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Comparison of three unionid mussel species in removing green microalgae grown in recirculating aquaculture system effluent

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Abstract Global increase in aquaculture production has created a need to reduce its environmental impacts. Nutrients could be recycled especially at land-based recirculating aquaculture systems (RAS) by cultivating green microalgae in aquaculture effluent. However, microalgae are difficult to harvest. As a multi-trophic solution, mussels could be used in harvesting microalgae. We tested three European freshwater mussels (duck mussel *Anodonta anatina*, swan mussel *A. cygnea*, and swollen river mussel *Unio tumidus*) for filtering two common green microalgae (*Monoraphidium griffithii* and *Selenastrum* sp.) grown in RAS effluent. Mussels decreased microalgal concentrations in the tanks 42–83% over three consecutive trials. Algal concentrations at the end of each trial were lowest for both microalgae in tanks containing *Anodonta* mussels. Clearance rates were higher for *Anodonta* mussels than for *U. tumidus*. Mussels biodeposited more microalgae to tank bottoms when *M. griffithii* was filtered. Ammonium concentration decreased or did not change in tanks with *M. griffithii*, but increased in tanks containing *Selenastrum* sp. These results suggest that of the tested species *Anodonta* mussels and *M. griffithii* show best potential for RAS effluent bioremediation application. We conclude that a co-culture of microalgae and unionid mussels could be used for recycling nutrients in aquaculture.

Introduction

Aquaculture production increased threefold during the first two decades of twenty-first century and the increase in production has created problems such as nutrient pollution (Naylor et al., 2021). Traditional cage or flow-through aquaculture systems offer few opportunities for nutrient recovery, because nutrients released from waste and fish excreta are diluted in a large volume of water. Recirculating aquaculture systems (RAS) are land-based systems for farming aquatic organisms where water use is significantly reduced compared to traditional flow-through systems due to constant effluent treatment and recycling (Bregnballe, 2015). In RAS, typically only 5–10% of the total water volume is renewed daily with fresh water to prevent the accumulation of dissolved

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nutrients and suspended solids originating from feces, metabolic waste products, and uneaten feed to levels harmful to fish (Piedrahita, 2003; Bregnballe, 2015). As a result, the concentration of dissolved nutrients is much higher in RAS effluent than in flow-through aquaculture, enabling nutrient recovery from the effluent for recycling (Piedrahita, 2003; Martins et al., 2010).

One of the ongoing developments in RAS is the uptake of nutrients from effluent via the integrated multi-trophic aquaculture (IMTA) approach (Martins et al., 2010). IMTA refers to the simultaneous cultivation of two or more compatible organisms from different trophic levels in a single system, where the wastes from the fed organism, usually fish, are used as sources of nutrition for other cultured organisms such as microalgae or filter-feeders (Troell et al., 2009; Neori et al., 2017). Microalgae are among the most suitable organisms for IMTA as they offer sustainable and cost-efficient bioremediation of aquaculture effluent, and important biomolecules from the produced biomass (proteins, lipids, carbohydrates, pigments) can be later used in human foods, animal feeds, fertilizers, biofuels, and other products (Mata et al., 2010, 2012; Arbib et al., 2012).

Microalgal harvesting is the main limitation for the utilization of microalgae in IMTA as chemical and mechanical harvesting of microalgae can be up to 90% of the total investment costs (Molina Grima et al., 2003; Lavrinovičs & Juhna, 2017). The direct use of microalgae as fish feed in IMTA is partly limited by the small cell size of microalgae and its poor digestibility, especially for some carnivorous fish species (Shah et al., 2018; Tibbetts, 2018; Dourou et al., 2020). Consequently, these constraints could be mitigated with biological harvesting of microalgae by filter-feeding organisms such as mussels and utilizing mussels as a fishmeal replacement in feeds for fish (Sicuro et al., 2010) or chicken (McLaughlan et al., 2014). Concentrated nutrient-rich feces and pseudofeces produced by mussels filtering microalgae grown in the effluent (van der Meer et al., 2023) could be a promising resource to be used as fertilizer and in biofuel and biogas production (Vaughn et al., 2004).

Bivalves, including the freshwater mussels of the order Unionida, are highly efficient in filtering all particulate matter from their surroundings (Vaughn et al., 2004; Vaughn, 2018). While filtering the surrounding water, mussels remove inorganic and organic particles, such as green microalgae and diatoms (Kryger & Riisgård, 1988; Vaughn & Hakenkamp, 2001; Bontes et al., 2007), but also harmful microorganisms and particles, such as flavobacteria (Hajisafarali et al., 2022), toxic cyanobacteria (Bontes et al., 2007), fish parasites (Gopko et al., 2017), and microplastics (Berglund et al., 2019). Freshwater mussels can select which of the filtered particles they ingest (Lopes-Lima et al., 2014). This enables the mussels to select food particles they require at any given time and discard potentially harmful or non-nutritional particles encapsulated in mucus as pseudofeces (Bontes et al., 2007). Pseudofeces production is a method by which some bivalves, including all unionid mussels, deposit particles from the surrounding water to the sediment without the particles going through the mussel gut. Freshwater mussels can be opportunistic in their grazing and can use the organic material in the sediment, including previously deposited pseudofeces, as food (Nichols et al., 2005).

