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Impact on Wastewater Quality of Biopellets Composed of *Chlorella vulgaris* and *Aspergillus niger* and Lipid Content in the Harvested Biomass

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Abstract

Filamentous fungi can be used to form easily harvested pellets with microalgae (fungal-assisted algal harvesting) in order to advance the sustainability and economic feasibility of wastewater treatment using microalgae. In experiments employing the microalga *Chlorella vulgaris* and using the filamentous fungus *Aspergillus niger* for harvesting, this study investigated the effect on water quality and the quantity and quality of lipids in the biomass produced. Major reductions in the concentrations of total nitrogen, ammonium-nitrogen and total phosphorus were observed after addition of the fungal spores (day 5) and during fungal growth and entrapment of the algal cells. At harvest (day 8), the decrease in total nitrogen was 47.4% ± 18.4% of the initial value, corresponding to a reduction of 41.9 ± 17.1 mg·nitrogen·L^{-1}. For total phosphorus, the decrease was 94.4% ± 3.2%, corresponding to a reduction of 6.4 ± 0.2 mg·phosphorus·L^{-1}. A significant decrease in concentration of the micropollutant diclofenac was observed at harvest, to 5.1 ± 4.0 μg·L^{-1} compared with an initial concentration of 9.5 ± 0.6 μg·L^{-1}. A significant decrease in total lipids in the biomass was observed after fungal-assisted algal harvesting, from 58.7 ± 2.7 μg·mg^{-1} at day 5 (algal biomass only) to 34.2 ± 2.7 μg·mg^{-1} at day 8 (fungal-algal biomass). However, because of high biomass production, the amount of lipids produced per litre of wastewater increased from 5.6 ± 0.9 mg on day 5 to 20.6 ± 4.9 mg on day 8.

Keywords

Aspergillus niger, Bioremediation, *Chlorella vulgaris*, Diclofenac, Water Quality

1. Introduction

Waste streams such as municipal wastewater are currently being produced at increasing rates and quantities worldwide, due to rapid human population growth. Consequently, there has been a rise in the need for novel sustainable technologies for municipal wastewater treatment. Such technologies should be characterised by low energy demand and simultaneously allow for recirculation of resources, such as nutrients for crop production. Algae-based technologies such as high-rate algal ponds offer one possibility to achieve sustainability within the wastewater treatment sector, since these systems are well-known for removal of inorganic nutrients from wastewater [1]. Another positive aspect of using microalgae for wastewater treatment is that the biomass obtained after treatment can be used within the emerging biofuel sector, with the quantity and quality of lipids produced being key factors [2].

However, the incentive for using microalgae for wastewater treatment and associated biofuel production is hampered by the high costs associated with the algal harvesting techniques currently in use, commonly filtration, chemical flocculation and centrifugation. These techniques are normally very energy-demanding, constituting 20% - 30% of the total costs for algal biomass production [3]. Research in the past few years has indicated that filamentous fungi may form pellets with microalgae (biopellets). During the pelletisation process, the microalgal cells become entrapped inside the fungal pellets and can then be removed from the wastewater by coarse filtration. Thus, the fungal-assisted algal harvesting technique may lessen the costs associated with production of microalgal biomass in wastewater and associated biofuel production [4] [5].

In the past decade, several studies have found that conventional wastewater treatment processes do not remove organic pollutants such as pesticides and pharmaceuticals to satisfactory levels [6]. Unsurprisingly, these substances are now regularly found in aquatic ecosystems and groundwater [7] [8]. Efficient but costly methods currently used for removal of organic pollutants in wastewater are ozonation and active carbon filtration [6]. In some situations, wastewater treatment techniques based on biological processes, e.g. bioremediation, could be a potential low-cost alternative. Microorganisms such as microalgae and fungi can achieve reductions in organic pollutant concentrations in water via initial and rapid biosorption onto their biomass and through biodegradation of the pollutant [9]. In fact, both microalgae and fungi have been demonstrated separately to remove organic pollutants from water [10] [11] [12].

Use of filamentous fungi for microalgae harvesting, through the formation of biopellets, is now an intensely researched area [5], due to its potential for development into a sustainable wastewater treatment technology. The aim of the present study was to increase knowledge of fungal-assisted algal harvesting from wastewater and to investigate its effect in reducing the concentrations in water of nutrients and the micropollutant diclofenac, which is included in the EU watch-

list of priority substances [13]. Also biomass production and the quantity and quality of lipids accumulated in the biomass were evaluated.

