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Biogas slurry – An alternative growth media for algae cultivation in algiculture systems with simultaneous reduction algal predation

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Abstract: Biogas slurry provides a complete and high growth rate producing algal cultivation medium thus offsetting the high cultivation costs of raising algal cultures in synthetic growth media. Robust mixed algal consortia can also be cultivated in non-traditional media such as wastewater or biogas slurry along with flooded agricultural crops such as paddy (Algiculture). This greatly enhances the N-sustainability of the algae cultivation. However, since the biogas slurry contains its own set of predators of algal biomass (grazers), it is now important to study the productivities attainable by using slurry in the conventional open ponds and Algiculture setup and understand its impact on grazer population dynamics. In this study we have used diluted biogas slurry (1:10) as growth media and compared algal growth and grazing /predation characteristics for algae cultivated under four treatments namely, Bold's basal media (M), biogas slurry (S), Algiculture (ALGI), Algiculture with biogas slurry as growth stimulant (ALGI-S). The algal biomass cultivated in slurry based systems gave rise to a productivity of 6.8 g/m²/d as harvestable biomass with simultaneous reduction in the algal mass sacrificed to grazers. We thus attempt to show that biogas slurry may be used as a potential growth media especially in Algiculture system which tends to be vulnerable to grazer attack and loss of algal population.

Keywords: Algae, Slurry, Biogas, Nitrogen, Algal grazers, Algal biofuel

1. Introduction: Large-scale sustainable algae production has many challenges of which two most important are a.sustainable nutrient source and b.prevention of algal predation. Algiculture was reported to be a simple method of cultivating adapted algal consortia in flooded paddy lands when the leaf area and therefore shading was low at the early stages of paddy cultivation. Close to 18Million ha of land under flooded paddy in India can thus adopt double cropping thereby creating a potential to double the biomass yields from flooded paddies. Algiculture was thus reported to be one of most economic lage-scale algae cultivation technique [1] where the nutrient required for algae cultivation (various macro and micro nutrients) can be recycled from one crop to another in order to enable nutrient sustainability as well [2]. Fertilizers destined for the paddy crop could be applied to the algal crop. When the algal crop is harvested, its lipid and biomethane potential extracted could be returned to paddy land so as to fertilize the paddy crop at appropriate stages. To make available small losses that are expected as well as provide other nutrients, there is a need to identify a sustainable, naturally available, nutrient-rich source for algal production by both the conventional open pond as well as Algiculture systems [3]. A significant extent of N and P content of biogas slurry is in soluble form and has the potential to be lost or leached when fresh slurry is applied to soil or filtered to extract storable compost. Trapping this in algae makes both the technologies paddy cultivation and biogas plant operation more sustainable – resource conservation wise speaking.

Modern anaerobic digesters, especially ones using kitchen wastes or other pulverized solid and liquid wastes do not produce an output slurry with high particulate content for latter use in agriculture just as it is done for cattle dung slurry (Gobar-gas plant slurry). Further, when digested cattle dung slurry is concentrated for storage and re-use later, a lot of centrate/filtrate is generated and there is generally no immediate use for it. Biogas slurry and urban wastewater provide a complete, low cost and high growth rate producing algal cultivation media thus offsetting the high cultivation costs of raising algal cultures in synthetic growth media [4]. Much of the plant nutrition such as N, P and K of this so called slurry (digestate) are in soluble or micro-particulate forms and are therefore highly labile and mobile [5]. During anaerobic digestion, most of these macro and micro nutrients become readily available (labile) which in turn enables their usage as fertilizer in various agricultural applications [6]. Unless capture of these nutrients in reusable forms are brought into effect within 1-5d after release of the biogas “slurry” these will be rapidly and permanently lost or immobilized into forms that will make it difficult to reuse and impacts overall sustainability in a drastic way. Earlier studies have suggested that mixotrophic growth of algae would aid their recovery in a reusable form of harvestable algal biomass. This algal biomass upon energy recovery as algal lipid (biodiesel) followed by biomethanation, leaves behind recalcitrant C and a large pool of nutrients that needs to be returned to the flooded paddy crop nearby. The anaerobic processes conserve most of the labile N and that small fraction as ammoniacal would be quickly picked up by the algae. Such a system completes the nutrient cycle with very low losses in the overall path /cycle [7]. It is well known that the flooded paddy systems loses between 60-70% of added N alone and algae can be good trap to these potential losses when handled appropriately. This schema thus converts an emerging pollution problem to that of a protection solution [8]. Algae take up both ammoniacal forms emerging from biogas slurry application as well as nitrate-N forms emerging from nitrification processes and are therefore ideal candidate organisms for picking up such N without need for nitrification to set in and make it plant available [9].

