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Effects of humic acid on nutrient removal efficiency of aquatic duckweed (*Lemna minor*) and both growth performance, and hemato-biochemical parameters of Nile tilapia (*Oreochromis niloticus*) cultured in water recirculating system

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ABSTRACT

This study was carried out to evaluate the effects of humic acid (HA) on the nutrient removal efficiencies of aquatic duckweed plant (*Lemna minor*) from a water recirculating system used to culture Nile tilapia (*Oreochromis niloticus*) fish for 30 days. The HA was added to water at three concentrations of 0 (Control), 1.5, and 3 mg/L in triplicate. Water quality parameters, growth performance, and some hemato-biochemical parameters of the fish in variable HA concentrations were compared. The total ammonia nitrogen (TAN) and total phosphorous (TP) removal efficiency of *L. minor* increased with increasing the HA concentration from 0 mg/L to 3 mg/L ($p < 0.05$). The concentration of nitrate (NO_3^-) in the HA-3 mg/L was higher than that in the other groups on days 20 and 30 of the fish cultivation period ($p < 0.05$). The growth performance of fish improved in the HA-3 mg/L compared to the other groups. The addition of different concentrations of HA to water had no adverse effect on the hematological properties of the Nile tilapia. The plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels in the HA-0 mg/L and HA-1.5 mg/L groups were higher than in the HA-3 mg/L ($p < 0.05$). No significant differences in the plasma glucose and cholesterol levels were observed between the HA-groups ($p > 0.05$), while the triglyceride level increased in the HA-3 mg/L compared to the control ($p < 0.05$). These results indicated that adding HA to water could be an effective method to enhance the bioremediation performance of the aquatic duckweed plants as biofilter and thus improve water quality, subsequently, fish growth performance in RASs.

NOVELTY STATEMENT

The current study applied aquatic duckweed plant (*Lemna minor*) as a new biofilter in a water recirculating system used to culture Nile tilapia (*Oreochromis niloticus*) fish. The effects of three concentrations of humic acid (HA) as water additive on the nutrient removal efficiency of *L. minor* from water were investigated. HA improved bioremediation performance of the aquatic duckweed plant.

KEYWORDS

Aquaculture; humic acid; nutrient recovery; phytoremediation; water quality

Introduction

Aquaculture is one of the most essential sectors providing nutritious animal protein sources needed by humans (FAO 2020). The increasing demand for edible fish and reducing their catch from natural water resources has led to the rapid growth of the aquaculture industry in recent years. Nowadays, intensive cultivation systems like recirculating aquaculture systems (RASs) are used more in the aquaculture industry to meet the growing need of humans for aquatic animals, which leads to the production of more nitrogen, and phosphorus compounds (Ferdoushi *et al.* 2008). The exposure of fish to high concentrations of nitrogen compounds, such as ammonia, nitrite, and nitrate harmfully affects their welfare, and growth performance (Xu *et al.* 2021; Hu *et al.* 2022). The discharge of nitrogen and phosphorus-rich effluent from aquaculture systems into the surrounding environment also leads to eutrophication, and algal bloom, which will ultimately reduce water quality and cause other environmental problems (Selvarani *et al.* 2015; Gao *et al.* 2022). It is well known that nitrogen and phosphorus elements are the cellular components of all living organisms such as fish and plants. Therefore, the recovery of these nutrients by plants, known as phytoremediation, is a new strategy to improve fish production and diminish their adverse environmental impacts (Gao *et al.* 2022). The potential applications of aquatic plants as a biological process to remove nutrients and pollutants from the water of the aquaculture systems have received attention in recent

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years (Zhang *et al.* 2014; Nizam *et al.* 2020; Sarkheil and Safari 2020).

Phytoremediation is an energy-efficient, cost-effective, sustainable, affordable, and environmentally friendly technology that uses plant-based systems and microbiological processes for the remediation of various pollutants from the environment (Gupta P *et al.* 2012; Kinidi and Salleh 2017). Plants with quick growth rates, good root systems, high uptake capacity of nutrients and pollutants, high biomass yield, and high tolerance to different types of pollutants are suitable in phytoremediation technology (Arslan *et al.* 2017; Burges *et al.* 2018). The bioremediation performance of plants also depends on other factors, such as temperature, salinity, pH, nutrient availability, solar radiation, and type of plant species (Kinidi and Salleh 2017; Reeves *et al.* 2018; Tewes *et al.* 2018). The application of aquatic plants for the biological treatment of wastewater is expanding due to its ability to assimilate and degrade pollutants (*e.g.*, ammonium, nitrate, and phosphate) (Mohebi and Nazari 2021). Duckweed is an aquatic plant with floating leaves and submerged roots that belong to the family Araceae and subfamily Lemnoideae (Les *et al.* 2002). This plant is small, fast-growing and is found richly in many water resources. Duckweeds can withstand broad changes in temperature (7–35 °C), pH (3.5–10.5), and nutrient level, which makes them a suitable candidate for phytoremediation (Krishna and Polprasert 2008; Radić *et al.* 2010). Among aquatic plants, duckweeds can uptake ammonium ions as a preferred nitrogen source (Xu and Shen 2011). Therefore, the duckweed plant can be considered as a biofilter to reduce total ammonia nitrogen (TAN) in aquaculture systems.

Ammoniacal nitrogen is the primary nitrogen form excreted by aquatic animals, and also produces from the degradation of residual feed, feces, and other organic waste matters in the aquaculture systems. Molecular ammonia (NH_3) is very soluble in water, and can be converted into ionized ammonia (NH_4^+) under the influences of the water temperature, and pH (Clément and Merlin 1995; Rezagama *et al.* 2017). Unionized ammonia is more toxic than ionized ammonia due to its more penetration into biological membranes (Chen *et al.* 2006; Rezagama *et al.* 2017) and both of these forms are known as limiting factors in the aquaculture systems. Plants require ammonium and nitrate ions more than other mineral nutrients for growth (Kinidi and Salleh 2017). Nitrogen utilization by the plant occurs through three steps, including uptake, assimilation, and translocation (Masclaux-Daubresse *et al.* 2010). The plants utilize ammonium more quickly than nitrate because of the lower energy requirement for the uptake and assimilation of the ammonium ions compared to the nitrate ions (Jampeetong and Brix 2009). Hu *et al.* (2019) reported that removal efficiency of total ammonia nitrogen (TAN) by *Lemna aequinoctialis* within 70 days from anaerobically digested swine wastewater (ADSW) was 70.86%. Removal efficiencies of NH^{4+}-N by *Spirodela polyrrhiza* in swine wastewater also increased to 81.6% after 28 days (Li *et al.* 2023). Therefore, the use of plants with ammoniacal nitrogen removal potential can be helpful in the treatment of aquaculture wastewater. However, phytoremediation is both space and time-consuming than conventional remediation technology. Also, there is insufficient knowledge about the sustainable performance of aquatic plants as biological reactors in aquaculture systems. Thus, many studies are needed to ensure the efficiency of the phytoremediation of aquaculture wastewater.

