SUSTAINABLE BIOFUEL PRODUCTION FROM ESTUARINE DIATOMS

T V Ramachandra [a, b, c*], Saranya Gunasekaran [a, b]
[a] Energy & Wetlands Research Group, Centre for Ecological Sciences, [b] Centre for Sustainable Technologies (Astra),
[c] Centre for infrastructure, Sustainable Transportation and Urban Planning, Indian Institute of Science, Bangalore,
Karnataka, India
*Email: tvr@iisc.ac.in, energy.ces@iisc.ac.in

Abstract

Purpose of the study: Microalgae with better carbon sequestration potential, higher biomass, and lipid productivity in comparison to terrestrial counterparts is emerging as a viable sustainable alternative to fossil fuels. Diatoms, a subgroup of a broader category of microalgae are well-known for their role as a bio-indicator in palaeo-climatological studies across historical timelines. Understanding ecology, community structure, and habitat preference of diatoms are prerequisites for prioritizing diatom strains towards sustainable biofuel production along with value-added product extraction.

Method: Selection of appropriate strains, economically viable harvesting and environmentally sound transesterification are the challenges faced in microalgal industry. The present study focusses on understanding the variability in benthic diatom community assemblages across seasons and its affinity to different substrata at fixed sampling locations in an estuarine eco-system through field sampling for twelve months covering all seasons.

Main Findings: The results highlight the tolerance and resilience in select diatom strains in fluctuating water qualities and seasonal variations, the insights much needed during mass cultivation under open environments. Statistical data analyses revealed distinct demarcation between sensitive and tolerant species with selective habitat preferences and resilience to fluctuating environmental conditions. Variations of benthic diatom community structure across various substrata highlights substratum affinity of diatom strains. From the inferences derived through field studies, mixed consortia of diatoms were developed, monitored, and optimized for higher biomass and lipid productivity under controlled laboratory conditions.

Application of the Study: The results highlight the prospects of phyco-prospecting of indigenously grown diatom strains for sustainable biofuel production.

Keywords: benthic diatom, substrate specificity, seasonal dynamics, community structure, phyco-prospecting

INTRODUCTION

Benthic diatoms are highly sensitive to hydrogeological and environmental changes in the water body. Diatom community structure generally varies concerning seasons and land-based discharges with associated nutrient fluctuations to the system in the form of run-offs and point-source pollutions. Understanding diatoms and its community structure have been useful in aquatic health assessment and cost-effective eco-system monitoring studies during the last few decades. Diatoms have aided as bioindicators of environmental changes like eutrophication, metal contamination, salinization, acidification, and catchment alterations with land-use changes (Dixit et al., 1992). Seasonal variability at the regional scale and level of anthropogenic inputs influences community structure in lotic eco-systems, especially the estuaries (Saranya et al., 2018). Anthropogenic inputs include land-based discharges that alter nutrient levels of the aquatic eco-systems in its immediate vicinity. Benthic diatoms are sensitive to such impulsive habitat changes in terms of physico-chemical and hydrological parameters. Hence understanding the variations in community structure at different locations with varied anthropogenic stress levels across seasons would give insights on species preference to a particular substratum and its resilience to fluctuating environmental conditions.

Screening based on this criterion would help in the selection of season tolerant strains for biofuel production. In recent decades, there has been a greater convergence towards sustainable bioresource utilization especially for biofuel production given addressing the country's energy shortages. Dwindling stock of fossil fuels, fluctuating prices in the world oil market, and concurrence among sensible nations to reduce CO_2 levels necessitates focus towards renewable energy. Global biofuel production has risen by 2.6% in 2016, which is higher by 0.4% than 2015's average (BP World Renewable Energy Statistics report 2016). As per the IEA statistical report (2016), the world has relied up to 4% on bioenergy derived liquid transportation fuels and this Figure is expected to increase by 4.3% in 2020. There are numerous global endeavors, evident from the annual publication of 550 research papers after 2005 (Azadi et al., 2017). These endeavors highlight the interest in attaining energy self-reliance and energy security.

