demonstrated that there is no benefit of using such shading materials for *Azolla* growth in field conditions suggesting that outdoor fish ponds may be an appropriate culture structures for a mass production of *Azolla*.

The water fern *A. filiculoides* is known to be sensitive to air temperatures with optimal for growth ranging from 20 to 30°C (Hasan and Chakrabarti, 2009; Sadeghi et al., 2013). In our experiment, depending on the weather conditions and the shading materials used (Table 1), we observed air temperatures ranging from 23 to 40°C. As for light intensity, increasing temperatures had a positive effect on *Azolla* productivity, indicating that in our experimental conditions high temperature was not a limiting factor for *Azolla* growth. Our results suggest that light and temperature have positive effects on *Azolla* production within small-scale fish farms in tropical environments.

The influences of temperature and light depended on fertilizer concentrations. In the absence of added fertilizer (treatment "no"), light, air and water temperatures had no effect on growth. In this condition, *Azolla* productivity was low and highly variable with regular production collapses. There were significant positive linear relationships between the environmental variables (light, air and water temperatures) and the productivity of *Azolla* cultivated at the high fertilization level (treatment "high") and, to a lesser extent, for *Azolla* grown at the intermediate fertilizer level (treatment "low"). Based on our results, we estimated, for *Azolla,* cultivated under direct sunlight and high fertilizer addition, yields of 30 ± 9 t DW ha^{-1} $year^{-1}$.

In the present study, *Azolla* was harvested every week, and a minimum of 100 g m^{-2} was maintained in culture. As suggested by Brouwer et al. (2018), regular harvests of *Azolla* can be used to maintain cultures in a linear growth phase and to obtain predictable and high biomass yields. Our results confirm that the biomass production of *Azolla* depends on fertilizer concentrations and presumably by P concentrations because this nutrient is the most limiting in *Azolla* culture (Subudhi and Watanabe, 1981; Kushari and Watanabe, 1991; Temmink et al., 2018). Although production varied with environmental conditions (light and temperature), productivity remained high (median values: 44, 73 and 79 g FW m^{-2} d^{-1} for *Azolla* maintained under a shade net, clear plastic or exposed to direct sunlight, respectively) when plants were cultivated at high fertilizer concentrations (addition of 16, 10 and 7.5 μmol L^{-1} of N, P and K respectively), regardless of the environmental conditions.

Here, we used an inorganic multi-nutrient fertilizer rich in P (NPK = 1:2:1, see Table S1). Thus, other important nutrients for *Azolla*, such as iron (Fe), which may be limiting under certain conditions and cause chlorosis (Temmink et al., 2018), were already incorporated in the fertilizer. Although no visible evidence of Fe deficiency has been observed, it is possible that other trace elements like Co, Cu, Mn, Mn and Zn required for *Azolla* growth, particularly in relation with its nitrogen fixation metabolism (Sadeghi et al., 2013) might have been limiting in the experimental condition without fertilizer. The use of multi-nutrient fertilizers mitigated the risk of trace element deficiency. Overall, *Azolla* cultivated under direct sunlight and with a fertilization level equivalent to the "high" condition may presumably generate 6.9 ± 2.2 t protein ha^{-1} $year^{-1}$, about three-fold higher than soybean (*Glycine max*) without arable land use (Brouwer et al., 2018).

The morphology and chemical composition of *A. filiculoides* also changed in response to different experimental treatments. We observed that plants from the control were smaller and more fragile than those from the intermediate and high fertilizer treatments. Furthermore, control plants turned brownish whereas plants from treatments "low" and "high" stayed green. The chemical composition analyses indicated that P and K concentrations in plants increased with fertilizer concentrations. Similar results were observed for crude protein (from 14 to 23% of DW) and energy content (from 8.7 to 10.9 MJ kg^{-1} FW) while the fibre content decreased from 21 to 15% of DW. These findings corroborate observations of Subudhi and Watanabe (1981) on *A. pinnata* who found increased of N (from 2.78 to 4.53 % of DW) and P (from 0.03 to 0.11 % of DW) cultivated with 0, 1 and 2 μmol L^{-1} of P additions. All together, these results indicate that visual aspect of *Azolla* (size and colour) can be used as an indicator of its health and its nutritional quality.

