

Removal of nutrients from WWTP effluent by an algae-mussel trophic cascade

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ABSTRACT

New treatment steps are needed to better remove nutrients from WWTP effluents to prevent further eutrophication of the receiving waterbodies. Algae can be used to remove nutrients from effluent, but the subsequent removal of the algae biomass remains a challenge. Employing the high filtration capacity of dreissenid mussels may offer an efficient tool to remove effluent grown algae. The aim of the present study was therefore to evaluate if an algae-mussel trophic cascade allows an efficient removal of both nutrients and effluent-cultured algae. To this end, we inoculated WWTP effluent with the alga *Tetradesmus obliquus* in a batch system and monitored algal growth and nutrient concentrations for 11 days. Subsequently, we fed this algal-effluent solution to *Dreissena bugensis* for 3 days, after which we determined the amount of remaining algae and produced (pseudo)faeces. The algae-mussel set-up was able to remove 99% of P and 99% of N from the initial effluent, and the combination of algae settlement and mussel filtration resulted in a 94% removal of suspended algae after only 24 h. A total of 67% of the algae were consumed by the mussels after 3 days. Most filtered algae were deposited as pseudofaeces. An even higher efficiency may be achieved by selecting algae with a high nutrient removal capacity, but also with a high palatability for the mussels. Nonetheless, this is the first research that shows that an algae-mussel cascade could already offer an efficient solution to reduce nutrient concentrations in WWTP effluent. This may reduce costs, energy consumption and coagulants use at WWTPs, and could offer an alternative for high-tech treatment techniques, which may be more suitable in countries lacking the infrastructure to build these high-tech treatment steps.

1. Introduction

Wastewater treatment plant (WWTP) effluents have large negative impacts on the structure and functioning of aquatic communities in the receiving waterbodies (Burdon et al., 2020; dos Reis Oliveira et al., 2020; Mor et al., 2019; Pereda et al., 2020). A major part of these adverse effects can be ascribed to the high nutrient concentrations in the effluent, causing algae blooms (Heisler et al., 2008) and excess macrophyte growth (Chambers and Prepas, 1994), leading to the accumulation of dead organic matter and subsequent deleterious oxygen depletion (Huang et al., 2017; O'Connor et al., 2019). Additional treatment of the effluent to further remove nutrients before discharge into surface waters will therefore significantly lower the adverse effects on aquatic ecosystems.

To reduce the nutrient concentrations in WWTP effluent several physiochemical treatments are at hand, including dosing with metal

salts to induce P-precipitation (Gutierrez et al., 2010), and the use of absorptive media (Zietzschmann et al., 2014). Yet, these methods require the input of chemical agents or adsorptive materials, which may increase costs (Bunce et al., 2018). Alternatively, the use of algae as an additional treatment step has been garnering increased attention (Wollmann et al., 2019; Y. Shen et al., 2009), since algae have shown to be able to remove nutrients (Mohsenpour et al., 2021) and contaminants (Sutherland and Ralph, 2019) from WWTP effluent rather efficiently. Commonly used algae genera include *Chlorella* and *Scenedesmus*, which can remove considerable amounts of phosphorous and nitrogen from wastewater of different origins. The types of wastewater used for algae cultivation, and removal efficiencies of P & N, include pre-treated wastewater (93–96%) (Choi, 2015) and secondary treated municipal wastewater (100–95%) (Arbib et al., 2013), but also piggy wastewater (83–60%) (Ji et al., 2013). The removal of the produced algal biomass continues to be a challenge, however, requiring high energy inputs and/

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or chemical coagulants (Yin et al., 2020). Applying this biological treatment step employing algae would thus strongly benefit from an environmentally friendly way to remove the produced algae biomass.

Here we hypothesize that the impressive filtration capacity of freshwater dreissenid mussels may be applied to harvest these effluent-grown algae. In aquatic ecosystems, these mussels can remove substantial amounts of algae from the water column (Reeders and Bij de Vaate, 1992; Sousa et al., 2009; Waajen et al., 2016), being able to filter the whole volume of a waterbody in just a matter of weeks (Reeders et al., 1989). Using dreissenids as an additional step in the treatment of wastewater to remove different types of contaminants has already been proposed and applied on an experimental scale. These experiments assessed the effectiveness of dreissenids in removing metals (Magni et al., 2015) and pharmaceuticals (Binelli et al., 2014) from WWTP effluent, to reduce BOD and phosphate concentrations in activated sludge (Mackie and Wright, 1994), and to reduce the amount of suspended solids in industrial effluents (Diggin et al., 2002). Although some of these studies showed that the dreissenids could enhance the removal of a part of the targeted substances, none have coupled these dreissenid treatments to effluent cultured algae. Hence, the potential of dreissenids to remove substantial amounts of algae in a WWTP setting remained largely unexplored. However, there are perspectives to do so, since relatively low concentrations of algae naturally occurring in wastewater effluent can be removed very efficiently by dreissenids (Elliott et al., 2008), and when contaminants are associated to algae, these can be more efficiently assimilated by dreissenids (Gossiaux et al., 1998). The use of dreissenids to remove WWTP cultured algae in a trophic cascade may therefore offer a low cost, energy efficient and environmentally friendly additional wastewater treatment step. Such an algae-mussel cascade would reduce the nutrient loads of effluent discharges, thereby preventing further eutrophication of the receiving water bodies. Therefore, the aim of this study was to assess how well nutrients are removed from WWTP effluent by algae, and how well

mussels in turn can filter these algae from the effluent. To assess these removal efficiencies, we cultured algae on effluent, and subsequently fed these algae to dreissenid mussels. We expected that the algae would remove almost all of the nutrients present in the effluent, and that the dreissenids in turn would remove most of the algae from the water column.

2. Material and methods

2.1. Outline of the study

WWTP effluent was collected and poured into two glass tanks (Fig. 1a). One tank was inoculated with a small volume of an algae culture and illuminated (Fig. 1a1), while as a control, no algae were added to the other tank, which was kept in the dark (Fig. 1a2). The algae were allowed to grow for 11 days, during which algae density and nutrient concentrations were monitored daily. After this algae growth period, aliquots of either the effluent-grown algae or the control effluent were poured into smaller tanks. To half of the algae tanks and half of the effluent tanks, mesh cages containing mussels suspended above a funnel were added, whereas in the other half of the tanks a funnel without mussels was placed, resulting in four treatments: an algae-mussel (AM) treatment (Fig. 1c1), an algae-control (AC) treatment (Fig. 1c2), an effluent-mussel (EM) treatment (Fig. 1c3), and an effluent-control (EC) treatment (Fig. 1c4) (See Fig. S1 for pictures of experimental setup). The mussels were allowed to filter the water for a 3-day period, during which the algae density was measured. At the end of this period, mussel survival was noted and mussels and (pseudo)faeces were collected.