Further, high rate cultivation of algae (large population of algae) in the open always attract a large populations of grazers that decimate valuable algal populations to insignificant levels in a very short time of 72-96 h. This then requires strategies to control or escape grazer outgrowth for which primary information on the species involved, their feeding pattern, preferences and means of escape would be required [10]. Biogas slurry retains a significant population of grazers that grow quickly and are species specific and can outcompete the more damaging grazers in the wild. A combination of two strategies namely the cultivation of an algal consortia instead of pure culture and use of biogas slurry with species specific hardy grazers to outcompete natural grazers has been attempted. Therefore growth and functionality of algae grazers is important to determine the role of biogas slurry in establishing the sustainability of algal biomass recovery and reuse from various algal cultivation systems.

2. Materials and Methods

2.1. Biogas Slurry Setup: Biogas slurry was collected from a cattle-dung + biomass fed biogas plant at Centre for Sustainable Technologies, Indian Institute of Science, Bangalore. The digested slurry was allowed to clarify by allowing digested particles to settle for about 6hrs in a 0.5m long container and the clarified supernatant was diluted ten times and used as the culture media with and without soil base (SL and ALGI-SL, respectively).

2.2. Microalgae species and media composition: A mixed algal consortia, consisting of *Chlorella sp.* and *Chlorococcum sp.* in the ratio of 108:1 was collected from a wastewater treatment plant at Mysore, Karnataka (between 12.27 °–12.27 °N and 76.65 °–76.66 °E) and maintained as an outdoor culture in Bolds Basal media [11] at growth phase. Typically about 120kg per hectare of Urea is applied on the flooded paddy fields. In order to mimic a typical flooded paddy conditions, farm grade urea was applied to both the treatments at 1.2 g/0.1m² [12]. 0.75 g of KH₂PO₄ was added as a source of phosphate in the Algiculture setup. While in the Media setup, other macro and micro nutrients were added based

on the composition of Bold's basal media [11]. The inocula of the mixed consortia algal species mentioned above was thoroughly homogenized and added to the reactors ($\sim 10^8$ cells/ml, 100 mL inoculum volume). The reactors were placed in an open space under sunlight with 9:15 h light/dark period for a duration of 32 days, until three consecutive harvests of the auto-flocculated algal biomass was collected. The evaporative losses (about 300-700 ml) were compensated every day with the addition of equivalent amount of deionized water to the reactors. A sample of 50 mL of the culture media was collected every day at 12:30 PM and the volume was replaced immediately with deionized water.

2.3. Algiculture setup: In order to study the effect of soil on the algae growth, a reactor with 13 L capacity and with a surface area of 0.1 m^2 was used. 10 L of algal media (as mentioned in the previous section) was added above 5 kgs of soil after saturating the soil with water (Figure 1). This was done to ensure that the added media was not absorbed by soil and was available for algal growth for a long time. The overall system was designed such that about 10 cm standing water (as in a flooded paddy field) was available for algal growth. Prior to inoculation, water was allowed to stand on the soil for 4 days for the settling of soil particles such that light penetration is not hampered for algal cultivation. The initial and final carbon and nitrogen contents of the soil were quantified as mentioned in the following sections.

2.4. Biomass estimation: To estimate the dry biomass of the algae, a known quantity of the sampled broth (10 ml) was centrifuged at 5000 rpm for 10 mins and the pellets were repeatedly washed with distilled water (at least twice) and dried to a constant weight at $60 \text{ }^\circ\text{C}$ [13, 14]. The biomass productivity was calculated using the equation

$$\text{Biomass productivity (g/l/d)} = (N - N_0) / T \quad \text{Eq. (1)}$$

Where, N (in g/L) was the concentration of the biomass at the end of the cultivation period, N_0 (in g/L) was the concentration of the biomass at the beginning of the cultivation period and T was the duration of the cultivation in days [11].