Fossil fuel-dependent electric power generation and petroleum products driven transportation sectors especially in India significantly contribute to the greenhouse gas footprint (Ramachandra and Shwetmala, 2012; Ramachandra and Shwetmala, 2009; Ramachandra and Hegde, 2015). Bioethanol and biodiesel are two primary liquid biofuels that are gaining momentum in the country's energy scenario. Lignocellulosic biomasses and seaweeds are considered for bioethanol production, while microalgae are considered as significant lipid resources for biodiesel production. The algae constitute third-generation biofuel feedstock. Figure 1 represents the share of different algal species (family-wise) experimented so far for biofuel production. A major share in the exploitation of algae as lipid precursors is from green microalgae than diatoms to produce

biofuel. Diatoms belonging to the class Bacillariophyceae have a prominent advantage in terms of its ubiquitous presence and are benthic with substratum attachment characteristics, which would considerably lower the cost associated with microalgae harvesting.

Diatoms have proven biofuel characteristics, evident from prioritizing 40 diatom strains (out of 50 microalgae strains) with higher lipid potential among the total 3000 screened strains by Aquatic Species Program hosted by the US Department of Energy (Sheehan et al., 1998). However, the economic viability of microalgae-based biofuel production systems is a major challenge in commercializing diatoms-based biofuels. Hence it is a rationale to choose a diatom strain or a consortium of strains that are consistently productive during all seasons under fluctuating environmental conditions, then a strain that gives higher productivity only under optimal conditions (Hildebrand et al., 2012). Prioritizing potential strains entails understanding respective strains/species' ecological preference under varying field conditions.
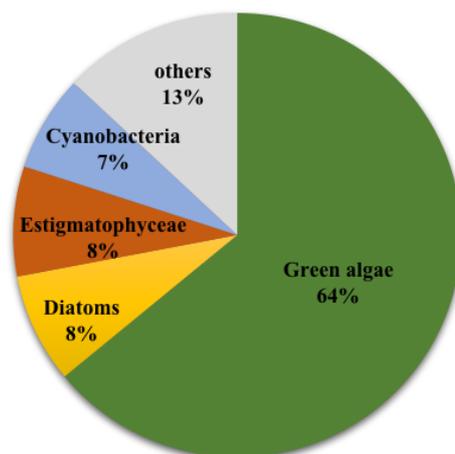

Figure 1: Share of Different Microalgae as Renewable Energy Feedstock

LITERATURE REVIEWS

Estuaries are transition zones or biologically productive ecotones with a constant confluence of fresh and saline waters (Kennish, 2002). They act as filters/buffer zones that trap natural and anthropogenic pollution causing materials between continents and pelagic environments (Qiu et al., 2010). They are dynamic water bodies characterized by strong gradients of salinity and nutrients due to land-based discharges (Lohrenz et al., 1990). Estuaries experience seasonal variations in irradiance, temperature, nutrients, and freshwater inflow, which influences the microalgal community structure (Seguro et al., 2015). Notable contributions to benthic diatom community structure as a part of aquatic health assessment programs in eco-system monitoring investigations are now available (Bere, 2016; Dalu and Froneman, 2016; Hering et al., 2006; Kelly et al., 1998; Reid et al., 1995; Reid, 2005; Schoeman, 1979; Tan et al., 2017; Townsend and Gell, 2005). Diatoms are highly resilient group of micro-organisms (Ramachandra et al., 2009; Ramachandra et al., 2011) with higher productivity, that dominates the world oceans with approximately 40% of the marine primary productivity and 20% of global carbon fixation (Hildebrand et al., 2012; Leblanc et al., 2018; Levitan et al., 2014). Nearly one-fifth of world's photosynthesis is carried out by diatoms (Armbrust, 2009; Nelson et al., 1995). Diatoms comprise of the lowest level marine food web and are responsible for export of carbon from surface to deeper oceans (Tréguer et al., 2018). Moreover, Diatoms gain importance at myriad dimensions as nano-fabricators of biogenic silica, progenitor elements of present day's crude petroleum reserves (Levitan et al., 2014), biofuel precursors, live feed for aquaculture, bio-active compound synthesizers, bio-based silicon semiconductors for solar photovoltaics (Jeffryes et al., 2011; Zhu et al., 2013) eminent source of carotenoids and essential fatty acids, subject of clue in the field of criminology and forensic sciences (Sun et al., 2015). Diatoms not only are the sources of lipids but also has potential scope for value added products due to the presence of high value bio active compounds such as carotenoids, pigments, neuro-excitatory amino acid derivative toxin – domoic acid and Naviculan – a sulphated polysaccharide having antiviral property (Prestegard et al., 2009).