2.2. Methods

2.2.1. Collection of WWTP effluent

Secondary treated effluent was collected from the WWTP of Rhenen

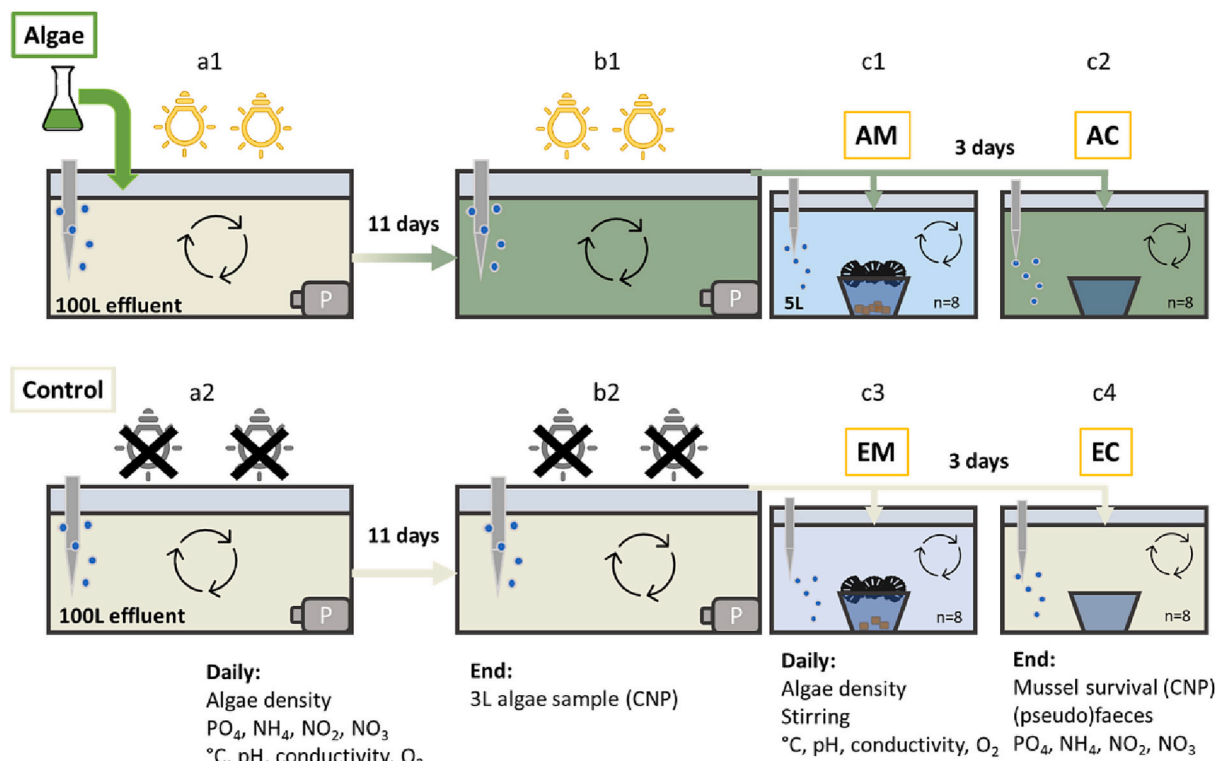


Fig. 1. Schematic overview of the experimental setup. Algae growth on effluent (a1), and control effluent without algae (a2). End of the 11-day algae growth period (b1 and b2). Mussel filtration phase: algae and mussels present (c1 AM), algae present but no mussels (c2 AC), effluent with mussels (c3 EM), and effluent without mussels (c4 EC). P: aquarium pump.

(46,000 population equivalents, Remmerden, The Netherlands (51°58'27.6"N 5°31'55.6"E)), which was a conventional UCT carousel with an additional filtration step (100 µm). A total of 240 L of effluent was collected and transported to a climate-controlled chamber at the University of Amsterdam, where 100 L was directly poured into each of two glass aquarium tanks (Juwel, 110 L, 81x36x45 cm (l*w*h)). The physiochemical characteristics of the initial effluent are shown in Table 1.

2.2.2. Phytoplankton culture

Tetrademus obliquus (formerly known as *Scenedesmus obliquus*) was selected because of its efficacy in removing nutrients from different sources of polluted wastewater water, and as it is one of the most commonly used species in algae cultivation systems (Oliveira et al., 2021). Moreover, *T. obliquus* has often been used in mussel filtration experiments (e.g. (Kraak et al., 1999; Stuijzand et al., 1995, 1998)). *T. obliquus* (CCAP 276/3a) was cultured axenic in 300 mL algae culturing solution (BG11) in a continuously swirled (100 rpm) 1 L Erlenmeyer flask, at a temperature of 20 °C. Dry weight of the algae at the start of the experiment was 407 mg/L $\pm$ 7 SD, with an OD₇₅₀ of 1.2 $\pm$ 0.02 SD.

2.2.3. Algae growth on WWTP effluent

To safeguard the homogeneity of the effluent in the glass aquarium tanks, four aquarium pumps (Eheim 1000 L/h) per tank were placed at the bottom in the four corners. To provide aeration, a glass pipette at each short side of each tank was installed. Because the pumps generated heat, the temperature of the climate-controlled room was set at 16 °C, which resulted in an average effluent temperature of 24.4 °C (SD: 1.7). One tank was used as a control, kept in the dark without algae. The other tank was used to grow the algae, which was therefore illuminated with 4 fluorescent tubes (58 W, 5200 lm) with a light-dark cycle of 16 h:8 h (Fig. S1a-b). To this tank, 240 mL of the *T. obliquus* culture was added, containing 98 mg DW algae, resulting in an initial algae density of 0.97 mg/L. The algae in this tank were allowed to grow for 11 days, since by then both phosphate and nitrogen were depleted from the tank with algae and effluent, and all NH₄ was oxidized into NO₃ in the tank with control effluent. From both tanks, three 15 mL water samples were collected daily. From each sample, 2 mL was used to determine the Optical Density at 750 nm (OD₇₅₀) and the remaining sample was filtered (0.45 µm) and stored at -20 °C until nutrient analysis. Each day, the pH, conductivity, and oxygen concentration were measured using a portable multi-meter (Hach HQ40) with the appropriate probes (PHC101, CDC401, LDO101, respectively). Deionized water was added daily to compensate for evaporation losses.

