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Research Article

Comparative Study on the Use of Untreated Water of Mahananda River in West Bengal, India and Household Gray Water for Cultivation of *Desmodesmus elegans*

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Abstract: This is the first study that has compared the use of untreated river and household gray water to obtain high-density cultures of the microalga *Desmodesmus elegans* under natural sunlight and at room temperatures. Cultivation of microalgae for all downstream applications requires the use of fresh water, inorganic nutrients, light, and energy to maintain optimal temperatures and harvest the biomass. To minimize our environmental footprint in terms of water, nutrients, and energy, untreated wastewater from two different sources were used. The Mahananda River receives unrestricted discharges of pollutants from urban activities, including the free movement of livestock. The household gray water produced from the laundry and kitchen sink consisted of a mixture of detergent, soap, oil, grease, and dirt from legumes, vegetables, and fruits. The gray water sample with high turbidity was filtered through an in-house dual-media filter made of sand and activated carbon. The filtration process significantly reduced the chemical oxygen demand (COD) of the gray water sample from 1,260 ppm to 546 ppm. Prior to algae cultivation, the initial measurements of pH, total dissolved solids (TDS), COD, phosphate, and ammonium for river and gray water were 7.44 and 8.5, 115 and 141 ppm, 350 and 546 ppm, 0.44 and 2.4 ppm, 2.5 and 1.7 ppm, respectively. The COD reduction of 74.89% and 54.31% in the river and household gray water respectively was achieved. The novelty of this study is that the cultivation of *D. elegans* was carried out under natural light and temperature conditions.

***Keywords*:** algae, *Desmodesmus*, lipids, river water, self-flocculation, wastewater

1. Introduction

The rivers provide habitat to multi-trophic aquatic organisms. Among these, the phytoplankton and periphyton algae are the only producers that support the trophic pyramid, which culminates in fishes as the third-order consumer [1]. These organisms interact with the water environment under a feedback regulation system that maintains the flow of materials and energy, which is necessary for the protection of aquatic ecosystems. An increase in human activities and climate change have altered the hydrological elements, which have directly impacted the quantity and quality of the river ecosystem, including a reduction in biodiversity [2]. In the absence of aquatic organism-driven restoration of

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rivers, the remedial measures, namely sewage interception, drug delivery, and sediment dredging, do not adequately reduce the pollution loadings [1].

The river Mahananda is an extensively used rain-fed river flowing through two Asian countries, India and Bangladesh [3, 4]. The untreated water from the Mahananda for this study was collected on the Indian side of the river in Siliguri City, West Bengal. In India, the river originates from the hills of the Himalayan range in the district of Darjeeling. Once the river enters the urban town of Siliguri, a significant number of pollutants get dumped into the river. The water pollutants arrive from functional activities like urban waste from individual families inhabiting the river bank, bathing, fishing, domestic waste, and cattle dung [3-5]. The agricultural and industrial pollutants are the two dominant factors that increase the greywater footprint on the Mahananda riverbank [5]. During water sampling, it was observed that there is a decrease in agricultural practices, a rise in urbanization, and river pollution caused due to road construction projects along and near river banks. The hydrological features of the Mahananda have been severely impacted by these forces, which are evident during the non-rainy seasons when the amount of water is noticeably decreased.

Most of India's seasonal rural rivers are suffering greatly as a result of widespread urbanization and the effects of climate change on global warming. For instance, the Indian monsoon pattern is altering as a result of climate change, and this causes the yearly fluctuation of stream flow in the river basins [6]. Uneven rainfall and decreased stream flow as a result of climate change make it difficult for India to meet its water sustainability goals [7]. On the other hand, urbanization is rapidly increasing water demand and contamination of waterways. The city water supply and sewerage boards in India's highly urbanized towns are unable to fulfill the rising demand for potable water and are rapidly approaching 'Day Zero' [8, 9]. The unrestrained and unplanned extension of civic space in Siliguri around Mahananda [10] is resulting in a surge in improperly managed solid and municipal wastes into the river body. Mismanaged plastic and urban solid wastes have catastrophic repercussions for local wildlife, as well as contributing to environmental injustice for the least responsible population in that geographical area [11]. As urban populations grow, so does the demand for and disposal of spent and contaminated water, necessitating the implementation of sustainable measures. Allowing water and nutrients recycling and reuse to provide safe urban water and chemical-free food production is a unique opportunity for the creation of resource-smart and sustainable flows of natural resources [12]. The avoidance of river water pollution can be achieved by implementing low-cost, plant-based technologies that will allow the treatment of spent water and the extraction of nutrients at the site of origin, as well as recycling for non-drinking purposes [13]. In this work, we investigated the ability of *Desmodesmus elegans*, a local strain of microalgae, to reduce chemical contaminants in untreated river and gray water by phytoremediation. We compared and optimized the use of wastewater with inadequate amounts of primary nutrients to achieve dense *D. elegans* cultures using sunlight as a source of light energy. The study's goal was to lower the ecological footprints (water, fertilizer, and energy) while increasing the sustainability of microalgal farming.