2. Material and Methods

2.1. Microorganisms

The microalgal species *Chlorella vulgaris* strain 211/11B was obtained from CCAP-SAMS (Culture Collection of Algae and Protozoa, Scottish Association for Marine Science), Scotland, and the filamentous fungus *Aspergillus niger* ATCC® 16888™ was obtained from the American Type Culture Collection, USA, for use in the study.

2.2. Experimental Design

Four treatments were included in the study (**Table 1**). The process used for fungal-assisted algal harvesting and the set-up for the sterile control treatments are described below. The pharmaceutical diclofenac (diclofenac sodium salt, Sigma D6899) was added from a methanol-based stock solution at the start of the experiment. All replicates were stirred (100 rpm) and sampled 5 minutes after addition, to determine initial concentrations of biomass, pH, nutrients and diclofenac.

In the biological treatment, the algal culture was started by inoculating synthetic wastewater [14] with 20% (v/v) of a *C. vulgaris* culture taken from a 5-day-old algal culture grown in BG-11 [4]. To prevent the algal cells from settling and to allow gas exchange, the culture was stirred at a speed of 100 rpm. The culture was grown for 5 days at 24˚C and illumination of 30 μmol/m^2 s (PAR) with a photoperiod of 16 h. Sterile control treatments consisting of 20% BG-11 and 80% synthetic wastewater were kept under the same conditions. After 5 days, the number of algal cells was determined by counting in a Bürkner chamber and spores of *A. niger* were added to obtain an algal cell:fungal spore ratio of 50:1. For spore production, *A. niger* was cultivated on Petri plates of potato dextrose agar at room temperature for 10 days. The spores were harvested by applying 2 × 10 mL of sterile distilled water directly onto the agar plate. The spore solution was filtered through a nylon filter (mesh size 100 μm) to remove mycelial fragments and the spore concentration was determined in a Bürkner chamber. After spore addition, the pH in each replicate was lowered to 4.0 with hydrochloric acid and glucose was added to reach a concentration of 3 g·L^{-1}. The controls were treated in the same way, but sterile distilled water was added instead of the spore solution.

The treatments were placed on a horizontal shaker (100 rpm) at room temperature without additional light for 3 days (days 5 - 8) to allow formation of fungal pellets and thus entrapment of algal cells. On day 8, no algal cells were observed in the water phase when studied under a microscope, so the treatments were removed from the shaker and the fungal-algal pellets produced were collected by filtration through a nylon filter (mesh size 100 μm).

2.3. Analysis

2.3.1. Biomass Production

At the beginning of the experiment (day 0), at day 5 of algal growth (before addition of the fungal spores) and at the end of the experiment (day 8), dry weight biomass in all treatments was determined. At day 0 and day 5, biomass was determined by sampling 50 mL from each replicate and centrifuging it at 3000 g (MegaStar 600, VWR) for 10 min, after which the pellets obtained were washed once with an equal volume of distilled water and lyophilised. At day 8, the fungal-algal pellets produced were removed by coarse filtration as described above, washed once with an equal volume of distilled water and lyophilised.

2.3.2. Diclofenac Analysis

Samples for analysis of diclofenac concentration were taken in parallel with biomass determination on day 0, day 5 and day 8. These water samples were stored at −20˚C before analysis, which was performed within two weeks. For analysis, the samples were filtered using a regenerated cellulose syringe filter (0.22 mm pores) and 1 mL of the filtered extract was placed in an autosampler vial with 10 ng of the internal standard (Diclofenac-13C6). The samples were analysed using a DIONEX UltiMate 3000 ultra-performance liquid chromatography (UPLC) system coupled to a TSQ QUANTIVA triple quadrupole mass spectrometer (MS/MS) system (both Thermo Scientific, Waltham, MA, USA). An Acquity UPLC BEH-C18 column (100 mm × 2.1 i.d., 1.7 µm particle size, Waters Corporation, Manchester, UK) was used as an analytical column. Data were evaluated using TraceFinder™ 3.3 software (Thermo Fisher). Detailed information about the analytical method can be found in Gago-Ferrero *et al.* [15].

2.3.3. Nutrient Analysis

To determine the effect of the treatment on total nitrogen (TN), ammonium-nitrogen (NH_4^+-N) and total phosphorus (TP), water samples were taken on day 0, day 3, day 4, day 5 and day 8. Biomass was removed as described above and concentration of TN was determined with Hach Lange LCK 338 (ISO 11905-1), concentration of NH_4^+-N with Hach Lange LCK 303 (ISO 7150-1) and concentration of TP with Hach Lange LCK 350 and LCK 349 (ISO 6878).