2.2.4. Collection and acclimatization of quagga mussels

One day prior to the start of the mussel part of the experiment, quagga mussels (*Dreissena bugensis*) were collected from lake Slotersplas (52°21'53.6"N 4°49'02.4"E), using a grab sampler in the middle of the lake. Furthermore, 40 L of Slotersplas water was collected, and mussels and water were transported in 10 L buckets to the laboratory on July

5th, 2021. *Dreissena polymorpha* mussels have previously been used in wastewater polishing experiments. In the present study, the closely related species *D. bugensis* was used, as *D. bugensis* is currently replacing *D. polymorpha*, which may be partly due its more efficient metabolism and thus faster growth and adaptation to lower temperatures (Karatayev et al., 2015).

Dreissenids were assessed for health by checking resistance to opening, and shell length was determined with digital callipers. Based on previous dreissenid filtration experiments, (Kraak et al., 1997, 1999), twenty-four mussels were assigned to each of the eight experimental replicates per mussel treatment. To ensure an equal size distribution over the replicates of the available mussels, three length classes were used: 10 individuals of the 24–26 mm length class, 10 individuals of the 26–28 mm length class, and 4 individuals of the 28–30 mm length class, respectively. Each batch of 24 mussels was placed on a half open cage made of a stainless-steel mesh (mesh size: 5 mm). To ensure a precise calculation of the produced (pseudo)faeces during the experiment, the cages with the mussels were first placed in two large trays with filtered (glass microfiber filters - 1.2 µm) Slotersplas water with constant aeration for 16 h at 16 °C, allowing the mussels to purge their gut. This also allowed the mussels to acclimatize to the experimental conditions, and to attach to the mesh cages before the start of the experiment, further ensuring mussel health. From some of the cages, 1 or 2 mussels moved out of the cage, and one mussel moved from one cage to the other during this time. This resulted in some cages having only 21 to 23 mussels, and one cage containing 25 mussels. However, there was no correlation between the number of mussels in a tank and the mussel filtration capacity (tested for AM, linear model; adj. R² = -0.16, F_{1,6} = 0.04, p = 0.86).

2.2.5. Filtration of algae by mussels

The four treatments were created by pouring 5 L aliquots of the effluent-grown algae (AM and AC, n = 8) into 16 7 L tanks (20x20x20 cm), and 5 L aliquots of the control effluent (EM and EC, n = 8) into 16 other 7 L tanks (Fig. S1c). To collect mussel (pseudo)faeces and/or settled algae, a sealed glass funnel (h = 9.5 cm, diameter 13 cm) was placed in the middle of these tanks. For the AM and EM treatments, a mesh-cage with mussels was placed over this funnel. To keep the algae and effluent in suspension, each tank was vigorously aerated with a glass pipette, taking care to leave any algae and (pseudo)faeces in the funnel undisturbed. To ensure temperature was not limiting for the filtration capacity of the mussels, the temperature was set at 16 °C, which is within their range for optimal filtration (16 °C–26 °C) (Gopalakrishnan and Kashian, 2020). To measure algae density, a 2 mL sample was collected after 24 h, after which all tanks were manually restirred carefully, to resuspend any settled algae outside the funnel, taking care to leave any algae and (pseudo)faeces in the funnel undisturbed. The sampling and manual stirring was repeated after 48 and 72 h. Before and directly after the last manual stirring, an additional 2 mL was used to determine algae density. Furthermore, directly after the last stirring, a 50 mL water sample was collected for the determination of algae/effluent dry weight in the tanks.

Table 1

Physiochemical characteristics of the initial effluent and the effluent after 11 days of algae growth.

Treatment	Temperature °C	pH	O ₂ concentration mg O ₂ *L ⁻¹	Conductivity µS*cm ⁻¹	COD mg O ₂ *L ⁻¹	BOD ₅ mg O ₂ *L ⁻¹	NH ₄ -N mg*L ⁻¹	NO ₂ -N mg*L ⁻¹	NO ₃ -N mg*L ⁻¹	TN mg*L ⁻¹	PO ₄ -P mg*L ⁻¹	TP mg*L ⁻¹
Initial effluent	19.9	6.6	4.9	352	32	4.1	2.76	0.023	0.57	3.8	0.48	0.63
Control 11 days	25.2	6.8	8.7	337	-	-	<0.006	<0.006	3.03 (0.08)	3.03 (0.08)	0.470 (0.009)	-
Algae 11 days	24.4	10.9	10.3	491	-	-	0.010 (0.002)	<0.006	0.045 (0.037)	0.055 (0.040)	0.008 (<0.001)	-

At the end of the 3-day filtration experiment, the cages were collected, mussel survival was noted, and the mussels were separated from the cage. The (pseudo)faeces and settled algae in the funnels were collected and frozen at -20°C until further analysis.

2.2.6. Test species performance and nutrient concentrations

2.2.6.1. Dissolved nutrients and algae density. To determine algae density, the OD_{750} of all water samples was determined from the 2 mL aliquots using a spectrophotometer (Novaspec 3) according to Chioccioli et al. (2014). To determine the dissolved PO_4 , NH_4 , NO_2 and NO_3 concentration of the effluent during the algae growth stage and at the end of the mussel filtration stage, the frozen filtered water samples were thawed and analysed on a continuous flow analyser (SAN++ system, Skalar Analytical B.V., Breda, The Netherlands). NO_2 and NH_4 concentrations below the determination limit ($0.04\ \mu\text{mol}$) were set at $0.02\ \mu\text{mol}$. The 50 mL samples collected at the end of the filtration phase were filtered through pre-weighed filters ($0.2\ \mu\text{m}$), dried at 75°C and reweighed to determine the algae/material DW in the tanks.

2.2.6.2. (Pseudo)faeces production. To determine the (pseudo)faeces production and algae settlement in the funnels, the frozen (pseudo) faeces and settled algae were freeze dried and weighed (Tang et al., 2014). Two replicates from the EM treatment were lost during processing. To assess which part of the settled material in the AM treatment

funnels could be assigned to the mussel filtration and deposition of algae, the average weight of the collected material in each of the EM and AC treatments was subtracted from the weight of the material in the AM treatment.