Biofiltering applications often use species that are easily available and have high filtering efficiency. For example, the dreissenid zebra mussel [*Dreissena polymorpha* (Pallas, 1771)] has been used in treating industrial and municipal wastewaters (Elliott et al., 2008; Mezzanotte et al., 2016), and in bioremediation of eutrophic natural waters (Pires et al., 2009). The dreissenid quagga mussel [*D. bugensis* (Andrusov, 1897)] has been used in harvesting green microalgae cultured in wastewater plant effluent for removal of nutrients from the effluent (van der Meer et al., 2023). However, *D. polymorpha* and *D. bugensis* are invasive species that can replace local fauna and cause detrimental changes in their invaded freshwater ecosystems (Strayer et al., 1998; Rowe et al., 2015; Ożgo et al., 2020). As there are still areas in Europe, where the invasive freshwater mussel is not present, native mussel species should be used in bivalve-based bioremediation applications. Duck mussel [*Anodonta anatina* (Linnaeus, 1758)], swan mussel [*A. cygnea* (Linnaeus, 1758)], and swollen river mussel (*Unio tumidus* Philipsson, 1788), all belonging to the family Unionidae, are common and readily available from lakes, ponds, and slow-flowing rivers in Northern Europe (Vaughn & Hakenkamp, 2001; Lopes-Lima et al., 2017). They have been shown to have high filtering capacities (Kryger & Riisgård, 1988; Pusch et al., 2001; Bontes et al., 2007; Moëzzi et al., 2013),

which makes them suitable candidates for testing bioremediation applications in the Nordic countries.

This experiment was carried out as a feasibility study to compare three native and common freshwater mussel species, *A. anatina*, *A. cygnea*, and *U. tumidus*, and two species of green microalgae in the removal of nutrients from RAS effluent. The two green microalgae tested, *Monoraphidium griffithii* (Berk.) Komárk.-Legn. and *Selenastrum* sp., are efficient at absorbing dissolved nutrients from RAS effluent and are common in European freshwater systems (Stevčić et al., 2019; Calderini et al., 2021). However, as these microalgae differ in shape and size and in their palatability to filter-feeders such as *Daphnia magna* Straus, 1820 (Stevčić et al., 2020), their suitability for bioremediation application with mussels was investigated. As the green microalgae are difficult and expensive to harvest, this study also aimed to determine if and to what extent the mussels would biodeposit the filtered microalgae into a highly condensed mucus-covered microalgae sludge that could be easily removed from the tank bottoms. Additionally, since the application aims to reduce the concentration of nutrients in the wastewater, levels of phosphate and ammonium were monitored as they are released by mussels and microbial metabolic activity.

Materials and methods

Microalgae cultivation

The green microalgae were cultured in wastewater originating from a laboratory-scale RAS located at the Department of Biological and Environmental Science, University of Jyväskylä, Finland. Fish kept in the RAS (total volume *c*. 4.5 m^3) were whitefish [*Coregonus lavaretus* (Linnaeus, 1758)] and rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792)], and they were fed with dry pellets (Circuit red, Raisioaqua, Raisio, Finland) using belt feeders. The microalgae were grown in 60-L tubular bubble-column photobioreactors (diameter 20 cm, height 200 cm) filled with RAS wastewater and inoculated with microalgae from stock cultures. The freshwater microalgal strains *M. griffithii* (NIVA-CHL 8) and *Selenastrum* sp. (K–1877 (NIVA)) were acquired from the Norwegian Culture Collection of Algae (NORCCA). Stock cultures were maintained in

microalgae medium MWC (Modified Wright's Cryptophyte based on Guillard & Lorenzen (1972) as described previously in Stevčić et al. (2019)). Two photobioreactors, each containing a monoculture of microalgae, were constantly illuminated with two vertically mounted LED grow lights (AP67 spectrum, 18 W, T8 tubes; Valoya Oy, Helsinki, Finland) with an intensity of 100 µmol photons m^{-2} s^{-1} measured at the side of the photobioreactor by a high-resolution spectroradiometer (HP-350; HiPoint Inc, Taichung City, Taiwan). The photobioreactors were covered to minimize evaporation and water spraying due to aeration. Systems were constantly supplied with compressed air from the bottom through perforated coiled tubes to keep the microalgae in suspension and to provide CO_2. The air supply was not measured but set to be similar in both photobioreactors based on visual water bubbling. The microalgae cultures used in the experiment were grown in the photobioreactors for four days until they had exhausted the phosphate in the wastewater (Stevčić et al., 2019) before each trial with the mussels.

Mussel collection and maintenance

Mussels were captured by snorkeling from the littoral zone of Lake Jyväsjärvi (62°14′31″N, 25°46′12″E) from the approximate depth of one meter on June 20th, 2019. Mussels were identified visually as *A. anatina*, *A. cygnea*, and *U. tumidus*. As mussels were captured haphazardly, they were assumed to represent typical adult specimens of each species regarding individual mussel size in the local population. The mussels were kept in two flow-through (400 mL min^{-1}) holding tanks in 40 L of borehole water aerated with air pumps (Eheim air 400; Eheim GmbH & Co. KG, Deizisau, Germany) at approx. 33 mL min^{-1}, in a temperature-controlled laboratory (17.0 ± 0.3 °C). Mussels used for *M. griffithii,* and *Selenastrum* sp. filtering trials were kept in separate tanks. No food was offered to mussels between the trials. Each mussel was marked by drilling a unique code on the shell with a rotary cutter (DremelStylo +; Robert Bosch GmbH, Gerlingen, Germany). Mussel lengths were measured before and after the series of three trials with Vernier calipers to the nearest mm. Dry weight estimates were calculated for each mussel from the average of the two shell length measurements (Table 1). The dry weight estimates for *A.*

anatina were calculated according to Zapitis et al. (2021)

$$dw = 9.66 * 10^{-4} * l^2 - 0.09l + 2.31$$

The dry weight estimates for *A. cygnea* and *U. tumidus* were calculated as

$$dw = 0.12e^{0.31l}$$

and

$$dw = 0.04e^{0.51l}$$

Respectively (Ravera & Sprocati, 1997). In the formulae, *l* is mussel length in cm.