RESEARCH PROBLEM

The study focusses on understanding the habitat wise ecological characteristics and diatom community structure. This not only aids in the selection of promising diatom species but also helps in understanding respective environmental conditions (which helps in the design of sustainable bioreactors). Environmental gradients and seasonal fluxes in water and biological parameters were monitored monthly for a period of one year to understand the influence of physico-chemical parameter and nutrient dynamics on benthic diatom community structure.

This study also intended to capture the seasonal variability with respect to substrata and habitat specificity of benthic diatoms and its relation to community structure. Insights into ecological perspectives would also lower the costs involved in the optimization of growth parameters. Seasons and environmental gradients play a pivotal role in richness, abundance, and community structure of diatoms especially in benthic diatoms - the most predominant group of all micro-organisms in the intertidal zones of the estuary. As benthic diatoms adhere to substrata and form biofilms with the help of exo-cellular

polysaccharide secretion, understanding substratum specificity could give additional insights on the type of community structure and the habitat preferences with substratum specificity of sensitive and tolerant species.

AIM OF THE STUDY

The objectives of the present study are to:

i. Understand the effect of seasonal fluctuations of water quality on diatom species composition;
ii. Investigate the variations in benthic diatom community structure in terms of tolerance and sensitivity through statistical analyses; and
iii. Estimate the biomass and lipid potential of enriched diatom from a particular substratum that had shown the highest species composition during ecological monitoring studies.

MATERIALS AND METHODS

Study Area

Benthic diatoms were sampled across salinity gradients along the shorelines (intertidal regions) of the Aghanashini estuary extending between 14° 27' 53.64" N, 74° 29' 26.88" E - 14° 31' 18.8" N, 74° 20' 54.44" E in the Indian west coast. Six different sampling stations were fixed after a reconnaissance survey of the study region and were categorized primarily based on varying salinity gradients as well as the levels of anthropogenic pressures prevalent at each station. Figure 2 maps the locations of sampling stations. Stations S1 and S2 are mesohaline (5 – 18 ppt) regions, S3 and S4 are polyhaline (8 – 25 ppt) regions at upper reaches, and S5 and S6 are polyhaline (25 – 30 ppt) regions at lower reaches of the estuary. The station S1 to S6 also vary in their levels of anthropogenic stresses evident based on the value of population density of each village around Aghanashini estuary. Station S1 has the second-highest population density among other villages with 14 – 18 persons per hectare. Stations S2 and S5 has a population density of approximately 9 – 14 persons per hectare. Stations S2 and S4 have the least population density of 0 – 4 persons per hectare. S6 falls in the range of 4 – 9 persons per hectare. The thematic layers based on population density (No of persons per Ha.) of the villages around the sampling locations are represented in Figure 2.
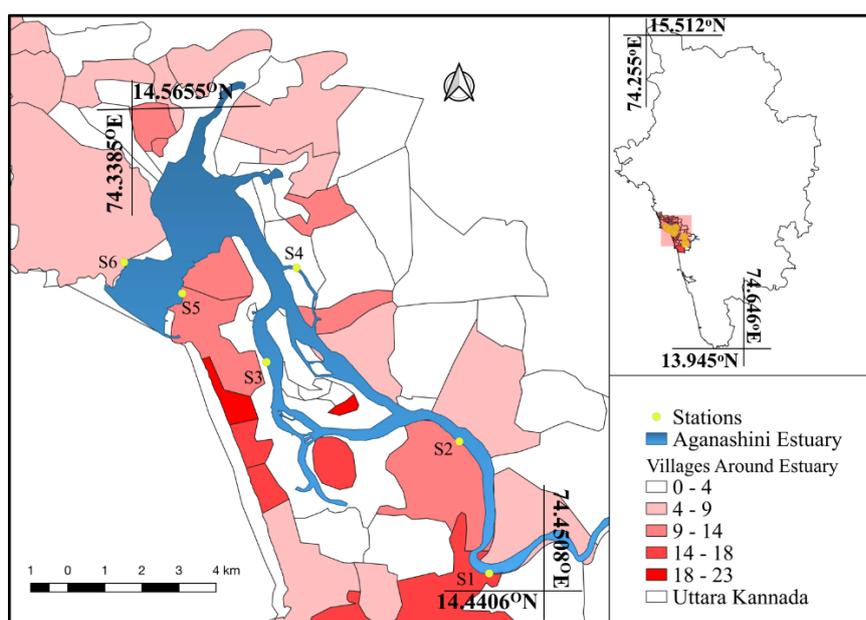