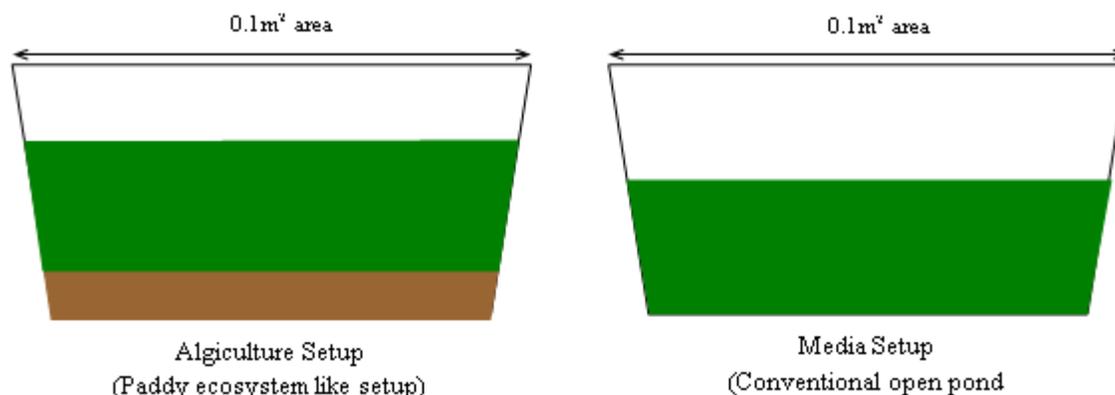


Figure (1): System design of Algiculture and media setups

2.5. Cell count: Algal cell count, of both individual species and total cell count was obtained on a daily basis. Species were identified using a light microscope (Labomed) at 400X magnification with the help of morphological keys as per literatures [15 - 18]. Drop count method was employed for counting algal population in five random fields in triplicate for each sample. The average count was then reported for every sample [11] and the algal counts were then expressed as their logarithmic values.

2.6. Chlorophyll and Carotenoid estimation: A 10 ml of the culture was centrifuged at 5000 rpm for 5 min and the pellet was treated with a known volume of methanol at the ratio of 1:1 and kept in water bath for 30 min at $60 \text{ }^\circ\text{C}$ [19]. Absorbance of the pooled extracts was measured at 450, 652 and 665 nm

in the UV-visible Hach spectrophotometer (DR5000-03) and chlorophyll-a, chlorophyll-b, total chlorophyll and total carotenoid were estimated using equations [20] which are as follows:

$$\text{Chlorophyll-a (Ca)} = 16.72A_{665.2} - 9.16A_{652} \quad \text{Eq. (2)}$$

$$\text{Chlorophyll-b (Cb)} = 34.09A_{652.4} - 15.28A_{665.2} \quad \text{Eq. (3)}$$

$$\text{Total Chlorophyll} = 1.44A_{665.2} - 24.93A_{652.4} \quad \text{Eq. (4)}$$

$$\text{Total Carotenoid} = (1000A_{470} - 1.63Ca - 104.96Cb)/221 \quad \text{Eq. (5)}$$

2.7. Analytical methods for TKN, TOC and total lipid estimation: Free or available ammonia was estimated on a daily basis to study the availability of nitrogen as ammonia for algal growth. Total Kjeldal Nitrogen (TKN) and nitrate of the soil and algal culture (initial and final) were estimated as per APHA [21]. Total lipids in the harvested algal biomass were extracted with chloroform-methanol at the ratio 2:1 and quantified gravimetrically [22]. The organic matter content in the soil used in the experiment and the total organic carbon in the algal biomass was determined by chromic oxidation method as described in Compendium of Indian Standard in Soil Engineering [23].

2.8. ORP, pH and DO: ORP, pH and DO was measured in all the setups using YSI probe (Model No.556), which was calibrated every week using appropriate standards.

2.9. Statistical Tests: ANOVA and all other statistical tests were carried out in the statistical language R, version 2.3.0 (R Development Core Team, 2006).

3. Results and Discussion

3.1. Biomass and growth rates of algae: The biomass, both in terms of dry weight and total cell count was highest in ALGI with a productivity of 5 g/m²/d, followed by ALGI-SL with 3.5 g/m²/d of suspended biomass (Figure 7). The algal biomass was harvestable in the soil base systems with harvestability of 61 g/m²/week in ALGI and 48 g/m²/week in ALGI-SL. In M and SL, the algal biomass never was harvestable as clumps or flocs. This suggests a key role of soil in clump formation and microscopic analysis indicated that *Cyanophyceans* in the soil played an important role in clump formation. The overall productivities of the soil base systems are on an average about 2.5 times greater than the average productivities of M and SL (Figure 2, 3, 4).