Experimental setup

The efficiency of the three mussel species in removing the two microalgal species cultivated in RAS wastewater was evaluated in a series of three trials. Before the first trial, the mussels were acclimated to the experimental setup for seven days. In each trial, mussels were placed individually in glass test tanks (width 17, length 17, height 24 cm), that were filled with 4L of algae-containing water taken from the photobioreactors (see Sect. "Calculations and data analyses".). Tanks were monitored for seven days for a change in algal density. The setup remained the same throughout the consecutive trials, and the same individual mussels were used in all trials and were provided with the same microalgae. The rationale for using the same individual in three separate trials was to monitor the consistency in the filtering behavior of the mussels. In each trial, there were three tanks for *A. anatina* and *A. cygnea* individuals, four tanks for *U. tumidus* individuals, and two tanks without mussels as controls for both species of microalgae, totaling 24 tanks. The laboratory was illuminated dimly by four fluorescent tubes (TL-D 36W/16 Yellow, T8;

Koninklijke Philips N.V., Eindhoven, The Netherlands) with 12:12 light:dark period, 0.09 ± 0.01 μmol photon m^{-2} s^{-1} light intensity at wavelength range of 570–590 nm. The dim lighting and the wavelength range were chosen to minimize microalgal growth in the test tanks and to mimic natural lighting conditions for the mussels. The tanks were covered with transparent plastic sheets to prevent water evaporation and microalgal cross-contamination between tanks. Test tanks had constant aeration from an integrated air compressor divided into each tank via tubing and air stones. The aeration rate was not measured but set visually to be similar in each tank and kept low to reduce stress on the mussels and spraying of water from the tanks. Test tanks were in the same laboratory as maintenance tanks, at 17.0 ± 0.3 °C.

Concentrations of microalgae were measured before, during, and after each trial from samples taken with a pipette from a depth of approximately 5 cm from the surface of the water so as not to disturb the sedimented microalgae on the bottom of the tanks. Sedimented microalgae deposited by mussels were quantified at the end of the third trial, and dissolved phosphate and ammonium in the water were analyzed at the beginning and the end of the second trial (Table 2).

Calculations and data analyses

Microalgal cell density and total cell volume (mL microalgae in mL H$_2$O) in the test tanks were measured with a CASY Electronic Cell Counter and Analyzer (OLS-OMNI Life Science GmbH & Co. Bremen, Germany, later referred to as CASY), with a capillary pore size of 60 μm. The selected measuring range was 3.5–7.5 μm for *M. griffithii* and 2.4–3.8 μm for *Selenastrum* sp.

Table 1 Dry weight (g) estimates (mean ± SE) for *Anodonta anatina*, *Anodonta cygnea*, and *Unio tumidus* used in *Monoraphidium griffithii* and *Selenastrum* sp. treatments based on their shell lengths (cm)

Mussel species	*Monoraphidium griffithii*		*Selenastrum* sp.	
	Length (cm)	Dry weight (g)	Length (cm)	Dry weight (g)
Anodonta anatina (*n* = 3)	9.07 ± 0.71	2.19 ± 0.58	9.10 ± 0.29	2.14 ± 0.25
Anodonta cygnea (*n* = 3)	11.10 ± 0.78	3.98 ± 1.04	10.87 ± 0.86	3.72 ± 0.87
Unio tumidus (*n* = 4)	7.44 ± 0.33	1.85 ± 0.31	7.70 ± 0.70	2.5 ± 0.99

Table 2 Starvation time of mussels before the start of the three trials and the measurements taken (denoted with x) during each trial

	Trial 1	Trial 2	Trial 3
Starvation time (days)	3	7	7
Microalgal cell volume change for sustained CR calculation	×	×	×
Microalgal cell volume change for CR_{max} calculation	–	×	×
Microalgal deposit area [cm^2]	–	–	×
PO_4-P	–	×	–
NH_4-N	–	×	–

CR clearance rate

Clearance rates (CR) of mussels were calculated for each combination of mussel and microalgal species from changes in total microalgal cell volume in the test tanks. The clearance rates were calculated using the formula (Frost, 1972):

$$CR = V/n * \Delta t * \left\{ lnA_0/A_t - lnA'_0/A'_t \right\}$$

in which V stands for test tank volume (4000 mL). For calculating individual CR, n is 1, as there was 1 mussel per test tank. For calculating the size-dependent CR, n denotes the calculated dry weight of a mussel in the test tank. Δt is the time period between measurements in hours. A_0 is the microalgal cell volume (mL microalgae in mL H_2O) in the test tank and A'_0 is the corresponding cell volume average in the control tanks at the beginning of the trial. For estimation of sustained CR, the starting point of the measurement for calculating Δt was day 0, and the cell volumes for A_0 and A'_0 were from samples of day 0. A_t and A'_t were the volumes at the time of sampling from the test tank and the average cell volume in the control tanks. In this study, A_t and A'_t were measured on days 4 and 7, to calculate the mussels' ability to maintain filtration for a period of 4 and 7 days of testing (sustained CRs). When calculating maximum CR (later referred to as CR_{max}), A_0 and A_t were the starting and the end volumes of the highest observed decrease in microalgal cell volume in each test tank in the daily sampling between days 1–4 of the second and third trials, with A'_0 and A'_t being the average values in the control tanks at corresponding time. The first trial was not included in the calculation of CR_{max} because of some missing data points on days 2–3.

Microalgal deposits were measured at the end of the third trial (Table 2) by photographing the test tanks after draining them of water. ImageJ (v1.50e, National Institute of Health, Bethesda, Maryland, USA) was used to determine the area of microalgal deposits in each tank, including the controls. Photos were cropped to include only the area of test tank bottoms (Fig. 1). For statistical analysis, the deposited microalgal area in the test tanks was calculated by multiplying the observed relative microalgal deposit area by the relative area free of microalgae in the control tanks to account for algal deposition not related to mussel presence in the test tanks.

