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Bioremediation and lipid synthesis through mixotrophic algal consortia in municipal wastewater

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HIGHLIGHTS

- Bioremediation by mixotrophic algal consortia grown in sewage fed bioreactor.
- Significantly removed TOC (86%), TN (90%), Amm.-N (89%), TP (70%) and OP (76%).
- Observed higher biomass productivity and lipid with FAME suitable for biofuel.
- Ca, Fe, P and Cl might have helped in cell clumping in the culture.
- Algae flocculated at the end of the culture.

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ABSTRACT

Algae grown in outdoor reactors (volume: 10 L and depth: 20 cm) were fed directly with filtered and sterilised municipal wastewater. The nutrient removal efficiencies were 86%, 90%, 89%, 70% and 76% for TOC, TN, NH_4-N, TP and OP, respectively, and lipid content varied from 18% to 28.5% of dry algal biomass. Biomass productivity of ~122 mg/l/d (surface productivity 24.4 g/m^2/d) and lipid productivity of ~32 mg/l/d were recorded. Gas chromatography and mass spectrometry (GC–MS) analyses of the fatty acid methyl esters (FAME) showed a higher content of desirable fatty acids (bearing biofuel properties) with major contributions from saturates such as palmitic acid [C16:0; ~40%] and stearic acid [C18:0; ~34%], followed by unsaturates such as oleic acid [C18:1(9); ~10%] and linoleic acid [C18:2(9,12); ~5%]. The decomposition of algal biomass and reactor residues with an exothermic heat content of 123.4 J/g provides the scope for further energy derivation.

1. Introduction

Treatment and disposal of wastewater generated in habitations are one of the key environmental challenges faced in urban localities due to a burgeoning population of the recent decade. Nutrient laden wastewater generated in municipalities has been either untreated or partially treated and directly fed into the nearby water bodies regularly, resulting in nutrient enrichment and algal blooms. Conventional wastewater treatment options are energy and capital intensive as well as inefficient in their capacity of removing nutrients completely. In this context, algal processes are beneficial in terms of removing nutrients through carbon sequestration and a resultant biomass production (Woertz et al., 2009). Algae grows rapidly and assimilate nutrients (C, N and P) available in wastewater (Mahapatra et al., 2013a,b) for which they are useful in nutrient remediation. This algal biomass provides aeration in the water body in addition to having a good valorisation potential with biofuel prospects (Mahapatra et al., 2013a,b).

Higher growth rates and the ability to accumulate lipids have made algae a viable substrate for biofuel when compared to other biofuel feed-stocks (Damiani et al., 2010; Chanakya et al., 2012; Ramachandra et al., 2013). Microalgae rich in lipids and hydrocarbons are being exploited now to mitigate the impending fuel oil

Abbreviations: TOC, Total Organic Carbon; TN, Total Nitrogen; NH_4-N, Ammonium Nitrogen; TP, Total Phosphate; OP, Ortho Phosphate.

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crisis (Ramachandra et al., 2009) as they are renewable, carbon neutral and viable substitutes for fossil fuels. Algae based biofuel generation coupled with wastewater treatment would also counter the diminution of environmental externalities and additional energy expenses (Chen et al., 2011).

The major aspects in establishing an integrated wastewater treatment and biofuel production system are the selection of appropriate species, requirement of modulated illuminating conditions, rapid nutrient removal ability, enhanced biomass productivity, and an efficient algal biomass harvesting capability. Native mixed algal species growing abundantly in wastewater are versatile to changing oxic-anoxic conditions, predominately following a mixotrophic mode of nutrition intake with high biomass productivity throughout the year. Mixotrophic species grow profusely in the carbon (organic C and CO_2) dominant environment. Mixotrophic algae unlike dense phototrophic cultures grew even in a light limiting environment (Wang and Lan, 2011), removing nutrients (C, N and P), thereby, possessing high cell densities and productivities (Woertz et al., 2009; Prathima Devi et al., 2012). These helped in the synthesis of higher lipids and removal of organics in wastewater (Bhatnagar et al., 2010). Earlier studies on nutrient removal and lipid production from wastewater were on uni-algal species like, *Chlorella* sp. (Wang et al., 2010), *Neochloris* sp. (Wang and Lan, 2011) etc. which grew under closed conditions in laboratories, wherein mixed cultivation systems of algal-bacterial consortium (Su et al., 2011) and algal-fungal (Zhou et al., 2012a) consortium existed as against mixed algal species/algal consortia (Perez-Garcia et al., 2010; Venkata Mohan et al., 2011). However, investigations on more efficient methods for algal biomass concentration and harvesting require further research.

Nutrient uptake by algae from wastewater has been assessed through cellular biochemical composition and elemental analysis. This helps in the understanding of nutrient transformations such as carbon allocation, lipid synthesis and accumulation. The spectroscopic techniques (like FTIR, etc. – Stehfest et al., 2005; Dean et al., 2010) are non-destructive, accurate, efficient and fast for compositional analysis. Algal cells, even after extracting lipid, would have fuel values, which is evident from thermal analysis of species, viz., *Chlorella* sp. (Sostaric et al., 2012) and derivatives from *Botyococcus braunii* (Salmon et al., 2009). This highlights that there is a scope for integrated energy analysis of mixed algal consortium of wastewater systems. The focus of the current work has been the assessment of bioremediation and biofuel potential of algal consortia, optimal harvest mechanism and derivation of thermal properties of algal biomass.

2. Methods

2.1. Wastewater sampling and analysis

Wastewater collected from the inflow channels (of Bellandur lake, Koramangla region, South of Bangalore, India) was allowed to settle for 2 days. The supernatant was sterilised, filtered and used as the culture media (as per Zhou et al., 2012b). Wastewater used for culturing were analysed through standard protocols (APHA, 2005) and the parameters were pH 6.3 ± 0.1, redox potential (ORP) -163 mV, total volatile solids (TVS) 432 ± 66 mg/l, total suspended solids (TSS) 540 ± 84 mg/l. Similarly, the analysis of wastewater broth through HACH protocol yielded Total Organic Carbon (TOC) of 219 ± 14 mg/l, Chemical Oxygen Demand (COD) of 676 ± 17.2 mg/l, Total Nitrogen (TN) of 64.6 ± 4.2 mg/l, Nitrate-Nitrogen (NO_3-N) of 1.07 ± 0.15 mg/l, Nitrite-Nitrogen (NO_2-N) of 0.32 ± 0.04 mg/l, Ammonium-Nitrogen (NH_4-N) of 51.5 ± 5.5 mg/l, Total Phosphorus (TP) of 28 ± 4.3 mg/l and Ortho-phosphates (OP) of 22.2 ± 2.4 mg/l.