In terms of algal species dynamics, the inoculum initially comprised of 99 % *Chlorella sp.* In setups with a soil base, *Diatom sp* were the first ones to appear in suspension on day-6 followed by *Scenedesmus sp* and *Anacystis sp* in ALGI and ALGI-SL respectively. By Day-11, *Chlorococcum sp* began to appear ALGI-SL, whereas, ALGI remained stable with *Chlorella sp*, *Anacystis sp* and *Diatom sp*. from day-6 [Figure 4 and 5]. In M the *Chlorella sp* population remained stable until day-10, after which appearance of *Anacystis sp* and *Scenedesmus sp* occurred on Day-11, followed by *Chlorococcum sp* on day-13. In SL, *Chlorella sp* stability was lost on day-8, when *Anacystis sp* appeared on day-8, followed by *Scenedesmus sp* on day-12 and *Chlorococcum sp* on day-13. On the final day of the experiment, *Anacystis sp* was the most dominant species in M, ALGI, and SL with the cell count being 66, 94 and 84 % of the total algal population, whereas in ALGI-SL, *Chlorella sp* continued to dominate (74 %) with *Chlorococcum sp* being the second most dominant (23 %). The presence of *Cyanophyceans* in the soil base setup namely ALGI and ALGI-SL enable the formation of clumps and flocs, thereby making them easily harvestable. We indicate that a soil base in the cultivation setup enables clumping and floc formation and therefore easy harvestability of the algal biomass.

Table (1): Growth rates of various algal species and their DOA in all the treatments

Algae Species	Parameters	M	SL	ALGI	ALGI-SL	Average Growth rate
<i>Chlorella sp.</i>	Growth Rate	0.26	1.16	1.09	0.29	0.7
	DOA	0	0	0	0	
<i>Scenedesmus sp.</i>	Growth Rate	1.8	0.46	NA	0.44	0.90
	DOA	11	12	NA	11	
<i>Diatom sp.</i>	Growth Rate	NA	NA	1.54	1.01	1.28
	DOA	NA	NA	6	6	
<i>Chlorococcum sp.</i>	Growth Rate	0.92	0.85	0.85	NA	0.88
	DOA	13	13	13	NA	
<i>Anacystis sp.</i>	Growth Rate	1.38	2.01	2.15	NA	1.85
	DOA	11	8	8	NA	
Average Growth Rate (all species)		1.08	1.12	1.14	0.58	