Phosphate-phosphorus (PO_4-P), nitrate-nitrogen (NO_3-N), and ammonium-nitrogen (NH_4-N) were measured at the beginning and at the end of the second trial from the tank water with a mobile

Fig. 1 Example of microalgal deposits on the bottom of a test tank at the end of the third 7-day trial with a tank containing *Monoraphidium griffithii* microalgae and an *Anodonta cygnea* mussel. (**a**) Original photo (dark green areas) and (**b**) photo after image analysis with ImageJ program (black areas cropped from (**a**)). Bottom areas with deposited feces and pseudofeces were included in the analyses
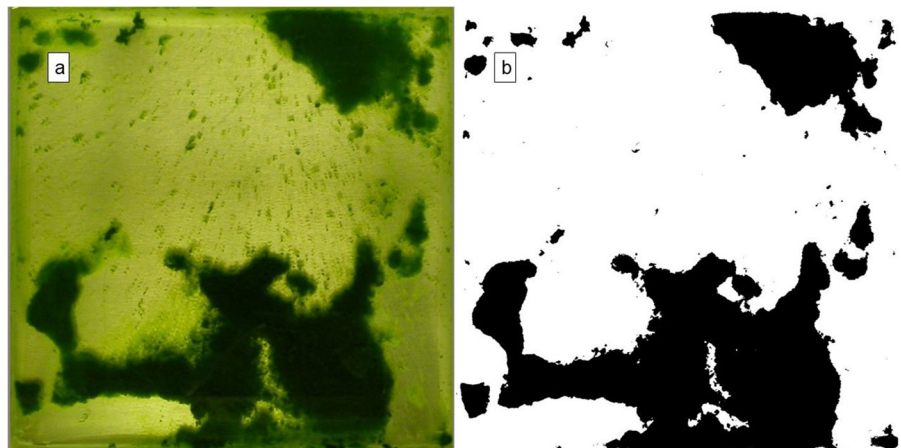
laboratory spectrophotometer (LASA 100, Dr. Lange, Germany) using testing kits (LCK 349 for PO_4-P, LCK 339 for NO_3-N, and LCK304 for NH_4-N; Hach Lange GmbH, Düsseldorf, Germany). The samples were pre-filtered through 0.22 μm syringe filters to separate microalgal biomass from the solution before the nutrient analyses of the filtered water. All nutrient analyses were made according to the manufacturer's instructions. Nutrient concentration at the beginning was measured only once before the water containing the microalgae was divided between the test tanks.

Statistical analyses were performed with IBM SPSS Statistics 26. As the same mussel individuals were used in repeated trials, the data from the three trials were considered dependent replicates. Differences in relative microalgal concentrations after day 7 and differences between observed maximum clearance rate and sustained clearance rates on days 4 and 7 were therefore analyzed with repeated measures ANOVA with microalgal treatments and mussel species as independent factors. Post hoc comparisons were made with Fisher's LSD test. Differences in microalgal deposits in the tanks were analyzed with one-way ANOVA. Differences in nutrient concentrations between day 0 and day 7 in each treatment were determined with one sample *t* tests. The threshold for statistical significance was 0.05.

Results

Relative concentrations of microalgae after 1, 4, and 7 days

Microalgal concentration in the test tanks relative to the starting level differed between mussel species on days 1, 4, and 7, and within mussel species on days 4 and 7 for both species of microalgae (Table 3). Microalgal concentration by day 7 was reduced to 17–58% relative to day 0, depending on the treatment (Fig. 2) i.e., mussels removed 42–83% of algae. *Anodonta cygnea* was the fastest to start filtering with some individuals clearing the test tanks to below 10% algal concentration during first 24 h, and both *Anodonta* species decreased algal concentrations more than *Unio tumidus* on all measurement days, except on day 1 in the *M. griffithii* trial, when the *A. anatina* treatment did not differ from the *U. tumidus* treatment (Fig. 2, Suppl. 1 and 2). By the end of the trials, relative concentrations were lowest in tanks with *Anodonta* mussels when compared to either *U. tumidus* or control tanks for both microalgae (Fig. 2).

Microalgal concentrations did not differ within each mussel treatment between the two microalgae (Fisher´s LSD, $P > 0.05$) for all three mussel species on relative concentrations measured at days 1, 4, and 7. Within control tanks, the microalgal concentrations were, however, higher for *M. griffithii* than for *Selenastrum* sp. on days 4 and 7 ($P = 0.038$ and 0.015, respectively) (Suppl. 1 and 2.).
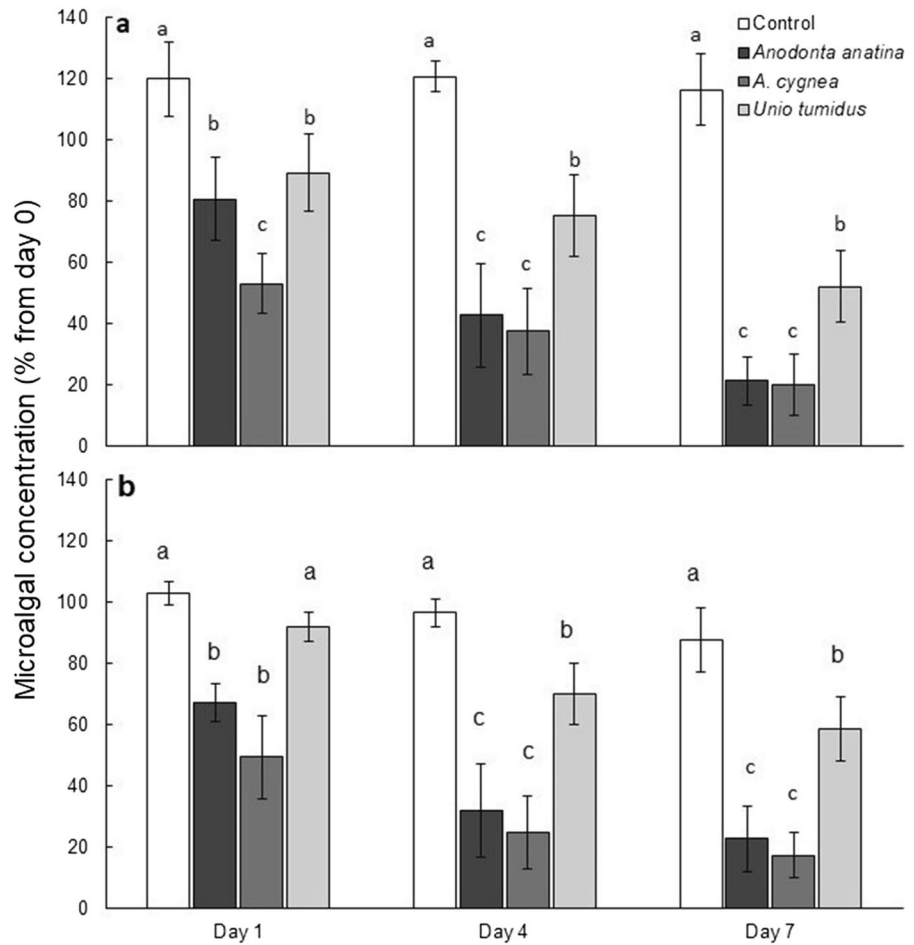
Table 3 Results of a repeated measures ANOVA between subject effects (BSE) tests to determine differences, and within subject effects (WSE) tests to determine consistency in micro-algal concentrations on days 1, 4, and 7 relative to starting concentration between used microalgal species, mussel species, and their interactions

