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Slurry-grown duckweed (*Spirodela polyrhiza*) as a means to recycle nitrogen into feed for rainbow trout fry

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ABSTRACT

Liquid manure from livestock production systems is a major source of nitrogen and phosphorus release from nutrient cycles and a cause of ecosystem eutrophication. Duckweeds, small aquatic plants, may be used to recover N and P from livestock slurry while producing high-quality protein feed. In order to assess N and P uptake efficiency and utility for fish feed, two duckweed species, *Landoltia punctata* and *Spirodela polyrhiza*, were grown in controlled climate chambers on two nutrient-rich media: diluted (1:10) cattle slurry and mechanically filtered household sewage. Treatments were in triplicate, each running in four cycles with fresh substrate (one week each). *Spirodela polyrhiza* exhibited the strongest growth (96 g fresh matter m^{-2} day^{-1}) and highest protein content (306 g per kg dry matter) on diluted slurry. The weakest growth was found for *L. punctata* on treated sewage (52 g fresh matter m^{-2} day^{-1}). Average removal of total provided and utilizable inorganic N from the media was 73.2% and 83.9% for sewage and diluted slurry, respectively. *Spirodela polyrhiza* grown on diluted slurry was subsequently tested as feed ingredient for rainbow trout (*Oncorhynchus mykiss*) fry. Two different ingredient levels of *S. polyrhiza* meal (6.25% and 12.5% of feed) were fed to rainbow trout fry for 4 weeks, during which fish growth, feed and nutrient utilization and gut health were assessed. Feed was accepted, but both duckweed meal treatments resulted in 5–10% poorer growth traits and feed efficiency compared to control. The intestine somatic index was not affected. This is the first time the potential of duckweed as feed for rainbow trout fry has been demonstrated. Furthermore, our experiments found considerable N and P uptake from diluted slurry by *S. polyrhiza,* which produced protein at a high rate per unit time and area.

1. Introduction

Over the last five decades, animal production in aquaculture (fish, crustaceans and molluscs) expanded from 3.25 million metric tons (mmt) in 1974 to 76.6 mmt in 2015, a 23.6-fold increase (FAO, 2017). No other food-producing sector parallels this steep growth. Traditionally, fish meal has been used as the major animal-based protein source for aqua feeds and soy bean meal as the main plant-based source (Tacon and Metian, 2008; Hardy, 2010). It is apparent, however, that sustainable growth of fed aquaculture production in the future will depend on an increase in production of fish at a lower trophic level, such as carps (Neori and Nobre, 2012). Another strategy would be to increase the proportion of more sustainable animal-based proteins such as insect meals (Henry et al., 2015; Stadtlander et al., 2017) and that of plant-based feed ingredients in the diets of carnivorous fish (Tacon et al., 2010). Further substitution of fish meal and fish oil in aqua feeds by plant-based products for fish at a higher trophic level, such as Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*), will effectively shift their production to lower trophic levels.

On the other hand, the production of plant-based protein sources for animal feed can itself generate negative global ecological impacts (Pelletier and Tyedmers, 2010; Schader et al., 2015). Feed sources that do not add to but rather reduce land-use impacts and inputs of biologically active nitrogen into ecosystems are therefore to be preferred. Duckweeds are one such potential source

Abbreviations: ANOVA, analysis of variance; CA, crude ash; CF, crude fiber; CL, crude lipids; CP, crude protein; DM, dry matter; DON, dissolved organic nitrogen; DS, diluted cattle slurry; FCR, feed conversion ratio; FGW, final group weight; FM, fresh matter; HSD, honest significant difference; IGW, initial group weight; ISI, intestine somatic index; kJ, kilojoule; LPV, lipid productive value; N, nitrogen; NFE, nitrogen free extract; P, phosphorus; PPV, protein productive value; PSB, primary sedimentation basin; PWG, percent weight gain; SD, standard deviation; SGR, specific growth rate.

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of protein in aquaculture. These are plants of the family Lemnaceae, classified in five different genera, namely *Lemna*, *Spirodela*, *Landoltia*, *Wolffia* and *Wolffiella,* altogether containing 38 species (Les et al., 2002). They can achieve very high growth rates with reported dry matter yields per hectare and year of up to 23 t under sub-optimal and up to 79 t under near-optimal conditions (Leng et al., 1995). Furthermore, duckweeds can be very rich in protein with up to 455 g crude protein (CP) per kg dry matter (DM) but are usually low in crude lipids (CL) with around 40–50 g kg^{-1} DM (Mbagwu and Adeniji, 1988; Appenroth et al., 2017). Rapid growth is usually achieved in media with a high nitrogen content (preferably as ammonium) at concentrations of 7–12 mg NH_4-N L^{-1} or higher (Leng et al., 1995).

Intensive animal production can produce animal manure and slurry in excess of the uptake capacity of the surrounding agricultural fields (Mallin and Cahoon, 2003), which in turn can lead to increased nitrate concentrations in groundwater. Especially in areas with intense agricultural activities such as livestock keeping (Mallin et al., 2015) and vegetable production (Ju et al., 2006), nitrate levels in groundwater frequently exceed statutory threshold levels due to application of nitrogen fertilizers or animal manure fertilization of fields and pastures.

In the past, various duckweed species have already been utilized as animal feed for poultry (Haustein et al., 1990), pigs (Rojas et al., 2014) and several commercially important food fish such as Nile tilapia (*Oreochromis niloticus*; De Matos et al., 2014), rohu (*Labeo rohita*; Bairagi et al., 2002), grass carp (*Ctenopharyngodon idella*; Cui et al., 1992), silver barb (*Barbodes gonionotus*; Noor et al., 2000), snakehead (*Channa striatus*; Raj et al., 2001) and striped catfish (*Pangasianodon hypophthalmus*; Da et al., 2013).