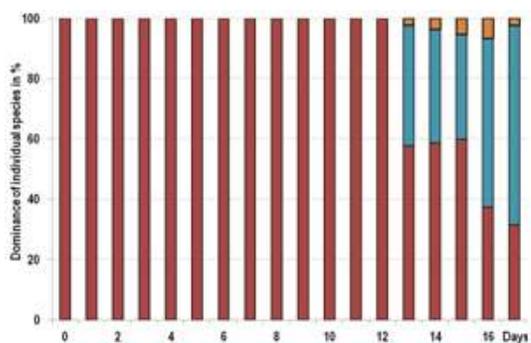


Figure (2): Algal species in Media

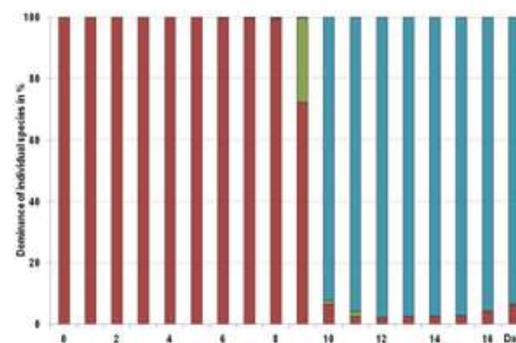


Figure (3): Algal species in ALGI

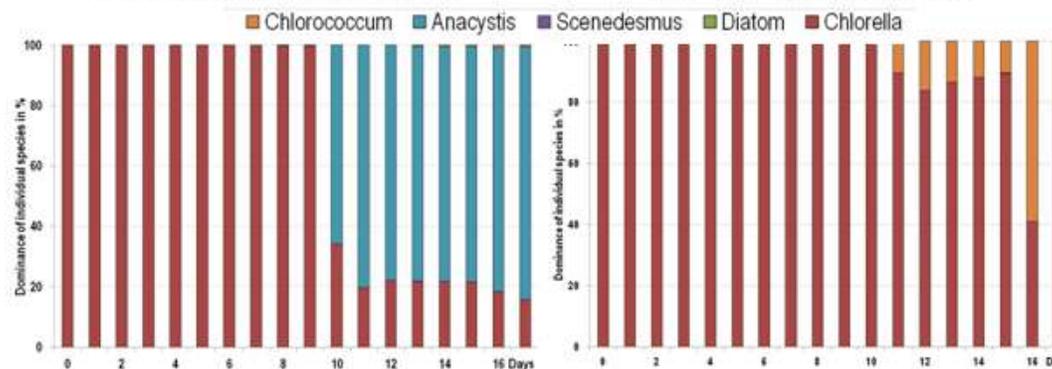


Figure (4): Algal species in SL

Figure (5): Algal species in ALGI-SL

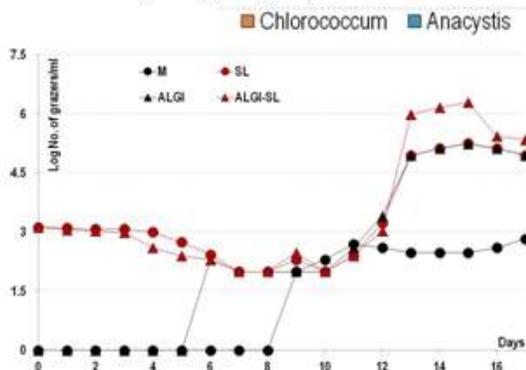


Figure (6): Total Algal Count

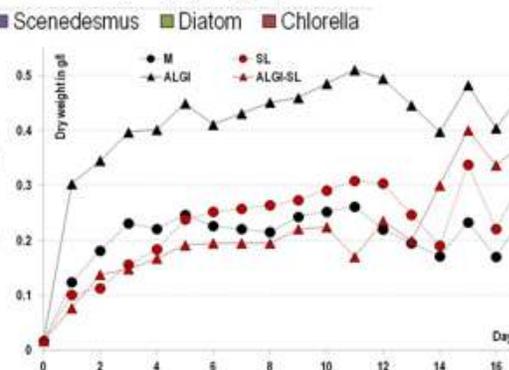


Figure (7): Biomass in suspension

Highest growth rate was observed for *Anacystis sp* in the soil base setups (< 2/d), with an average growth rate of 1.85/d, followed by *Diatom sp* with a growth rate of 1.3/d (Table 1). Soil has been observed to contribute to the *Diatom sp* population. *Chlorella sp* being the largest species in terms of size (> 4 µm), has the highest bio volume has the least growth rate as compared to all the other algae species in all the treatments. SL seems to have supported *Chlorella sp* growth to the maximum contributing to the highest growth rate of 1.16, as compared to all the other treatments. When the average growth rate of overall algae population was compares, it is seen that ALGI and SL had higher growth rates as compared to M, while ALGI-SL had the least [Figure 6]. From these results we infer that a soil base supports algal growth to a large extent as compared to conventional setup (M). ALGI-SL had high turbidity in suspension (> 900 NTU), which did not support easy penetration of light and hence algal growth in this setup was hindered.

Grazer count: Slurry, collected from the biogas plant contained *Ciliophora sp* (at 10⁴ individuals/ml) and facultative *nematodes* (9 /ml, Figure 8). Soon after inoculations, when the slurry was diluted and exposed to aerobic environment, immediately the *nematodes* failed to survive, while the population of *Ciliophora sp* drastically declined from 1000 to 0 within 7 days coinciding with the appearance of *Daphnia sp* (Figures 8 and 9). *Daphnia sp* being a natural predator of *Ciliophora sp*, there was a 99.9 % probability of *Daphnia sp* having preyed upon *Ciliophora sp*. (Figure (10)).

In ALGI and M, initially, there were no grazers. However, as the algal population attained a stationary phase (at around day-6 and day-10), *Daphnia sp* began to appear. The growth rate of *Daphnia sp*. was calculated to be 0.6 and 0.8 respectively, which shows that soil favours grazer growth in algae culturing systems. When the slurry based systems were compared, a growth rate of 0.85 and 0.76 respectively was observed in SL and ALGI-SL respectively. The overall comparison of growth rates of *Daphnia sp* shows that M is least supportive when compared to all treatments. Slurry based systems seem to enhance the growth of *Daphnia sp*.