Figure 2: Aghanashini estuary with sampling locations

Sampling

The water along with diatom samples was collected at select sampling locations in the intertidal regions of the estuary, once in every month, during July 2016 to June 2017 at low tide hours of the day as visibility and better accessibility of landmass, mud-flats, sediments, and stones was possible during low tide as the water recedes towards the downstream during low tide. Water and benthic diatom samples were collected from different substrata. Physico-chemical parameters like air temperature, water temperature, pH, DO and salinity were measured at respective sampling locations. Dissolved oxygen was estimated through Wrinkler's titration method following APHA 2005 protocol. Nutrient levels (Nitrates (NO_3^-), Phosphates (PO_4^{3-}) and silicates (SiO_4^{4-}) of the water samples was assessed in the laboratory within 4 hours of sampling, following the standard protocols of APHA (2005).

Diatom Sample Collection and Preparation

Diatom samples were collected at each sampling locations (from substrata such as stones, cobbles, boulders, macrophytes, sedges, shells, halophytes (mangrove plantations), sediments, and sand. Triplicate samples were collected from each

substratum. The epilithic (diatoms attached to stones) were collected by scraping using a clean spatula after washing the substrata under the water to remove the silt deposited on hard surfaces. Epiphytic (diatoms attached to plants) were collected mainly from small mangrove plants grown along the shoreline. A portion of sedges and the mangrove stems were cut and placed inside the sampling bottle containing distilled water and were shaken to dislodge the diatom cells attached on the surface of the plants. Epipelic (Diatoms attached to sediments) and Episammic (diatoms attached to sand) were collected from damp and moist surfaces exposed during low tide by moving the sampling container along the sand/sediment surface. The collected diatom samples were immediately subjected to fixation using 2% Lugol's Iodine to cease cell multiplication and preserved at 4ºC until analysis. Table 1 lists the details of study locations and different substrata considered at each station during the study. Diatom samples collected were pre-treated and processed to remove the organic contents inside the cells using potassium permanganate and hydrochloric acid as per the standard protocols of (Karthick et al., 2013; Kelly et al., 1998; Taylor et al., 2007).

Table 1: Different Substrata Considered for Diatom Sample Collection

Stations	Diatoms Sampled	Different Substrata considered
S1	Epilithic	laterite (Cobble) Stones
S2	Epipelic, Epilithic, and Epiphytic	Sediments, laterite (Boulders) and Sedges
S3	Epilithic, Epiphytic	Shells, Mangrove plants, sediments
S4	Epipelic, Epiphytic and Epilithic	Sediments, Mangrove plants, laterite (Boulders)
S5	Epilithic, Episammic	Rocks, Sand
S6	Epilithic and Episammic	Rocks, Sand

The processed diatom samples were observed under high-resolution fluorescence microscope (Model: Olympus BX51 integrated with Olympus camera: TV1X-2) 1000× magnification. The images of diatom frustules were captured by adjusting the dilution of the sample to a maximum of 2 - 5 valves per view using a digital camera fixed to the microscope. Diatoms were identified using standard identification keys (Van Heurck; Karthick et al., 2013; Krammer K and Bertolet H, 1986; Patrick and Reimer, 1966). The identified species were also enumerated as per DARES protocol by counting 400 valves per sample to determine the relative abundances, species diversity, and community composition. Light micrographic images of few diatom species were listed in Figure 3.

Statistical Analyses

Two-way ANOVA statistical analysis was carried out to estimate the level of significance of seasonal physico-chemical parameters on species richness (S). The data were compiled season-wise (pre-monsoon, post-monsoon and monsoon) and subjected to agglomerative hierarchical clustering for understanding the dynamics of diatom species' abundance w.r.t seasons. Non-metric Multi-Dimensional Scaling (MDS) technique was used to understand the species abundance pattern of benthic diatom communities' season-wise across stations. Agglomerative hierarchical clustering and nMDS analysis were performed using R Studio version 1.1.423. Season wise and station wise diatom diversity (Shannon – Wiener (H')) and Simpson's Dominance (D) were computed using Shannon diversity and Simpson's dominance indices. Overall species richness (SR) and species abundance (SA) with respect to different substrata sampled at each station are illustrated in the form of a pie chart. The species that had shown a relative abundance of >15% and recorded consistently during all three seasons were considered as tolerant species and radar plot was generated using season-wise relative abundances of those tolerant species at each study station.