Previous studies have shown that the amino-acid profile of *Azolla* is suitable for fish feed (see for review: Feedipedia, 2018). Given that the fibre content in *Azolla* cultivated under direct sunlight was the lowest that we measured (13% of DW), we also suggest that the recommended culture conditions can also improve the digestibility of *Azolla* for fish (Maina et al., 2002). However, culture conditions (CO_2 concentrations) also affected the total polyphenol content of *Azolla*, thereby affecting digestibility (Browner et al. 2018). In addition, plant-based feed may contain other anti-nutritional factors such as anti-vitamins, phytic acid protease inhibitors and tannins (Dersjant-Li, 2002; Drew et al., 2007). Such factors were not considered in our study and more research is needed to confirm the effects of the culture conditions on the concentrations of these compounds in *Azolla*.

Regarding the impacts of the golden apple snail on *Azolla* production, we found that snails were able to ingest daily up to 38% of their body weight (FW) of *Azolla,* with a preference for *Azolla* cultivated in high fertilization level. These results highlight the huge negative impact of the presence of the golden apple snail on *Azolla* productivity. The average biomass of *P. canaliculata* has been estimated at 0.07 kg m^{-2} (i.e. approx. 3-4 adult individuals m^{-2}) in Javanese aquaculture ponds (Pouil et al., 2019) which is in accordance with density observed in Philippines rice paddies (i.e. 1-5 individuals m^{-2}, Cowie, 2002) although higher densities up to 150 individuals m^{-2} have been reported (Halwart, 1994; Schnorbach, 1995). Based on this finding, the yield loss of *Azolla* due to ingestion by snails can reach about 12.3 t DW ha^{-1} $year^{-1}$ (i.e. 35% of the estimated average yield, see Section 3.2). Further large-scale investigations are needed to confirm our findings.

Based on our observations, even maintaining *Azolla* in optimal conditions for production (direct sunlight and addition of fertilizer with high P concentration), does not overcome the risk of drastic drops in yields or even total loss of *Azolla* due to snails. The golden apple snail is able to select among macrophytes and prefers macrophytes species with high N and Ca contents and low C:N ratios (Zhao et al., 2012). These facts may explain the preference of snails for *Azolla* cultivated in optimal culture conditions (i.e. with the highest nutritional content). Considering the high risk represented by snails in the mass culture of *Azolla* in aquaculture ponds, we recommend (1) cultivating *Azolla* in snail-free ponds, (2) installing protections (e.g. wire-mesh grills; Cowie, 2002) in association to snail predator fish such as common carp (Sin, 2006) to prevent access of snails to ponds and/or (3) regularly trapping snails (e.g. using baited traps), which are traditionally used for other purposes, such as feeding poultry or certain fish (Cowie, 2002).

The present study demonstrates some important requirements for the mass production of *Azolla* in aquaculture ponds. This aquatic plant crop is potentially useful to improve sustainability of tropical pond aquaculture requiring a limited production surface with only few inputs and no nitrogen fertilizer. Potential yields are very promising compared to terrestrial crops. However, several practices must be optimised, especially regarding fertilization and risk of grazing by snails to maintain yields over time and sustain a mass production.

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Conflict of Interest and Ethical statement

The authors declare that they have no conflict of interest. This article does not contain any studies with animals performed by any of the authors.