2.2. Culturing algal consortia and growth conditions and reactor design

Algal samples were collected from wastewater fed urban lakes. From these samples, algal species were identified by morphological keys using the light and electron microscopes. The water sample collected from the lake was centrifuged at 1816*g* and the algal pellet containing the algal consortia was washed and centrifuged repeatedly with deionised water. Subsequently, the algal cells were inoculated in Bolds Basal (BB) media. The algal consortia grown in BB media comprised of bacillariophyceae (*Cyclotella meneghiniana*, *Nitzschia palea*), chlorophyceae (*Chlorococcum* sp., *Scenedesmus quadricauda*, *Scenedesmus obliques*, *Arthrodesmus curvatus*, *Golenkinia radiata*, *Kirchneriella lunaris*, *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorococcum humicola*, *Chroococcus* sp., *Monoraphidium* sp., *Ankistrodesmus falcatus*), cyanophyceae (*Oocystis* sp., *Phormidium tenue*, *Spirulina maximus*) and euglenophyceae (*Trachelomonas* spp., *Euglena* spp., *Phacus longicauda*, *Phacus caudatus*). Inoculum comprising of these algal consortia were then added to the reactor ($\sim 10^6$ cells/ml; inoculum volume 40 ml; reactor working volume 10 L).

The algal reactor was made up of translucent polypropylene with a storage capacity >12 l (measuring 32 cm × 16 cm × 24 cm depth; working volume 10 L), covered with a glass sheet (the spacing between the glass sheet and the reactor was 20 cm). The reactor was kept on rooftop with a direct access to sunlight (light: dark period of 12 h: 12 h), and the culture was mixed uniformly thrice a day at regular intervals. The diurnal illuminance was recorded at the horizontal surface of the reactor using a lux meter. Illuminance ranges from 600 lux (during sunrise and sunset) to ~1,00,000 lux (during the mid-day). Algal cultures were maintained at ambient temperatures (22–30 °C) and the growth was monitored daily for two weeks. 100 ml of broth was collected daily and centrifuged. The reactor volume was maintained at 10 L with deionised water (to compensate evaporation losses and also 100 ml of broth). The filtered supernatant from centrifuged broth was used for nutrient analysis. The algal pellet was subjected to repeat washing following which it was dried and weighed (dry weight). Subsequently, the total lipid content, spectroscopic and elemental analyses were conducted. Algal biomass concentration (cell dry weight in the culturing medium in terms of g/l) was estimated by gravimetry.

2.3. Growth, productivity and lipid measurements

The probable model for biomass growth in 12 days is given by Eq. (1).

$$B_2 = B_1 e^{SGR(T_2 - T_1)} \quad (1)$$

where T_2–T_1 (day) represents the time difference between the two measurements,

B_2 and B_1 (g/L) represent the concentration of biomass at T_2 (12th day) and T_1 (1st day), respectively. SGR – specific growth rate helps in estimating likely biomass on the *n*th day.

The biomass productivity (B_{prod}) during culturing is given by Eq. (2)

$$B_{prod} = (B_2 - B_1)/T_2 - T_1 \quad (2)$$

where, B_{prod} is the biomass productivity (g/l/d), T_2–T_1 (day) represents the time difference between the two measurements, B_2 and B_1 (g/l) represent the concentration of biomass at T_2 (12th day) and T_1 (1st day), respectively.

Cell suspensions were pelletised through centrifugations at 7267*g* for 10 min, and stored at −20 °C for further use. Lipids from these pellets were extracted through Bligh Dyer's method which

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included solvent extraction followed by gravimetric analysis. The total lipid content of the algal biomass is given by Eq. (3).

$$LC = L_W/B_w \quad (3)$$

where, LC is the quantity of total lipid per unit of algae (g/g), L_W is lipid weight in grams (g), B_w is dry weight of algal biomass in grams (g).

Similarly, lipid productivity is given by Eq. (4).

$$L_{prod.} = (LC_2B_2 - LC_1B_1)/(T_2 - T_1) \quad (4)$$

where, $L_{prod.}$ = lipid productivity per litre of wastewater per day (g/l/d), LC_2 and LC_1 are the respective total lipid content of algae (g/g) at times T_2 (12th day) and T_1 (1st day). Similarly, B_2 and B_1 are the respective biomass (g) at times T_2 (12th day) and T_1 (1st day).

2.4. Composition analysis – Fourier Transform Infrared Spectroscopy (FTIR)

Attenuated total reflectance ATR-FTIR spectra were obtained to assess the composition of algal biomass based on bio-molecular functionalities. Freeze dried pulverised algal daily samples were observed through IR spectra in the Mid IR range (64 scans, wave number 4000–800 cm^{-1} and spectral resolution of 2 cm^{-1}) and data were analysed using Origin Pro 8 SR0, v8.0724 (B724) with an initial base line correction, and scaled up to Amide I_{max} (as per Stehfest et al., 2005). Spectral curves were fitted based on Gaussian distribution considering peak values with area for each being computed. The biochemical transitions in the algal cells were monitored by the analysis of carbohydrate to protein (C/P); lipid to protein (L/P) and lipid to phosphate (L/Phos.) ratio (as per Stehfest et al., 2005). In the spectrogram, ~1740 cm^{-1} represented ν(C=O) ester groups (based on stretching vibrations) primarily from lipids and fatty acids; similarly, ~1655 cm^{-1} represented ν(C=O) amides from proteins (Amide I); ~1240 cm^{-1} represented ν_{as}(>P=O) phosphoric compounds and ~1000 cm^{-1} represented ν(C–O–C) stretching from polysaccharides. Compared to these, ~1545 cm^{-1} represented δ(N–H) amides from proteins (Amide II) based on bending vibrations.