2.2.7. Data analysis

Filtration rates were calculated from the decrease in optical density for each day of the 3-day filtration experiment according to a formula from Coughlan (1969), which has been adapted to incorporate the effect of algae settlement in the tanks and the daily resuspension of algae:

$$m = \frac{M}{n \times (t - t_{lr})} \times \ln \left(\frac{C_{tAC}}{C_{tAM}} \right)$$

Where m is the filtration rate in $\text{mL} \cdot \text{mussel}^{-1} \cdot \text{hour}^{-1}$, M is the volume of the tank (5000 mL), n is the number of mussels in the tank, t is the time of the measurement and t_{lr} is the time of the last resuspension in hours ($t - t_{lr} = 24\ \text{h}$). C_{tAC} and C_{tAM} are the optical density of the respectively the control tanks without mussels and the tanks with mussels at time t . The ratio between the optical density in the control tanks and mussel tanks allows for a conservative estimate of the filtration rate, as the optical density was likely higher directly after resuspension than in the no mussel controls 24 h after resuspension.

Generalized linear models were used to determine the effect of the algae containing effluent vs control effluent on the optical density, and PO_4 , TN, NH_4 , NO_2 and NO_3 concentrations, where treatment was set as

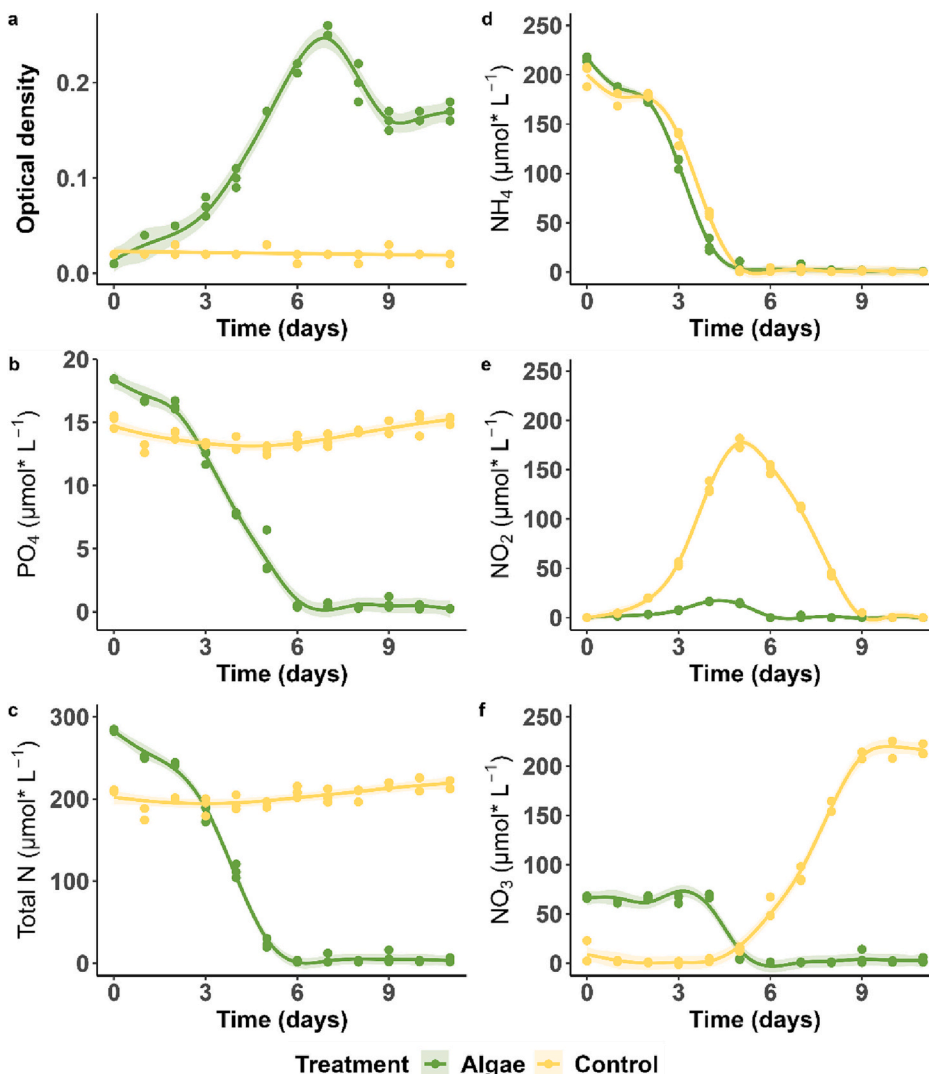


Fig. 2. Optical density and nutrient dynamics during the 11-day algae growth phase. Green lines and dots represent the algae treatment, yellow lines and dots represent the control effluent (pseudo- $n = 3$). Based on daily triplicate measurements, trends and confidence intervals were displayed by deriving thin plate regression splines with a generalized additive model that can handle non-linear data. The optimal smoothing of the data by the thin plate splines was determined using generalized cross validation. a: Optical density at $750\ \text{nm}$ (AU), b: PO_4 concentration ($\mu\text{mol} \cdot \text{L}^{-1}$), c: Total dissolved N concentration ($\mu\text{mol} \cdot \text{L}^{-1}$), d: NH_4 concentration ($\mu\text{mol} \cdot \text{L}^{-1}$), e: NO_2 concentration ($\mu\text{mol} \cdot \text{L}^{-1}$), f: NO_3 concentration ($\mu\text{mol} \cdot \text{L}^{-1}$).

a fixed effect, and the number of hours since inoculation was set as a random effect. Algae growth rate and generation time were calculated using the OD₇₅₀ data until day 7, to increase the fitness of the curve. For visual purposes, in Fig. 2, for each variable, trends and confidence intervals were displayed by deriving thin plate regression splines with a generalized additive model that can handle non-linear data, based on triplicate measurements. The optimal smoothing of the data by the thin plate splines was determined using generalized cross validation. To assess algae filtration by the mussels during the three-day period a generalized mixed linear model was used, to determine the effect of treatment (with start, AC and AM) as a fixed effect on the OD₇₅₀, and the time in hours (0, 24, 48, 72) as well as the container as random effects. Differences between treatments in algae density and settled material at the end of the filtration phase, were tested with a one-way ANOVA. The settled material data was log-transformed to meet normality and variance criteria. If treatment had a significant effect on a variable, a TukeyHSD was performed subsequently.

Data analyses were performed in R 4.2.1, using the packages “lme4”, “lmerTest”, “emmeans” for generalized mixed linear model analyses, the “GrowthCurver” package for algae growth rate and generation time, and the “ggplot2” package for visualization and thin plate splines (geom_smooth function with method = gam).