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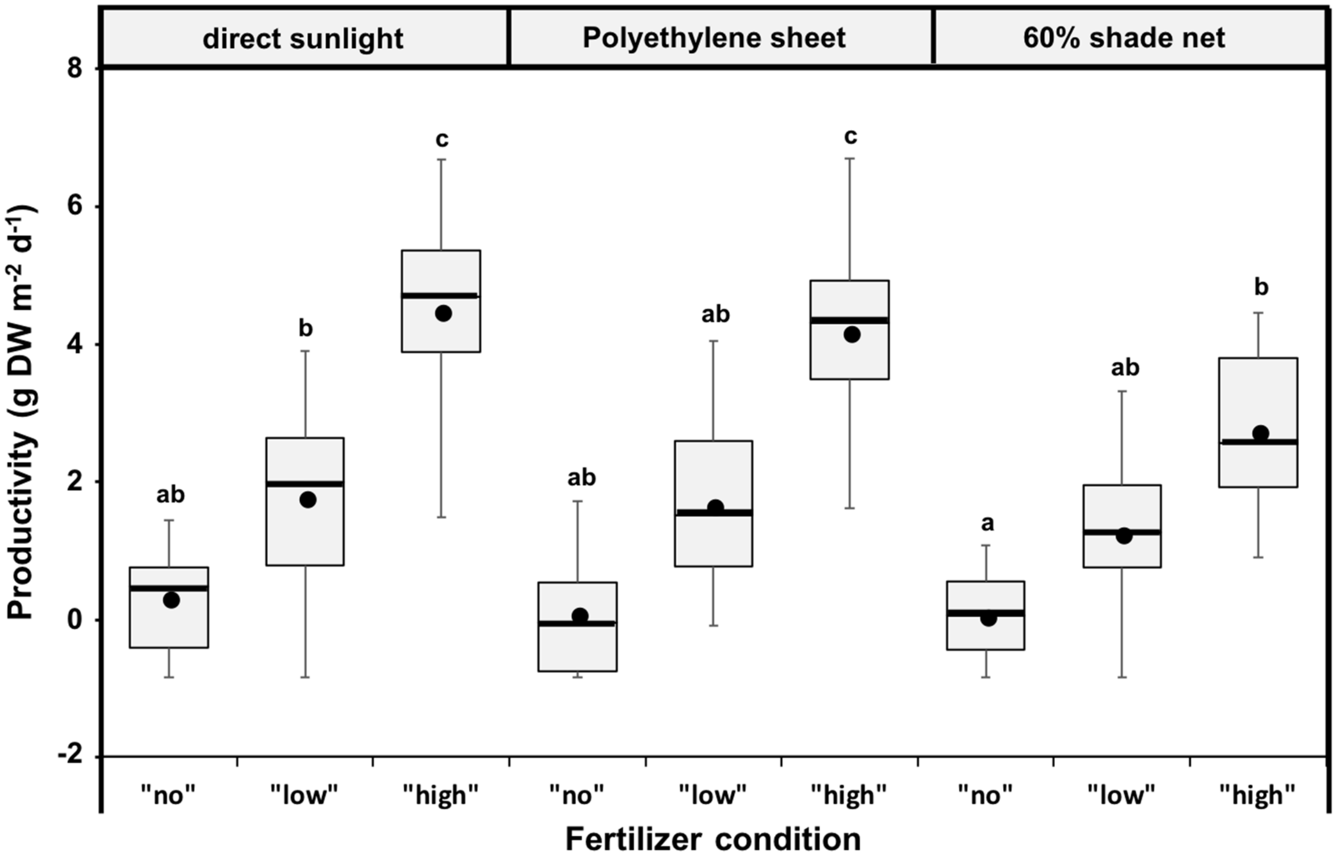
Captions to figures

Figure 1. Productivity of *Azolla* (g FW m^{-2} d^{-1}, n = 3 replicates x 6 weeks = 18 per treatment) cultivated at the three fertilization levels (treatments “no”, “low” and “high”) under three shading conditions: direct sunlight, polyethylene sheet and 60% shade net. Limits of the box indicate the first and the third quartiles. Whiskers indicate the maximum and minimum values, black lines and black dots indicate the median and mean values respectively. Different letters denote significant differences.