However, to the knowledge of the authors, no reports on duckweed inclusion in salmonid diets in general and rainbow trout fry diets in particular are available. Furthermore, feed production efficiency and inorganic N and P removal rates from media are not sufficiently elucidated under temperate climatic conditions. For this reason, a study was launched to evaluate duckweed species' growth capacity on different nutrient-rich liquids and investigate the effect of feeding this material to trout.

The study compared two different duckweed species on two different media in order to determine the fastest growing duckweed species with the highest protein content as a basis for producing larger amounts of duckweed for rainbow trout fry diets. As fry are more sensitive to nutritional imbalances than older fish it can be assumed that the effects of duckweed inclusion in the diet are more pronounced and can be observed in a shorter time. In the fish feeding experiment the goal was to evaluate growth and feed utilization in rainbow trout fry fed at two different dietary levels of duckweed compared to a control diet fed without duckweed. Due to a lack of relevant scientific data, the overall inclusion levels selected were rather low (6.25% and 12.5% of the diet) in order to ensure fish welfare.

2. Material and methods

2.1. Duckweed screening experiment

A screening experiment was conducted in a fully controlled climate chamber with relative humidity of 50%, photoperiod of 12 h light and 12 h dark and light intensity of 11,000 lx. Five sodium and mercury vapor lamps each provided the light. The Landolt Duckweed Collection (Zurich, Switzerland) provided two duckweed species, *Landoltia punctata* and *Spirodela polyrhiza*. The duckweed was grown in triplicate containers of approx. 440 cm^2 (16.5 × 26.5 cm) and 5.5 L (L) volume each and filled with 1.2 L of either the mechanically filtered inflow water of a sewage treatment plant for household sewage (primary sedimentation basin, PSB) or with 1:10 diluted cattle slurry (diluted slurry, DS). Prior to their use, both media were autoclaved for 15 min at 121 °C. Inorganic nitrogen (N) (ammonium-N, nitrite-N and nitrate-N) and orthophosphate phosphorus (P) concentrations during the screening experiment are presented in Figs. 1–4. For each species 4.0 g of fresh matter was inoculated into each container, and the media were exchanged completely once a week. One day before and one day after the exchange, media in each container were analyzed for ammonium-N, nitrate-N, nitrite-N, ortho-phosphate-P, temperature and pH. Duckweed growth was measured during the screening experiment as fresh matter gain (g m^{-2} day^{-1}) and also calculated as relative biomass increase (%) (Table 1). To estimate fresh matter yields, duckweed was harvested in a standardized way by scooping with a sieve; each scoop was shaken exactly 10 times to reduce attached water. However, as duckweed leaves have a comparatively high surface area, even after shaking 10 times some water was still attached, resulting in high variability of fresh matter yields. This effect also depended on the fact that *L. punctata* has smaller leaves and thus a higher surface area than *S. polyrhiza*. Due to the variability of attached water, dry matter content was also highly variable between 4.36% and 10.7% for both species. For that reason, and because most of the living plants were needed as inoculum for the stocking of the subsequent stage (polytunnel), total dry matter yields could not be determined during the screening experiment. Therefore N disappearance and protein accretion rates were estimated twice, under two different assumptions: either low (4.5%, Appenroth et al., 2017) or high (11%, Xu et al., 2012) dry matter contents were assumed, which almost exactly covered the lower and upper range found in the present study.

Evaporated medium was replaced by de-ionized water. After 4 weeks, duckweed samples were analyzed for crude protein and crude ash content while most of the biomass was kept alive for inoculation in the subsequent experiment. Ammonium-N, nitrite-N and nitrate-N disappearance were calculated based on concentrations at the beginning and end of each single week (Table 1). To calculate protein-N a nitrogen to protein conversion factor of CP = 6.25 x N was used.

Based on growth rates and protein content during the screening experiment, *Spirodela polyrhiza* was chosen to be reared on diluted slurry (DS) in a greenhouse to produce material for inclusion in fish feed and chemical analysis of crude protein, crude lipids, crude ash and amino acids. The duckweed was grown in four 600 L containers filled with 400 L of DS for around 10 weeks in order to grow enough dry matter for the trout feeding trial and detailed chemical analysis,

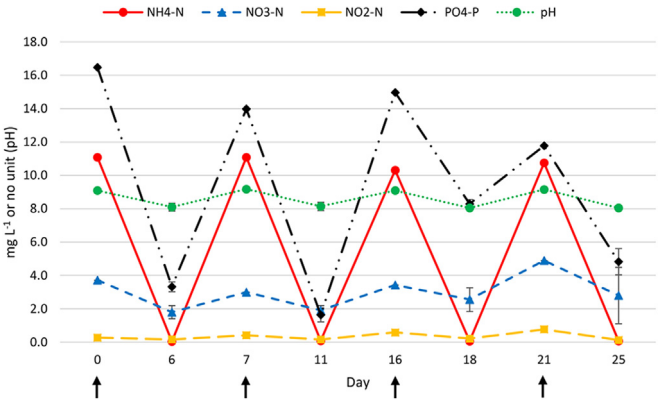

Fig. 1. Development of NH_4-N, NO_3-N, NO_2-N and PO_4-P concentrations and pH for *L. punctata* cultured on DS (diluted slurry) over 25 days. Values are mean of N = 3 ± SD. Arrows indicate measurements immediately after replacement with fresh substrate.