3. Results

3.1. Algae growth on WWTP effluent

3.1.1. Experimental conditions

During the algae growth period, the oxygen concentration increased from 5 to 9 mg/L O₂ during the first day, after which it remained stable in the effluent control (8–9 mg/L), and slightly further increased in the algae treatment (10–12 mg/L) (Fig. S2). Conductivity remained stable in the effluent control at 344 µS/cm (SD: 6.8), and slightly increased from 437 to 550 µS/cm from day 5 onward in the algae treatment. The pH remained stable in the effluent control between 6.5 and 8, while the pH in the algae treatment increased to 11 during the growth period. Temperature was stable at around 24.5–25.0 °C, after a small increase (to 27.8 °C) in the first 3 days in the control, due to a temporal lack of sufficient ventilation.

3.1.2. Algae growth and nutrient dynamics

During the algae growth stage, the optical density in the algae treatment increased to a maximum of 0.256 (SE:0.003) at day 7, followed by a small drop to 0.158 (SE:0.004) from day 8 to 11 (Fig. 2a). The specific growth rate of the algae was 0.74 (SE:0.06), with a doubling time of 0.9 days. In the control treatment on the other hand, optical density remained constantly low (0.02 SE:0.002). Visual microscopical inspection of the effluent-algae mixture showed that nearly all cells were *T. obliquus*. Both the phosphate concentration as well as the total dissolved nitrogen concentration in the algae treatment were exhausted at day 6 (Fig. 2c,e), which may explain the slight decrease in algae density from day 7 onward. At the end of the 11-day growth period, the concentrations of phosphate and total dissolved nitrogen were 0.25 µM (SE:0.01) and 3.92 µM (SE:1.63), respectively, which meant that the algae had removed 99% of the initial phosphate and also 99% of the total dissolved N from the effluent. The phosphate and total dissolved nitrogen concentration in the effluent control on the other hand remained virtually unchanged at 15.2 µM P (SE: 0.2) and 216 µM N (SE: 3.3), respectively (Fig. 2c,e).

Whereas the ammonium in the effluent disappeared in both treatments (Fig. 2b), in the control effluent, most was oxidized to nitrite, but in the algae treatment, this increase in nitrite was barely present (Fig. 2d). The nitrite in the control effluent was subsequently oxidized to nitrate, giving a textbook example of nitrification (Fig. 2f). It must be noted that both the phosphate and nitrate concentration were higher in the algae treatment at the start of the algae growth phase, which was

likely due to the inoculation of the initial algae suspended in algal culture medium.

3.2. Filtration of algae by mussels

3.2.1. Experimental conditions

During the filtration of the algae by the mussels, the oxygen concentration in all treatments remained stable at 10.1 mg O₂/L (SD:0.1) (Fig. S3). Conductivity was not affected by the mussels and remained stable in both the EC and EM treatment at 346 µS/cm (SD: 4.8). The pH decreased from 8.5–9.4 at the start to 7.3–7.8 in all treatments. The temperature was stable in all treatments at around 16.7 °C (SD: 0.4) (Fig. S3). Mussel survival was high in both the AM (99%) and EM treatment (97%).

3.2.2. Removal of suspended algae

In the two treatments containing algae (AC and AM) the sedimentation of the algae resulted in a lower optical density (AC: 0.059 SE: 0.005; AM: 0.009 SE: 0.005) 24 h after each resuspension (all $p < 0.001$) when compared to the initial optical density (0.157; SE = 0.011). On top of that, the mussels were able to remove significantly more algae from the water column, reflected by the lower optical density 24 h after each resuspension (AC-AM OD₇₅₀ difference of 0.050, $p < 0.001$) (Fig. 3). This indicated that on average 94.3% of the initial algae were removed by mussels and settlement from the water column after each 24-h period in the AM treatment, and only 5.7% of the algae remained in the water column. Furthermore, the average OD₇₅₀ of the AM treatment after 72 h (0.006; SE:0.0006), indicated that even 96.1% of the algae were removed by mussels and settlement, with only 3.9% of the algae remaining in suspension. The 72 h AM OD₇₅₀ was also very close to the average OD₇₅₀ of the EC treatment at that time (0.004; SE:0.0004) (Fig. S1e), also indicating a near complete removal of algae from the water column. The mean filtration rates of the mussels were 14.3 ± 1.3 , 18.5 ± 1.5 and 19.2 ± 2.3 (mean $\pm$ SD) mL*mussel⁻¹*hour⁻¹ after 24, 48 and 72 h respectively.