Humic substances (HS) are one of the most essential components of all aquatic ecosystems, so they represent about 50–70% of dissolved organic matter (DOM) in freshwater (Thurman 1985), and 0.7–2.4% of DOM in marine water (Paul *et al.* 2004). HSs are brown-black, and are usually divided into three fractions according to their aqueous solubility, including humic acid (HA), fulvic acid (FA), and humin. Potential applications of HS for the treatment of wastewater contaminated with metals and organic pollutants have received much attention in the last two decades (Gupta VK *et al.* 2009; Sannino *et al.* 2013; Lipczynska-Kochany 2018). HA directly improve plant growth through mechanisms, such as increasing the permeability of cell membrane, root cell growth, photosynthesis, respiration, ion uptake, nucleic acid synthesis, and regulating hormone level (Russo and Berlyn 1991; Nardi *et al.* 2002; Paksoy *et al.* 2010). EL-Sayed *et al.* (2014) reported that HA at a rate of 0.1% w increased nitrogen, phosphorus, and potassium uptake by the Radish plant. HS also enhances the growth of microorganisms as a source of nutrient or biologically active compounds (Kulikova and Perminova 2021). Vallini *et al.* (1997) found that humic acids increased the growth of nitrifying bacteria probably due to an increase in bacterial cell membrane permeability, thus better uptake of nutrients. Some studies have demonstrated the beneficial effects of dietary humic substances administration on growth performance, and immune status, as well as reducing the toxicity effects of heavy metals, and organic pollutants, and increasing disease resistance in different fish species (Abdel-Wahab et al. 2012; Soytaş 2015; Costa *et al.* 2016; Yilmaz *et al.* 2018). To the best of our knowledge, there is insufficient information on the administration of humic substances as a water additive on the growth performance of fish and shellfish, water quality and bioremediation performance of the aquatic plants in the aquaculture systems. Therefore, in the current study, a water recirculating system containing duckweed plants as a biological filter was set up to culture juveniles Nile tilapia (*Oreochromis niloticus*). Next, we investigated the potential effects of different concentrations of humic acid added to water on the removal efficiency of *Lemna minor* for nitrogen, and phosphorous compounds, the growth performance, and some hematological and blood biochemical parameters of Nile tilapia.

Materials and methods

Plant materials

Duckweed plants were obtained from a local aquatic plant farm in Mashhad, Razavi Khorasan province, Iran. They were transferred to the Aquatic Laboratory of Ferdowsi University of Mashhad, Iran, washed with tap water to eliminate undesired organisms, and particles, and then placed in

glass aquariums filled with aerated tap water and equipped with a LED grow light lamp for two weeks. The duckweed was identified as *Lemna minor* by taxonomy experts.

Humic acid (HA) powder (Humi-Grow WSG-The Plant's Choice., USA) was purchased from Iran Keshavarzi Co. (Alborz Province, Iran). Plant's Choice Organic 100% Soluble Humic Acid Powder derived from sub-bltuminous coal contained 60% humic acid and 12% K_2O.

Experimental units design and setup

In this work, nine experimental units were designed to investigate three concentrations of humic acid on the efficiency of the free-floating aquatic plant *L. minor* in removing nitrogen and phosphorus compounds from water in triplicate. Each experimental unit holding 283 L of water consisted of a plastic tank (100 cm × 50 cm × 40 cm) filled with 120 L of water to stock fish; a cylindrical plastic tank (35 cm × 45 cm) filled with aquarium filter pad and sponge filter foam (43 L) as a mechanical filter to eliminate suspended solid particles from water; a glass-aquarium (100 cm × 40 cm × 35 cm) filled with 120 L of water to stock the duckweed plants (600 g wet weight) as a biofilter; a LED grow light lamp placed on the top of glass-aquarium to supply 16-h light/8-h dark cycle for duckweed; a water pump; a controllable valve and a pipe to transfer water from fish tank to mechanical filter; an air stone connected to a central air blower by a perforated pipe (Figure 1). In each unit, the water of fish tank was pumped to the bottom of the mechanical filter at a flow rate of 1.3 L/min, passed slowly through the filter materials to the top of the filter and entered into glass-aquarium. Finally, the water passing through the biofilter returned to the fish tank by gravity. Physicochemical properties of used water were presented in Table 1.

Fish culture

In this study, 250 fish (*O. niloticus*) were purchased from a local tilapia farm in khaf, Razavi Khorasan province, Iran and transferred to the Aquatic Laboratory of Ferdowsi University of Mashhad, Iran. The fish were stocked in four plastic tanks (500 L) with intense aeration and fed with commercial pellets (Faradaneh, Aquatic Animals Feed Producer., Iran) for two weeks to acclimate to laboratory conditions. In the following, 20 fish with an average weight of 62.60 ± 0.79 g and an average length of 14.52 ± 0.14 cm were randomly stocked in the fish tank of each experimental unit. The duckweed plants were introduced to each unit after launching the systems.

Humic acid (HA) powder (Humi-Grow WSG-The Plant's Choice., USA) was dissolved in the water and added to each unit at concentrations of 0 (control), 1.5, and 3 mg/L in three replicates. HA concentrations were chosen according to the results of a series of pretests. Adding higher concentrations of humic acid caused the water to turn black, and subsequently, the fish food intakes decreased. Fish were fed on commercial pellets (Faradaneh, Aquatic Animals Feed Producer., Iran) three times daily for appetite satiation. The proximate composition of pellets was determined as 38% crude protein, 13% crude lipid, 2% crude fiber, and 7% ash. During the cultivation period, the water of each unit was replaced daily by 10% with freshwater, and the HA was added to the water to maintain its concentration constant.

Water quality analysis

The water samples were collected every ten days from the fish tank of each experimental unit to measure total ammonia nitrogen (TAN), nitrate (NO_3^-), and total phosphorus (TP). The TAN and NO_3^- were analyzed using HACH Ammonia-Nessler (NitrogenAmm_8038_NES.fm),

Table 1. The physicochemical properties of Tap water used in the experiment.