2.5. Elemental analysis and imaging: SEM-EDXA field emission scanning electron-microscopy

Algal cell imaging involves (i) fixing algal cells in 2.5% of glutaraldehyde, (ii) dehydration by ethanol (from 30% till 100% concentration), (iii) drying algal cells, (iv) mounting on carbon tapes (over aluminium stub), gold-sputtered and (v) observation and imaging using FEI ESEM Quanta 200 and FEI Sirion XL30 FEG SEM (in the ultra high range) with a schottky field emission source with high voltage variable between 200 V and 300 kV. EDS Detector EDAX Genesis with working resolution of 0.5 nm at >10 kV, 2.5 nm at 1 kV, 3.5 nm at 500 V with accelerating voltage, 2–30 kV were used for elemental analysis.

2.6. Fatty acid (FAME) composition analysis through GCMS

Algal fatty acid composition determination comprised of (i) cell disruption, done by ultra-sonication using ultrasonic bath (frequency 35 kHz) for 30 min, (ii) extraction of lipid with chloroform–methanol (2:1, v/v) and (iii) determination of total lipids through Bligh and Dyer's method (1959). Methylation of lipids was performed using Boron-trifluoride–Methanol (BF_3–MeOH) that yields corresponding methyl esters from fatty acids. FAME composition was assessed through gas chromatograph mass spectrometry (Agilent Technologies 7890C, GC System; Agilent Technologies 5975C insert MSD with Triple-Axis Detector) with helium gas as carrier in split-less mode. The components were identified based on their retention times, abundance and fragmentation patterns by comparing them with a known standard.

2.7. Thermal analysis – differential scanning calorimetry (DSC)

Thermal analysis was done with METTLER-TOLEDO DSC1 instrument in N_2 atmosphere (30–50 mL/min) to determine energy transitions (exothermic/endothermic) and thermal behaviour. Samples include (a) algal matter, (b) spent algal matter after lipid extraction, (c) algal mass floc floating on the surface of the reactor and (d) settled sludge in the reactor. 5–10 mg samples placed in 40 μg aluminium crucibles were heated up to 500 °C at a constant heating rate of 10 °C/min. DSC thermograms were analysed for energy values with the help of Lab METTLER STAR 9.3 and Origin Pro 8 software.

3. Results and discussion

3.1. Assessment of nutrient uptake capability of algal consortia

The growth of the algal consortia monitored for 12 days as shown in Fig. 1a depicts the growth profile of mixed algae in wastewater. The algal consortia grew exponentially at a mean specific growth rate of 0.2/d in mixotrophic conditions. The consortia exhibited exponential growth ($p < 0.05$) with two distinct phases immediately after inoculation without lag phase. This is further substantiated by the similar decline pattern in TOC and TN due to algal growth with the uptake of nutrients. In the first phase, biomass increased from 0.17 ± 0.046 to 0.66 ± 0.042 g/l (30%) during the initial 4 days due to heterotrophy with the assimilation of easily available organic C. The second exponential phase, from day 6 to day 11, shows a biomass increase from 0.71 ± 0.037 to 1.64 ± 0.043 g/l (57%), which can be attributed to mixotrophy involving available residual organic C and dissolved CO_2 that is significantly higher than the initial phase. The algal consortium predominantly comprising of *Euglena* spp., *Phacus* spp., *Chlorella* sp., *Chlorococcum* sp. and *Phormidium* sp. has played a decisive role in the assimilation of carbon. pH at the beginning of the experiment was 6.3 that indicated a slightly acidic media largely due to the presence of semi decomposed carbon substrates in the forms of acetate, propionate, butyrate etc. as reported earlier (Zhou et al., 2012b). pH gradually increased to 8.6 with algal growth and a simultaneous increase in photosynthesis and accumulation of OH^- ions.

The biomass growth and productivities are shown in Table 1. Due to availability of abundant organic carbon, the biomass productivity was 122.5 mg/l/d in the reactor, which is higher than an earlier report (Chinnasamy et al., 2010) of 57 mg/l/d (in raceway pond) and 70 mg/l/d (mixed algal species cultured in poly bags). Compared to these, *Chlorella minutissima* grown in an optimised media showed a biomass productivity of 1780 mg/l/d (Li et al., 2011) in suspended algal systems, which seems quite high and rare.

TOC reduced from 219 ± 14 to 31 ± 12 mg/L in 12 days with a removal efficiency of ~86% due to an initial heterotrophic mechanism (with the abundant organic load). This was comparable to 86% carbon removal with *Chlorella vulgaris* (Feng et al., 2011), 98% carbon removal in mixed algal culture (Prathima Devi et al., 2012) and ~ 36–57% carbon removal in diluted piggery wastewater (Wang and Lan, 2011). Incomplete removal of TOC could be attributed to growth inhibition due to either light limitation or presence of refractory carbon/algal polysaccharide secretions. Due to the filtration of cultured broth, visible suspended matter was found to be less (absorbance = 0.04) with ample light penetration during initial stages. Higher biomass densities (absorbance = 2.9) after the day 10