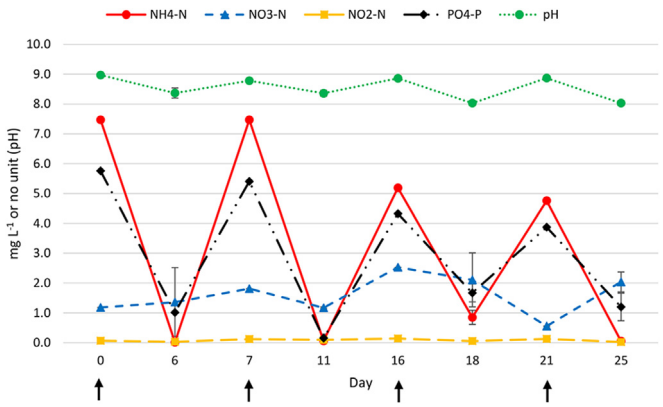

Fig. 2. Development of NH_4-N, NO_3-N, NO_2-N and PO_4-P concentrations and pH for *L. punctata* cultured on PSB (primary sedimentation basin) over 25 days. Values are mean of N = 3 ± SD. Arrows indicate measurements immediately after replacement with fresh substrate.

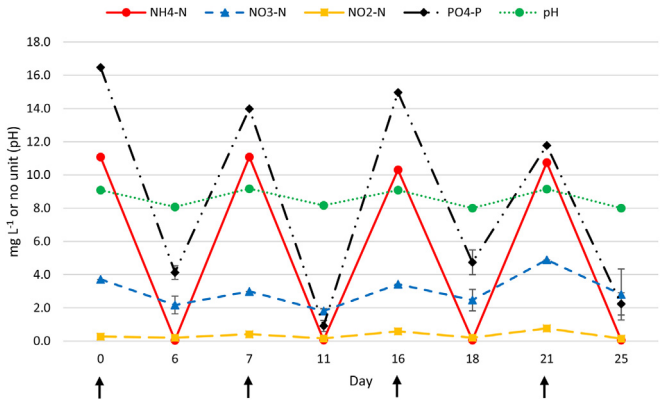

Fig. 3. Development of NH_4-N, NO_3-N, NO_2-N and PO_4-P concentrations and pH for *S. polyrhiza* cultured on DS (diluted slurry) over 25 days. Values are mean of N = 3 ± SD. Arrows indicate measurements immediately after replacement with fresh substrate.

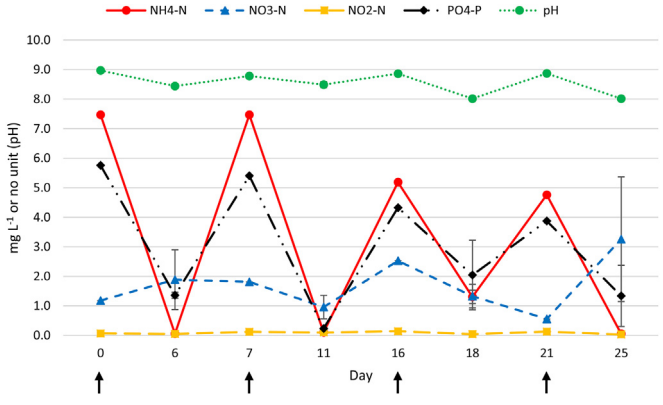

Fig. 4. Development of NH_4-N, NO_3-N, NO_2-N and PO_4-P concentrations and pH for *S. polyrhiza* cultured on PSB (primary sedimentation basin) over 25 days. Values are mean of N = 3 ± SD. Arrows indicate measurements immediately after replacement with fresh substrate.

including amino acid determination. The growth rate of *S. polyrhiza* was not recorded in the greenhouse. The DS medium was neither autoclaved nor changed during the greenhouse phase for reasons of practicability (volumes too large). The greenhouse phase started on 31 July 2015 and ended in the first week of October 2015. Once a week, temperature, pH, ammonium-N, nitrite-N, nitrate-N and ortho-phosphate-P were measured. At the end of the 10 weeks all duckweed was harvested, weighed, dried in a drying cabinet at 40 °C, autoclaved for 15 min at 121 °C and afterwards frozen at −20 °C before being used either in the fish feed or for proximate composition and amino acid analysis.

2.2. Feed production

The diets were calculated to contain approximately 470 g kg^{-1} crude protein, 130 g kg^{-1} crude lipids and 20 kJ g^{-1} of gross energy, corresponding to an estimated 17.3 kJ g^{-1} of digestible energy (assuming a caloric value of 19.6 kJ g^{-1} for protein, 33.5 kJ g^{-1} for lipids and 16.7 kJ g^{-1} for carbohydrates; Brett and Groves, 1979). The feed ingredients (Table 2) were mixed thoroughly before addition of water and oil and pelleting through a 1 mm die of a standard kitchen meat grinder. Afterwards, the pellets were dried in a drying cabinet for approximately 36 h at 40 °C, finely ground and sieved with a mesh size of 0.6–0.8 mm to receive feed granules with the appropriate size for the mouth gape of the trout fry.

2.3. Rainbow trout and experimental design

A total of 560 rainbow trout fry were purchased from a commercial trout farm (Forellenfarm Störk, Bad Saulgau, Germany) and acclimatized in a glass aquarium of 55 L capacity with flow-through fresh water for a week. Of those fish, 360 fry with an average individual body weight of 0.28 ± 0.01 g (mean ± SD) were divided equally into 12 different aquaria (30 fish per aquarium) with individual aeration and 5 L volume connected to a flow-through system and a water flow rate of 600 L day^{-1} for each aquarium. A day later, the remaining 200 fry were euthanized as explained below, separated into two groups of 100 animals each and frozen until further analysis. The three different experimental diets (Control (Con), 6.25% *Spirodela polyrhiza* (SPlow), 12.5% *S. polyrhiza* (SPhigh)) were distributed randomly to four aquaria each and the fish were hand fed four times a day for 6 day at 4.5% of their body mass. On the last day of the week the fish were group weighed and the new feeding rations calculated according to the new body weights. The whole experimental feeding lasted for four weeks. The mean temperature over the whole experimental period was 12.8 °C, oxygen saturation was > 90% (approximately 9 mg L^{-1}) and pH was on average 8.3. Ammonium, nitrite and nitrate concentrations were on average 0.1 mg L^{-1}, 0.04 mg L^{-1} and 13.7 mg L^{-1}, respectively. Photoperiod was 12 h dark and 12 h light with fluorescent light.