Parameters	Unit	Concentration
Total ammonia nitrogen (TAN)	mg L^{-1}	0
Nitrate (NO_3^--N)	mg L^{-1}	5.26 ± 0.081
Total phosphorus	mg L^{-1}	0.024 ± 0.006
pH	–	7.88 ± 0.008
Dissolved oxygen	mg L^{-1}	6.86 ± 0.17
Electrical conductivity (EC)	µS cm^{-1}	1105.77 ± 9.83

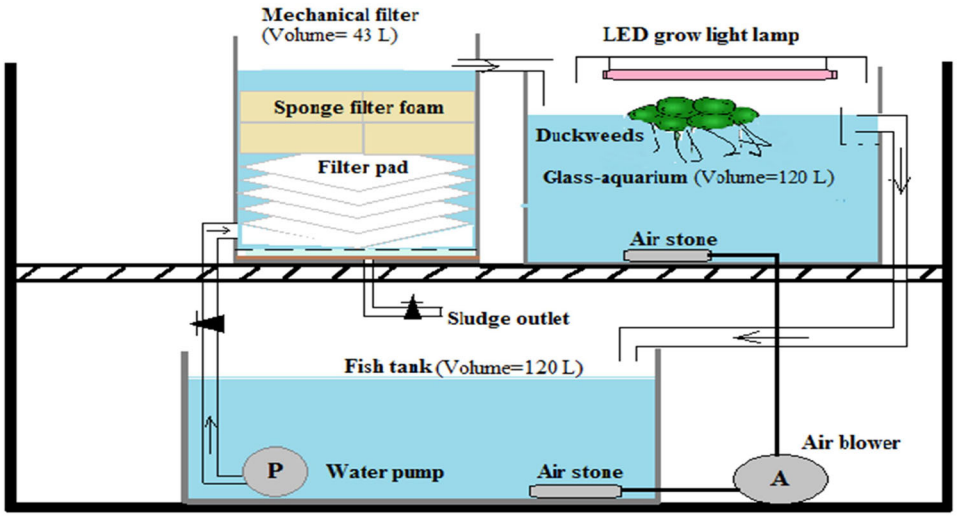

Figure 1. Scheme of a water recirculating system used to culture Nile tilapia (*O. niloticus*) fish.

and HACH Cadmium Reduction (Nitrate_8039_AVPP_HR.fm) methods using an ultraviolet-visible spectrophotometer (DR 5000 TM model, HACH Co., USA) at the wavelength of 425 nm, and 500 nm, respectively. The TP concentration was determined using an inductively coupled plasma-optical emission spectrometry (ICP-OES, Spectro Arcos-76004555 plasma model, Germany). The removal efficiency of the *L. minor* for the TAN, NO_3^- and TP in different HA groups were calculated using the following formula (Can-Terzi *et al.* 2021):

$$R\ (\%) = \left[(CA - CB)/CA\right] \times 100$$

Where R is removal efficiency (%), CA, and CB are the concentrations of the parameter (mg/L) in control and treated groups, respectively.

Water temperature and dissolved oxygen (DO) of the fish tanks were monitored daily using a portable multi-meter (AZ-8603 model), and their fluctuations were recorded between 26–28.5 °C and 6.7–7.4 mg/L, respectively. Variations of electrical conductivity (EC) and pH were also recorded every ten days using a conductivity meter (JENWAY 4510), and a pH meter (Crison, Basic 20+ model), respectively.

Fish growth performance

To calculate the growth performance indices from the following equations, the fish were starved for 24 h at the end of the feeding trial, anesthetized individually with clove oil (20 mg/L), and biometrically measured using an electronic scale (AND GF-6100), and a measuring board.

$$\text{Weight gain (g)} = (\text{final weight} - \text{initial weight})$$

$$\text{Specific growth rate (SGR; \% body weight day}^{-1}) = \left[(\text{Ln final weight} - \text{Ln initial weight})/\text{Time}\right] \times 100$$

$$\text{Daily growth index (DGI) (g)} = \left[(\text{final weight} - \text{initial weight})/\text{Time}\right]$$

$$\text{Condition factor (CF) (g/cm)} = \left[\text{final weight (g)}/\text{final length}^3\text{(cm)}\right] \times 100$$

$$\text{Feed conversion ratio (FCR)} = \left[\text{feed consumed (g)}/\text{weight gain (g)}\right]$$

$$\text{Survival rate (\%)} = (\text{final number of fish}/\text{initial number of fish}) \times 100$$

Chemical composition analysis

At the end of the experimental period, three fish were randomly sampled from each fish tank to analyze the proximate chemical composition of the fish carcass according to the standard method (AOAC, 2005). The proximate analysis of the commercial diet was also conducted in triplicate. The crude protein content ($N \times 6.25$) of the samples was measured by the Kjeldahl procedure. Crude lipid was obtained based on the Soxhlet extraction method. Moisture content was estimated by drying the samples at 105 °C for 24 h using an oven (Behdad, BC OVEN 50, Iran). Ash content was also determined by incineration of the dry samples at 550 °C for 6 h using a muffle furnace (Exciton Co., 1200 °C model, Iran).

The *L. minor* samples were analyzed for total nitrogen (TN) and total phosphorus (TP) contents at the start and the end of the experiment. For this purpose, 200 g (wet weight) of duckweed was collected from the glass-aquarium of each unit, rinsed with distilled water to remove any undesired particles, and dried at 70 °C for 48 h in an oven (Behdad, BC OVEN 50, Iran). The dried samples were ground and sieved using a 300 µm mesh to obtain fine powder. The TN content of the powder was measured based on the Kjeldahl method. For the TP content, 0.5 g of powder was kept in the presence of $HClO_4$ and HF (10:4) at room temperature for 48 h. Then, HNO_3 and $HClO_4$ (4:2) were added to the mixture, and heated at 150 °C for 2 h (Westerman 1990). The digested sample was filtered using a Whatman paper (45 µm), diluted to 50 mL using double distilled water, and the TP concentration was measured using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Spectro Acros, Germany).

Hematological and blood biochemical analysis

Fish were starved for 24 h after 30 days of the feeding trial. Six fish from each fish tank were randomly sampled and anesthetized with clove oil at 20 mg/L. Blood was taken from the caudal vein using a 2-mL syringe and transferred to a heparinized tube. Three blood samples from each tank were used to measure hematological parameters. The other blood samples were centrifuged at 6000 g for 5 min. Plasma was removed and stored at −80 °C until analysis of the biochemical parameters.

Red blood cell (RBC) and white blood cell (WBC) were counted, hemoglobin (Hb) concentration was measured, and hematocrit (Ht), mean corpuscular hemoglobin volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) values were calculated using a hematology analyzer (Sysmex KX 21 Cell Counter). The plasma biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose, triglyceride, and cholesterol were measured based on the protocols of commercial kits (Darman Faraz Kave Co., Iran) using an ultraviolet-visible spectrophotometer (DR 5000 TM model, HACH Co., USA).

Statistical analysis

Data were presented as mean ± standard deviation (SD). All statistical analyses were performed using SPSS 19 software.

The normality of distributions was evaluated using the Kolmogorov-Smirnov test. Statistical differences between means were determined by one-way analysis of variance (ANOVA) followed by Duncan's New Multiple Range test. Significant differences between means in each group at different times of the experiment were determined by subjecting the data to one-way repeated measures analysis of variance (ANOVA with repeated measures). Significant differences between means were considered at p value of <0.05.