Statistical test	Day	Algae			Mussel species			Algae × Mussel species		
		df, Error	*F* value	*P*	df, Error	*F* value	*P*	df, Error	*F* value	*P*
BSE	1	1, 16	2.522	0.132	3, 16	24.327	**< 0.001**	3, 16	0.893	0.466
	4	1, 16	8.710	**0.009**	3, 16	55.409	**< 0.001**	3, 16	0.699	0.566
	7	1, 16	1.772	0.202	3, 16	64.101	**< 0.001**	3, 16	2.618	0.087
WSE	1	2, 32	19.787	**< 0.001**	6, 32	2.034	0.090	6, 32	1.927	0.107
	4	2, 32	1.894	0.167	6, 32	4.318	**0.003**	6, 32	1.538	0.198
	7	2, 32	0.901	0.413	6, 32	5.404	**< 0.001**	6, 32	1.915	0.109

Statistically significant differences ($P < 0.05$) are bolded

Fig. 2 Relative microalgal concentration on days 1, 4, and 7 (mean % of day 0 ± SE of 3 trials) of control ($n=2$), *Anodonta anatina* ($n=3$), *A. cygnea* ($n=3$), and *Unio tumidus* ($n=4$) treatments for (**a**) *Monoraphidium griffithii* and (**b**) *Selenastrum* sp. microalgae. Different letters (a–c) denote significant differences (Fisher's LSD, $P<0.05$) between mussel and control treatments within used microalgae

Maximum and sustained clearance rates (CR) per individual mussel

Both the CR_{max} per individual mussel calculated from trials 2–3 and the sustained CRs per individual mussel over 4 or 7 days differed between the mussel species (Table 4). Both CR_{max} and sustained CRs were higher for *A. cygnea* and *A. anatina* when compared to *U. tumidus* for both microalgal species (Fig. 3). The clearance rates did not differ between

Table 4 Results of a repeated measures ANOVA between subject effects (BSE) tests to determine differences, and within subject effects (WSE) tests to determine consistency in individual maximum clearance rate, and sustained clearance rates from day 0 to day 4 and from day 0 to day 7, between microalgal species, mussel species and their interactions

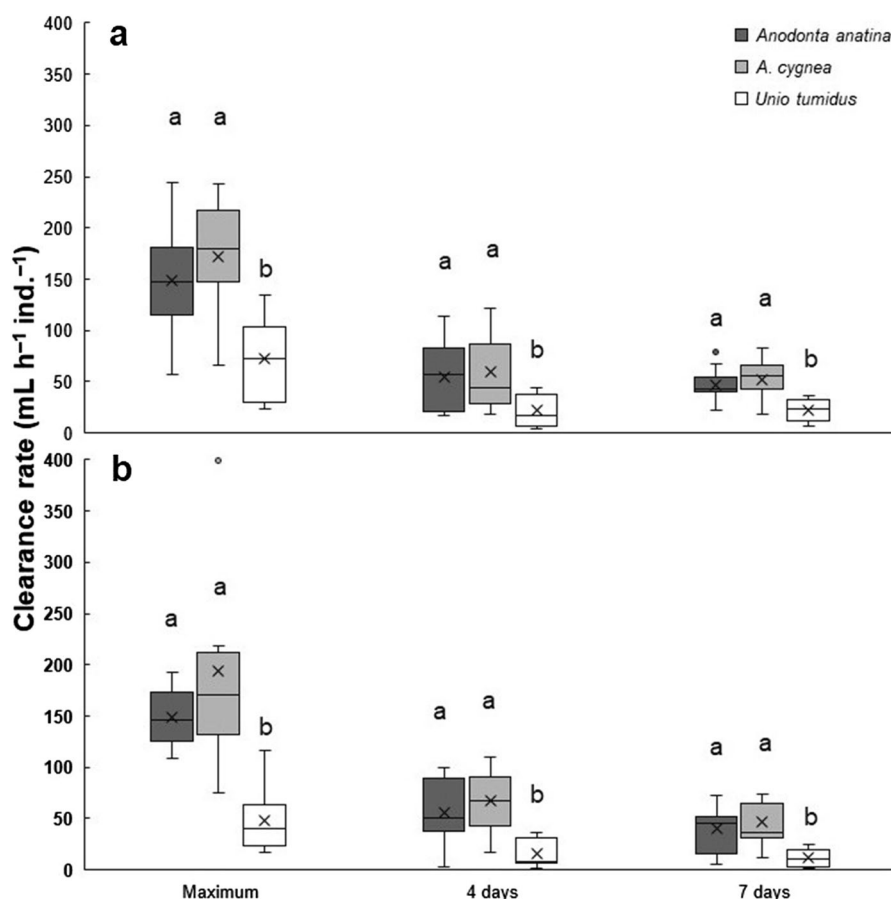| Statistical test	Clearance rate	Algae			Mussel species			Algae × Mussel species		
		df, Error	*F* value	*P*	df, Error	*F* value	*P*	df, Error	*F* value	*P*
BSE	Maximum	1, 14	0.005	0.946	2, 14	14.199	**<0.001**	2, 14	0.462	0.640
	4 days sustained	1, 14	0.029	0.868	2, 14	31.889	**<0.001**	2, 14	0.659	0.516
	7 days sustained	1, 14	2.615	0.128	2, 14	20.187	**<0.001**	2, 14	0.118	0.889
WSE	Maximum	1, 14	9.535	**0.008**	2, 14	3.782	**0.049**	2, 14	2.239	0.143
	4 days sustained	2, 28	8.520	**0.001**	4, 28	2.828	0.053	4, 28	2.226	0.092
	7 days sustained	2, 28	15.279	**<0.001**	4, 28	4.693	**0.005**	4, 28	3.144	**0.030**

Statistically significant differences ($P<0.05$) are bolded

the *Anodonta* species for either of the microalgal species (Fig. 3).