2.4. Duckweed and fish sampling

At the end of the duckweed screening experiment a small quantity (around 10 g of dried material) of each treatment (duckweed species and medium) was collected for protein and ash determination. At the end of the greenhouse phase the total *S. polyrhiza* biomass was harvested and used either as fish feed or for analysis (protein, ash, lipids and amino acids).

At the beginning of the fish feeding experiment two initial groups of 100 fish each had been euthanized with 150 mg L^{-1} MS-222 buffered with 300 mg L^{-1} sodiumhydrogencarbonate. At the end of the four-week experiment, all fish were similarly euthanized and the group weight for all aquaria was measured as well as the length (to the nearest mm), individual body weight and intestine weight of the two largest fish of each aquarium. Euthanized fish were frozen at −20 °C, freeze-dried and their moisture determined

Table 1
Absolute and relative duckweed growth, final crude protein content, inorganic N and P disappearance and estimated crude protein accretion in both duckweed species at the end of the screening experiment (N = 3 ± SD, except for protein content and inoculated fresh matter). Different superscripts in the same row indicate statistically significant differences in the screening experiment ($p < 0.05$; Brown-Forsythe test).

Species	*L. punctata*		*S. polyrhiza*	
Medium	PSB	DS	PSB	DS
Inoculated fresh matter (g)	4.0	4.0	4.0	4.0
Total harvest (g FM)	70.1 ± 14.5^{a}	111.2 ± 7.32^{bc}	97.0 ± 15.2^{ab}	129.6 ± 9.11^{c}
Biomass gain (g FM m^{-2} day^{-1})	52.0 ± 10.8^{a}	82.5 ± 5.43^{bc}	72.0 ± 11.3^{ab}	96.2 ± 6.76^{c}
Relative growth (%)	1853 ± 362^{a}	2881 ± 181^{bc}	2525 ± 381^{ab}	3340 ± 228^{c}
Crude protein content (g kg^{-1} DM)	163	138	275	306
Inorganic P[1] disappearance (%, w/w)[3]	79.2 ± 4.23	68.4 ± 1.97	74.3 ± 11.8	79.0 ± 2.34
Inorganic N[2] disappearance (%, w/w)[3]	75.0 ± 5.92^{ab}	83.5 ± 4.42^{b}	70.8 ± 4.71^{a}	83.1 ± 2.73^{b}
Inorganic N disappearance (mg)	28.0 ± 2.23^{a}	60.4 ± 3.20^{b}	26.7 ± 1.78^{a}	60.1 ± 1.98^{b}
Total N in crude protein (mg, 4.5% DM assumed)[4]	82.3 ± 17.0^{a}	111 ± 7.28^{a}	192 ± 30.2^{b}	286 ± 20.1^{c}
Total N in crude protein (mg, 11% DM assumed)[4]	201 ± 41.6^{a}	270 ± 17.8^{a}	469 ± 73.8^{b}	698 ± 69.1^{c}

[1]Inorganic P = PO_4-P.
[2]Inorganic N = NH_4-N, NO_2-N and NO_3-N.
[3]Average disappearance measured over the whole experimental period.
[4]To estimate protein-N accretion either low (4.5%) or high (11%) dry matter contents were assumed.

Table 2
Feed formulation and proximate composition of the differently produced duckweed and the three different diets.

Ingredients (g kg^{-1})	*Spirodela polyrhiza*		Diets		
	Screening	Greenhouse	Con	SPlow	SPhigh
Fish meal			666	660.7	655.2
Spirodela polyrhiza			0	62.5	125
Whole wheat meal			208.4	151.2	94.5
Bentonite			10	10	10
Sunflower oil			75.6	75.6	75.3
Vitamins[a]			20	20	20
Minerals[a]			20	20	20
Proximate composition					
Dry matter (g kg^{-1})			956	947	956
Crude protein (g kg^{-1} DM)	306	180	475	475	481
Crude lipids (g kg^{-1} DM)	n.d.	31	133	125	120
Crude ash (g kg^{-1} DM)	196	218	145	153	165
Crude fiber (g kg^{-1} DM)	n.d.	n.d.	0.86	0.89	1.20
NFE (g kg^{-1} DM)	n.d.	n.d.	238	238	222
Gross energy (kJ g^{-1} DM)[b]	n.d.	n.d.	20.6	20.2	19.9

n.d = not determined.
[a] Commercial trout vitamin and mineral premix (Hokovit Hofman Nutrition AG, Bützberg, Switzerland).
[b] Gross energy estimated with caloric values of 22.4 kJ g^{-1} for crude protein, 40 kJ g^{-1} for crude lipids and 20 kJ g^{-1} for crude fiber and nitrogen free extract (NFE).

by difference before they were ground for later proximate composition analysis.

2.5. Proximate composition analysis

For proximate composition analysis, the duckweed and fish samples were milled homogenously in an IKA M23 mill (IKA®-Werke GmbH & CO KG, Staufen, Germany). Samples were dried for 5 h at 105 °C to determine dry matter content followed by ashing in a muffle oven for 5 h at 550 °C to determine crude ash (CA) content. Nitrogen was determined by the Dumas method (MB III 4.1.2 VDLUFA) and crude protein calculated as CP = N * 6.25. Crude lipids (CL) were determined by Soxhlet extraction with petrol-ether. Crude fiber (CF) content was determined only in the three different fish feeds by the EC 152/2009 III I method. Gross energy values for feed and fish were estimated by assuming a gross energy content of 22.4 kJ g^{-1} for proteins, 40 kJ g^{-1} for lipids and 20 kJ g^{-1} for carbohydrates (nitrogen free extract, NFE), which were estimated by difference (NFE = 100 − CP − CA − CL − CF). For fish, NFE was estimated without the CF fraction.