Results

Effects of HA on water quality

Figure 2 shows the fluctuations of TAN, nitrate, and total phosphorus of the water in the RAS with different concentrations of HA during the 30 day of cultivation period. On day 10, the TAN concentrations in the HA groups decreased significantly compared to the control ($p < 0.05$). Water treatment with HA for 20 days showed lower TAN concentrations than the control, and the lowest TAN concentration was observed in the HA-3 mg/L group ($p < 0.05$). On day 30, the treated water with 3 mg/L of HA had lower TAN concentrations than other groups ($p < 0.05$). There was no significant difference between the HA-1.5 mg/L and the control. The TAN concentration increased significantly in the control and the HA-1.5 mg/L group from day 0 to day 30 ($p < 0.05$). In the HA-3 mg/L, the TAN concentration elevated significantly from day 0 to day 20 ($p < 0.05$) and this value showed no significant change until day 30 (Figure 2a).

The nitrate concentration showed no significant differences between the HA-groups and the control on days 0 and 10. The nitrate concentration in the HA-3 mg/L was higher than in the other groups on days 20 and 30 ($p < 0.05$). The NO_3^- concentration in the control group decreased significantly from day 0 to day 30 ($p < 0.05$). In the HA-1.5 mg/L, the nitrate concentration showed a decreasing trend until

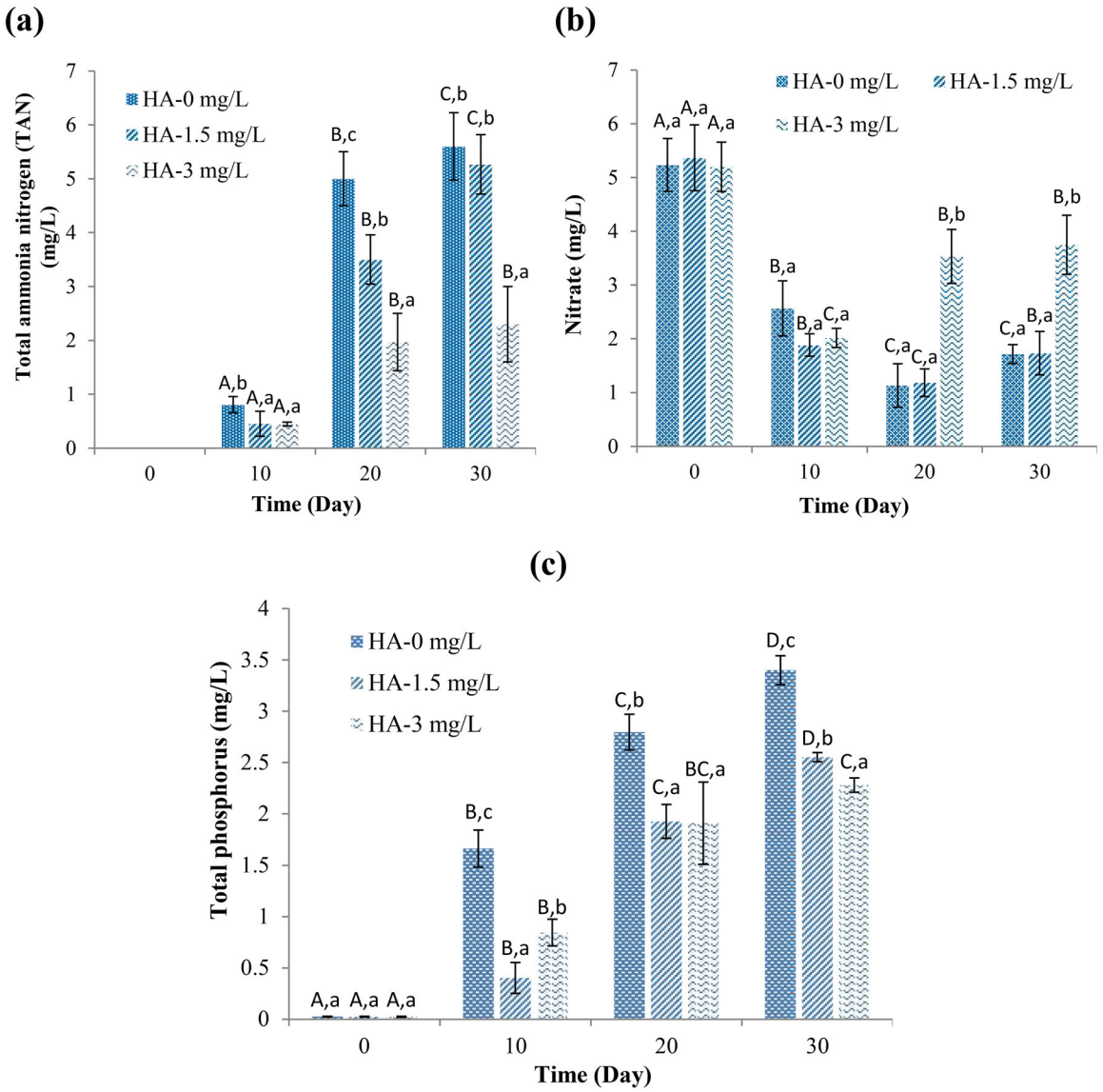

Figure 2. Variations of the total ammonia nitrogen (TAN) (a), nitrate (b) and total phosphorus (c) of the water in the RAS with different concentrations of humic acid (HA) used for cultivation of Nile tilapia (O. niluticus) fish for 30 days (mean ± SD). Bars marked with different lowercase letters at each time are significantly different (ANOVA, $p < 0.05$). Bars with capital letters in each group are significantly different (ANOVA with repeated measures, $p < 0.05$).