RESULTS AND DISCUSSION

Variations in Hydrological Parameters

Figure 4.a-d reflects the variability of physical parameters (AT, WT, pH, and Salinity) at sampling locations. Temperature is a critical physical factor that controls the rate of photosynthesis in addition to influencing many other chemical and biological reactions (Fatema et al., 2014). Temperature variations are influenced by prevailing precipitation and humidity levels. In this study, air temperature during pre-monsoon season was varying across stations with a mean temperature of (av. 31.36 ± 1.20 ºC). During pre-monsoon, the estuary was characterized by warm (31.42 ± 0.66 ºC) with high saline (30.20 ± 3.45 ppt) water in all stations (S1 to S6): lower (stations S5, S6), central (stations S3, S4) and upper reaches (stations S1, S2).

During monsoon season, the estuary had relatively lower variations of air and surface water temperatures (~ 1 to 3 ºC), with air temperature of (29.06 ± 0.67ºC) and water temperature (28.11 ± 0.87 ºC) respectively. The mean air and water temperature across the stations during post-monsoon season was (31.73 ± 0.82 ºC) and (29.04 ± 0.56 ºC). This drop-in water temperature during post-monsoon season could be attributed to heavy precipitation that resulted in the inflow of cold freshwater from the Ghats. Generally, in a tropical estuary, the temperature is inversely correlated with salinity and water transparency (Fatema et al., 2014).
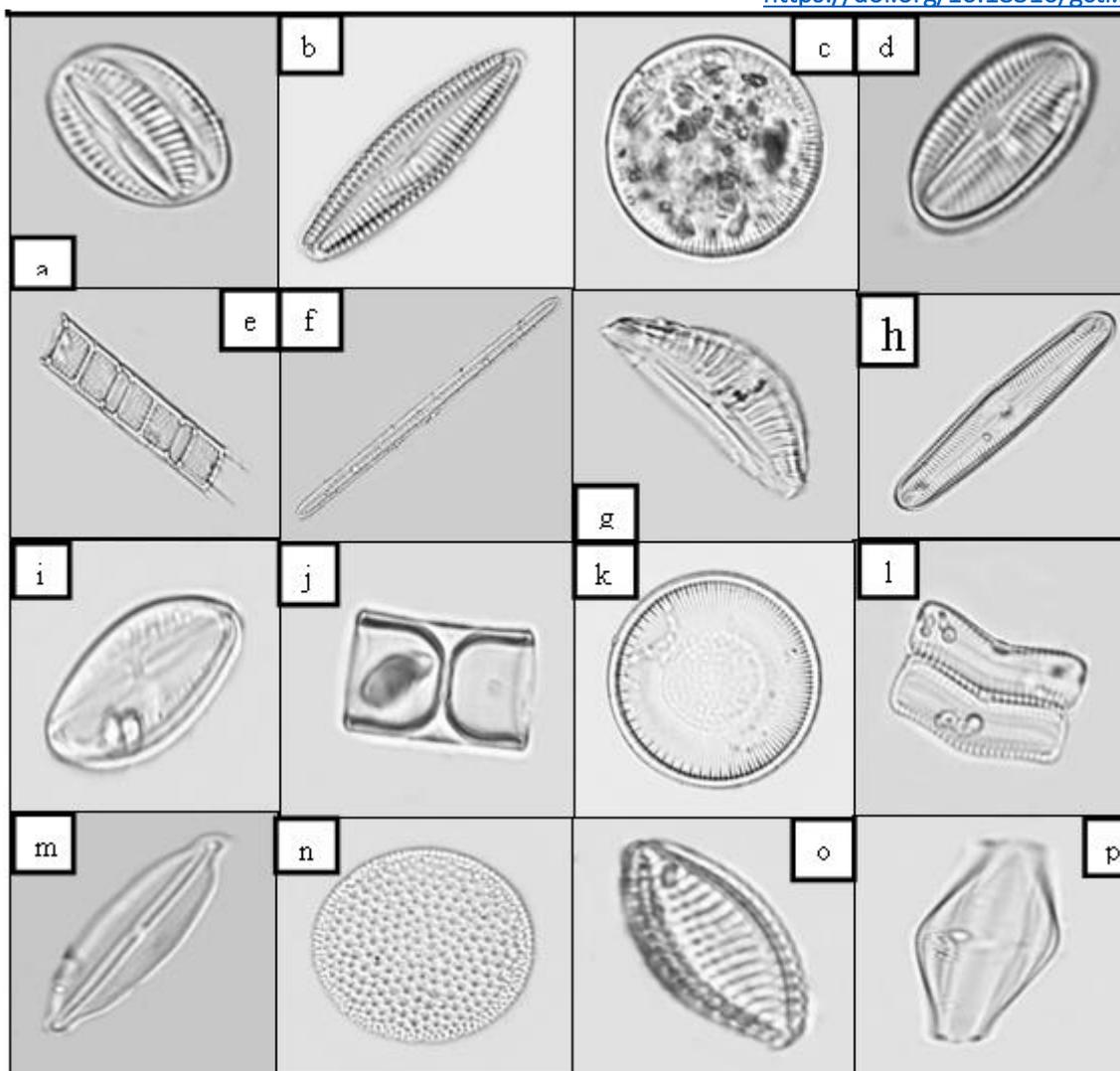
Figure 3: Light Micrographs of Select Diatom Species (a) Navicula forcipata (b) Gomphonema sp. (c) Cyclotella operculata (d) Diploneis subovalis (e) Aulacoseira granulata (f) Bacillaria paradoxa (g) Epithema gibberula (h) Pinnularia sp. (i) Navicula integra (j) Melosira jurgensii (k) Cyclotella meneghiniana (l) Achnanthes brevipes (m) Frustulia crassinervia (n) Coscinodiscus radiatus (o) Raphoneis amphiceros (p) Amphora salina