2.6. Analysis of media and water parameters

Weekly routine measurements for oxygen, pH and temperature were conducted using a WTW Multi 3410 IDS hand-held multimeter (WTW, Weilheim, Germany). Ammonium-N, nitrite-N, nitrate-N and phosphate-P were measured by photometer using commercial test kits (Merck, Darmstadt, Germany).

For the fish feeding trial, dissolved oxygen and pH were measured daily while ammonium, nitrite and nitrate were measured once weekly in the water of each aquarium.

2.7. Calculations and statistics

In order to evaluate the growth and feed utilization response of the trout fry, the following calculations were conducted:

Percent weight gain (PWG; %)

$$\frac{final\ body\ weight\ (g) - initial\ body\ weight\ (g)}{initial\ body\ weight\ (g)} \times 100 \quad (1)$$

Specific growth rate (SGR; % day^{-1})

$$\frac{\ln final\ body\ weight\ (g) - \ln initial\ body\ weight\ (g)}{days\ of\ experiment} \times 100 \quad (2)$$

Intestine somatic index (ISI; %)

$$\frac{intestine\ weight\ (g)}{whole\ body\ weight\ (g)} \times 100 \quad (3)$$

Feed conversion ratio (FCR)

$$\frac{total\ dry\ feed\ intake\ (g)}{final\ body\ weight\ (g) - initial\ body\ weight\ (g)} \quad (4)$$

Protein productive value (PPV; %)

$$\frac{final\ fish\ protein\ content\ (g) - initial\ fish\ protein\ content\ (g)}{total\ protein\ intake\ (g)} \times 100 \quad (5)$$

Lipid productive value (LPV; %)

$$\frac{final\ fish\ lipid\ content\ (g) - initial\ fish\ lipid\ content\ (g)}{total\ lipid\ intake\ (g)} \times 100 \quad (6)$$

To compare whether or not observed differences between the treatments of the duckweed growth experiment and between the different feeding groups in the fish feeding experiment were statistically significant, all data were tested for normal distribution by means of a Kolmogorov-Smirnoff test and for homogeneity of variance by a Levene test. Growth and nutrient utilization data was compared using a one-way ANOVA followed by Tukey HSD post-hoc test. For comparison of duckweed growth between treatments during the screening experiment the Brown-Forsythe test was applied, followed by a Tukey HSD. For histological data of the fish intestines and mortality, the non-parametric Kruskal-Wallis H test was applied. All statistical calculations were conducted with SPSS version 21 (IBM Corporation, Armonk, USA).

3. Results

3.1. Screening experiment

In terms of nutrient concentrations, the media differed substantially. Ammonium-N and ortho-phosphate-P concentrations were higher in DS compared to PSB (Figs. 1–4). In both media the N and P concentrations dropped during one week. The ammonium-N values in DS fell close to zero (0.04 mg L^{-1}), while ortho-phosphate-P only dropped below 1 mg L^{-1} on one occasion. The pH value fluctuated between 8 and 9 with a higher value at the beginning of each new week and a lower value towards the end of the week.

Both duckweed species, *L. punctata* and *S. polyrhiza*, grew better on DS compared to PSB. *Spirodela polyrhiza* exhibited better growth than *L. punctata* in both absolute and relative terms. The best growth was achieved by *S. polyrhiza* on DS with a daily fresh matter (FM) gain of 96 g m^{-2} and a 33.4-fold increase over the four weeks. The protein content was also remarkably higher in *S. polyrhiza*, independent of medium, compared to *L. punctata*; for both species it was higher in DS compared to PSB (Table 1). As can be seen in Figs. 1–4, both species utilized ammonium-N very effectively, nitrate-N and ortho-phosphate-P partly. Nitrogen disappearance in the media attributable to duckweed differs significantly between media, DS being substantially more efficiently cleared compared to PSB. A comparison of estimated protein accretion in the different species on the different media shows that there was no difference for *L. punctata* grown on either medium. *Spirodela polyrhiza* grown on PSB, however, exhibited significantly lower protein-N accretion compared to *S. polyrhiza* grown on DS (Table 1). Similarly, the difference between ammonium-N, nitrite-N and nitrate-N uptake and N fixed in protein was equal in *L. punctata* grown on PSB and on DS, while it was found to be higher in *S. polyrhiza* grown on DS than on PSB. The difference between measured inorganic N uptake and N fixed in protein was lower when assuming a dry matter of 4.5% compared to an assumed dry matter content of 11%. Independent of dry matter content, for all treatments except *L. punctata* grown on DS the estimated N uptake from organic sources was several times higher than the measured inorganic N uptake (Table 1).

3.2. Greenhouse phase

During the greenhouse phase, initial values for ammonium-N, nitrate-N and nitrite-N were very low at around or below 5 mg L^{-1} (Fig. 5). Ammonium-N steadily increased to values around 6–7.5 mg L^{-1} before declining again until it reached minimum concentrations of 0.2 mg L^{-1} after 7 weeks. Ortho-phosphate-P declined steeply during the first 12 days with a reduced decline afterwards. From 45 days onwards, ortho-phosphate-P declined slowly further towards a minimum concentration of 3.3 mg L^{-1} at the end of the greenhouse phase. The temperature declined steadily over the 10 weeks from around 25 °C to around 15 °C (Fig. 5). The pH was more or less steady over the whole greenhouse phase at around 8.4 (data not shown). The protein content of *S. polyrhiza* produced in the greenhouse was 180 g kg^{-1} DM, lipid content was 31 g kg^{-1} DM and ash content was 218 g kg^{-1} DM (Table 2). The amino acid content, along with that of other typical plant protein sources, is presented in Table 3. The two essential amino acids methionine and tryptophan were both higher compared to soybean and lupine (both 1.72 g per 100 g crude protein) while tyrosine and histidine were lower compared to soybean and lupine (3.00 and 1.89 g per 100 g crude protein, respectively).