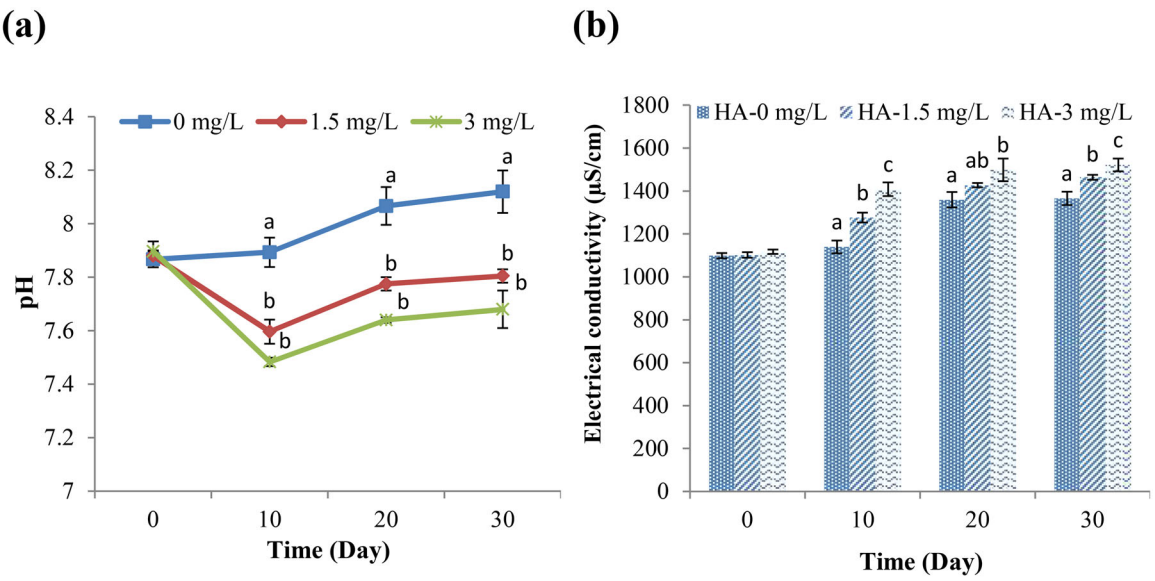

Figure 3. Fluctuations of the pH (a) and electrical conductivity (EC) (b) of the water in the RAS with different concentrations of humic acid (HA) used for cultivation of Nile tilapia (*O. niluticus*) fish for 30 days. Bars marked with different lowercase letters in each time are significantly different (ANOVA, $p < 0.05$).

Table 2. Growth performance, feed utilization indices and survival rate of Nile tilapia (*O. niloticus*) fish reared in the RAS with different concentrations of humic acid for 30 days (mean ± SD, $n = 3$).

	Waterborne humic acid (HA) groups		
Parameters	HA-0 mg/L (Control)	HA-1.5 mg/L	HA-3 mg/L
Initial weight (g)	63.26 ± 1.9^{a}	61.72 ± 1.81^{a}	62.82 ± 1.06^{a}
Initial length (cm)	14.66 ± 0.14^{a}	14.38 ± 0.33^{a}	14.52 ± 0.12^{a}
Final weight (g)	102.27 ± 2.91^{a}	105.46 ± 3.10^{a}	113.93 ± 2.91^{b}
Final length (cm)	17.56 ± 0.77^{a}	17.34 ± 0.18^{a}	18.14 ± 0.50^{a}
Weight gain (g)	39.01 ± 3.72^{a}	43.73 ± 1.4^{a}	51.10 ± 3.82^{b}
SGR (%BW day^{-1})	1.60 ± 0.15^{a}	1.78 ± 0.03^{ab}	1.98 ± 0.14^{b}
DGI (g)	1.30 ± 0.12^{a}	1.45 ± 0.05^{a}	1.70 ± 0.12^{b}
CF (g cm^{-3})	1.60 ± 0.16^{a}	1.70 ± 0.012^{a}	1.62 ± 0.10^{a}
FCR	1.44 ± 0.14^{b}	1.27 ± 0.021^{ab}	1.11 ± 0.096^{a}
Survival rate (%)	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

SGR: specific growth rate; DGI: daily growth index; CF: condition factor; FCR: food conversion ratio. Data assigned with different letters in a row are significantly different (ANOVA, $p < 0.05$).

the 20th day, but showed a significant increase on the 30th day ($p < 0.05$). Treatment of water with 3 mg/L of HA decreased the NO_3^- concentration to the lowest level on day 10, but increased its concentration significantly until the 30th day ($p < 0.05$) (Figure 2b)

The total phosphorus (TP) concentration decreased in the HA-groups compared to the control on day 10 ($p < 0.05$). The lowest TP concentration was observed in the HA-1.5 mg/L group ($p < 0.05$). On day 20, the TP concentration in the HA- groups was lower than the control ($p < 0.05$). After 30 days of the cultivation period, the HA-groups had lower TP concentration than the control, and the lowest concentration was observed in the HA-3 mg/L ($p < 0.05$). The TP concentration in the control and the HA-groups increased significantly during the 30 days of the experiment period ($p < 0.05$) (Figure 2c).

The pH of water in the HA-1.5 mg/L and HA-3 mg/L groups was lower than that in the HA-0 mg/L on days 10, 20, and 30 ($p < 0.05$) (Figure 3a). The electrical conductivity (EC) of water increased in the HA-1.5 mg/L and HA-3 mg/L groups compared to the control on days 10, 20, and 30 ($p < 0.05$) (Figure 3b).

Effects of HA on growth performance of fish

The effects of three concentrations of waterborne humic acid (HA) on the growth performance, and survival rate of Nile tilapia fish reared in the RAS for 30 days are shown in Table 2. The final weight, weight gain (WG), and daily growth index (DGI) of the fish in the HA-3 mg/L group increased compared to the other groups ($p < 0.05$). The specific growth rate (SGR %) of the fish in the HA-3 mg/L group was significantly higher than the control group ($p < 0.05$), while it showed no significant difference with the HA-1.5 mg/L group. Feed conversion ratio (FCR) decreased in the HA-3 mg/L group compared to the control ($p < 0.05$), while the FCR value in the HA-1.5 mg/L showed no significant difference with the HA-3 mg/L and the control. There were no significant differences in the initial weight, initial length, final length, condition factor (CF), and survival rate of the fish between different groups.

Effects of HA on fish and plant's chemical composition

The final mean carcass proximate composition of Nile tilapia reared in the RAS with different concentrations of HA is shown in Table 3. No significant differences in the carcass dry matter, the crude protein, and the ash contents were observed in the three experimental groups. The crude lipid content in the HA-3 mg/L group was higher than HA-0 mg/L, and HA-1.5 mg/L groups ($p < 0.05$), while there was no significant difference between the HA-0 mg/L, and the HA-1.5 mg/L.

Table 4 shows the changes in the fresh weight, the total nitrogen (TN) and the total phosphorus (TP) contents of *L. minor* cultured in the RAS with different concentrations of HA at the start and end of the experiment. The fresh

weight, TN and TP contents of duckweeds (dry weight) increased significantly in all HA-groups during the experimental period ($p < 0.05$). The fresh weight, TN and TP contents of *L. minor* were significantly higher in the HA-1.5 mg/L and HA-3 mg/L groups than in the control group at the end of the experiment ($p < 0.05$). There were no significant differences in the TN and TP contents between the HA-1.5 mg/L and HA-3 mg/L groups.

Effects of HA on hematological parameters

The hematological variables of Nile tilapia reared in the RAS with different concentrations of HA are shown in Table 5. There were no significant differences in these variables between different HA groups at the end of the experiment.

Effects of HA on blood biochemical parameters

Figure 4a–f shows the changes in the plasma biochemical parameters of the Nile tilapia reared in the RAS containing different concentrations of HA. The aspartate aminotransferase (AST), alanine aminotransferase (ALT), and the alkaline phosphatase (ALP) levels decreased significantly in the HA-3 mg/L group compared to the other groups ($p < 0.05$) (Figure 4a–c). No significant differences were observed in the plasma glucose and cholesterol levels among different HA groups (Figure 4d,e). In addition, the triglyceride level in the HA-3 mg/L group was significantly higher than that in the control ($p < 0.05$) (Figure 4f).