2. Materials and methods

2.1 *Sampling of river water*

The origin and geographical characteristics of the Mahananda River are well explained in [3, 4, 14]. The two different spots, sampling site 1 (SS1) (upstream) and sampling site 2 (SS2) (downstream) (26.723494, 88.422061, 26.737869, 88.422710) that were selected for the collection of water samples showed run-off of urban pollution from human settlements on either side of the river bank, plastic garbage, fishing, and animal activities (Figure 1). The water samples were collected in clean 5-liter plastic tanks, and pH, total dissolved solids (TDS), and electrical conductivity (EC) were recorded on-site with hand-held probes. The samples were immediately brought back to the laboratory, which is around 2.7 km from sampling site 1 (SS1) and kept in the refrigerator until further use for algae cultivation and nutrient analysis. Water samples were collected from both sites and as there were no discernible differences in their chemical makeup, they were combined and used in the experiment.

Figure 1. The sampling sites in Mahananda River. A) Satellite image from Google Maps showing two sampling spots namely SS1 and SS2 which are 2 km apart in the river. B) Photograph image of SS1, C) Photograph image of SS2

2.2 *Sampling of household grey water*

The kitchen sink and laundry were the sources of household gray water. Specifically, the kitchen wastewater included the washing of dirty and greasy utensils and cookware and the rinsing of vegetables, fruits, and legumes, whereas the laundry wastewater contained dirty water from the washing machine that used detergents as well as soap-based water from hand washing of the clothes. The wastewater from a single household from both the kitchen and laundry was mixed in a 10-liter plastic bucket, from which 5 liters were collected in a clean plastic tank and brought to the laboratory on the same day for physio-chemical analysis. This mixed gray water displayed a high turbidity.

2.3 *Dual media filter*

To reduce the high turbidity of the household gray water, an in-house dual media filter was made. The dual-media filter was fabricated from a specialized ultra violet (UV)-resistant hollow and transparent algae culture tube. One end of the tube was sealed (Figure 2) with an open valve to create the outlet for the filtered gray water.

Figure 2. The domestic gray water with high turbidity (kept in the beaker) was run through the dual media filter composed of sand and activated charcoal

2.4 *Microalgae cultivation and experimental set up*

The pure cultures of *Desmodesmus elegans* were maintained in the laboratory in 500 and 1,000 ml conical flasks containing BG-11 (N+) growth medium [15]. To reduce the environmental footprint, the study was carried out under natural sunlight and at room temperature. The experimental set-up for microalgae cultivation consisted of six 1.25 L culture tubes (Figure 3). Each culture tube received a continuous and regulated supply of air bubbling through silicon tubing connected to the air stones, which were kept at the bottom of each tube. With the objective of comparing the adequacy of the untreated river and household grey water, the microalgae cultures were grown under three different nutrient regimes. The untreated river and grey water in the control set of culture tubes, R3 and G3, displayed differences in their nutrient content (Table 1). Compared to river water, the higher levels of phosphate in grey water were due to the presence of laundry detergent and kitchen washings. To understand the efficacy of different nutrients, 100 ppm nitrate in the form of $NaNO_3$ and 2.6 ppm PO_4^{3-} were added into the experimental tubes R1 and G1; the culture tubes R2 and G2, received 0.1X BG-11 (N-) on the third day of cultivation. On the ninth day of cultivation, the phosphate levels in all culture tubes were exhausted. Consequently, to sustain growth, 3 ppm of phosphate was introduced to each of the R1, R2, G1, and G2 culture tubes. The culture tubes R3 and G3 were kept as a 'no addition' control to compare the growth of microalgae in the absence of any external nutrient inputs. After observing the growth for 10 days and to further enhance it, 0.1X BG-11 with 100 ppm nitrate and 3 ppm PO_4^{3-} were added to the R2 and G2, whereas, the R1 and G1cultures were supplemented with a second dose of 100 ppm nitrate and 3 ppm PO_4^{3-} on day 11. The photobioreactor was set up near the north-facing window, which received ample amounts of sunlight throughout the day. Daily measurements of light in terms of lux received during the day were taken. The lowest amounts of light were received during evening hours, and the average lowest was found to be 41.625 µmol/sec/m^2. The highest intensity of light was recorded after noon and before 4 p.m. The average of the highest sunlight received by the cultures was 103.94 µmol/sec/m^2. The study was conducted in our laboratory in Siliguri, West Bengal, India, during the winter season, specifically from February 27 to March 18, 2024, and the average room temperature recorded was 24.2 °C, attained without the use of air conditioning.