2.3.4. Fatty Acid Methyl Ester (FAME) Content Analysis

The algal biomass collected on day 5 and the fungal-algal pellets collected on day 8 were used to determine the quantity and quality of lipids in the biomass. For this analysis, the lyophilised biomass was treated with methanolic H_2SO_4 (2% v/v) for 60 minutes at 90˚C. Fatty acid methyl esters (FAME) were then extracted and analysed as described in Hultberg *et al.* [16]. For quantification, nonadecanoic acid (19:0) was added before esterification as an internal standard.

2.3.5. Statistics

The experiment was set up with three replicates in each treatment and the data

obtained were analysed statistically using Minitab 18 for Windows. All data were analysed using analysis of variance (ANOVA) and Fisher's LSD with $P < 0.05$ considered significant.

3. Results

3.1. Biomass Production

For the algal biomass was harvested on day 5, a fivefold increase in the amount of biomass compared with the initial value on day 0 was observed in both the treatment exposed to diclofenac and the unexposed treatment. On day 8, similar biomass production was again observed in both treatments, with a sixfold increase compared with the amount on day 5. As expected, the pH increased during algal growth, to a value of 9.4 - 9.5 at day 5 in the algal treatments. At harvest (day 8), the pH in the algal-fungal treatments had decreased from the set value of 4.0 to 2.4 - 2.5. No biomass production or changes in the set pH were observed in the sterile control treatments during the experiment (Table 1).

3.2. Reduction in Nutrient and Diclofenac Concentrations

Major reductions in TN, NH_4^+-N and TP concentrations were observed between day 5 and day 8 in the biological treatments (Figure 1). On day 5, the decrease in TN was 9.3% ± 3.1% of the initial value and on day 8 it was 47.4% ± 18.4% of the initial value. The latter corresponded to removal of 41.9 ± 17.1 mg $N \cdot L^{-1}$ since the start. For NH_4^+-N, an initial increase in concentration was observed in both the biological treatment and the control, probably due to biotic and abiotic degradation of urea present in the synthetic wastewater medium 14. A steep decrease in NH_4^+-N concentration was observed after fungus addition on day 5 and a final reduction of 84.4% ± 2.9% compared with the initial value was recorded on day 8. For TP, a very steep decrease was observed between day 5 and day 8, resulting in a decrease of 94.4% ± 3.2% at harvest compared with the initial value, corresponding to a reduction of 6.4 ± 0.2 mg $\cdot P \cdot L^{-1}$.

The fungal-algal treatment also had an effect on the concentration of diclofenac (Figure 2). Compared with the initial diclofenac concentration (9.5 ±

Table 1. Dry weight biomass and pH in the different treatments during the experiment. Mean ± standard deviation is shown.

Treatment	Biomass (mg·L^{-1}, dwt)			pH		
	Day 0	Day 5	Day 8	Day 0	Day 5	Day 8
Algal-fungal+[1]	18.7 ± 3.1	95.3 ± 12.1	610.7 ± 191.3	7.8 ± 0.04	9.5 ± 0.1	2.5 ± 0.2
Algal-fungal-[2]	18.0 ± 4.0	99.3 ± 15.1	649 ± 207.0	7.8 ± 0.1	9.4 ± 0.3	2.4 ± 0.2
Control+	0	0	0	7.4 ± 0.01	7.4 ± 0.1	4.2 ± 0.1
Control-	0	0	0	7.4 ± 0.01	7.4 ± 0.2	4.3 ± 0.2

[1]The micropollutant diclofenac was included in the experiment. [2]The micropollutant diclofenac was not included in the experiment.