Size-dependent maximum and sustained clearance rates

Size-dependent CR_{max} calculated from trials 2–3 did not differ between the treatments, but the size-dependent CR sustained either for 4- or 7-day periods differed between the mussel species for both species of microalgae (Table 5). *Unio tumidus* had lower sustained CR when compared to *Anodonta anatina* over both 4- and 7-day periods when filtering *M. griffithii*, and over 7 days when filtering *Selenastrum* sp. (Fig. 4). Sustained CRs did not differ between *U. tumidus* and *A. cygnea*, nor between the two *Anodonta* species (Fig. 4).

Microalgal deposition in the test tanks after 7 days

The area of deposited microalgae on the bottom of the test tanks at the end of the third trial differed between microalgal species (ANOVA $F_{1,18} = 13.165$, $P = 0.002$). The microalgal deposit area was significantly larger for *M. griffithii* (ca. 15–30%) than for *Selenastrum* sp. (ca. 5–15% of tank bottom area) in *Anodonta* mussel tanks, but not in tanks containing *U. tumidus* (Fig. 5).

Nutrients

All measured PO_4-P concentrations on days 0 and 7 were below the lowest detection limit of the used test kit (0.05 mg L^{-1}), hence no statistical tests were performed. There was no statistically significant change during the 7-day experiment in NO_3-N in any of the tanks, with nitrate-nitrogen levels being undetectable in tanks containing *M. griffithii* and

Fig. 3 Boxplots of maximum and 4- and 7-day sustained individual average clearance rates over 3 trials for (**a**) *Monoraphidium griffithii* and (**b**) *Selenastrum* sp. microalgae for *Anodonta anatina* ($n = 3$), *A. cygnea* ($n = 3$) and *Unio tumidus* ($n = 4$). Different letters (a and b) denote statistically significant differences (Fisher's LSD, $P < 0.05$) between the mussel species for data sets with statistical differences between subjects. Median, interquartile range, min/max, and outliers are displayed, with average indicated with ×

Table 5 Results of a repeated measures ANOVA between subject effects (BSE) tests to determine differences, and within subject effects (WSE) tests to determine consistency in size-dependent maximum clearance rate, and sustained clearance rates from day 0 to day 4 and from day 0 to day 7, between microalgal species, mussel species, and their interactions

Statistical test	Clearance rate	Algae			Mussel species			Algae × Mussel species		
		df, Error	F value	P	df, Error	F value	P	df, Error	F value	P
BSE	Maximum	1, 14	0.317	0.582	2, 14	3.040	0.080	2, 14	0.258	0.776
	4 days sustained	1, 14	0.117	0.737	2, 14	4.426	**0.032**	2, 14	0.199	0.822
	7 days sustained	1, 14	0.268	0.268	2, 14	4.847	**0.025**	2, 14	0.351	0.710
WSE	Maximum	1, 14	9.570	**0.008**	2, 14	4.955	**0.024**	2, 14	1.532	0.250
	4 days sustained	2, 28	3.493	**0.044**	4, 28	2.032	0.117	4, 28	0.829	0.518
	7 days sustained	2, 28	8.754	**0.001**	4, 28	3.805	**0.014**	4, 28	1.996	0.126

Statistically significant differences ($P < 0.05$) are bolded

Fig. 4 Boxplots of maximum and 4- and 7-day sustained size-dependent (dry weight) clearance rates over 3 trials for (**a**) *Monoraphidium griffithii* and (**b**) *Selenastrum* sp. microalgae for *Anodonta anatina* ($n = 3$), *A. cygnea* ($n = 3$), and *Unio tumidus* ($n = 4$). Different letters (a and b) denote significant differences (Fisher's LSD, $P < 0.05$) between the mussel species for data sets with statistical differences between subjects. Median, interquartile range, min/ max, and outliers are displayed, with average indicated with ×
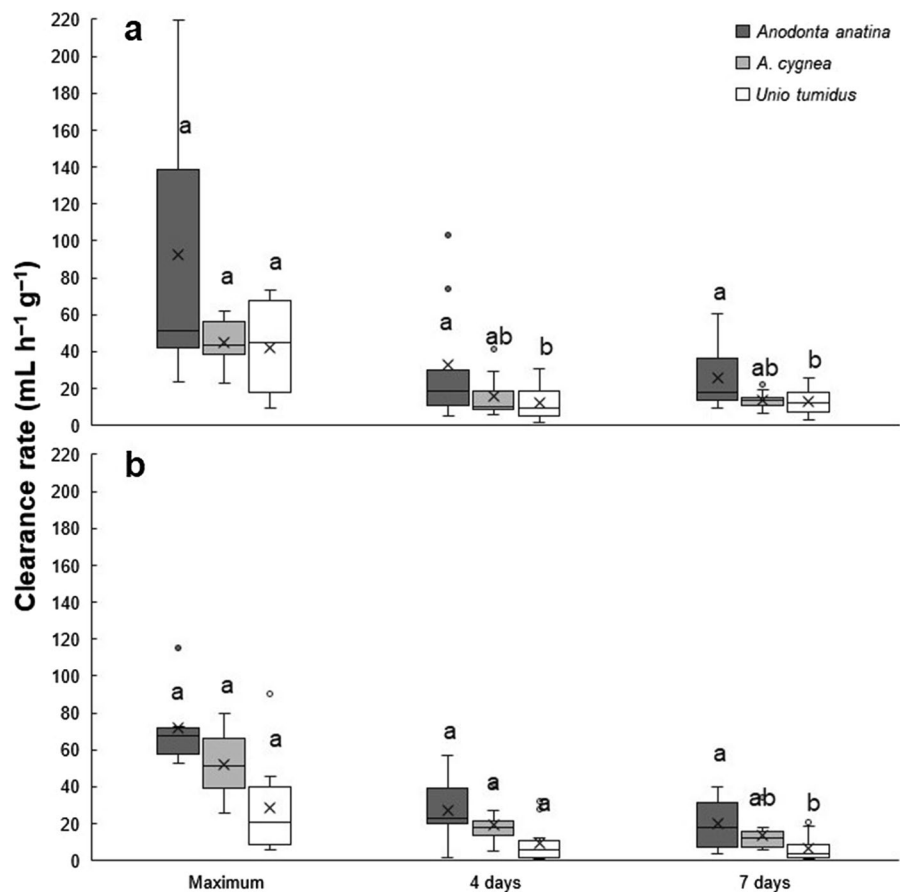
remaining at 1.9 ± 0.2 mg L^{-1} in tanks containing *Selenastrum* sp. Similarly, NH$_4$-N levels did not change in tanks containing *M. griffithii* and *Anodonta* mussels, but the concentration decreased significantly ($P = 0.006$) in tanks containing *M. griffithii* and *U. tumidus*, and in *M. griffithii* control tanks. NH$_4$-N increased significantly ($P < 0.005$) in all *Selenastrum* sp. containing tanks (Table 6).