Figure 3. Microalgae culture tubes. From left to right: *D. elegans* grown in river water (R1)-supplemented only with N, P; (R2)-supplemented with 0.1X BG-11 medium; (R3)-control without any supplementation and in household grey water (G1)-supplemented only with N, P (G1); (G2)-supplemented with 0.1X BG-11 medium; (G3)-control without any supplementation

2.5 *Chemical and nutrient analysis*

The quantitative measurement of chemical oxygen demand (COD) in the water sample was performed in COD MR Reagent Vials H193754B-25 by Hanna Instruments. This is an adaptation of the USEPA 410.4 approved method for the COD determination on surfaces and wastewater. The instruction manual accompanied by the reagent kit was followed; briefly, upon addition of 2 ml of water samples, the reagent vials were mixed and inserted into the digester for 2 hours at 150 °C. After cooling down the vials, the absorbance at 610 nm was measured in HI83306 Environmental Analysis

Photometer, Hanna Instruments, Inc., USA.

The chemical test kits HI93733, HI93728, and HI93713 from Hanna Instruments, Inc., USA were used to conduct ammonia, nitrate, and phosphate tests respectively, in the water samples. Upon reaction time, the concentrations for each of these chemicals were measured in the HI83306 Environmental Analysis Photometer, Hanna Instruments, Inc., USA.

Table 1. The chemical composition of the water samples used for the cultivation of microalga

	pH	NO_3^-	NO_3^--N	PO_4^{3-}	P	NH_3	NH_4	NH_3-N	TDS/EC	COD
Units		ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm/µs/cm	ppm
River water	7.44	1.1	0.3	0.44	0.14	2.3	2.5	1.9	115/230	350
Household gray water	7.35	N.D	N.D	2.5	0.8	1.6	1.7	1.4	237/474	546

N.D: not detected, TDS: total dissolved solids, EC: electrical conductivity

2.6 *Biomass harvesting and lipid extractions*

After 20 days of cultivation, the aeration in each of the photobioreactor tubes was stopped to allow the overnight settlement of the microalgae culture. The *D. elegans* cultures showed efficient settlement (Figure 4) which made the harvesting process less energy-intensive. The supernatant fluid was discarded by decantation, and the remaining liquid containing the cellular biomass was centrifuged at 4,000 rpm for 5 minutes in the Remi R-8C Plus centrifuge machine (Remi Electrotechnik Ltd., India).

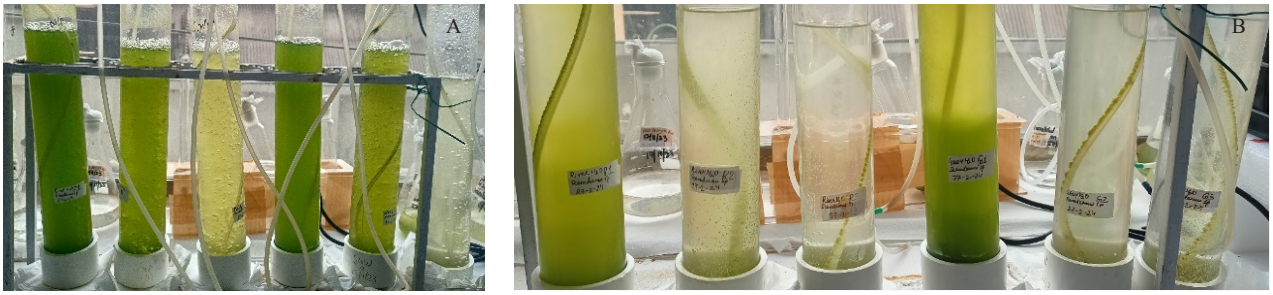

Figure 4. Sedimentation of *Desmodesmus elegans* in photobioreactor tubes. A) Self-flocculation during growth period in presence of aeration-the first culture tube from right G3 showed maximum settlement. B) Self-flocculation during harvest, when aeration was stopped for several hours

The fresh biomass pellets were dried for 5-7 hours at 70 °C in a food-grade dehydrator. The dried biomass was crushed into a fine powder using a pestle and mortar. The powdered biomass was used for the gravimetric estimation of microalgae lipids. The extraction of lipids was performed following the conventional Folch Method using a 2:1 chloroform-methanol solvent mixture [16].