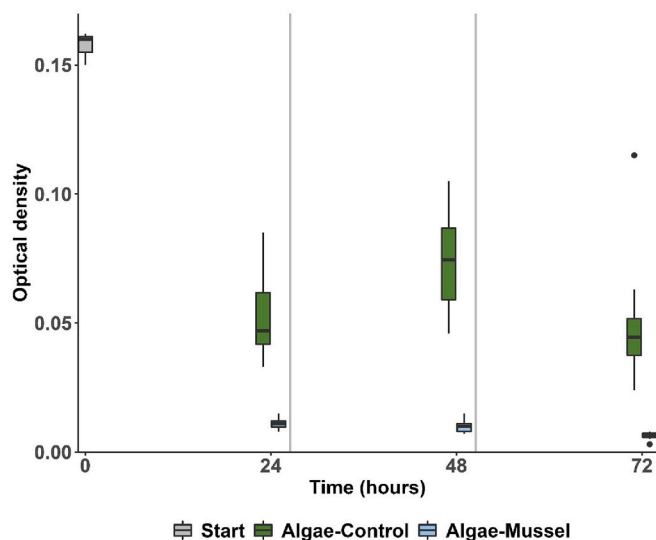


Fig. 3. Boxplots of the optical density during the filtration phase, for the AC treatment (in green; $n = 8$) and the AM treatment (in blue; $n = 8$). The optical density of the algae-effluent suspension at the start of the filtration phase was also included (grey). The optical density was determined at 24, 48 and 72 h after the start of this phase. After the first 24 h, the settled material outside the funnels was manually resuspended (grey lines). Boxes show interquartile range, bold lines represent the median, whiskers indicate the lowest and highest values within a 1.5× interquartile range from the box, dots represent data-points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Comparing the OD₇₅₀ of the four treatments directly after the 72-h resuspension with the starting OD₇₅₀ of the effluent-cultured algae and the control effluent revealed that the OD₇₅₀ was significantly influenced by treatment ($F_{5,32} = 272.6$, $p < 0.001$). The optical density did not differ between the effluent-cultured algae (0.157; SE = 0.004) and the AC treatment (0.156; SE = 0.004) ($p < 0.001$), implying that the algae density remained unchanged in the AC treatment. In contrast, in the AM treatment the optical density was approximately three times lower (0.051; SE = 0.005) than in both the AC treatment and the effluent-cultured algae ($p < 0.001$), which indicated that the mussels removed 67.3% of the total initial algae. The OD₇₅₀ of the control effluent (0.004; SE = 0.002), the EC (0.013; SE = 0.003) and the EM (0.013; SE = 0.003) treatments was significantly lower ($p < 0.001$) than of the treatments containing algae and did not differ among each other. The algae DW in the tanks after the 72 h resuspension showed the same pattern as the OD₇₅₀ values: AM 14.5 mg/L (SE = 1.9), AC 36.2 mg/L (SE = 3.2), EM 3.3 mg/L (SE = 1.2) and EC 1.2 mg/L (SE = 0.3). This resulted in the following correlation between the OD₇₅₀ and the algae dry weight biomass (mg/L): $C_{\text{algae}} = \text{OD}_{750} * 236.3$ ($R^2 = 0.94$).

3.2.3. (pseudo)Faeces production

The weight of the settled material ((pseudo)faeces plus attached algae) in the funnels differed between treatments ($F_{3,26} = 418.5$, $p < 0.001$), and was >3 times higher ($p < 0.001$) in the AM treatment (181.4 mg; SE = 10.4) than in the AC treatment (57.8 mg; SE = 4.4) (Fig. 4b). When subtracting the settled material in the EM (23.3 mg; SE = 1.8) and AC treatments from this amount, the contribution of mussels to the settled algae material (as (pseudo)faeces) in the funnel was 100.5 mg (SD ± 32.1). The settled material in the EC treatment was very low (7.8 mg; SE = 0.4).

The sum of the settled algae in the funnel and the total suspended algae in the AC treatment was 238.5 mg (SE = 16.1), whereas the sum of the settled (pseudo)faeces (minus EM (pseudo)faeces) and the total suspended algae in the AM treatment was 230.5 mg (SE = 16.3). This indicated that almost all of the algae filtered from the water column were deposited as (pseudo)faeces, and assimilation by the mussels was low (Fig. 5).

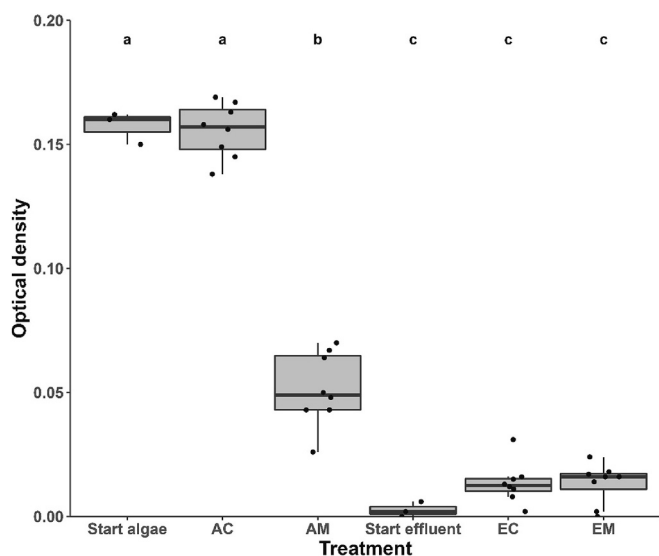


Fig. 4. Optical density of the effluent-cultured algae ($n = 3$), the control effluent ($n = 3$), and the overlying water directly after resuspension at 72 h of the AC, AM, EC and EM treatment ($n = 8$). Boxes show interquartile range, bold lines represent the median, whiskers indicate the lowest and highest values within a $1.5 \times$ interquartile range from the box, dots represent datapoints. Letters indicate significant ($p < 0.05$) differences between treatments.

4. Discussion

In line with our expectations, the algae were able to remove over 99% of the nutrients during the algae growth phase, and the mussels were able to remove 67% of the algae from the effluent. To our knowledge, this is the first example of the filtration of effluent cultured algae by mussels, demonstrating that the algae-mussel trophic cascade was rather efficient.

4.1. Removal of nutrients and algae

With a removal of 99% TN and PO₄ within 11 days, the nutrient removal efficiency of the algae was comparable to previous observations (Mohsenpour et al., 2021; Oliveira et al., 2021). This may be due to the N:P ratio of the effluent being 15:1, which is close to the optimal N:P ratio of 9–13 for *T. obliquus* (Arbib et al., 2013). As the starting nutrient concentrations in our experiment were generally much lower than in those studies (0.6 mg PO₄-P, 4 mg TN vs 1.9–27.9 TP and 20–1280 mg TN), the high removal efficiency in our experiment indicates that *T. obliquus* is very well capable of purifying WWTP-effluent to a high standard. Their effectiveness in removing nutrients from effluent might also be linked to their facilitative mixotrophy (Wu et al., 2014), as at very low nutrient concentrations higher algae densities might sustain due to feeding on bacteria present in the effluent. This may also aid *Scenedesmus* spp. to remain the most prevalent algae during culturing in wastewater effluents (di Termini et al., 2011). The low initial nutrient concentrations in our experiment resulted in an algal biomass in the lower end of the range (36.2 mg/L) when compared to other experiments using different types of wastewater (5–3800 mg/L). Depletion of nutrients rather than slow growth was indeed most likely, as the doubling time of our algae (0.9 days) did not differ from doubling times in other experiments (0.7–3 days) (Çelekli et al., 2008; Gatamaneni Loganathan et al., 2020). Nonetheless, the primary aim of this cascade was to efficiently remove nutrients, which it successfully did, rather than producing a high algal biomass.