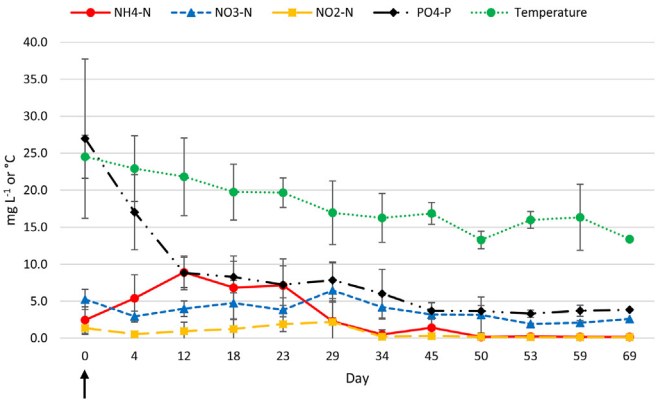

Fig. 5. Development of NH_4-N, NO_3-N, NO_2-N, PO_4-P and temperature of *S. polyrhiza* cultured in the greenhouse over 69 days. Values are mean of N = 4 ± SD. Arrow indicates time of addition of fresh substrate.

3.3. Fish feeding experiment

The experimental diets were similar in protein content

Table 3
Amino acid composition of *S. polyrhiza* (g per 100 g crude protein) grown on diluted slurry in the greenhouse in comparison to other common protein sources.

Amino acid	*S. polyrhiza* (this study)	Soybean meal[1]	Lupine meal[2]
	g 100 g^{-1} crude protein		
Aspartic acid	8.50		
Threonine	4.17	3.93	3.95
Serine	4.39		
Glutamic acid	9.22		
Glycine	4.89		
Alanine	5.56		
Cysteine	1.11	1.59	1.68
Valine	6.17	5.45	4.24
Methionine	1.72	1.39	0.89
Isoleucine	3.67	4.52	4.54
Leucine	7.39	7.77	7.99
Tyrosine	3.00	3.84	4.44
Phenylalanine	4.50	4.95	4.01
Histidine	1.89	2.66	2.53
Lysine	5.11	6.43	5.07
Arginine	6.78	7.34	11.1
Proline	4.39		
Tryptophan	1.72	1.39	0.86

[1]Soybean meal, 44% crude protein (CP), solvent extracted.
[2]Lupine meal, sweet white, 30.4% CP.
[1, 2]NRC (2011).

(475–481 g kg^{-1} DM) but decreased in lipid (133 g kg^{-1} DM to 120 g kg^{-1} DM), NFE (238 g kg^{-1} DM to 222 g kg^{-1} DM) and energy (20.6 kJ g^{-1} DM to 19.9 kJ g^{-1} DM) and increased in ash (145 g kg^{-1} DM to 165 g kg^{-1} DM) and fiber content (0.86 g kg^{-1} DM to 1.20 g kg^{-1} DM) with increasing dietary *S. polyrhiza* level (Table 2).

All groups accepted all diets immediately and consumed the food offered during the first minute after feeding. Mortality increased with increasing dietary duckweed level, with no mortality in the control group and 3.33% in the SPhigh group (Table 4). It appeared that the mortality was not related to the feeds themselves but rather to underdeveloped and weak fish and coincidence of distribution, as there were 2 out of 4 replicates in SPlow and SPhigh each, respectively, in which no mortality occurred while in the other two aquaria one fish each (SPlow) and two fish each (SPhigh) died. Overall the growth responses of the different groups were very good and the fish grew 4.6 (SPlow and SPhigh) to 5.0 (Con) times their initial size during the four weeks. At the end of the feeding trial, fish fed with control feed exhibited a significantly higher group weight and PPV and a significantly lower FCR compared to groups fed with SPlow and SPhigh (Table 4). No differences in terms of relative and absolute growth, SGR, FCR or PPV were observed between both duckweed groups. A significantly lower LPV compared to Con and SPlow fed fish was observed in fish fed with SPhigh (Table 4). The ANOVA detected a significant difference in PWG and SGR but the subsequent Tukey HSD post-hoc showed only a trend in both parameters between Con fed fish and SPlow and Con fed fish and SPhigh, respectively. No difference was observed in ISI between the treatment groups (Table 4). Feed had no impact on the whole body composition of trout fry as no changes were observed in crude protein, crude lipids, crude ash or gross energy.

4. Discussion

Duckweed species have been studied over at least the past four decades for applications such as wastewater treatment (Culley and Epps, 1973; Zhao et al., 2014), animal feed (Haustein et al., 1990; Xu et al., 2012), green manure for rice paddies (Yao et al., 2017) and bioenergy crop production (Tonon et al., 2017; Verma and Suthar, 2015). Depending on the intended purpose, targeted agricultural production as crop plant aims at a number of different final product traits. For utilization as feed ingredient, a high protein content with good amino acid profiles is desirable. For utilization as bioenergy crop, a high starch content with lower protein content is preferable. Utilization for wastewater treatment seeks to reduce nutrient concentrations and increase uptake rates into duckweed with maximum efficiency.