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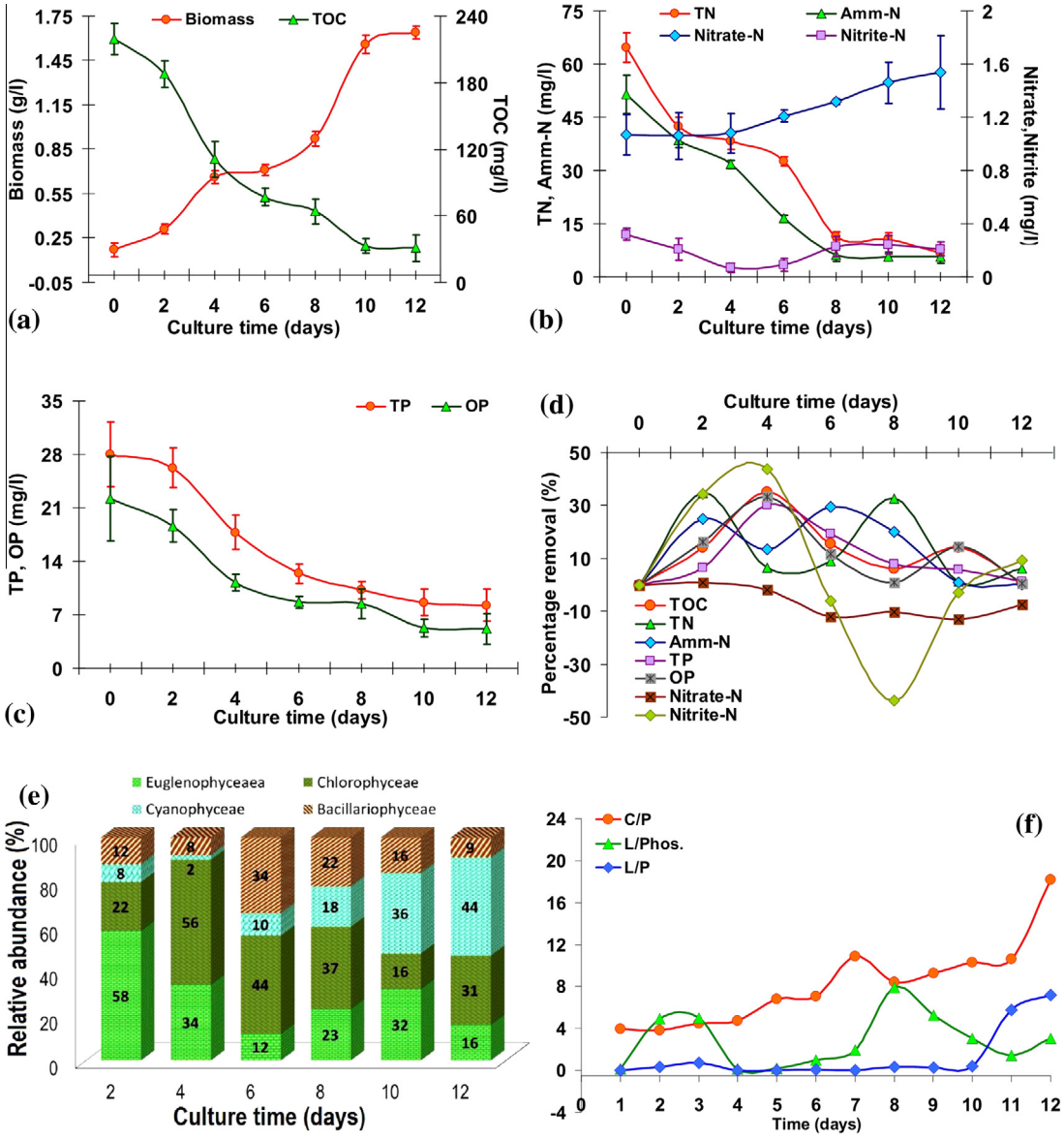

Fig. 1. (a) Biomass growth and Total Organic Carbon (TOC) removal during culture; (b) TN, Amm.-N, nitrate-N and nitrite-N removal curve; (c) TP and OP removal by algal consortia during culture; (d) percentage nutrient removal of the mixed algal consortia during culture; (e) algal species composition variation provided as relative abundance during culture period; (f) ratio's of macromolecular composition and their changes across the growth period.

Table 1
Specific growth rate and biomass productivity.

Day	Algal biomass dry wt. (g)					
	μ	σ	Inc. growth (%)	$\%_{age}$ Growth	SGR (/d)	Biomass productivity (g/l/d)
0	0.17	0.046	–	–	–	–
2	0.31	0.033	45.16	11.38	0.30	0.07
4	0.66	0.042	53.03	28.46	0.38	0.18
6	0.71	0.037	7.04	4.07	0.04	0.03
8	0.92	0.048	22.83	17.07	0.13	0.11
10	1.56	0.062	41.03	52.03	0.26	0.32
12	1.64	0.043	4.88	6.50	0.03	0.04

μ – mean; σ – standard deviation.

have contributed to a light restricted environment (with 5–7% light penetration) ceasing algal growth and consequent TOC removal. Specific growth rates differed from 0.38/d (initial 4 days) to 0.26/d (second phase corresponding to day 6 to day 12) that correlated with corresponding TOC removal of 219 to 111 mg/l ($r = 0.9$, $p < 0.01$) during the initial period and 77–33 mg/L ($r = 0.99$, $p < 0.01$) during the second phase of the exponential growth during algal culture.

Decline of TN from 64.6 ± 4.2 to 6.66 ± 2.1 mg/l (Table 2; Fig. 1b) during the growth period indicate ~90% of nitrogen assimilation

(in the form of ammonium, ~80%) by growing algal cells in the presence of higher quantum of organic carbon. Nitrogen removal of 34.5% was observed during the first 2 days (heterotrophic phase) followed by 32.5% from day 6 to day 8 (mixotrophic phase) and overall removal of 82% was observed during all the 8 days (Fig. 1d). These were comparable to an earlier report (Cho et al., 2011) and they were also relatively higher compared to 78% TN removal of diluted primary wastewater and 54–75% of digested manure (Wang et al., 2010).

NH_4-N had dropped from 51.5 ± 5.5 to 5.76 ± 2 mg/l (89% removal) during 12 days of batch experiment (Table 2; Fig. 1b). This was lower compared to the values in the earlier studies carried out with *Chlorella vulgaris* in artificial wastewater (97% NH_4-N removal) conducted in heterotrophic/mixotrophic mode (Feng et al., 2011), and 96% removal in mixed algal culture grown in municipal wastewaters (Woertz et al., 2009). The incomplete removal of NH_4-N could have been due to carbon limitation or light interception as dense algal cultures were floating (of large and filamentous algae *Spirulina* and *Phormidium,* respectively) thereby interrupting light penetration. However, the current report of NH_4-N removal is better than the reported NH_4-N removal (69%) by *Auxenochlorella protothecoides* UMN280 grown in municipal wastewaters (Zhou et al., 2012b).