3. Results and discussion

3.1 *Comparison of growth of D. elegans in river and household grey water*

The river water carrying pollutants from city discharge and domestic waste has been successfully used for growing different species of microalgae such as *Selenastrum* sp., *Chlorella* sp., and *Arthrospira platensis* [17-19]. When compared to untreated domestic gray water, Mahananda River water facilitated the highest cell growth in *D. elegans*

cultures with an additional supplementation of nitrate and phosphate (Figure 5). The nutrient supplementation in R1 cultures allowed it to attain the highest cell densities (Figure 6). In contrast to the R3 and G3 control cultures, there was no discernible growth observed in the BG-11 (N-) enriched R2 and G2 cultures (Figure 5). This study found that nitrate, a growth-limiting nutrient, allowed the uptake of other necessary nutrients for microalgae growth, as confirmed by BG-11 (N-) and 'no-nutrient' cultures. Investigating the need for additional micronutrients present in the standard nutrient media was made possible by the initial use of a BG-11 synthetic medium without nitrate. Apart from N and P, the other micronutrients are only required in trace amounts. The microalga *Scenedesmus obtusiusculus* A189 was cultivated in natural river water to replace the micronutrients in BG-11 and also produced high yields of carbohydrates and fats [20]. The culture tubes used in this study were arranged to examine the function of micronutrients in the microalgae cultivation process while excluding the need for analytical estimation of remaining nutrient components present in both river water and household greywater. Given that river water does not consistently contain high levels of nitrate and phosphorus, a comparable approach was employed, which involved blending synthetic media with river water and its effluents, to facilitate the cost-effective cultivation of microalgae aimed at promoting a sustainable bio-based economy [21].

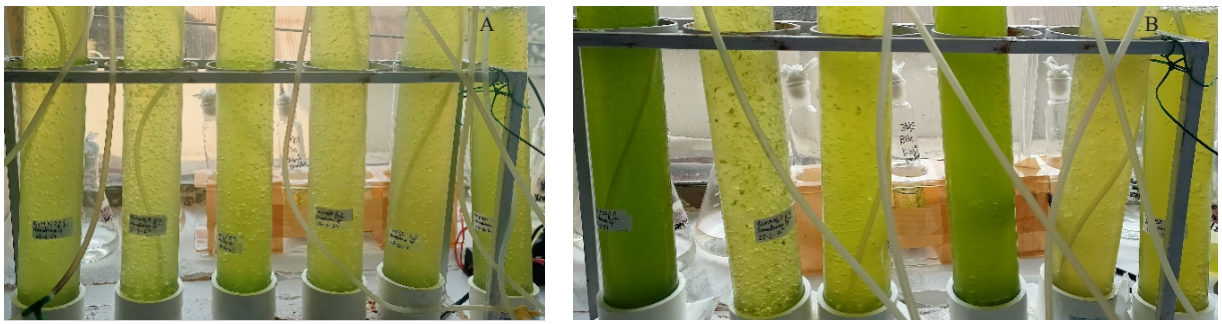

Figure 5. Growth of *D. elegans* in photobioreactor tubes (left to right: R1, R2, R3, G1, G2, G3) bubbled with ambient air. A) Growth before addition of nutrients. B) Increased growth in R1 and G1 after addition of nutrients

An impediment to the improved treatment of municipal wastewater using microalgae is the lower nutrient concentrations, particularly lower N:P, in secondary domestic wastewater and natural water sources compared to the standard microalgae growing medium, agriculture, and livestock wastewaters [22]. Therefore, the strategy of nutrient supplementation was adopted. In both river and grey water, the additions of NO_3 and PO_4^{3-} produced increased *D. elegans* cell counts (Figure 6) which resulted in dense green cultures (Figure 5). However, adding 0.1X BG-11 (N-) medium to cultures did not increase cell numbers or culture growth (Figure 6). During the first seven days, there was a surge in the growth of G3 cultures relative to R3 which could be caused by the presence of greater phosphate levels (Table 1) in the greywater because cell division in microalgae cultures depends on phosphate concentrations. In addition to being a crucial structural element, phosphorous is necessary for energy transduction and storage at the cellular level in all living things [23]. Because the geological reserves of inorganic P fertilizers are not distributed evenly [23], it is imperative to use wastewater in place of inorganic P fertilizers and to use it sparingly from an ecological and environmental standpoint. After the initial growth surge in G3, the performance of *D. elegans* in grey water remained stagnant (Figure 6). When microalgae are used for the biological wastewater treatment process, their growth is influenced by the various types of nutrients that are present in the wastewater. The efficacy of biological wastewater treatment is adversely affected by the overabundance of nutrients that are otherwise used to promote growth [24]. Thus, while wastewater is being phycoremediated, it is essential to regularly check the amount of nutrients present. Nitrogen and phosphorous are the two primary elements that significantly affect the microalgae growing process, in comparison to other physical parameters such as light, temperature, photoperiod, salinity, pH, air mixing, and CO_2 [25]. Every culture except for R1 (0.06 ppm) and G1 (0.14 ppm) had a decrease in PO_4^{3-} concentration to 0 ppm on the 9th day of cultivation. The result was a yellowing of the R3, G2, and G3 cultures (Figure 5). Phosphorous is an essential