Of the two species, *S. polyrhiza* achieved the higher biomass gain on diluted slurry (DS) (96 g m^{-2} d^{-1} fresh matter), which is lower than that reported by Xu et al. (2012) (145 g m^{-2} d^{-1} fresh matter). However, the ammonium-N concentration reported by Xu and colleagues in swine manure was, at 1.5 mmol L^{-1} (corresponding to approximately 27 mg L^{-1}), more than twice as high as the ammonium-N concentration in our DS treatment (around 10–11 mg L^{-1} ammonium-N after each medium change). Besides producing more biomass than *L. punctata*, *S. polyrhiza* also contained more protein (306 g kg^{-1}) when grown on DS, a content well above the 226–265 g kg^{-1} reported elsewhere (Xu et al., 2012; Appenroth et al., 2017) for *S. polyrhiza* grown under different conditions. A substantially higher protein content (455 g kg^{-1}) was reported for another duckweed species, *Lemna paucicostata*, by Mbagwu and Adeniji (1988) from a cow-manure enriched culture in Kenya. Generally, a positive correlation between ammonium levels in the medium and protein content has been reported by Leng et al. (1995). That correlation likely contributes to the low protein content of the duckweed harvested from the greenhouse (180 g kg^{-1}), as ammonium-N was diminished there after around 50 days because the medium was not exchanged in the greenhouse for reasons of practicability. Phosphorus, on the other hand, does not appear to have been a limiting factor in the whole project. Only at two measurements did the ortho-phosphate-P concentration reach values close to zero; it usually stayed well above 1 mg L^{-1} (Figs. 1–5). High efficiencies of nitrogen and phosphorus removal from swine wastewater by *Spirodela punctata* (Cheng et al., 2002), *S. olygorrhiza* (Xu and Shen, 2011) and *Landoltia punctata* (Mohedano et al., 2012) have been reported. That is one of the reasons for the strong interest in duckweed for wastewater treatment, given that phosphorus is a limited resource with its global peak in production expected to occur around 2030 (Cordell et al., 2009). Furthermore, anthropogenic reactive nitrogen emissions are projected to rise, primarily with increasing livestock production, and may surpass environmentally safe boundaries for sustainable food production in 2050 by 294% (Pelletier and Tyedmers, 2010). A comparison of protein-N accretion during the screening experiment with the available inorganic N shows clearly that, even when the lowest dry matter content (4.5%) in duckweed is assumed, more N has been fixed in the protein than inorganic N was available (Table 1). Some aquatic plants such as *Azolla* sp. are known for their ability to fix atmospheric N at levels similar to legumes, yet duckweeds are not. Therefore the only likely explanation for the discrepancy between protein-N accretion and N availability in the screening experiment is that dissolved organic N (DON) was utilized by both duckweed species. Dissolved organic N is a heterogeneous term describing all particles smaller 0.2 μm. It includes urea, amino acids, peptides, proteins, viruses and bacteria (Jørgensen, 2009). Up to now, no other publication has reported potential utilization of DON by duckweed. The available literature only states inorganic (ammonium, nitrite and nitrate) N concentrations or uptake rates. It is therefore possible that reporting only inorganic N concentrations leads to underestimation of available N for the growth and protein production of duckweed.

The palatability of the different diets to trout fry appeared

Table 4
Growth, feed conversion, protein and lipid utilization, mortality and proximate composition of the differently fed rainbow trout fry. Different superscripts in the same row indicate statistically significant differences (N = 4 ± SD, except for analysis of initial fish).

	Con	SPlow	SPhigh
IGW[1] (g)	8.35 ± 0.33	8.35 ± 0.26	8.35 ± 0.37
FGW[1] (g)	41.9 ± 1.61[a]	38.4 ± 2.32[b]	37.5 ± 0.76[b]
PWG[1] (%)[2]	502 ± 25	467 ± 19	465 ± 14
SGR[1] (% day^{-1})[2]	5.76 ± 0.18	5.50 ± 0.14	5.49 ± 0.11
ISI[1] (%)	19.5 ± 1.29	18.5 ± 1.35	19.0 ± 0.27
FCR[1] (g/g)	1.08 ± 0.03[a]	1.13 ± 0.02[b]	1.16 ± 0.03[b]
PPV[1] (%)	26.0 ± 0.64[a]	24.9 ± 0.48[b]	23.9 ± 0.50[b]
LPV[1] (%)	31.7 ± 0.65[a]	32.1 ± 0.49[a]	29.7 ± 0.57[b]
Mortality (%)[3]	0.00 ± 0.00	1.67 ± 1.92	3.33 ± 3.85

Proximate composition	Initial fish	Con fed fish	SPlow fed fish	SPhigh fed fish
Moisture (%)	83.5	80.5 ± 0.40	80.6 ± 0.38	80.6 ± 0.51
Crude protein (% g^{-1} FM)	11.0	12.4 ± 2.75	12.3 ± 2.88	12.3 ± 2.58
Crude lipids (% g^{-1} FM)	15.8	37.8 ± 0.94	37.0 ± 2.40	34.2 ± 2.56
Crude ash (% g^{-1} FM)	16.8	18.0 ± 0.29	17.8 ± 0.70	18.2 ± 0.61
Gross energy (kJ g^{-1} FM)	3.55	4.59 ± 0.08	4.56 ± 0.09	4.50 ± 0.11

[a,b]Values within the same row carrying different superscripts differ at P < 0.05.
[1]FM: Fresh matter, IGW: Initial group weight, FGW: Final group weight, PWG: Percent weight gain, SGR: Specific growth rate, ISI: Intestine somatic index, FCR: Feed conversion ratio, PPV: Protein productive value, LPV: Lipid productive value.
[2]One-way ANOVA significant, group-wise comparison with Tukey HSD showed only trends.
[3]Kruskal-Wallis test.

similar, as all feed offered was consumed in not more than 2–3 min after provision. Nevertheless, feed intake was not measured directly because a restrictive feeding regime was applied. It can be expected that with an increasing inclusion of *S. polyrhiza* in rainbow trout fry diets the potential negative effects on growth, feed and nutrient utilization become more prominent, as observed with the highest duckweed inclusion level in striped snakehead (*Channa striatus*) diets (Raj et al., 2001). However, the pattern of the observed effects was somewhat surprising. Besides lipid utilization, which was significantly lower in SPhigh-fed fish, basically no differences in terms of growth and feed conversion between both *S. polyrhiza* fed groups were observed, although both duckweed-fed groups grew slower than the control group. The relative and absolute weight gains were very good in all treatments. The specific growth rates (SGRs) in our experiment (5.49% day^{-1} to 5.76% day^{-1}) at 12.8 °C water temperature were well above the 3.5% day^{-1} to 4% day^{-1} reported by Bagheri et al. (2008) for rainbow trout fry at around 14 °C and those reported for fry of another salmonid, Atlantic salmon (*Salmo salar*), of 2.63% day^{-1} to 3.23% day^{-1} (Berge and Storebakken, 1996) at 12 °C water temperature. That points to good growth and a good nutritional status of all fish.