Discussion

The bioremediation performance of aquatic plants as biological reactors in aquaculture systems can be very effective in improving water quality, increasing fish production, and reducing wastewater discharge to the environment. In the present study, we aimed to examine the effects of humic acid (HA) addition to water on the TAN, nitrate, and total phosphorous removal efficiency of *L. minor* in a water recirculation system used to culture Nile tilapia fish. The results showed that the TAN concentration in the RAS with the 1.5, and 3 mg/L of HA was lower than those with the 0 mg/L of HA on days 20, and 30 of the fish cultivation period. The TAN removal efficiency of *L. minor* in the RAS with 1.5 mg/L of HA was 43.75%, 30%, and 6%, while these values increased to 45%, 60.6%, and 58.92% in the RAS with 3 mg/L of HA on days 10, 20, and 30 of the experiment, respectively. These results indicated the positive effects of HA on the TAN removal efficiency of duckweeds, especially in the 3 mg/L HA. The results also showed the changes in nitrate (NO_3^-) concentration in the RAS systems. It decreased from the 1st to the 30th day in all systems regardless of HA concentration. Also, the total nitrogen content of *L. minor* at the end of the experiment was significantly higher than that on the first day of the experiment. These findings indicated that ammonium (NH_4^+) and nitrate (NO_3^-) ions of water could be absorbed by duckweeds. The previous study also showed that *L. minor* as a biological filter in a water recirculating system used in the rearing of African cichlid (*Labidochromis lividus*) fish decreased the TAN and total phosphorus by 41%, and 37.8% compared to the control, respectively (Sarkheil and Safari 2020). It was reported that HA mediates and improves nutrients uptake, such as nitrogen and phosphorous, by the plants (Hofman and Cleemput 2004). The role of humic substances in stimulating plant nutrient uptake is mainly related to the increase of plasma membrane H^+-ATP activity (Canellas *et al.* 2002; EL-Sayed *et al.* 2014). Gao *et al.* (2022) reported that the total nitrogen content of lettuce increased with increasing HA added to the aquaponic systems from 0 to 15 ppm. They also found that the TAN concentration was lower in the HA-based aquaponic system with 15 ppm concentration than those with 0 ppm, and 30 ppm. In the current study, the nitrate concentration in the RAS with 3 mg/L of HA was higher than that in the systems with 0, and 1.5 mg/L of HA

Table 3. Proximate chemical composition of Nile tilapia (*O. niluticus*) carcass reared in the RAS with different concentrations of humic acid (HA) for 30 days (mean ± SD, $n = 3$).

	Waterborne humic acid (HA) groups		
Parameters	HA-0 mg/L (Control)	HA-1.5 mg/L	HA-3 mg/L
Dry matter (%)	23.94 ± 0.95^a	24.19 ± 0.79^a	24.30 ± 1.04^a
Crude protein (%)	17.71 ± 0.29^a	18.07 ± 0.08^a	18.12 ± 0.28^a
Crude lipid (%)	3.06 ± 0.29^a	3.09 ± 0.42^a	4.16 ± 0.19^b
Ash (%)	4.25 ± 1.5^a	4.75 ± 0.95^a	5.75 ± 0.96^a

The data assigned with different letters in each raw are significantly different (ANOVA, $p < 0.05$).

Table 5. Effect of waterborne humic acid (HA) on hematological parameters of Nile tilapia (*O. niluticus*) reared in the RAS for 30 days (mean ± SD, $n = 3$).

	Waterborne humic acid (HA) groups		
Parameters	HA-0 mg/L (Control)	HA-1.5 mg/L	HA-3 mg/L
WBCs ($\times 10^3/\mu L$)	275.51 ± 18.17^a	272.90 ± 13.24^a	280.58 ± 6.32^a
RBCs ($\times 10^6/\mu L$)	1.74 ± 0.47^a	1.84 ± 0.21^a	1.76 ± 0.25^a
Hb (g/dL)	9.36 ± 0.96^a	9.71 ± 0.29^a	9.78 ± 0.76^a
Ht (%)	28.25 ± 4.51^a	30.11 ± 2.58^a	29.73 ± 3.48^a
MCV (FL)	159.10 ± 9.93^a	163.60 ± 5.03^a	160.51 ± 5.86^a
MCH (pg)	57.33 ± 8.15^a	53.21 ± 5.91^a	56.40 ± 7.83^a
MCHC (g/dL)	36.18 ± 6.88^a	32.45 ± 2.77^a	34.40 ± 4.50^a

Means in the same row with different letters are significantly different (ANOVA, $p < 0.05$).

Table 4. Fresh weight, total nitrogen and phosphorous contents of *L. minor* (dry weight) in the RAS with different concentrations of humic acid (HA) at the start and the end of the experiment (mean ± SD, $n = 3$).

		End of exp.		
Parameter	Start of exp.	HA-0 mg/L (Control)	HA-1.5 mg/L	HA-3 mg/L
Fresh weight (g)	600.67 ± 2.92^a	889.03 ± 21.88^b	985.14 ± 30.39^c	1064.83 ± 17.77^d
Total nitrogen (mg/g D.W)	28.36 ± 0.68^a	30.35 ± 0.15^b	32.86 ± 1.22^c	32.70 ± 0.90^c
Total phosphorus (mg/g D.W)	4.06 ± 0.18^a	$6.03 \pm 0.0.7^b$	6.38 ± 0.2^c	6.63 ± 0.11^c

The data assigned with different letters in each raw are significantly different (ANOVA, $p < 0.05$).