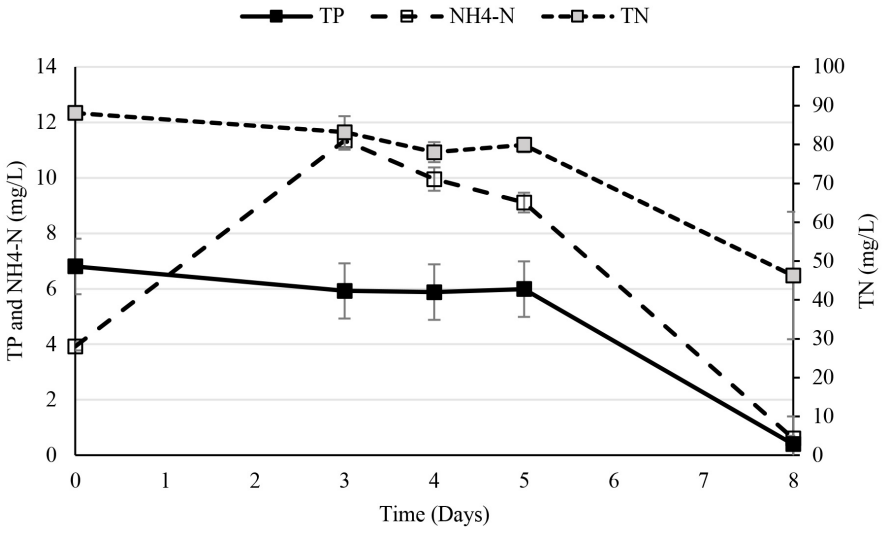

Figure 1. Mean concentration of remaining total nitrogen (TN), ammonium-nitrogen (NH^{4+}-N) and total phosphorus (TP) in synthetic wastewater over time. Bars show standard deviation.

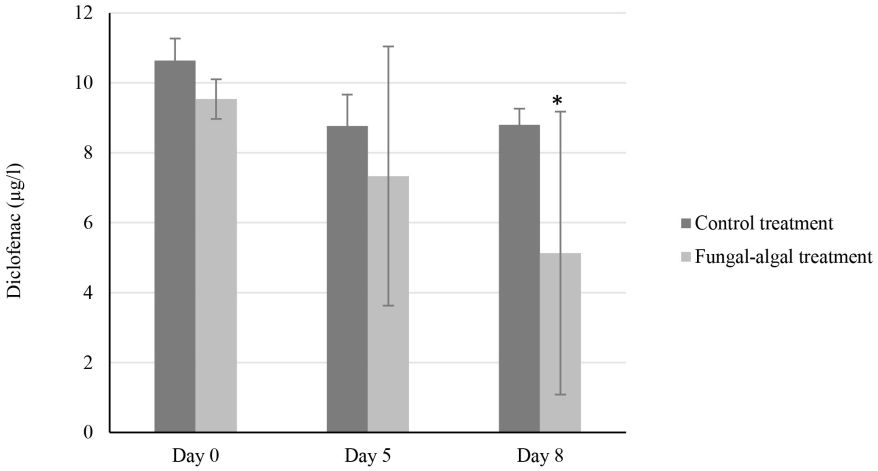

Figure 2. Mean concentration of diclofenac in the control treatment and in the algal-fungal treatment on day 0, day 5 and day 8. Bars show standard deviation, *indicates a significant difference compared with initial concentration ($P < 0.05$, Fisher's LSD).

0.6 µg·L^{-1}), a significant decrease was observed on day 8 (5.1 ± 4.0 µg·L^{-1}) in the biological treatment. In the control treatment, no significant change in the diclofenac concentration was observed.

3.3. Fatty Acid Methyl Ester Analysis

Total lipid content, measured as sum of FAMEs, was significantly lower in the biomass harvested on day 8 than in the biomass harvested on day 5. This decrease, of approximately 40%, was consistent for both the treatment exposed to diclofenac and the unexposed treatment (Table 2). No significant effect on total lipid content due to exposure to diclofenac was observed.

Table 2. Total lipid content (μg·mg^{-1}, dwt) and relative proportions (% of total amount of fatty acids) of the major fatty acids in the biomass sampled on day 5 (A, algal biomass) and day 8 (B, algal-fungal pellets) in treatments exposed to diclofenac (+) or unexposed (−).

Lipid Content	A (−)	A (+)	B (−)	B (+)
	58.7 ± 2.7a*	58.5 ± 7.1a	34.2 ± 2.7b	33.5 ± 2.0b
Fatty Acid				
14:0	0.1a	0.2a	0.5a	0.3a
15:0	0.6a	1.5b	0.7a	0.7a
16:0	21.1a	20.7a	18.9a	18.5a
16:1	1.9a	1.7a	0.7b	0.4b
16:2	7.4a	6.9a	0.8b	0.7b
16:3	14.2a	14.8a	0.9b	0.7b
17:0	0.2a	1.1a	5.0b	3.6b
18:0	0.9a	0.4a	9.0b	7.8b
18:1	10.7a	4.7a	3.7a	16.8a
18:2	13.0a	12.8a	29.2b	19.9ab
18:3	29.9a	35.2a	30.5a	30.5a
ΣSFA	22.9a	23.9a	34.1b	30.9b
ΣUFA	77.1a	76.1a	65.8b	69.0b

*Values within rows followed by different letters are significantly different ($P < 0.05$, Fisher's LSD).