Fish are generally more efficient nutrient converters than terrestrial livestock (Tidwell and Allan, 2001); the latter have rather low feed conversion efficiencies. The observed feed conversion ratios (FCRs) were between 1.08 and 1.16, which is below the reported global average of 1.3 for rainbow trout (Tacon and Metian, 2008) but above the FCR of 0.9–1 reported by Bagheri et al. (2008). With increasing duckweed content in diets, the lipid content and also the estimated energy tended to decrease while crude fiber increased. Although the differences were minor, they probably contribute to decreased lipid utilization in SPhigh-fed fish. Dietary differences and the level of duckweed did not lead to differences in whole body chemical composition. The findings of feeding studies with various fish and duckweed species are diverse and inconclusive. The only other study reporting on duckweed fed to carnivorous fish stated no differences in FCR between control-fed and duckweed-fed groups of snakehead (*Chianna striatus*) when up to 75% *Lemna minor* was included in formulated diets (Raj et al., 2001). In Nile tilapia (*O. niloticus*), increasing levels of dietary duckweed led to decreasing final weight, increasing FCR and, at the highest inclusion level, to reduced protein utilization (Fasakin et al., 1999). In another study in which Nile tilapia were fed with 15% dietary *Lemna minor*, the growth, feed conversion and nutrient conversion of fish were superior compared to most other diets and were only inferior compared to a wheat-gluten diet and the fishmeal-based control diet (Schneider et al., 2004). Feeding red tilapia (*O. mossambicus* × *O. niloticus*) compressed pellets of *Lemna valdiviana* resulted in a similar FCR and SGR compared with commercial pellets but showed a tendency for both values to be smaller when fish were fed duckweed pellets (De Matos et al., 2014). In grass carp (*Ctenopharyngodon idella*), a herbivorous fish species, feeding a duckweed mixture consisting of *L. minor* and *S. polyrhiza* resulted in inferior growth compared to fish fed with tubificid worms (Cui et al., 1992). Increasing dietary duckweed levels in the feed of silver barbs (*Barbonymus gonionotus*) led to decreasing final weights, weight gains, feed and protein utilization with concomitant accumulation of leukocytes and hemorrhagic lesions in the liver of the fish with highest dietary duckweed content (Noor et al., 2000). Generally, plants contain various types and concentrations of anti-nutritional factors (ANF) which can severely influence the utilization of plant-based feed ingredients (Francis et al., 2001). Although we did not analyze for ANF in our duckweed, some potential ANFs such as tannins, phytic acid and cyanide have been reported to be present at low doses in *S. polyrhiza* (Fasakin, 1999) and might influence growth and nutrient utilization, especially at higher dietary duckweed levels. The significantly lower growth, feed and protein utilization of both duckweed treatments in comparison to the control treatment indicates that some negative effect of duckweed inclusion is present but the similarity between both duckweed groups suggests that the concentration of potential ANFs was not high enough to result in a concentration-dependent effect. One important factor for feed and nutrient utilization is the digestibility of feed ingredients and whole diet (NRC, 2011). To our knowledge, only one detailed study has investigated the digestibility of duckweed in striped catfish *Pangasianodon hypophthalmus*. Da et al. (2013) report a crude protein digestibility of 81.7% and a gross energy digestibility of 69.8% for *Lemna polyrhiza* meal. By comparison, the same study found the digestibility of soybean meal crude protein to be 91.3% and that of soybean meal gross energy to be 87.3%. Soybean meal is the quantitatively most utilized plant-based feedstuff for aquafeeds (Troell et al., 2014) and contains several ANF such as protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins and allergens (Francis et al., 2001) which can partly be removed by roasting. Post-harvest treatment of duckweed could also improve utilization parameters as would a more sophisticated feed manufacturing method like extrusion cooking. Extrusion generally increases digestibility and nutrient utilization considerably. Moreover, due to the high temperature and pressure applied, it is effectively a simultaneous disinfection step (Sørensen, 2012). The autoclaving of *S. polyrhiza* in our experiment may have had a positive influence on utilization parameters.

5. Conclusion

Spirodela polyrhiza can be grown on diluted cattle slurry and has fairly high protein contents as well as a satisfactory amino acid profile with regard to fish feed. However, care needs to be taken to avoid ammonium deficiency in the medium to keep growth and, more importantly, protein content high. This feeding experiment, the first of its kind conducted, revealed that duckweed inclusion in trout fry diets can have negative effects on growth, feed and nutrient utilization, although the effects do not increase with a doubling of *S. polyrhiza* inclusion. Given the simple feed preparation process used in the experiment, it can be assumed that more sophisticated feed manufacturing would remedy some of the negative impacts and that larger fish may not be impacted as strongly. Growing duckweed on diluted slurry has the potential to utilize the high nitrogen and phosphorus contents in slurry and thus reduce the impact of livestock excreta on the environment, while simultaneously generating a high-protein feedstuff usable as animal feed ingredient. Furthermore, the current study gives reason to assume a considerable N uptake potential of duckweed from the DON fraction of the media. The latter finding needs to be verified by future experiments.

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