Figure 2. Relationships between light intensity (µmol m^{-2} s^{-1}), air temperature (°C) and average productivity (g FW m^{-2} d^{-1}) of *Azolla* cultivated at the three fertilization levels (treatments “no”, “low” and “high”) under three shading conditions: direct sunlight, polyethylene sheet and 60% shade net. For light intensity and air temperature, data are the average values recorded during the 7 d before harvest. Regression lines are shown in black and the dotted lines indicate the upper and lower 95% confidence levels. Equations, determination coefficients (R^2) and significance (p-values) are also given.

Figure 3. Daily ingestion (g g^{-1} DW d^{-1}, n = 6 per day) of fresh *Azolla* cultivated at fertilization levels (treatments “no” and “high”) by the golden apple snail (A) and feed preference (expressed as percentage of ingestion over 72 h, n = 3) between the two fertilization levels N0 and N2 (B). Data are means ± SD. Letters denote significant differences.


direct sunlight
Polyethylene sheet
60% shade net
8
6
4
2
0
-2
Productivity (g DW m-2 d-1)
ab
b
c
ab
ab
c
a
ab
b
"no"
"low"
"high"
"no"
"low"
"high"
"no"
"low"
"high"
Fertilizer condition

581 Figure 1

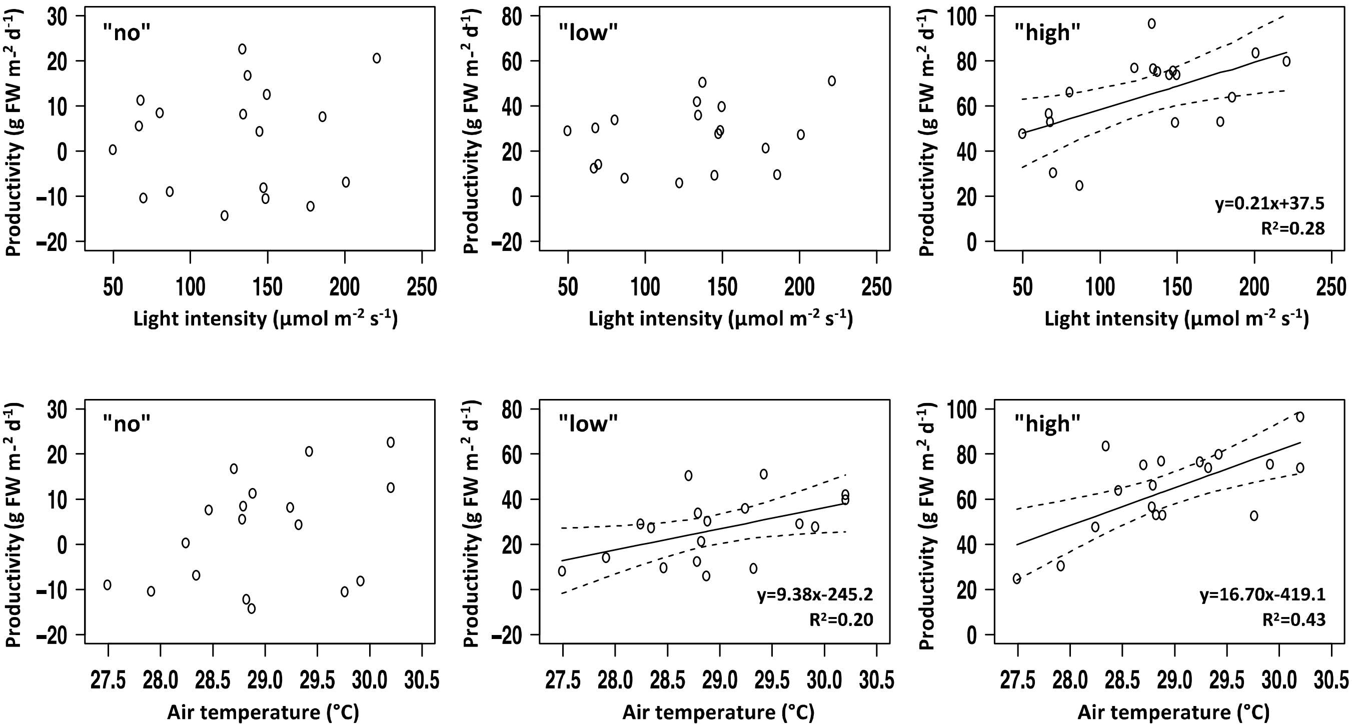
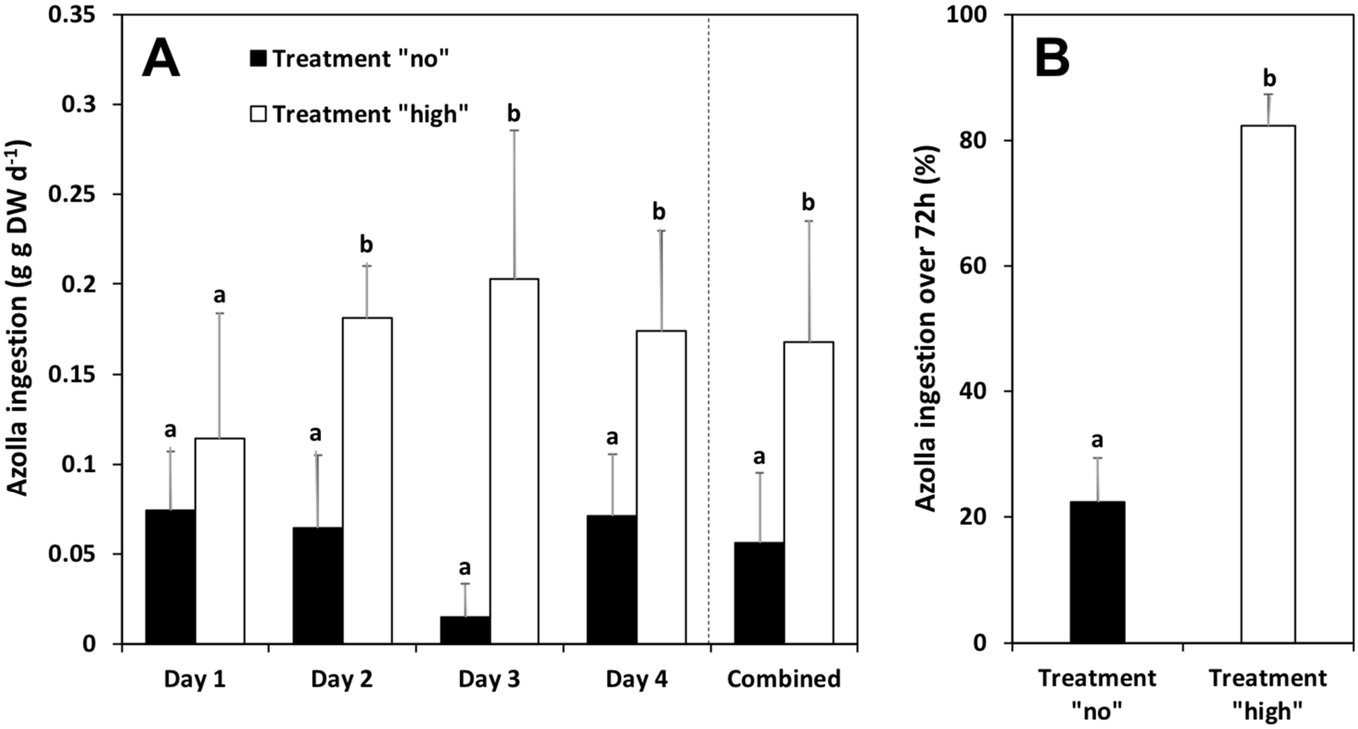

"no"
"low"
"high"
Productivity (g FW m-2 d-1)
Light intensity (µmol m-2 s-1)
Air temperature (°C)
y=0.21x+37.5
R²=0.28
y=9.38x-245.2
R²=0.20
y=16.70x-419.1
R²=0.43