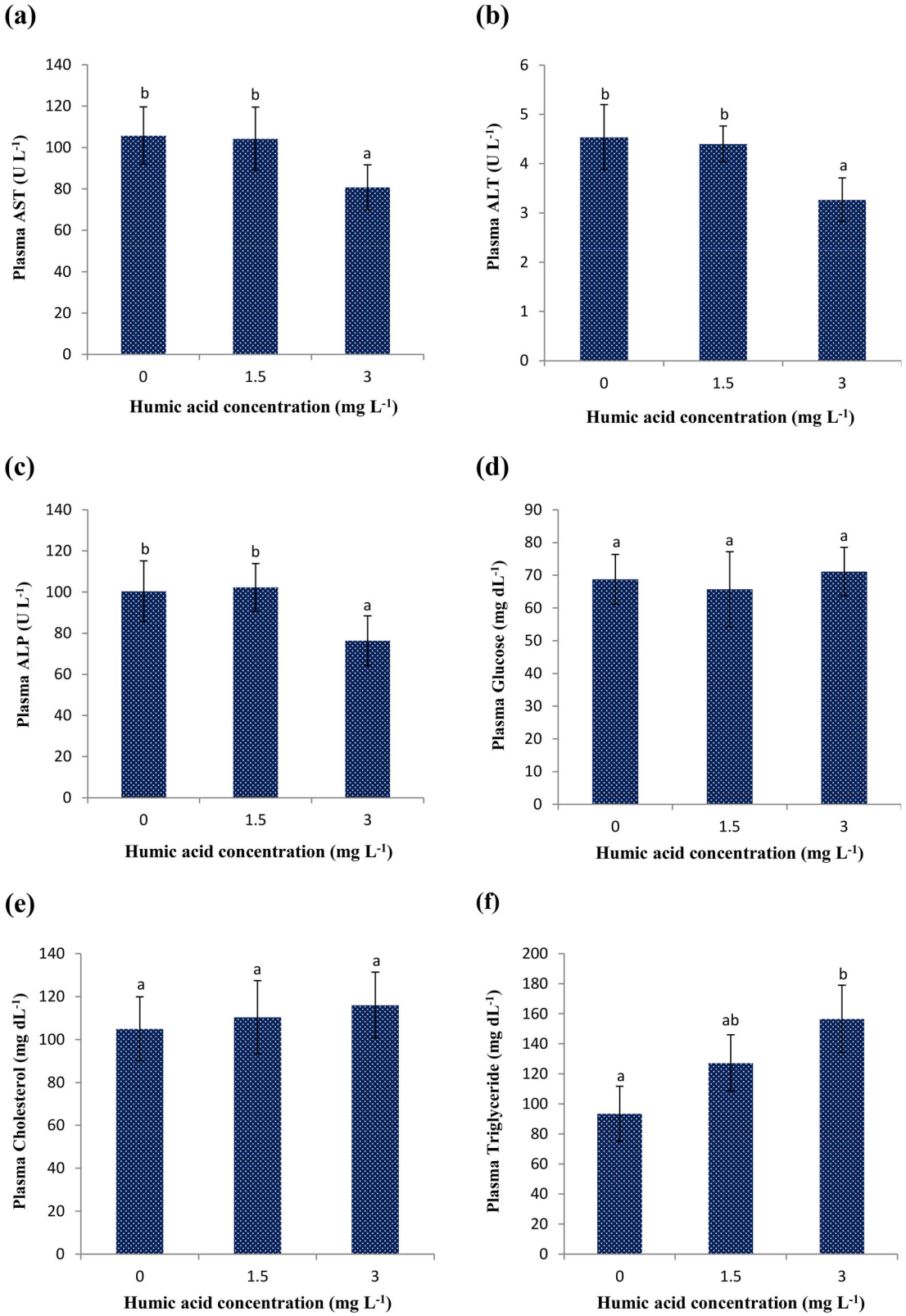

Figure 4. Changes in the plasma aspartate aminotransferase (AST) (a), alanine aminotransferase (ALT) (b), alkaline phosphatase (ALP) (c), glucose (d), cholesterol (e), and triglyceride (f) of the Nile tilapia (*O. niluticus*) reared in the RAS with different concentrations of humic acid (HA) for 30 days (mean ± SD). Bars marked with different lowercase letters are significantly different (ANOVA, $p < 0.05$).

on days 20 and 30 of the experiment. In another study, Gao *et al.* (2022) showed that the nitrate concentration increased in the HA-based aquaponic system with 15 ppm compared to other systems with 0 ppm, and 30 ppm. They found that 15 ppm HA increased nitrifying bacterial communities of the plant roots involved in ammonia oxidation to nitrate. The

accumulation of nitrate is beneficial to promote plant growth.

Phosphorous is one of the essential macronutrients for plant growth. It is a component of adenosine triphosphate (ATP), phospholipids, and nucleic acids and is involved in transfer's sugar molecules to plant leaves (Abbo 2020). It is estimated that 82% of phosphorous from total feed used in aquaculture can be released into the environment (Holby and Hall 1991). Aquaculture effluent is deficient in phosphorous, but fish sludge, which constitutes feces and uneaten feed contains a substantial amount of phosphorous (Delaide *et al.* 2017). However, phosphorous is bound in organic solid waste and is not readily available to plants (Abbo 2020). Dissolved inorganic phosphate (PO_4^{3-}) is a form of phosphorous that can be assimilated by plants (Monsees *et al.* 2017). It has been reported that adding acids and lowering of pH increase phosphorous leaching from fish sludge (Conroy and Couturier 2010; Monsees *et al.* 2017). In the present study, the total phosphorous (TP) concentration in the RAS with 1.5 mg/L of HA decreased by 75.90%, 31.33%, and 25% compared to the RAS without HA on days 10, 20, and 30, respectively. The reduction percentages of 49.40%, 31.70%, and 32.94% were also recorded in the RAS with 3 mg/L of HA. Furthermore, the TP content of *L. minor* in all experimental groups increased during the cultivation period. The TP content of *L. minor* in the HA-1.5 mg/L and HA-3 mg/L groups was also significantly higher than in the HA-0 mg/L. These results show more uptake of phosphorous by duckweeds in the RAS with HA most likely due to the increase in phosphorous solubility resulting from the lowering pH and/or the stimulating effect of HA on the plant roots. The pH in the RAS with HA was lower than that in the RAS without HA during the 30 days of the experiment (Figure 3a). It was reported that humic acids increased phosphate (P_i) solubility in soil (Erro *et al.* 2012). Jindo et al. (2020) also found that humic acid increased the root and shoot biomass, and the expression of high-affinity P_i transporters in the root cells of the tomato plant.