nutrient for both freshwater and marine microalgae; its depletion could result in the start of cellular stress and a halt to cell division [26]. Phosphorous deficiency in microalgae can have a variety of negative effects, such as oxidative damage to structural organelles like cell membranes and a decrease in chlorophyll content, which in turn can affect the algae's ability to absorb nitrogen and carbon [27]. For wastewater to be treated by phytoremediation, it is essential to supplement with nutrients to maximize biomass production, reduce heavy metals toxicity, increase lipid and protein production, and absorb other nutrients or pollutants [27, 28]. Therefore, to sustain microalgae growth, 3 ppm of PO_4^{3-} was added to the R1, R2, G1, and G2 culture tubes on day 9, as shown by the arrows on the growth curve graph (Figure 6). At the outset of the experiment, occurring within two days, all four cultures exhibited a depletion of phosphate, likely attributable to the phenomenon known as "luxury P uptake" [29]. On the eleventh day, R1 and G1 were given a second dose of nutrients, which included 100 ppm NO^{3-} and 2.6 ppm PO_4^{3-}. At the same time, R2 and G2 received a second dose of 0.1 X BG-11 (N+) with the same concentrations of nitrate and phosphate as those provided to R1 and G1, to maximize and attain higher cell densities. The microalgae growth improved with supplementation, but the final cell densities were noticeably different between R1-G1 and R2-G2. This clearly shows that in both wastewaters, nitrate, and phosphate were the main nutrients that can restrict the microalgae growth, rather than the other ingredients in the BG-11 medium. A great way to supply only the limiting nutrient and reduce reliance on inorganic fertilizers when growing microalgae is to use untreated wastewater instead of freshwater. The use of river water for microalgae cultivation plays dual roles in the remediation of river water pollution and low-cost biomass production for various industrial applications [19].

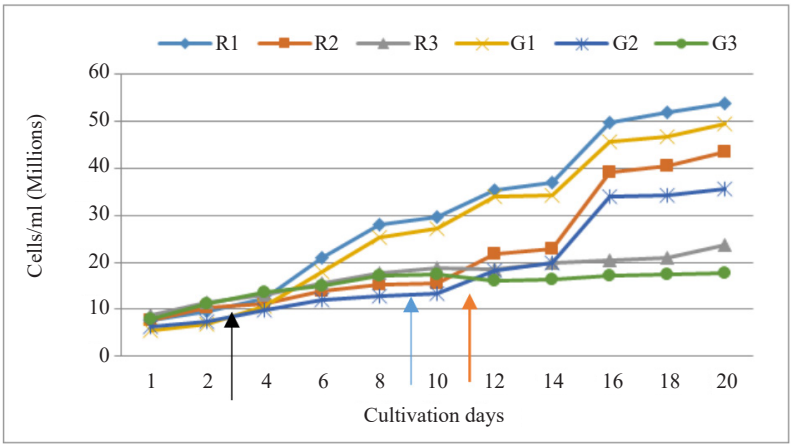

Figure 6. Comparison of cell counts of *D. elegans* grown in different nutrient regimes of River Mahananda and household grey water. Black arrow represents addition of nutrients in R1, R2, G1, G2; Blue arrow represents addition of 3 ppm PO_4^{3-} in R1, R2, G1, G2 and orange arrow represent second shot of nutrients in R1, R2, G1 and G2

3.2 *Nutrient uptake dynamics in D. elegans grown in wastewater*

Different nutrient enrichments in wastewater medium are needed depending on the type of microalgae to obtain a significant volume of lipid-rich biomass with improved nutrient removal efficiency [30]. The species of *Scenedesmus* and *Desmodesmus* are proficient in the removal of phosphate from any type of wastewater [31]. The removal of phosphorous from different types of wastewater using microalgae offers several advantages, such as the destruction of organic pollutants, suppression of pathogenic microflora, sequestration of greenhouse gases, and the formation of slow-release P-biofertilizers. The presence of indoor compressed air, combined with high phosphorus levels in the growth medium, may promote the growth of cyanobacteria. Therefore, to reduce the risk of contamination, we ensured a low concentration of phosphorus and implemented intermittent feeding for the cultures. The establishment of a natural ecosystem or mesocosm environment within the photobioreactor was a significant sustainability element taken into account in this study. Therefore, the microalgae were grown in the untreated river and household gray water without the need for energy-intensive inputs such as artificial lighting, air conditioning in the chamber, or the injection of

clean and sterile CO_2. The diverse types and amounts of nutrients found in wastewater sources can affect the growth of microalgae. The high concentration of ammonium in anaerobic effluents may pose a threat to certain algal species, including *Chlorella vulgaris*; consequently, it is essential to either dilute the wastewater or subject it to nitrification and enhancement to optimize the phytoremediation process [32].