The average C/N and N/P ratio of the media were 3.77 ± 1.14 and 1.69 ± 0.67 respectively that favoured nitrogen removal. The decrease in nitrogen values correlated with the increased biomass ($r = -0.9$; $p < 0.005$) indicating nitrogen assimilation during algal growth. Highest NH_4-N removal was observed during the first 2 days (25.04%) and from day 6 to 8 (29.51%), which were similar to nitrogen removal (Fig. 1d).

NH_4-N removal happens due to ammonia volatilisation under high pH and temperature. However, in the present study the major fraction of NH_4-N removal was due to assimilation by algae as pH and temperature were moderate (pH was <8.5 and temperature was 26 ± 4 °C) in the reactor. Fig. 1b shows a gradual increase of nitrate-N concentrations from1.07 ± 0.15 to 1.54 ± 0.28 mg/l due to nitrification. This was lower than 67% of nitrate-N removal in wastewater through mixed algal cultures isolated from wastewaters (Prathima Devi et al., 2012). On the other hand, Nitrite-N decreased slightly from 0.32 ± 0.05 to 0.21 ± 0.05 mg/l due to rapid assimilation by algae and slower nitrification process.

TP values listed in Table 2 showed a decrease from 28 ± 4.3 to 8.26 ± 2.4 mg/l (70% removal) compared to ~72% (Wang et al., 2010), 31–77% (Wang and Lan, 2011), ~84% removal (Cho et al., 2011); ~90% (Feng et al., 2011; Zhou et al., 2012a,b) and ~95% (Yujie et al., 2011). Phosphorus concentration in wastewater was relatively higher (Fig. 1c) than that of carbon and nitrogen (C:N:P = 7.8:2.3:1). Algae used only a small fraction of phosphorus that was available in the system, as a result of which ~30% of phosphorus remained residual in the culture. This fraction was unutilised as algal growth reached saturation due to carbon and nitrogen limitations as well as light limitations in the dense algal culture. Higher phosphorus removal on day 4 (30%) coincided with carbon assimilation that had resulted in a higher biomass growth (Fig. 1d). Excess phosphorus was also revealed by a lower N/P ratio (range 0.8–2.6) of 1.69 ± 0.67, which had not affected either biomass growth or productivity. Ortho phosphate removal (Fig. 1c) efficiency was 78% (22.2 ± 2.4 to 5.13 ± 1.67 mg/l) that was relatively higher than phosphates removal (40–65%) by mixed algal cultures grown in wastewater (Prathima Devi et al., 2012). Higher OP removal (33.15%) had occurred on day 4 (Fig. 1d). Organic phosphates varied from 7.6 to 1.78 mg/l, which constituted to about 16% of TP. Fig. 1c highlights this decline of TP that reached a minimum of ~2 mg/L (on day 8, after which, it stabilised to ~3 mg/l). The conversion of organic phosphorus into inorganic forms aided in the assimilation of phosphorous by algae.

The species composition and dynamics are elucidated in Fig. 1e. Euglenophyceae members such as *Euglena* sp., *Phacus* sp. etc. dominated (58%) during the early culture period up to the first 4 days that coincided with carbon uptake and higher biomass growth. In high organic environment, euglenoids grow in heterotrophic mode (Mahapatra et al., 2013a,b; Ramachandra et al., 2013). Chlorophyceae (especially *Chlorella* sp. and *Chlorococum* sp.) increased from 22% to 56% (day 4), and then, showed a declining trend. Mixotrophic chlorophycean members (*Chlorococcum* sp.) are known to grow faster in nutrient rich conditions (Mahapatra and Ramachandra, 2013).

Bacillariophyceae (*Cyclotella* sp. and *Nitzschia* sp.) considered as facultative heterotrophs (Mahapatra et al., 2013b) increased to 34% (day 6) and showed a gradual decline to 9% (day 12). The species turnover indicated changes in nutrient availability and reactor conditions that was suitable for assimilation and growth of chlorophyceae and bacillariophyceae members resulting in switch over from heterotrophy to mixotrophy. Cyanophyceae (blue green algae as *Phormidium* sp. (filamentous) and *Spirulina* sp. (spiral shaped)) showed an increasing trend (Fig. 1e) from 2% (day 4) to 44% (day 12). These algae are often found as naturally floating (due to dense gas vacuoles) masses in urban lakes and wastewater treatment lagoons (Mahapatra et al., 2013b). The dense gas vacuoles in filamentous algae help in floatation. This highlighted that algal consortia of varied proportion depending on the nutrient availability enables the treatment of wastewater. Diverse species with differential interactions/competition also contributes to the system stability with enhanced biomass growth and efficient removal of nutrients. This required further investigation, which is being undertaken.

3.2. Mechanisms for optimal harvesting of algae – algal floc formation, P accumulation and elemental composition

SEM-EDXA analysis of suspended algal biomass revealed higher organic content comprising ~55% C; 7% N with a higher P

Table 2
Decrease in nutrients and nutrient removal rates.

Day	TOC (mg/l)				TN (mg/l)				Amm.-N (mg/l)				TP (mg/l)				OP (mg/l)			
	μ	σ	Inc. Rem.	% Rem.	μ	σ	Inc. Rem.	% Rem.	μ	σ	Inc. Rem.	% Rem.	μ	σ	Inc. Rem.	% Rem.	μ	σ	Inc. Rem.	% Rem.
0	219	14	–	–	64.6	4	–	–	51.5	5.5	–	–	28	4.3	–	–	22.2	2.4	–	–
2	188	12	14.16	12.16	42.3	2.6	34.52	76.90	38.6	2.12	25.05	49.62	26.2	2.26	6.43	8.18	18.6	1.64	16.22	22.50
4	111	16	40.96	30.20	38.2	2.22	9.69	14.14	31.7	1.11	17.88	26.54	17.8	1.64	32.06	38.18	11.24	1.77	39.57	46.00
6	77	8	30.63	13.33	32.5	1.26	14.92	19.66	16.5	0.76	47.95	58.46	12.4	1.77	30.34	24.55	8.66	0.26	22.95	16.13
8	64	11	16.88	5.10	11.5	1.11	64.62	72.41	6.24	1.89	62.18	39.46	10.22	2.43	17.58	9.91	8.44	0.69	2.54	1.38
10	33	7	48.44	12.16	10.66	1.76	7.30	2.90	5.77	1.17	7.53	1.81	8.62	1.08	15.66	7.27	5.26	1.04	37.68	19.88
12	31	12	6.06	0.78	6.66	2	37.52	13.79	5.76	2	0.17	0.04	8.26	2.4	4.18	1.64	5.13	1.67	2.47	0.81

μ – mean; σ – standard deviation.