Comparison of the fatty acid profile in the biomass harvested on day 5 and the biomass harvested on day 8 revealed a significant difference (Table 2). The most pronounced change was an increase in the proportion of the saturated octadecanoic acid (18:0) and a decrease in unsaturated hexadecatrienoic acid (16:3) and hexadecadienoic acid (16:2) on day 8. Lower amounts of the unsaturated linoleic acid (18:2) were observed on day 5 compared with day 8. However, in total a significant increase in sum of saturated fatty acids was observed in the biomass harvested on day 8 compared with the biomass harvested on day 5 (Table 2).

Low amounts of the odd-chain fatty acids 15:0 and 17:0, indicating bacterial contamination, were detected, with higher levels of 17:0 in the biomass harvested on day 8. Also, low amounts, approximately 0.1% of total lipid content, of the long-chain fatty acids 20:0 and 22:0 were detected in the biomass harvested on day 8.

4. Discussion

The use of microalgae for treatment of wastewater, followed by a deliberate strategy to use the biomass produced, provides substantial benefits in terms of sustainability, but the cost of microalgae harvesting is a major issue hampering development of this technique. Due to the formation of large, easily harvested

algal pellets, bioflocculation by various methods has been suggested as an efficient low-cost technology for this purpose [17]. In the present study, flocculation was obtained by formation of fungal-algal pellets. Complete removal of algal cells from the water phase was observed after three days of fungal growth and high efficiency in algal harvesting was achieved, which is well in line with previous findings on the use of *A. niger* for this purpose [4]. A recent study suggests that specific surface proteins in the hyphae of fungi are important for achieving algal pelleting [18].

The pelleting process used in the present study was based on fungal spore addition, accompanied by addition of glucose as a carbon source. This approach resulted in a strong increase in biomass production between day 5 and day 8, as can be seen in **Table 1**. In parallel with the increase in biomass production, a major increase in nutrient removal from wastewater was also observed (**Figure 1**). A particularly notable finding was a steep decrease in the concentration of total phosphorus. In general, the rate of removal of phosphorus from wastewater by algal treatment is reported to be high, commonly between 60% - 100% [19]. This removal effect is attributable to assimilation into the biomass and precipitation of phosphorus. The main factors inducing phosphorus precipitation are increased pH due to algal growth, assimilation of dissolved carbon dioxide during algal photosynthesis and the concentration of calcium and magnesium ions [19].

However, in the present study the main reduction in phosphorus concentrations was observed under the acidic conditions that prevailed between day 5 and day 8. *Aspergillus niger* is well-known for production of citric acid [20], which explains this decrease in pH during its growth (**Table 1**). As discussed above, phosphorus precipitation is more likely to occur at alkaline pH, so cellular uptake is a possible explanation for the observed decrease. In a recent study on carbon:nitrogen:phosphorus ratio in fungal biomass, variations were observed between different phyla, but on an average the stoichiometry was similar to the Redfield value, with an atomic nitrogen:phosphorus ratio of 16:1 [21]. This value is close to the 14.2:1 atomic ratio of reduced nitrogen:reduced phosphorus observed in the present study, further supporting cellular uptake as the explanation for the steep decrease in phosphorus concentration in the wastewater.

The concentration of diclofenac used here (10 $\mu g \cdot L^{-1}$) appeared to have no effect on algal or fungal growth. This confirms the low toxicity reported for non-steroidal anti-inflammatory drugs, including diclofenac, in algal tests [22]. Diclofenac is used for a broad range of medical treatments and is thus frequently detected in wastewater. It is also poorly eliminated in wastewater treatment plants, with concentrations of almost 1 $\mu g \cdot L^{-1}$ being reported in treated wastewater [23]. Advanced oxidation techniques, such as sonolysis, anodic oxidation and electro-Fenton treatment, are currently being developed for removal of diclofenac from wastewater and promising results have been reported [24]. In the present study, no significant effect was observed on diclofenac concentration after five days of algal growth (**Figure 2**). Reductions in diclofenac concentrations by algal treatment systems have been observed elsewhere, but with a considera-

bly longer treatment period than applied in this study [25]. After fungal-assisted algal harvesting (day 8), a significant decrease in diclofenac concentration was observed in the present study. This is in agreement with results reported by Lucas *et al.* [26], who tested a similar concentration range of the pollutant and observed high sorption of diclofenac to fungal biomass due to its hydrophobic character.