Table 2. Changes in the nutrient concentrations during cultivation of *D. elegans* in river and grey water

	NH_4 (ppm)			PO_4^{3-} (ppm)								COD (ppm)	
Days	0	2	3	0	2	3	3 (after nutrients addition)	4	9	9 (after nutrients addition)	20	0	20
R1	2.5	0.9	n.d	0.44	0.25	0.1	1.4	0.8	0.06	1.36	0.66	350	92
R2		0.85	n.d		0.28	0.1	2.4	0.0	0.00	2.30	0.08		150
R3		0.9	n.d		0.3	0.2	0.0	0.06	0.00	0.00	0.00		88
G1	1.7	1.1	n.d	2.5	0.55	0.25	1.55	0.34	0.14	1.44	0.08	546	250
G2		1.1	n.d		0.6	0.41	2.7	0.16	0.0	2.30	0.02		331
G3		1.0	n.d		0.45	0.32	0.32	0.33	0.00	0.00	0.00		347

The untreated Mahananda River water contained a higher concentration of ammonium than the household gray water (Table 1). In the wastewater treatment process, microalgae alone or in symbiotic association with bacteria can uptake ammonia, nitrite, nitrate, and nitrogen from other organic sources. Ammonia is favored as a nitrogen source for both microalgae and cyanobacteria due to its efficient cellular absorption, and it is also found in significant quantities in various forms of wastewater [33]. In the present study, due to the low concentrations of ammonium in both river and grey water, it was readily consumed by *D. elegans* in less than 3 days (Table 2). The genus *Desmodesmus* is routinely used for nutrient recovery and treatment of various types of wastewater, such as anaerobic digestion [34], secondary effluents [35], and cosmetic wastewater [31]. Other genera of microalgae that are extensively considered for the wastewater phytoremediation process are *Chlamydomonas*, *Chlorella*, and *Scenedesmus* [24, 36]. One of the reasons phytoremediation is preferable to non-biological tertiary wastewater treatment processes is that microalgae can sequester heavy metals and harmful organic compounds without producing secondary contaminants in the effluents [24]. Microalgae-based technologies can be utilized globally due to the widespread distribution of various species. Notably, frequently used cyanobacteria include *Arthrospira*, *Leptolyngbya*, *Anabaena*, *Oscillatoria*, *Limnosira*, and *Phormidium*. In addition, autotrophic species such as *Chlamydomonas*, *Botryococcus*, *Chlorella*, and *Scenedesmus*, along with mixotrophic microalga *Euglena* are of particular significance [37]. Furthermore, extremophilic microalgae contribute substantially to mitigating eutrophication and addressing the challenges posed by emerging contaminants in the context of increasing global urbanization [38]. By tracking changes in COD, this study examined the decrease in chemical pollutants in the untreated river and gray water. In both river and gray water, the microalga *D. elegans* efficiently reduced the COD levels (Table 2). *D. elegans* was able to lower the COD of untreated gray water to levels that were all lower than the initial COD. The initial COD concentration and the levels of nutrients, specifically N and P, present in the influent determine the COD reduction efficiency in the microalgae wastewater treatment process [39, 40]. The lowest COD value of 88 ppm (equivalent to 75% reduction) was achieved in R3 cultures, where no nutrients were added to the river water. In field trial experiments, native green microalga *Chlorella* sp. from an industrially and urbanely polluted river in Tamil Nadu, India, reduced COD by 32.53% [18]. The R1 cultures supplemented with N and P demonstrated the highest COD removal efficiency of 54.3%, while the "no-nutrient" household greywater culture (G3) showed the lowest COD removal efficiency of 36.5% (Table 2). Following the extraction of suspended solids through centrifugation, the

untreated bathroom greywater, which contained 10.6 ppm of total nitrogen and 1.67 ppm of total phosphorus, was subjected to treatment with *Chlorella variabilis* for 19 days at a temperature of 27 °C in a shaking incubator. This process resulted in a significant decrease in chemical oxygen demand (COD) from 654 ppm to 69.2 ppm [41]. Different factors like the type of greywater, the source of illumination, the mode of aeration, and temperature are responsible for the variation in COD removal efficiency between *Chlorella variabilis* [41] and *D. elegans* in this study. The two key factors that distinctly influenced the efficiency of COD reduction in household greywater treated with *Botryococcus* sp. were the low initial COD concentration and the elevated levels of both nitrates and phosphates [40]. The implementation of phytoremediation, combined with wastewater reuse strategies, presents a straightforward and cost-effective primary treatment method that is carbon-neutral. This approach is effective in reducing the gray water footprint in residential settings and contributes to achieving the United Nations' sustainable development goals [42].