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accumulation of ~4% (Fig. S1 provided in the supplementary material). Higher phosphorus accumulation (in the form of Poly-P; Chen et al., 2011) in this biomass was due to algal habitat that stored enormous phosphorus which was coupled with a higher metabolic phosphorus demand to fulfill the cellular phosphorus requirement. Further, phosphorus fractionation was required to understand the metabolic phosphorus demand and phosphorus storage, which would help in estimating phosphorus holding capacity and its relevance to phosphorus recovery and recycling in the environment. In the present case, phosphorus accumulation in the biomass was attributed to higher ortho phosphate concentration remaining in the media (~22%) that had not precipitated out because of low alkaline conditions. In the present study, the suspended matter were mostly in the form of granules (15–45 μm) that comprised of ~50% C and ~33% oxygen with higher levels of Ca (~5%) indicating the formation of $CaCO_3$ salts that get adsorbed over other organic granules or over biogenic Si (from diatoms) in the reactor. The reactor sludge in the final stages showed abundant organic matter with inorganic elements like Si, Al, Mg etc.

The analysis of elemental composition of the algal flocs/scum (helpful in algal harvest), floating on the reactor surface and the surface morphology (Fig. S1 provided in the supplementary material), was done through SEM-EDXA. Smaller flocs (<0.5 cm) mainly composed of green algal cells were flocculated by probable algal derived flocculant (unpublished data) in combination with metal ion. Algal flocculation was due to the negative charges on the cell surfaces during the peak period of algal growth (Chen et al., 2011). Floc formations or auto-flocculation (Salim et al., 2011) have been attributed to pH transitions (Wu et al., 2012) and availability of higher cations (divalent) which lead to mineralisation at the mature stages of algal growth. Filamentous cyanophycean masses as *Phormidium* sp. (1–4 cm diameter) with gas bubbles float in large masses on the surface. This natural phenomenon of aggregation and floatation was useful for efficient algal harvesting. Harvesting of algae has been a major constraint for its commercial use as existing mechano-chemical processes are energy and also time consuming when compared to bio flocculation (Park et al., 2012). Therefore, there were attempts to harvest algal biomass through bio-flocculation involving algal-bacterial interaction (Su et al., 2011) and fungal palletisation-assisted bio-flocculation of algal cells (Zhou et al., 2012a). In such cases, the culture environment plays a major role in phase separations in flocculation and sedimentation.

Table 3 lists the elemental composition of Algal scum (flocs) and other substrates (reactor sludge, naive algal biomass and suspended matter). The elemental analysis showed high concentration of Ca (~26%), Fe (~5%), K (~8%) and Cl (~8%) in the algal surface flocs. However, these elements were low in other substrates as they were primarily organic. The elemental peaks indicated the presence of Ca and P (Fig. S1 provided in supplementary material). Higher froth or algal floc formations had been attributed to Ca, Al, Cl (Chen et al., 2011; Park et al., 2012; Wu et al., 2012). Phosphorus helps in coagulation while $CaCO_3$ (calcium, carbon and oxygen) and $FeCl_3$ (due to higher chlorine and iron) aid in the formation of flocks in algal scum.

SEM studies highlighted diverse surface morphologies of algae with vast surface area that helped in adsorption, and uptake of elements. Algal cell surface with extracellular polymeric secretions (mucilage) bind the cells together, while the gas vacuoles aid in floatation and auto flocculation. Surface complexation by attachment of metals on cell surfaces also helped in floc formation. However, this required further research focussing on surface morphologies, texture and ionic interactions with cells surfaces.

3.3. FTIR analysis

ATR-FTIR spectra of the algal consortia showed five distinct absorption bands (corresponding to wave numbers 1800–800 cm^{-1}) representing specific functional groups as per biochemical standards (Stehfest et al., 2005). FTIR spectrogram of the algal consortia showed the quantitative changes in the macromolecular compositions with respect to the nutrient content, etc. during the culture period. The algal consortia showed significant variation in the carbohydrate, protein, lipid and phosphate contents (Table T1, Supplementary material) with an increase in L/P ratio during the final stages.

The daily observations of the variations in cellular macromolecular composition (Table T1) and Fig. 1f highlighted a peak for carbohydrate/amide (C/P) ratio on day 7 and day 12 (highest), inferring a gradual increase in polysaccharides with TOC assimilation. High C/P ratio indicated a net decrease in the protein content (with TN and NH_4-N removal, as discussed earlier) and the synthesis of polymeric carbon secretions helped in aggregation of algae during floc formation. This further demonstrated the potential NH_4-N limitations in triggering the lipid synthesis and accumulation. In the present experiment, the C/P ratio of the algal consortia showed 4.58-fold increase that varied from 3.95 to 18.15 on day 12. This was higher compared to an increase of 1.14 and 2.24 times in *Phaedactylum tricornitum* (Stehfest et al., 2005) and lower than 7- and 9.6-fold increase in *Chlamydomonas reinhardtii* (Dean et al., 2010) and *Microcystis auregonosa* (Stehfest et al., 2005) respectively. Nitrogen starvation leads to diversion of carboxyl part (precursor to protein) to lipid and polysaccharides enhancing C/P values. This is in contrast to low C/P (0.6) in *Pediastrum duplex* under natural conditions (Dean et al., 2012).