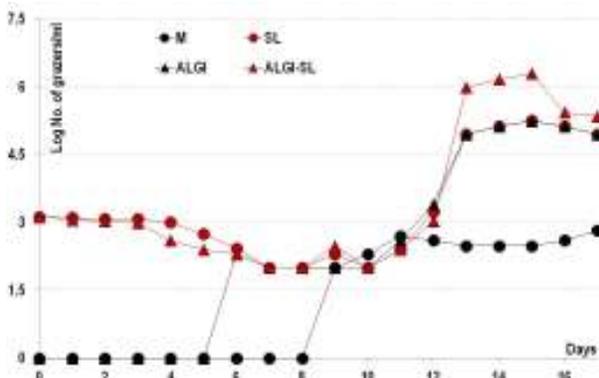


Figure (8): Total grazer count

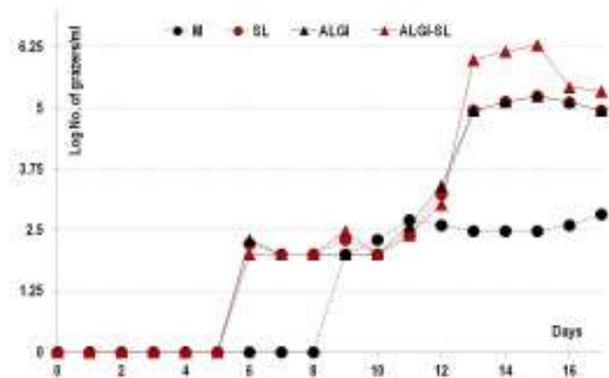


Figure (9): Population of *Daphnia sp*

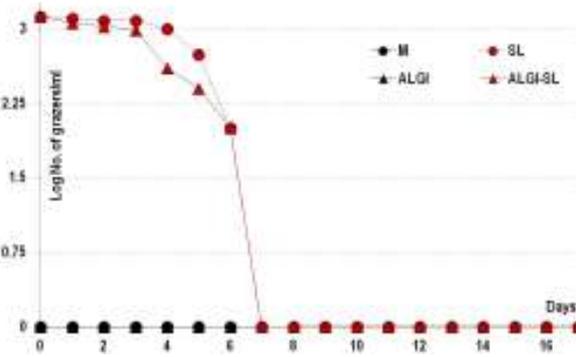


Figure (10): Population of *Ciliophora sp*.

3.2. Nitrogen: An alga’s most preferred form of N is ammonia. The ammonia peaks in M and ALGI correspond to the release of ammonia from urea, which was added as the N source in these two treatments (Figure (11)). In SL and ALGI-SL, the peaks are observed till day-4, after which the peak declines but neither of the peaks touch zero and a minimum residual free ammonia of a least 1.2mg/l was found in all the treatments suggesting that N as ammonia was never limiting the algal growth in any of these treatments. When the TKN levels were plotted (Figure 12) it showed two distinct peaks in all the treatments, except in M. The first peak is a characteristic of free ammonia release in the culture broth, with simultaneous reduction of ammonia uptake by algae as the algal biomass enter a stationary phase. Although not distinct, a second peak of TKN without accompanying peak of free ammonia may be attributed to contribution from the bacterial or grazer biomass as this peak coincides with the appearance of *Daphnia sp.* In general, it can be seen that the overall algal uptake of N reduces by day-8 when the algal biomass appears to enter a stationary phase. The N content of algal biomass did not match the TKN pattern for all treatments. The algal N content in the soil based treatments resembled the TKN and showed a continuous reduction of their N content from day-3 onwards. On the whole, slurry has proven to be a good source of ammonia-N for algal growth in SL and ALGI-SL (Figure (13)).