3.3 *Factors influencing self-flocculation tendencies in different cultures of D. elegans*

There are abundant species of microalgae that are rich in valuable compounds and could be used for nutrient recovery in wastewater. In general, preference is given to those that are abundantly found in natural ecosystems and can self-flocculate. For example, the use of microalgae, namely *Ankistrodesmus falcatus*, *Chlorella vulgaris*, *Scenedesmus* spp., *Chlorococcum* sp. [43], and the cyanobacterium *Oscillatoria* sp. [15], for nutrient recovery in wastewater treatment provides low-cost, environment conducive, and less energy-intensive harvesting or effluent separation methods. The following trend (G3 > R3 > G2 > R2 > G1 > R1) was observed concerning the tendency of self-flocculation by *D. elegans* during the entire period of cultivation.

Daily observations of the cultures indicated that when *D. elegans* is cultivated in untreated gray water, there is an increased propensity for self-flocculation. Consequently, the cultures in G3 settled at the bottom each day. To mitigate the impact of sedimentation on cellular growth, the cultures were manually agitated by vertically repositioning the aeration tube and briefly enhancing the air sparging. Cell settlements were noted in all tubes except for R1 and G1 throughout the growth period; however, the highest levels of flocculation and sedimentation were observed exclusively in G3 (Figure 4). Harvesting microalgae requires a lot of energy, which is the main obstacle to mass cultivation for biotechnological uses. When self-flocculation-based harvesting is used, algal farming for biodiesel feedstock in open cultivation systems is more sustainable than nutraceutical applications. Various species of microalgae namely *Scenedesmus subspicatus* and *Parachlorella kessleri* demonstrate tendencies toward segregation, colony formation, and flocculation in the presence of various stress factors such as predators [34], nutrient limitation, and culture turbulence [35]. As most microalgae media contain nitrate as the source of nitrogen, the increased ammonia concentrations of up to 50 ppm can cause stress such as observed in the *Chlorococcum* sp. that resulted in self-flocculation attributed to the induction of extracellular proteins in the cultures [44]. Microalgae cultures undergo flocculation as a result of self-flocculating species or symbiotic relationships with microorganisms that secrete a variety of biomolecules, including lipids, polysaccharides, extracellular polymeric substances, and their complexes [45]. Our study conforms with these results as the culture tubes R3 and G3 cultures tubes which were set up as 'no-nutrients' control showed higher contamination and lowest microalgae growth among all other experimental tubes. Optimized aeration intensity serves as a crucial abiotic factor influencing the self-flocculation of symbiotic algae-bacterial cultures, alongside the impact of biotic factors [46]. In the current study, it was observed that reduced bubbling resulted in increased sedimentation of the algae-microorganism flocs within *D. elegans* cultures, which were established through the introduction of non-sterilized indoor air (Figure 4).

Small and large ciliates (zooplanktons), fungal spores and hyphae, and small flagellates were among the biological contaminants observed in this study. Microscopic examination of the cultures was performed regularly, and it was discovered that on the second day of cultivation, cultures in R3 and G3 (Figure 7C, F) included a large number of fungal spores and flagellates in G2. Contamination eventually emerged in every tube, albeit at varying levels. *D. elegans* cells in R2, G2, R3, and G3 were observed entangled between some fungal hyphae, resulting in floc formation and subsequent settlements in the culture tubes (Figure 7). Contaminating organisms do not need to be eradicated from microalgae cultures through chemical treatments, provided they do not lead to a culture crash, because such treatments can adversely impact the microalgae. Conversely, recognizing the presence of these organisms is essential in algae farming, as it facilitates a successful and efficient transition from laboratory conditions to field applications. Consortia of several types of microorganisms increase biomass production through cooperative interactions, which improves

pollution reduction efficiency in the phytoremediation process [24].