In this study, the maximum values of the final body weight, weight gain (WG), DGI, and SGR% and the minimum FCR value were observed in the HA-3 mg/L group, which indicated that adding HA into the RAS at a concentration of 3 mg/L improved the growth performance of the Nile tilapia. These results can be attributed to better water quality in the HA-3 mg/L, so that the TAN concentration in this group was reduced by 58.92% and 55.75% compared to the HA-0 mg/L and HA-1.5 mg/L after 30 days of the fish cultivation period, respectively. In intensive aquaculture systems like RAS, ammonia is one of the most common pollutants and stressors to fish health. The lethal concentrations of ammonia induce nervous disorders, gill and kidney damages, severe respiratory problems, and, finally death (Lin and Chen 2003; Zeitoun *et al.* 2016). Exposure to sub-lethal concentrations of ammonia reduces growth rate, disease resistance, and induces poor food conversion (Kuttchantran 2013). Zeitoun *et al.* (2016) reported a reduction in the final body weight, specific growth rate (SGR), and average daily gain (ADG) of Nile tilapia (*O. niloticus*) exposed to 3 mg/L of ammonium chloride for 4 days compared to the control. They attributed the poor growth performance of fish exposed to ammonia to the inhibition of the fish appetite leading to a reduction of food intake. In the current study, the TAN concentration in the HA-0 mg/L and the HA-1.5 mg/L increased to 5.60 mg/L and 5.26 mg/L after 30 days, respectively, which led to a decrease in the fish growth performance compared to that in the HA-3 mg/L with 2.3 mg/L of TAN. To the best of our knowledge, there are insufficient reports on the effects of HA as a water additive on the growth performance of aquatic organisms. Some studies have investigated the potential growth-promoting effect of humic substances as a feed additive in different fish species (Sharaf and Tag 2011; Yılmaz *et al.* 2018; Prokešová *et al.* 2021). Gao *et al.* (2022) found that weight gain and SGR of Nile tilapia (*O. niloticus*) increased with adding HA at concentration of 15 mg/L into the aquaponic system. They concluded that dissolved HA in water can be taken up by fish and can improve their growth at a suitable concentration. In the present study, adding HA into the RAS had no significant effect on the carcass dry matter (%), crude protein, and ash contents of the Nile tilapia, while the crude lipid content increased in the RAS with HA-3 mg/L. This increase in the crude lipid content can be related to the direct effect of HA on fish health and, or its effect on the TAN reduction in this HA group. Few studies have provided data regarding the effect of adding HA into the culture water on the proximate composition of aquatic organisms. However, adding HA into the water increased the protein content of *Dunaliella salina* and *Nannochloropsis salina* algae (Mohammady 2008). In contrast, adding HA at different rates (0, 150, 300, and 450 ppm) to drink water of broilers did not affect the dry matter, protein, fat, and ash contents of breast meat (Ozturk *et al.* 2010). Regarding the effect of ammonia on the proximate composition of fish species, Shalaby *et al.* (2021) reported that whole-body dry matter, protein, and lipid contents of Nile tilapia (*O. niloticus*) decreased significantly with increasing ammonia concentration.

Changes in the hematological and blood's biochemical indices are important indicators for assessing stress, and health status in fish (Yilmaz *et al.* 2012; Elbialy *et al.* 2021). Ammonia enters the fish circulation system, binds to hemoglobin and affects some hematological properties (Kim *et al.* 2019). Shalaby *et al.* (2021) reported significant decreases in the RBCs, Hb, and Ht in the Nile tilapia (*O. niloticus*) after exposure to the high ammonia concentration. In the present study, the hematological parameters of the fish reared in the RAS with different concentrations of the HA did not change significantly. The TAN concentration in the HA groups varied between 0 to 5.6 mg/L during 30 days of the fish cultivation period, which did not cause adverse effect on the hematological parameters. In addition, these results indicated that adding HA (1.5 mg/L and 3 mg/L) into the water had no adverse effects on the hematological properties of the Nile tilapia. Yilmaz et al. (2018) also reported no changes in the RBCs, Hb, and Ht of rainbow trout

(*Oncorhynchus mykiss*) fed diets supplemented with 0.3%, 0.6%, and 1.2% HA for 60 days.

Plasma AST, ALT, and ALP enzymes are used as sensitive indicators for determining damage to the liver and other fish tissues are caused by environmental stress (Fırat *et al.* 2011; Kim and Kang 2016). Elevated levels of ALT, AST, and ALP have been reported in *Cyprinus carpio* (Hoseini *et al.* 2019), Nile tilapia (*O. niloticus*) (Shalaby *et al.* 2021), and triangle sail mussels (*Hyriopsis cumingii*) (Zhao *et al.* 2021) following ammonia exposure, which may be attributed to damage to the cell membrane and liver tissue. In the current study, the plasma AST, ALT, and ALP levels in the fish exposed to higher TAN concentration in the HA-0 mg/L, and HA-1.5 mg/L groups increased significantly compared to those exposed to lower TAN in the HA-3 mg/L.

In the present study, adding HA into the water did not change the plasma glucose, and cholesterol levels of Nile tilapia. In contrast, the triglyceride level increased at 3 mg/L of HA compared to the control. In agreement with our findings, Yilmaz *et al.* (2018) did not find any changes in the serum glucose, and cholesterol levels of rainbow trout (*O. mykiss*) fed HA-supplemented diets. In contrast, they reported that the triglyceride level decreased significantly when fish fed on diets supplemented with HA. Prokešová *et al.* (2021) reported that dietary HA had no significant effect on the plasma cholesterol and triglyceride levels of African Catfish (*Clarias gariepinus*), while the glucose level significantly decreased. These different results may be due to differences in fish species, the route of HA administration and its different components. In general, exposure of fish to ammonia also affects the blood's biochemical properties. Ammonia as a stressor elevates blood levels of catecholamines which in turn leads to lipolysis, gluconeogenesis, and glycogenolysis, and ultimately increases plasma glucose levels to provide the energy requirement of the organs and tissues (Shalaby *et al.* 2021). It was reported that the plasma glucose, cholesterol, and triglyceride levels increased in Nile tilapia (*O.niluticus*) exposed to 18 mg NH_4Cl/L. At the same time, the addition of zeolite to the ammonia-treated group decreased these values to those of the control as a result of reduction of ammonia concentration (Shalaby *et al.* 2021). The results of the current study indicated that the plasma glucose, cholesterol, and triglyceride levels were not affected by the TAN concentrations in different HA-groups.

Conclusion

This study was conducted to evaluate the effects of adding different concentrations of humic acid (HA) into water recirculating systems on the removal efficiency of the free-floating aquatic plant *L. minor* for nitrogen and phosphorus compounds from water, the growth performance, carcass proximate composition, some hematological and blood's biochemical parameters of Nile tilapia. From the results, it can be concluded that the addition of HA to the water (a) increased the TAN and phosphorous removal efficiency of *L. minor* in a concentration-dependent manner, (b) improved the growth performance of the Nile tilapia at concentration of 3 mg/L, (c) had no adverse effect on hematological parameters, and (d) improved some plasma biochemical parameters at 3 mg/L of HA. The present study suggests that adding HA into the water can be effective in improving the performance of the duckweed plants as a biofilter and fish growth performance in recirculating aquaculture systems (RASs). Furthermore research, however, is needed to identify mechanisms of waterborne HA affecting aquatic plants and fish species.

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Ethics statement

The experiments on fish in this study were performed based on FUM animal ethic rights and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals.

Authors' contributions

Mehrdad Sarkheil: Conceptualization, Design of the experiments, Statistical analysis of data, Writing the first draft of the manuscript; Saeed Zahedi: Contribution to design of the experiments, Preparation of fish feed, Contribution to manuscript revision; Omid Safari: Conceptualization, Methodology, Statistical analysis of data; Hamidreza Ahmadniaye Motlagh: Hematological and biochemical analysis, Contribution to manuscript revision. All authors read and approved the submitted version.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data were included in the article. Supplemental data are available from the corresponding author upon reasonable request.

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