Fig. 5 Area of mussel produced microalgal deposit as percentage of tank bottom area at the end of the third 7-day trial (mean ± SE). Asterisks denote significant differences (Fisher's LSD, $P < 0.05$) between the two microalgal species (*Monoraphidium griffithii* and *Selenastrum* sp.) within the tested mussel species: *Anodonta anatina* ($n = 3$), and *A. cygnea* ($n = 3$). The difference between microalgal species was not statistically significant (ns) for *Unio tumidus* ($n = 4$)
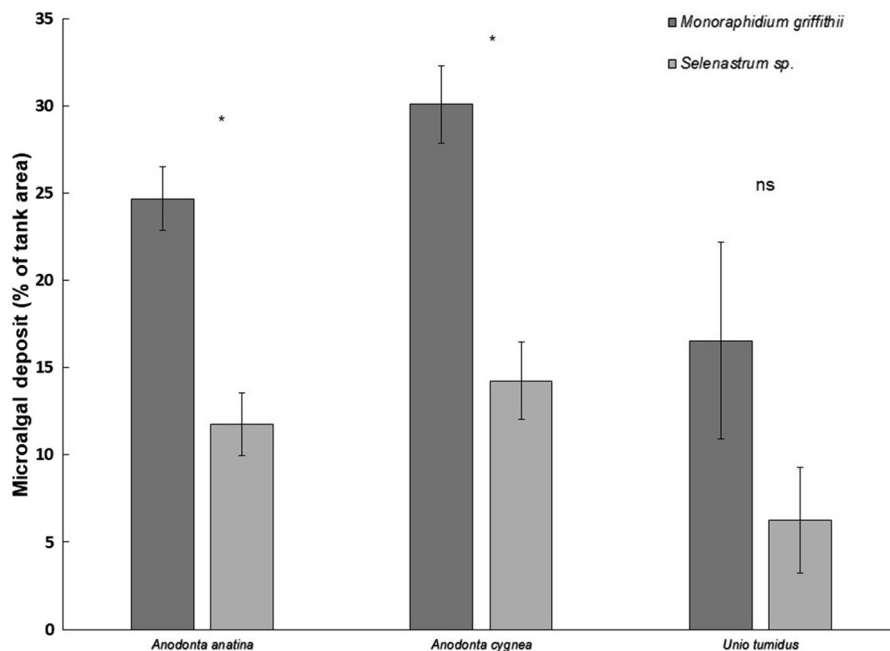

Table 6 NH$_4$-N (mg L^{-1}) on day 7 (mean ± SE) in tanks containing one of the three Unionid mussel species and one microalgal species (*Monoraphidium griffithii* or *Selenastrum* sp.) in the second trial

Treatment	*Monoraphidium griffithii*			*Selenastrum* sp.		
	NH$_4$-N d0 (mg L^{-1})	NH$_4$-N d7 (mg L^{-1})	p	NH$_4$-N d0 (mg L^{-1})	NH$_4$-N (mg L^{-1})	p
Control ($n = 2$)	0.09	0.02 ± 0.00	**0.018**	0.01	0.27 ± 0.00	**0.002**
Anodonta anatina ($n = 3$)	0.09	0.17 ± 0.05	0.258	0.01	1.09 ± 0.17	**0.024**
Anodonta cygnea ($n = 3$)	0.09	0.33 ± 0.16	0.281	0.01	0.59 ± 0.03	**0.003**
Unio tumidus ($n = 4$)	0.09	0.03 ± 0.01	**0.006**	0.01	0.27 ± 0.04	**0.008**

Control tanks were without a mussel. Statistically significant differences (*t* test, $P < 0.05$) from day 0 concentrations are bolded

Discussion

Mussels can be used in biofiltering and bioremediation of waters in different freshwater environments (e.g., Bontes et al., 2007; Gopko et al., 2017; Hajisafarali et al., 2022; van der Meer et al., 2023). The goal of this study was to test the use of green microalgae and unionid mussels native to Europe in bioremediation of recirculation aquaculture system (RAS) wastewater. In this application, the nutrients in the water are taken up by the green microalgae and the microalgae are harvested and deposited by the mussels. The tested mussel species were able to reduce the microalgal concentrations in the water 42–83% from the starting concentration in seven days, depending on the mussel and microalgal species. This suggests that green microalgae and unionid mussels can potentially be used as a multi-trophic option for bioremediation of RAS wastewater.