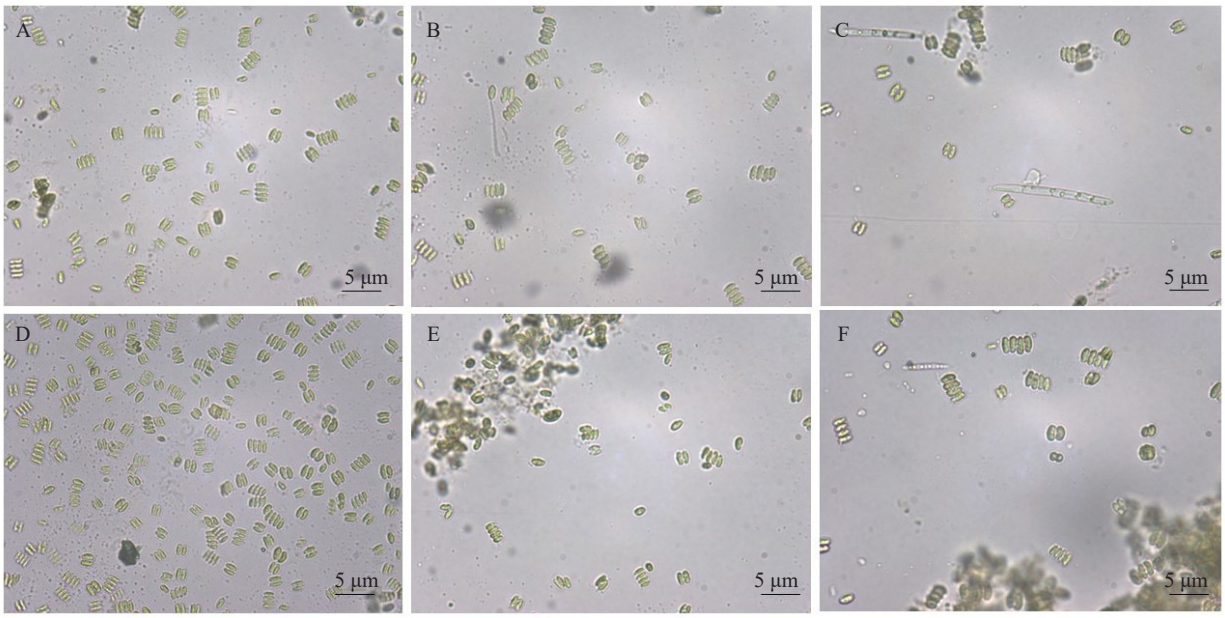

Figure 7. Microscopic images of *D. elegans* in different culture tubes. A-F) Cultures in R1, R2, R3, G1, G2 and G3

3.4 *Comparison of biomass and lipid production in D. elegans grown in river and household gray water*

R1 cultures grown in river water with NO_3 and PO_4^{3-} exhibited the highest biomass and lipids (Table 3). Although biomass production in river and gray water was comparable, G3 cultures produced significantly lower lipids (Table 3). In a study of polyculture microalgae grown in gray water with natural temperature changes, the greatest biomass concentration reached was 0.82 g/L [47], which is equivalent to the biomass concentrations achieved in R2 and G2 of the present study. After removing organic pollutants from polluted river water, microalgae biomass can be utilized to produce feed, biofuels [48], and slow-release P-biofertilizers [29]. In our investigation of lipid production, the primary objective was to monitor lipid content changes rather than to analyze the potential for biodiesel generation. This study clearly showed changes in lipid content, which were associated with the source of wastewater. The greater lipid content in river water could be attributed to specific impurities that acted as cellular stressors, as diverse types of pollutants were discharged unrestrictedly in the Mahananda River. Although the biomass concentrations in R3 and G3 were similar, G3 had the lowest percentage of dry-weight lipids. Further investigations are needed to correlate the amounts of lipids to different water sources. Further speculations on these correlations are beyond the scope of this study.

Table 3. Comparison of *D. elegans* biomass production and total lipid content in river and grey water

	River water			Household gray water		
Culture tubes	R1	R2	R3	G1	G2	G3
Biomass production (g/l)	1.34	0.84	0.35	1.04	0.71	0.30
Dry weight lipids (%)	18.8	17.7	13.6	16.9	15.3	8

4. Conclusions

The uncontrolled release of urban pollutants into rain-fed rivers can be significantly reduced by implementing decentralized treatment systems for household greywater, utilizing microalgae to absorb nutrients and decrease pollutant concentrations in the effluents. In this research, we employed untreated water from the Mahananda River and household gray water to cultivate the indigenous strain of the microalga *Desmodesmus elegans* within a tubular photobioreactor, utilizing natural light and variations in temperature. Due to the scarcity of essential nutrients in both water sources, we utilized a nutrient enrichment strategy to explore the significance of nutrients that are vital for the growth of microalgae. The periodic addition of essential nutrients, specifically nitrate and phosphate, to the river and household gray water led to the maximum biomass concentrations of 1.34 g/L and dry-weight lipids of 18.8%. The addition of a complete BG-11 medium was determined to be ineffective in enhancing the growth of microalgae. Concerning the reduction of Chemical Oxygen Demand (COD), *D. elegans* efficiently diminished COD concentrations from 350 to 88 ppm in river water and from 546 to 250 ppm in residential gray water, even in the absence of any additional supplementation. This research illustrates that untreated river water and domestic gray water can serve as a growth medium for microalgae when supplemented periodically with nitrate and phosphate, eliminating the necessity for heavy inorganic fertilizers. The results indicate that phytoremediation utilizing self-flocculating microalgae presents an effective strategy for addressing the challenges posed by rapidly increasing urban wastewater.

Conflict of interest

The authors declare that they have no conflict of interest.

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