The produced algae biomass was efficiently filtered by the mussels and deposited as (pseudo)faeces, and combined with the settlement of the algae in our experimental setup, this resulted in a near complete removal of suspended algae already after 24 h, which is in line with the

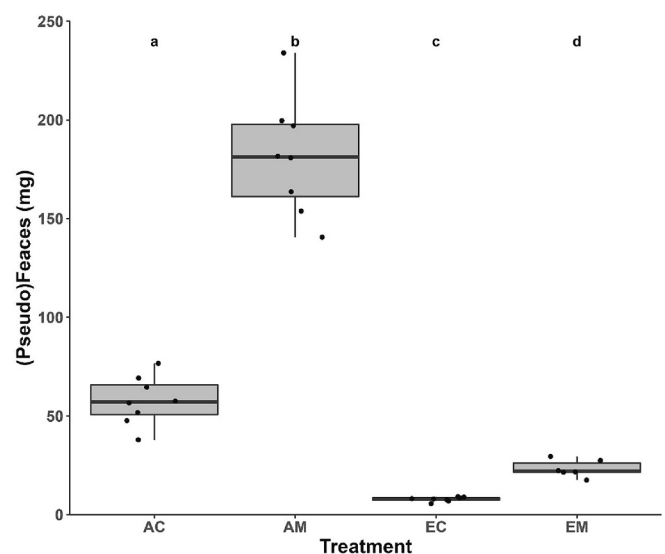


Fig. 5. Material deposited in the funnels (mg DW) after 72 h of mussel filtration for the AC ($n = 8$), AM ($n = 8$), EC ($n = 8$) and EM ($n = 6$) treatment. Boxes show interquartile range, bold lines represent the median, whiskers indicate the lowest and highest values within a $1.5 \times$ interquartile range from the box, dots represent datapoints. Letters indicate significant ($p < 0.05$) differences between treatments.

readily ingestion of *T. obliquus* by *D. polymorpha* (Vanderploeg et al., 2013). The net filtration rates of the mussels were lower in our experiment ($\pm 17 \text{ mL} \cdot \text{mussel}^{-1} \cdot \text{hour}^{-1}$ or $18 \text{ mL} \cdot \text{g}^{-1} \text{ FW} \cdot \text{hour}^{-1}$) compared to other observations (e.a. $50\text{--}110 \text{ mL} \cdot \text{mussel}^{-1} \cdot \text{hour}^{-1}$ (Kraak et al., 1997) or $574 \text{ mL} \cdot \text{g}^{-1} \text{ FW} \cdot \text{hour}^{-1}$ (Elliott et al., 2008)). This was likely due the relatively quick settlement and thus depletion of the algae, as the OD₇₅₀ of the AM treatments 24 h after each resuspension was comparable to the OD₇₅₀ in the EC treatment.

The mussels could have filtered even more algae, since the settlement of 29% of the algae initially present prevented further filtration. Hence, an increased resuspension or continuous water flow might have increased the filtration capacity of the mussels even further. This would have resulted in an even higher filtration percentage at the end of the experiment. Nonetheless, even without increased resuspension, the combination of mussel filtration and algae settlement now already resulted in a 96.1% removal of suspended algae. Furthermore, the effect of discharging the remaining algae-containing effluent would likely be minimal, as the non-treated effluent may cause significantly higher algae blooms in the receiving surface waters.

4.2. Optimizing nutrient removal by algae and assimilation efficiency of mussels

The preferred algae species for dreissenid culture may not necessarily be the best species for the removal of nutrients from WWTP effluent, while some algae species that are most promising regarding the polishing of effluent may not be a suitable food for dreissenids. Thus, there may be a trade-off between the nutrient removal potential and the assimilation efficiency/long term mussel health (Table 2).

In the present study, the filtered algae biomass in the mussel treatment was almost completely deposited as (pseudo)faeces, which might indicate that the algae cells were rejected and not assimilated and incorporated into biomass. This rejection might have been post-ingestion due to the tough cell walls of *T. obliquus* (Tang et al., 2014), which keep the algae cells viable, and the Chl-a intact (Waaen et al., 2016). Although *Scenedesmus* has been used to culture adult *D. polymorpha*, which even reproduced on this diet (Wacker and von Elert, 2003), and *Scenedesmus* are also often used to determine dreissenid filtration rate (Kraak et al., 1997), it might not be the most suitable algae species for long term culturing of *D. bugensis*.

Table 2

Preferred algae species for mussel culture and their WWTP effluent polishing potential. Last row includes *Chlorella vulgaris* for comparison, low culture potential, but often used in WWTP effluent treatment. * Indicates response measured for non-mussel aquatic invertebrates.

Preferred Algae	Measured mussel response	Explanatory variable	Reference	Wastewater treatment potential	Reference
<i>Nannochloropsis limnetica</i>	Increased egg masses	Presence of LC-PUFA, lack of DHA	(Wacker and von Elert, 2003)	Suggested but not tested; marine species used to treat wastewater.	(Freire et al., 2016; Ledda et al., 2015)
<i>Isochrus galbena</i>	Survival and growth	Presence of Saturated FA, PUFA, DHA	(Wacker et al., 2002; Wright et al., 1996)	Yes, but marine species	(Wang et al., 2021)
<i>Thalassiosira</i> sp.	Increased assimilation efficiency, shell growth, body mass	Presence of LC-PUFA	(Stoeckmann and Garton, 2001)	Yes, but marine species	(Wang et al., 2021)
<i>Ankistodesmus falcatus</i>	High assimilation efficiency*, often used in cultures	High in PUFAs, lack of EDH, DHA	(Demott et al., 2010; Kainz et al., 2010)* (Boegehold et al., 2018)	Yes, slightly slower P removal than <i>S. obliquus</i>	(Ansari et al., 2017)
<i>Scenedesmus</i> sp.	Mussel reproduction observed, often used in filtration efficiency tests	Poor in LC-PUFAs, tough cell wall	(Kraak et al., 1997; Tang et al., 2014; Wacker and von Elert, 2003)	Yes, efficient nutrient removal, can act as mixotroph	(Mohsenpour et al., 2021; Wu et al., 2014); this study
<i>Cryptomonas</i> sp. <i>Cryptomonas erosa</i>	Electivity index, reproduction	"Chemical" selection, presence of LC-PUFA	(ten Winkel and Davids, 1982; Wacker and von Elert, 2003)	Can be dominant if nitrification prevented in effluent	(Krustok et al., 2016)
<i>Chlorella minutissima</i> (Freshwater strain)	Increased survival and growth rate, lower growth rate	LC-PUFA, lack of EDH, DHA	(Vanderploeg et al., 1996; Wacker et al., 2002)	Yes	(Malla et al., 2015)
<i>Rhodomonas minuta</i>	Increased survival and growth rate	LC-PUFA	(Vanderploeg et al., 1996)	No information	NA
<i>Chlorella vulgaris</i>	Failed to culture	PUFAs present, lacking LC-PUFA	(Wright et al., 1996)	Often used in WWTP treatment	(Mohsenpour et al., 2021)

The suitability of algae as food for mussels depends on multiple factors; first the mussels need to be able to ingest and assimilate the algae, in which the size and physical and chemical properties of the algae play a significant role (Ward and Shumway, 2004). Tough cell walls or gelatinous layers, for instance, may have a low sensory quality, and limit the ability of the mussels to break down the algae (Tang et al., 2014). Secondly, the nutritional quality of the algae need be sufficient to sustain all life stages of the mussels. A sufficient concentration of long-chained poly unsaturated fatty acids (LC-PUFA's) promotes reproduction (Wacker and von Elert, 2003) and is essential to larval survival (Vanderploeg et al., 1996), which may especially be the case at lower temperatures (Martin-Creuzburg et al., 2012).