Lipid to amide (L/P) ratio increased on day 3 and reached threshold on day 10 as the culture inched towards the stationary phase (Fig. 1f) due to accumulation of lipid at the expense of protein. L/P ratio increased from 0.014 to 3.08 during the culture period and was higher compared to 1.27- and 2.42-fold under nutrient sufficient and deficient conditions in *Phaedactylum tricornitum* (Stehfest et al., 2005), 1.5-fold in *Scenedesmus subspicatus* (Dean et al., 2010) and 6.75-fold in *Chlamydomonas reinhardtii* (Dean et al., 2010). However, *Pediastrum duplex* under natural conditions, showed a minimal L/P of 0.2 (Dean et al., 2012). These results further substantiated that lipid accumulation was a function of nutrient deprivation at an advanced stage of the cell culture.

Increased lipid/phosphates (L/Phos.) exhibit bimodal pattern with the first peak during the initial 4 days and the second one spanning from day 7 to 10 (Fig. 1f). *Phaeodactylum tricornitum*

Table 3
SEM EDXA elemental analysis of algal biomass, flocs, suspended matter and reactor sludge.

	C	N	O	P	S	Cl	K	Ca	Fe	Na	Mg	Al	Si	F	Total
Algal scum	36.97	0.01	10.64	2.36	2.72	7.85	8.31	26.31	4.83	–	–	–	–	–	100
Reactor sludge	61.39	3.45	17.65	2.32	0.61	3.7	0.63	5.54	–	0.99	1.81	0.56	1.35	–	100
Algal biomass	69.49	6.7	14.77	3.54	1.69	–	0.58	2.69	–	–	0.54	–	–	–	100
Suspended matter	50.64	–	32.72	2.53	0.63	–	0.07	5.25	1.61	0.25	0.15	1.97	2.54	1.64	100

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had L/Phos ratio increased by 1.23 and 1.2 times under nutrient sufficient and stress conditions on day 14 (Stehfest et al., 2005).

3.4. total lipid content, productivity, potential yield and FAME composition

Algae grown in carbon rich wastewater stored more lipids through heterotrophy (Bhatnagar et al., 2010). Organic and inorganic forms of carbon get transformed into algal biomass primarily due to mixotrophy and subsequently get accumulated as lipids. Total lipid in the current study was significantly contributed by the dominant algae -euglenoids, chlorococcales and *Phormidium* sp. as had been reported earlier (Ramachandra et al., 2013; Mahapatra and Ramachandra, 2013). Maximum total lipid content of the algal consortia was 28.5%, which was higher compared to 16% in *Chlorella minutissima* UTEX2341 grown in optimised media (Li et al., 2011), 20–42% in *Chlorella vulgaris* grown in artificial wastewaters (Feng et al., 2011) and 12–80% in mixed algae grown in untreated carpet mill effluents (Chinnasamy et al., 2010).

Algal consortia of euglenoids, chlorococcales and *Phormidium* sp. were suitable to treat Indian municipal wastewaters at a decentralised level. Higher biomass growth with moderate lipid content (28.5%) amounted to lipid productivity of ~31.77 mg/l/d suggesting higher lipid content with biomass increase. This was higher compared to 24 mg/l/d in dairy and municipal wastewaters (Woertz et al., 2009), ~23 mg/l/d in wastewater effluent (Cho et al., 2011), 4–4.5 mg/l/d in wastewater (Chinnasamy et al., 2010); 2–6 mg/l/d in *Chlorella pyrenoidosa* (Wang and Lan, 2011); 7–23 mg/l/d in filtered secondary treated wastewater (Cho et al., 2011) and lower compared to 40 mg/l/d from *Chlorella vulgaris* grown in artificial wastewater (Yujie et al., 2011), 44–147 mg/l/d in a semi-continuous system (Feng et al., 2011) and 200 mg/l/d in *Chlamydomonas reinhardtii* grown in concentrated wastewater (Li et al., 2011).

The surface productivity of algal biomass was 6.36 g/m^2/d accounting to a lipid productivity of ~19.08 tonnes/hectare (Ha)/yr (or 17.69 kl/Ha/yr considering 300 sunny days per year (as in Bangalore), which was higher compared to 11 kl/Ha/yr (Woertz et al., 2009). However, this yield depends on outdoor conditions like the season, temperature, nutrient availability as well as predation by zooplanktons and grazers, seasonal algal succession, wash-outs, etc. The utility of algal fuel depends on lipid and thermal characteristics.

Table 4 lists the percentage composition of respective FAME with the time of retention, peak area, and percentage correlation maxima. Composition of methyl esters in lipids in addition to the chemical characteristics like, number of carbon atoms, degree of saturation/unsaturation etc. governs the quality of biofuel (Ramachandra et al., 2009). Eighteen different fatty acids were identified in the FAME mixture extracted from wastewater algal consortia. The lipid profile of the algae showed a dominance of C16:0 and C18:0 methyl esters, and moreover, greater than 91% of the FAME comprised of C16 to C18 FAME indicating good quality biofuel (Table 4). C18 fatty acids are very important from a fuel perspective (Cho et al., 2011) as it is an integral part of vegetative oil comprising of stearic, oleic, linoleic and linolenic acids. However, <25% of unsaturated fatty acids was recorded in the FAME mix. Higher abundance of saturates as palmitate and stearate signify better fuel properties with smooth ignition (Feng et al., 2011) and higher cetane values which provide good combustion with lower emissions (Li et al., 2011). The unsaturated fraction of biodiesel is useful for achieving increased cold filter plugging point and hence higher stability at low temperatures. The susceptibility of polymerisation (Venkata Mohan et al., 2011) and oxidation of fuel (Damiani et al., 2010) during storage is low as the algal oil has low PUFA (~10%) content. The FAME composition of algal consortia (in the present study) follows an order, with major contributions from saturates as palmitic acid C16:0 (42.30%) > stearic acid C18:0 (25.69%) > unsaturates as oleic acid C18:1(11) (10.87%) > C18:2(9) (5.27%). This is comparable to algae grown heterotrophically in piggery wastewaters with – *Chlorella pyrenoidosa* C18:2(30%) > C16:0(28%) > C18:3(27%) > C16:1(10%) (Wang and Lan, 2011) and *Chlorella minutissima* UTEX2341 C18:1(9) (46.13%) > C18:2 (26.7%) > C16:0 (13.5%) > C18:0 (3.4%) and 16% lipid (Li et al., 2011). About 76% of the total FAME comprised of saturated fatty acids contrary to studies (Li et al., 2011; Wang and Lan,

Table 4
Percentage composition of FAME with the retention time in columns.