Figure 2


A
Treatment "no"
Treatment "high"
Azolla ingestion (g g DW d-1)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
b
a
b
a
b
a
b
Day 1
Day 2
Day 3
Day 4
Combined
B
Azolla ingestion over 72h (%)
100
80
60
40
20
0
a
b
Treatment "no"
Treatment "high"

583 Figure 3

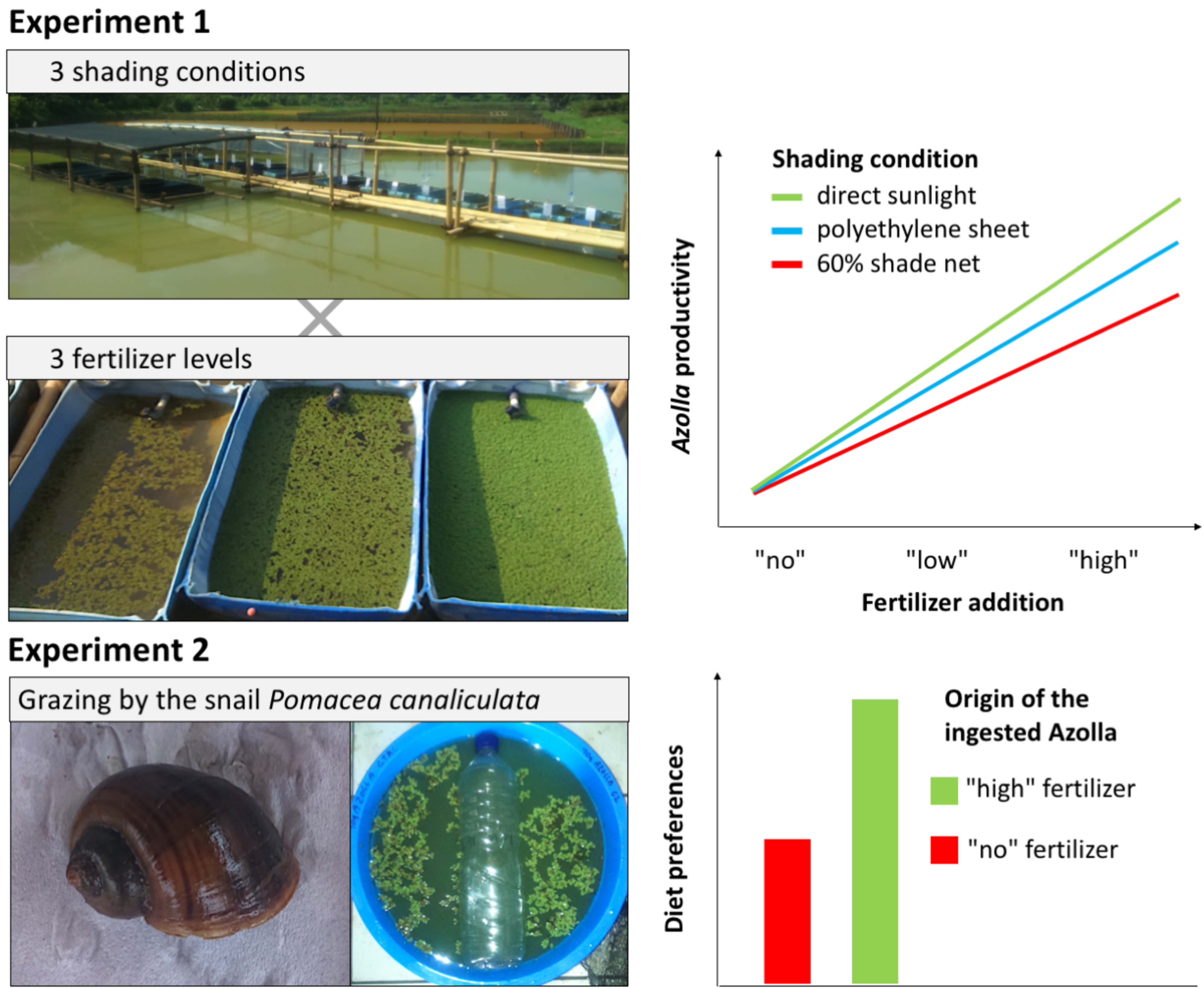
Table 1. Summary of the environmental conditions measured throughout the experiment in the three shading conditions (n = 99 for discrete measurements): direct sunlight, polyethylene sheet and 60% shade net. Data are means ± SD. Letters denote significant differences.