The species that seems to be most fitted for culturing mussels, containing LC-PUFAs, including DHA, is *Isochrus galbena*. This species has an optimal growth at salinities between 15 and 35‰ (Fabregas et al., 1985), and thus, is not suitable for municipal WWTP effluent treatment, nor to be fed to mussels together with the marine culture medium. Freshwater algae that may be suitable for culturing mussels are *Rhodomonas* and *Cryptomonas*. Although *Cryptomonas* may be suitable to be cultured on modified effluent (Krustok et al., 2016), little information is available on the nutrient removal rates of these algae when grown directly on effluent. On the other hand, algae often used in WWTP effluent treatment, such as *Chlorella vulgaris*, are not suitable to support mussel cultures due to their low nutritional value (Wright et al., 1996). An algae species that may both be suitable for the long-term culturing of mussels, as well as the removal of nutrients from WWTP effluent is *Nannochloropsis limnetica*, which has sufficient LC-PUFA's to sustain mussel cultures (Wacker and von Elert, 2003). While related marine *Nannochloropsis* species are used for treatment of wastewater (Ledda et al., 2015), the freshwater species *N. limnetica* has potential to remove contaminants from the water (Encarnação et al., 2020), and its potential to treat WWTP effluent has been suggested, but not tested (Freire et al., 2016).

4.3. Perspectives for practical application in WWTP effluents

The present study has shown that mussels can efficiently filter effluent-cultured algae without prior treatment of this effluent-algae mixture. This may allow for a relatively easy implementation of a larger algae-mussel cascade experiment with an increased duration,

comparable to experiments performed by Binelli et al. (2014) and Magni et al. (2015) at WWTPs that already used algae as a post-treatment step to remove nutrients. Experiments to determine the best performing (combination of) algae species for the long-term nutrient removal and mussel health and filtration efficiency may thus not necessarily be restricted to the laboratory, but may also be performed on-site. However, with long-term application of mussels and algae as an effluent treatment step, the plethora of contaminants in the WWTP effluent may eventually affect the health and performance of both algae and mussels. For example, a 112-day exposure to WWTP effluent had a negative effect on body weight and an endocrine disrupting effect on *D. polymorpha*, but mortality and shell gaping were negligible (Quinn et al., 2004). Nonetheless, these results could also be interpreted positively, as they show long term mussel survival in pure effluent. Moreover, the negative effects of the effluent may also be partly explained by other stressors than contaminants, including a lack of edible food particles in the effluent. Furthermore, both algae (Hejna et al., 2022; Sutherland and Ralph, 2019) and mussels (Gomes et al., 2018) themselves can remove and biodegrade part of the contaminants present in the effluent. Year-round algae filtration by dreissenid mussels would preferably occur with a limited input of heating energy. For WWTPs outside a Mediterranean or tropical climate, this would imply that the dreissenid mussels may experience low temperatures during a part of the year. Although optimum filtration rates may be in the 16–26 °C range (Gopalakrishnan and Kashian, 2020), dreissenid filtration may not be severely impacted by temperatures as low as 8 °C (Reeders and Bij De Vaate, 1990) and *D. bugensis* filtration rates at 1–7 °C are comparable to those at higher temperatures (Vanderploeg et al., 2010). Furthermore, *D. bugensis* can survive and grow at temperatures as low as 6 °C in their natural environment (Karatayev et al., 2011; Karatayev and Burlakova, 2022). Nonetheless, future experiments should also study the effects of season and temperature on survival, growth, and filtration rates of the dreissenids.

The (pseudo)faeces produced by the algae-mussel cascade may be added to the excess sludge for further treatment. Alternatively, it could be used to extend the cascade, by using this material as a food source for chironomid larvae, which can feed on dreissenid faeces (Izvekova and Lvova-Katchanova, 1972), and can also grow on activated sludge from a WWTP (van der Meer et al., 2022). This extension of the trophic cascade may facilitate further degradation or biomagnification of organic contaminants, which would allow for an easier removal of these contaminants from the effluent. As contaminants also pose a risk for receiving water bodies (Burdon et al., 2020), the algae-mussel cascade might also be able to prevent further deterioration of these water bodies, as it may reduce effluent contaminant concentrations.

As the algae-mussel cascade requires relatively simple techniques, and mussel and algae species occur globally, this setup adds to the arsenal of low-cost and easy to maintain treatments units, such as upflow anaerobic sludge blankets and downflow hanging sponge-cubes (Machdar et al., 1997). Such low-cost wastewater treatment techniques may be especially beneficial for countries that lack the infrastructure to use other treatment steps, as 48% of the globally produced wastewater is still released into the environment untreated (Jones et al., 2021). Hence, further experiments with different algae species, setups and continuous cultures may lead to an increased understanding of nutrient-algae-mussel dynamics, paving the way for practical application of this trophic cascade in WWTPs. Nonetheless, our study showed that the current algae-mussel cascade was already quite efficient in nutrient and algae removal.

5. Conclusions

To our knowledge, the present study is the first to show that an algae-mussel cascade may offer a way to efficiently remove nutrients and algae from secondary treated effluent without the use of coagulants or adsorptive media. Harnessing the traits of invertebrates and their biotic

interactions may thus provide environmentally friendly ways to mitigate a part of the anthropogenic impact on aquatic ecosystems.

CRedit authorship contribution statement

Tom V. van der Meer: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Charlie J.E. Davey:** Methodology, Investigation, Writing – review & editing. **Piet F.M. Verdonchot:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Michiel H.S. Kraak:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

Tom V. van der Meer reports financial support was provided by Water Authority of the Stichtse Rijnlanden. Tom V. van der Meer reports financial support was provided by Water Board Hollands Noorderkwartier. Tom V. van der Meer reports financial support was provided by Waterschap Rivierenland.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecoleng.2023.106930>.

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