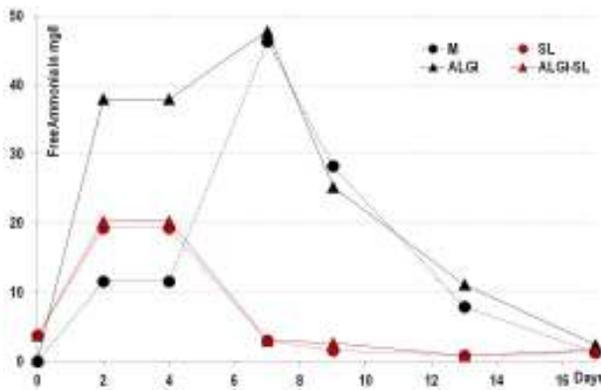


Figure (11): Free ammonia concentrations across all treatments

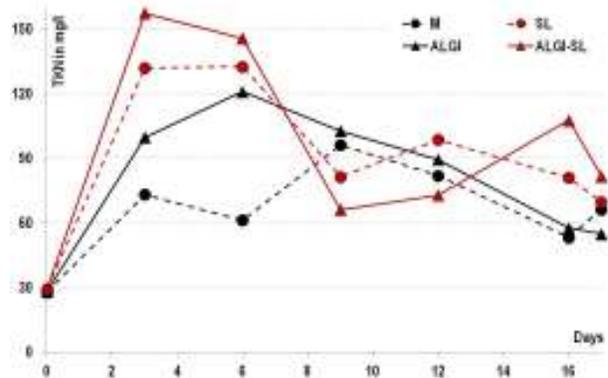


Figure (12): TKN across all treatments

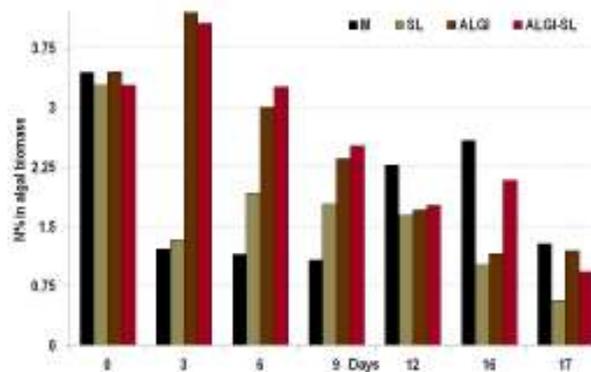


Figure (13): N content of algal biomass in all the treatments

3.3. N-losses and Nitrogen efficiency: The overall concentration of nitrate across all the treatments had never risen above 3 mg/l and does not appear to be significant (Figure 14 and 15). The concentration of nitrate in media accumulated till day-9 and fell to low levels thereafter. Whereas in SL, ALGI and ALGI_SL it continues to build up till day 15 before reducing. This increase occurs in spite of falling ORP levels in most cases. The ORP values in all these treatments range between -50 to -100 mV. As all these data were monitored at mid-day the ORP-NO₃ relations could be poorly brought out and these need to be measured at mid-night to determine the reasons for small levels of build up of NO₃. The

corresponding DO levels were not appreciably high and peaks were measured between 4-12d and levels remained < 4 mg/l in all the treatments after day-12 (at 12.00 PM). The decline in the DO concentration shows that there has not been any significant photosynthetic DO contribution from algal biomass after day-10. The pH values during this period, after day 4, has remained high at 9-10 suggesting CO₂ starvation and indicate conditions that are favourable to the loss of N as ammonia volatilization (Figure 17).