Biosorption is of interest in removal of micropollutants from wastewater due to the potential for removal of biomass and thereby of adsorbed pollutants. Certain fungal species are also of high interest for bioremediation due to their production of extracellular enzymes, such as laccases, capable of degrading recalcitrant xenobiotics [27]. Laccase-mediated degradation of diclofenac, using laccase from the white-rot fungi *Trametes versicolor*, has been demonstrated [28]. Decreased toxicity was also observed after treatment in that study, which is important as biodegradation processes are required not only to remove the target compound, but also its potentially toxic metabolites. *Aspergillus niger* is suggested to be a good source of laccase [29]. However, previous evaluations of the strain of *A. niger* used in the present study for laccase production, under similar conditions as applied in the present study, have detected no extracellular production of laccase [30]. Further removal of diclofenac could possibly be obtained developing laccase-producing fungal strains for algal harvesting. It should also be pointed out that wild strains of *A. niger* have recently been categorised as class 2 microorganisms [31], making development of algal harvesting techniques using other fungal strains highly relevant.

Lipid accumulation, in both microalgae and fungi, is dependent on the growing conditions, with stress conditions being favourable for lipid accumulation by microalgae [32] and with the quantity and quality of carbon and nitrogen sources suggested to be important factors for *A. niger* [33]. The lipid content of the microalgal biomass harvested on day 5 was 5.9% of dry biomass and that of the algal-fungal biomass harvested on day 8 was 3.4% of dry biomass (**Table 2**). These values are low compared with reported values from a similar experimental set-up [4], where a lipid concentration of 37.4% was detected in the microalgal biomass. For the algal biomass harvested on day 5, both the quantity and quality of lipids produced are well in line with results obtained using the same strain of *C. vulgaris* for treatment of real wastewater [16]. This suggests that nutrient availability in the synthetic wastewater used in the present study resembles that in real wastewater and also that it may be less conducive to lipid production. However, inter-strain variation may also explain the difference in total lipid concentration.

In the present study, fungal-assisted algal harvesting resulted in a significant decrease in total lipid content in biomass and a significant change in lipid quality towards saturation. However, because of high biomass production, the amount of lipids produced per litre of wastewater increased, from 5.6 ± 0.9 mg on day 5 to 20.6 ± 4.9 mg on day 8, when calculated based on the data presented in **Table 1** and **Table 2**. In a study using the related fungi *A. fumigatus* for har-

vesting different microalgae, increased total lipid yield was reported for certain treatments, with the total lipid yield varying between 17.8 and 246.0 $mg \cdot L^{-1}$ [34]. Furthermore, a fatty acid chain length of between 16 and 18 C and a low concentration of unsaturated fatty acids are reported to promote biodiesel production [35]. Thus, the significant increase in saturated fatty acids obtained after fungal growth in this study represents an advantage for biofuel production.

Prolonging the algal cultivation period, thereby causing possible exposure to nutrient deficiency, might have resulted in increased amounts of lipids in the algal cells [36]. However, the batch reaction time represents a considerable cost and prolonged cultivation needs to be balanced against the value of the biomass. Considering the reaction time for pelleting microalgae, the use of spores, as in the present study, should be weighed against the use of pre-grown fungal pellets. It is suggested that direct use of pre-grown fungal pellets is favourable from a resource perspective [37]. Moreover, developing a harvesting process using pre-grown fungal pellets would allow optimisation of the growing conditions in order to achieve maximal lipid content in the mycelium and this would provide a benefit from a biofuel perspective. On the other hand, the use of pre-grown fungal pellets for algal harvesting would probably decrease the efficiency of nutrient removal from wastewater, as the main reduction in nutrient concentrations in the present study was observed during fungal growth.

5. Conclusion

Under the conditions applied in the present study, high nutrient removal rates from wastewater and a significant decrease in the concentration of the micropollutant diclofenac were observed after fungal-assisted algal harvesting. However, the lipid content in the biomass produced was low. Nevertheless, fungal-assisted algal harvesting has the potential to be developed into a sustainable technology for wastewater treatment. Future work could be directed at selection and development of fungal strains in which laccase production and potential for high lipid accumulation are important factors.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical Approval

This work did not involve any studies with human participants or animals performed by any of the authors

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