No.	FAME (chemical name)	Chemical formula	Retention time	Peak height ($\times 10^6$)	Corr. area ($\times 10^6$)	Corr. % Max.	% FAME
1	Butanedioic acid, dimethyl ester	C6:0	7.49	2.54	10.86	6.51	2.76
2	Methyl tetradecanoate	C14:0	24.92	0.75	3.31	1.99	0.84
3	Tetradecanoic acid, 12-methyl ester	C14:0-12 CH_3	26.91	1.12	6.33	3.79	1.61
4	Methyl 4,7,10,13-Hexadecatetraenoate	C16:4(4,7,10,13)	29.99	1.19	7.49	4.49	1.90
5	7,10-Hexadecadienoic acid, methyl ester	C16:2(7,10)	30.27	0.99	4.50	2.7	1.14
6	7,10,13-Hexadectrienoic acid, methyl ester	C16:3(7,10,13)	30.47	1.33	6.57	3.94	1.67
7	7-Hexadecenoic acid, methyl ester	C16:1(7)	30.66	1.32	5.43	3.26	1.38
8	Hexadecanoic acid, methyl ester	C16:0	31.61	12.88	167	100	42.30
9	Heptadecanoic acid, methyl ester	C17:0	34.42	0.49	3.72	2.23	0.95
10	8,11,14-Eicosatrienoic acid, methyl ester	C18:3(8,11,14)	35.92	0.06	0.19	0.12	0.05
11	Methyl Octadecatetraenoate	C18:4(6,9,12,15)	35.94	0.28	1.19	0.71	0.30
12	9,12-Octadecadienoic acid, methyl ester	C18:2(9,12)	36.49	3.72	20.78	12.46	5.27
13	9-Octadecadienoic acid (Z)-metyl ester	C18:1(9)	36.74	7.30	42.85	25.7	10.87
14	11-Octadecadienoic acid, metyl ester	C18:1(11)	36.84	1.85	5.59	3.36	1.42
11	Octadecanoic acid, methyl ester	C18:0	37.63	10.9	101	60.73	25.69
14	Eicosanoic acid, methyl ester	C20:0	43.08	0.35	1.66	1	0.42
15	Docosanoic acid, methyl ester	C22:0	48.39	0.35	1.48	0.89	0.38
16	Tetracosanoic acid, methyl ester	C24:0	51.69	0.51	1.42	0.85	0.36
17	Hexadecanoic acid, methyl ester	C26:0	53.80	0.48	1.51	0.91	0.38
18	Octacosanoic acid, methyl ester	C28:0	55.52	0.46	1.28	0.77	0.32
	Saturates						76.00
	Unsaturates						24.00
	Monounsaturated FA						13.67
	Polyunsaturated FA						10.33
	Unsaturate to Saturate ratio						0.32
	C16–C18						91.04

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2011) that showed the dominance of unsaturated fatty acids. This was due to the extent of light exposure or high light intensities with larger dark periods that influenced nature and quality of fatty acids (Su et al., 2011; Seyfabadi et al., 2011). Culture experiments were conducted in outdoor open reactors with a 12 h light: 12 h dark photoperiods and the lipid profile showed that fatty acids with ~84% contributions from saturated (C16:0, C18:0) and unsaturated (C18:1, C18:2) fatty acids that are considered key components for biodiesel.

3.5. DSC analysis

Thermal analysis through DSC provides quantitative information about the substrate from the perspective of energy generation. Thermal decomposition was assessed through thermogram for (a) algal matter, (b) spent algal matter after lipid extraction, (c) algal mass floc floating on the surface of the reactor and (d) reactor bottom sludge. This comprised of three different stages (i) initial stage (5 °C to ~175 °C) characterised by a large endothermic peak near 80 °C, corresponding to dehydration (Salmon et al., 2009), (ii) intermediate stage (175 °C to 350 °C) comprising of broad exothermic peak due to thermal decomposition of polymers as carbohydrates/lipids and to certain extent decarboxylation of carboxylic groups and aliphatic group dehydration (Silva et al., 2012) and (iii) final stage (at 350 °C to >500 °C) comprising of thermal degradation of aromatic substances (Sostaric et al., 2012; Silva et al., 2012), with a large hike in the exothermic region (and peak >500 °C). Algae sample with two exothermic peaks jointly accounted for 123.4 J/g, which was higher than that of *Chlorella vulgaris* (82 J/g), (Sostaric et al., 2012) and sewage sludge (11.2 J/g) (Silva et al., 2012).

Algal biomass and spent biomass after extraction showed better thermal properties as compared to the surface algal floc and algal sludge (less peaks between 175 and 350 °C). However, the reactor sludge sample and algal spent biomass (after extraction) showed increased heat flow beyond 380 °C due to net decrease in the carbohydrate content with increased molar weight, stability and more aromatic conversions of the organic content. Algae and spent algal biomass (extract) after cell disruption and lipid extraction showed a significant difference in the exothermic peaks ($p < 0.01$) among various substrates considered for the study, suggesting scope for energy derivation with various by-products of the reactor and algal spent biomass.

4. Conclusion

The study demonstrated mixed algal consortia's bioremediation potential (removal of nutrients) with the scope for biofuel production. Mixotrophy with self flocculating abilities of algal consortia aided in the effective treatment of wastewater and algal harvest. This technique if replicated in sewage fed ponds and lagoons helps in the decentralised treatment of water with the value added option for energy generation. This would cater to the regional energy demand while mitigating GHG emissions. Thus, bioremediation through mixed algal consortia meets multiple objectives of sustainable development that would help in treatment of wastewater and avert water crisis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.03.130.

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