Microalgal concentrations were reduced fastest in the test tanks with *A. cygnea*. However, by day 4, there were no significant differences in microalgal concentrations between the two *Anodonta* species. When comparing CR of individual mussels, the two *Anodonta* species filtered both microalgae more effectively than *U. tumidus*. *Anodonta* species are less sensitive to disturbances in their environment and have a lesser need for a substrate to burrow in when compared to the mussels of the genus *Unio* (Lorenz et al., 2013), which could contribute to the differences

observed in our experimental setup in clear glass aquaria. The differences in size-dependent clearance rates between mussel species were less distinct, but observable for both microalgae, even though there was marked lack of consistency for the individual mussels to maintain the clearance rates throughout the trials (Suppl. 1).

Size-dependent CR_{max} observed in this study ranged between 30 and 60 mL h^{-1} g^{-1}. Earlier laboratory studies have shown that *A. anatina* and *U. tumidus* can reach size-dependent clearance rates up to 830–920 and 870–990 mL h^{-1} g^{-1}, respectively, when filtering green microalgae *Chlorella vulgaris* Beij. (Kryger & Riisgård, 1988) and 170–620 mL h^{-1} g^{-1} for *A. anatina* and 150–480 mL h^{-1} g^{-1} for *U. tumidus*, when filtering natural seston (Pusch et al., 2001). In a study by Moëzzi et al. (2013), the clearance rate of *A. cygnea* was 120–180 mL h^{-1} g^{-1}. Differences between clearance rates observed in the studies mentioned above can be explained by differences in the test setup, such as suspended solid concentration and type, temperature, the substrate in the tank bottoms, and measurement intervals in relation to test tank volume. The filtration rates reported in previous studies indicate a high potential for water bioremediation application based on unionid mussels.

Microalgal deposits differed between the microalgal species, with larger deposit areas for *M. griffithii* than for *Selenastrum* sp. Since the microalgal concentrations in the tank water between microalgal species differed much less than the microalgal deposit areas, it suggests that deposits containing *M. griffithii* cells are less susceptible to being mixed back into the water from the tank bottoms than those with *Selenastrum* sp. cells. This is an important finding, as the filtered and deposited microalgae should be collected from the tank bottom to recycle the nutrients that the microalgae have removed from the wastewater. Under conditions of high concentrations of edible particles, most of the sedimented deposits are mucus-covered pseudofeces (Nichols et al., 2005; Bontes et al., 2007). However, as noted by Bontes et al. (2007) and van der Meer et al. (2023), a high yield of pseudofeces might also indicate that the available food is unsuitable for mussels. Additionally, it has been shown in previous studies (Donk et al., 1997) that nutrient deficiency thickens the cell wall, which might affect the digestibility of

the microalgae to filter-feeding organisms. N:P ratio in the RAS effluent used in our experiment might not be optimal for microalgae cultivation (Stevčić et al., 2019). Further studies would be required to determine the long-term suitability of the *M. griffithii* diet for freshwater mussels in a multi-trophic biofiltration application.

It was assumed that at least some portion of phosphorus and nitrogen taken up by the microalgae during 4 days of cultivation would return to the water as a byproduct of mussel and microbial metabolism within the tank. After cultivation, the PO$_4$-P concentrations of the algal water in the tanks were depleted below detection limit of the test kit. Concentrations did not increase to detectable levels in the mussel tanks after 7 days, indicating that phosphorus was not released back to water. The increase in ammonium levels in *Selenastrum* sp. tanks, unlike in *M. griffithii* tanks, suggests that *M. griffithii* can utilize NH$_4$-N as a nitrogen source, while *Selenastrum* sp. cannot. This could reduce the need for maintenance in the system using *M. griffithii* and mussels, as part of ammonium will appear as ammonia when pH rises above 7 and due to its toxicity to aquatic fauna, should be kept in low concentrations (Beggel et al., 2017). Also, the increase of microalgal concentration in *M. griffithii* control tanks and the decrease in *Selenastrum* sp. tanks suggest that *M. griffithii* can grow in a system without further nutrient addition, and with moderate temperature and very dim light.

The bioremediation of large effluent volumes from commercial aquaculture facilities would require large quantities of mussels. For commercial use of European freshwater mussels in biofiltering applications, the mussels should hence be cultured artificially. Freshwater mussels spend the first stage of their life as parasitic larvae requiring a host fish, which makes artificial rearing challenging (Wächtler et al., 2001). In vitro culture (Lima et al., 2012; Sicuro, 2015) and captive breeding of freshwater mussels (Geist et al., 2023) has been studied and implemented mostly for the conservation of endangered species. Of the mussel species used in this study, the *Anodonta* mussels would be most suitable for artificial culturing, as they have a large range of suitable hosts, and a relatively short larval period of 9–14 days in in vitro culturing at room temperature. (Wächtler et al., 2001; Taskinen et al., 2011).

In conclusion, it was shown that a co-culture of native freshwater mussels and green microalgae can remove nutrients from RAS wastewater on a laboratory scale. Of the mussel and microalgal species used, the most promising results were gained with a combination of *M. griffithii* microalgae and *Anodonta* mussels. This combination provided the highest measured clearance rates and the largest amount of sedimented pseudofeces, containing microalgae in easily harvestable form, without increasing concentrations of nutrients in the water. However, for the system to be commercially applicable, there would need to be a means of producing large quantities of mussels. Additionally, methods for collecting concentrated microalgae from the bottom of the aquaculture tanks should be developed. With these challenges resolved, green microalgae and mussels can be utilized as a practical means of enhancing circular economy in RAS.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by VJ and ČS. Tables and figures were formulated by VJ. Data analyses were done by VJ and KP. The first draft of the manuscript was written by VJ and ČS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability Data will be provided upon reasonable request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Consent for publication All authors have given their consent for publication.

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