Parameters	Shading condition		
	direct sunlight	polyethylene sheet	60% shade net
Continuous records (data loggers)			
Diurnal light intensity (μmol m^{-2} s^{-1})	359 ± 403^{a}	324 ±288^{b}	153 ± 133^{c}
Atmospheric temperature (°C)	33.25 ± 4.78^{a}	34.66 ± 5.19^{b}	31.84 ± 4.03^{c}
Water temperature (°C)	29.17 ± 2.65^{a}	29.35 ± 2.35^{b}	28.72 ± 1.94^{c}
Discrete measurements (performed between 08:00 and 10:00 AM)			
pH	7.09 ± 0.19^{a}	7.05 ± 0.20^{ab}	6.99 ± 0.22^{b}
DO (mg L^{-1})	5.95 ± 1.02^{a}	5.62 ± 0.65^{b}	5.22 ± 0.59^{b}
Conductivity (μS cm^{-2})	74.19 ± 10.05^{a}	74.48 ± 10.49^{a}	74.62 ± 10.29^{a}
TDS (mg L^{-1})	37.08 ± 5.03^{a}	37.17 ± 5.18^{a}	37.31 ± 5.15^{a}

Table 2. Chemical composition of *Azolla* (n = 1 per experimental treatment) cultivated at the three fertilization levels (treatments “no”, “low” and “high”) under three shading conditions: direct sunlight, polyethylene sheet and 60% shade net. Analysis were performed after the 12-d acclimation period.

	Shading conditions								
	direct sunlight			polyethylene sheet			60% shade net		
Fertilizer condition	“no”	“low”	“high”	“no”	“low”	“high”	“no”	“low”	“high”
Proximate									
Humidity (% FW)	90.41	90.27	89.17	89.68	88.48	87.74	92.66	90.58	89.76
Ash (% DW)	25.00	24.03	24.00	29.46	29.9	25.77	23.61	29.58	26.98
Fibre (% DW)	20.28	16.16	13.00	20.02	15.71	15.02	21.45	18.75	15.06
Lipid (% DW)	1.10	1.08	1.57	0.99	1.27	1.65	0.95	1.62	1.41
NFE* (% DW)	39.96	43.07	38.50	35.44	36.45	35.72	38.49	31.94	34.95
Protein (% DW)	13.66	15.66	22.93	14.09	16.67	21.84	15.50	18.11	21.60
Energy (MJ kg^{-1} FW)	9.39	10.24	10.87	8.66	9.37	10.25	9.39	8.99	10.00
Chlorophyll and nutrients									
Chlorophyll (mg kg^{-1} FW)	290	370	430	350	370	460	390	410	460
K (mg kg^{-1} FW)	683.7	817.9	947.5	616.2	692.1	949.4	618.5	683.2	1112.8
P (mg kg^{-1} FW)	121.4	169.3	318.5	129.0	168.8	280.6	131.3	153.7	330.9

* Nitrogen Free Extract.


Experiment 1
3 shading conditions
3 fertilizer levels
Shading condition
direct sunlight
polyethylene sheet
60% shade net
Azolla productivity
"no"
"low"
"high"
Fertilizer addition
Experiment 2
Grazing by the snail Pomacea canaliculata
Diet preferences
Origin of the ingested Azolla
"high" fertilizer
"no" fertilizer