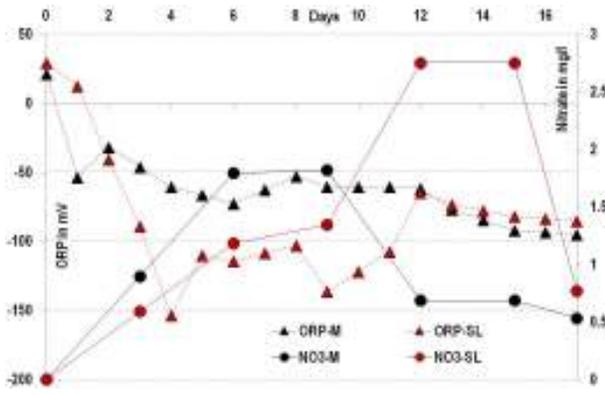


Figure (14): Nitrate concentration and ORP in M and SL

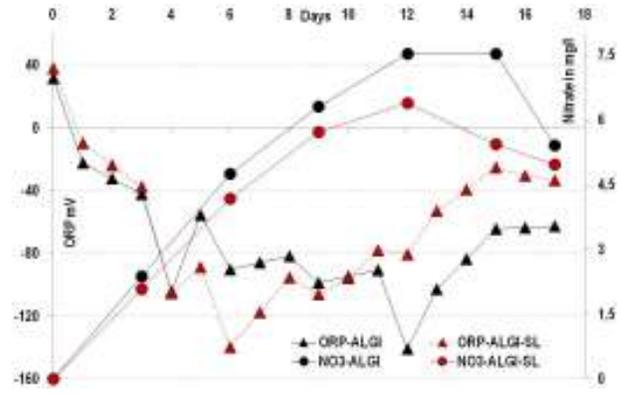


Figure (15): Nitrate concentration and ORP in ALGI and ALGI-SL

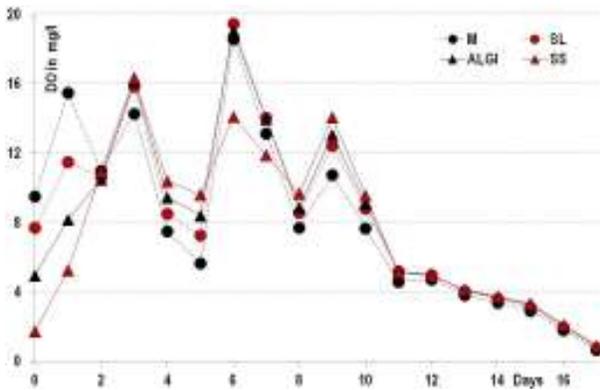


Figure (16): DO concentration across all treatments

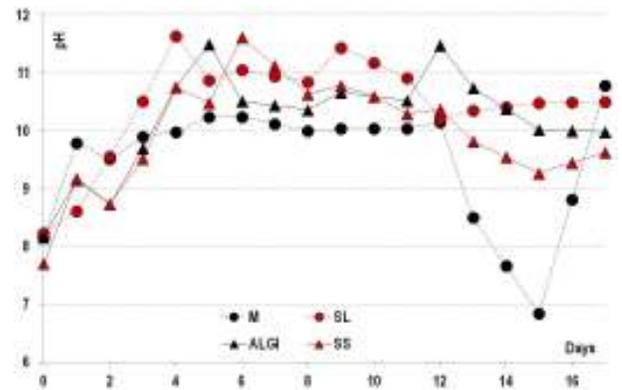


Figure (17): pH concentration across all treatments

The mass balance of N, calculated in terms of N as algal biomass, TKN (with and without algal biomass) and nitrate, and the N capture efficiencies of algal biomass have been depicted in Figure (18). It is seen that ALGI is the most efficient treatment in terms of N capture as algal biomass with 23% of applied N captured in the biomass, while M and ALGI-SL had similar N capture as algal biomass though the quantity of algal biomass was more than 3 times greater. This is because; the N-content of algal biomass in M is two times greater than ALGI-SL And this needs to be explained with more research efforts The overall N-loss has been quantified to be the least in M, however, the N as TKN-in suspension (without algal biomass) has been quantified to contain about 90% of the applied N. This N is mostly not accessible by algal biomass for their growth, unless bacterial degradation of dead cells of algal biomass and various particulates takes place to replenish the locked up nitrogen into the media for algal growth. Both the slurry based systems have relatively lower N-capture efficiency with greater N-losses. Yet, ALGI-SL has a high harvestability of algal biomass with a recovery of about 6.8g/m²/d with somewhat low-N content.

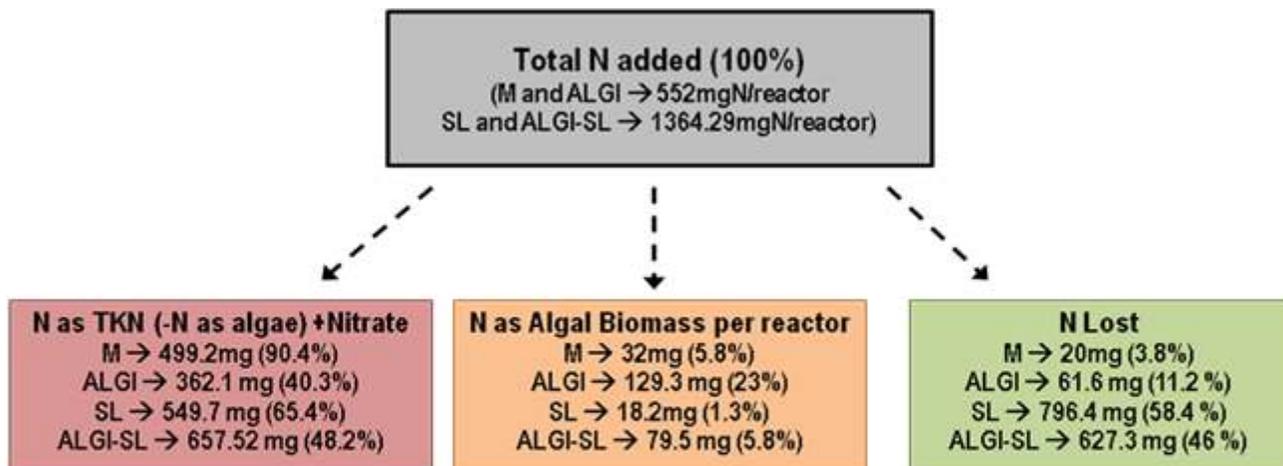


Figure (18): N Balance of the treatments

4. Conclusion: This study has provided a new insight into the possibility of using biogas slurry to augment conventional media for algal growth. When conventional open pond setup is compared with slurry substituted growth media, the growth rate and productivities pick up slowly till 8d after which the biomass productivity seems to be at least 60 % higher than synthetic media and is favourable for use in conventional open ponds, raceways and photobioreactors. When soil alone (ALGI) and soil with slurry (ALGI-SL) are compared, though ALGI (soil alone) proved to be the most efficient system in terms of total biomass productivity and N-capture into algal biomass, ALGI-SL (soil culture with slurry, Algiculture) gave more easily harvestable algae as well as algae without need for synthetic fertilizers – a sustainable growth media in various algae cultivation systems.

The grazer population dynamics in this study shows that the algal sacrifice to grazer is reduced with the use of slurry. This is apparently achieved by the introduction of another trophic level between algae and the most predominant grazer, *Daphnia sp.*, thereby giving the algal biomass an extra window of time to multiply. It appears that with higher algal biodiversity lower is the algal biomass sacrificed to grazers and needs to be confirmed with more research.

This study shows that in conventional paddy ecosystem, when algae is cultivated as a multi-tier crop along with paddy, slurry can be used to substitute the additional N required to be applied to the paddy fields for algal cultivation with minimal sacrifice of algal biomass to grazers. This hence addresses the N-sustainability aspect of simultaneous paddy and algae culture.

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