The water quality results depicted the seasonal variability. Air temperature showed wider fluctuations primarily due to higher insolation during pre-monsoon seasons followed by reduced levels due to precipitation. Air and water temperature variations were comparable to earlier studies in tropical estuaries of the south-west coast of India (Madhu et al., 2007; Martin et al., 2008). pH values in water samples in all sampling locations across seasons were neutral to slightly basic (7.88 ± 0.04) with no significant fluctuations. pH of a waterbody is mainly influenced by induced anthropogenic disturbances in the form of domestic or industrial effluents. Variations in pH were minimal as the study locations were mainly oligotrophic to mesotrophic. The salinity during monsoon season in central and upper reaches (stations S1 – S4) was lower (5.6 ± 2.58 ppt) compared to lower reaches (stations S5 and S6) with (18.1 ± 2.9 ppt). A marked wider salinity gradient was observed in all the stations during different seasons. The upper (S1 and S2) and the middle reaches (S3 and S4) with an influx of freshwater during monsoon had freshwater regime. However, the salinity levels peaked to 30 ppt with the onset of the summer season (pre-monsoon). The salinity levels were distinctly different across stations during post-monsoon with low salinity levels (6.25 ± 2.47 ppt) in upper reaches, mid salinity levels (18.12 ± 2.29 ppt) in central reaches and high salinity (25.37 ± 1.59 ppt) in lower reaches of the estuary.

Nutrient levels across the stations were more of temporal dilutions due to anthropogenic inputs. There was no trend in nutrient variation across seasons among the stations and the results were highly location specific and prone to non-point source pollutions. Higher nitrate levels were observed (5.12 ± 0.57 µg/L) during pre-monsoon season followed by post-monsoon (4.875 ± 1.11 µg/L) and monsoon (4.36 ± 0.58 µg/L). Phosphates showed fluctuations within stations across different seasons from (1.43 ± 0.09 µg/L) to (2.43 ± 0.54 µg/L). Silicate levels showed distinct variability (Figure 4.b-d) across seasons among stations, with higher silicate levels observed during monsoon (6.40 ± 1.82 µg/L), followed by post-monsoon (5.04 ± 2.66 µg/L) and pre-monsoon (av. 3.90 ± 2.34 µg/L). The variation in silicate range was both stations as well as season-specific

and comparable to earlier studies (Madhu et al., 2007; Srinivas et al., 2003) in the two tropical estuaries along the Indian west coast.
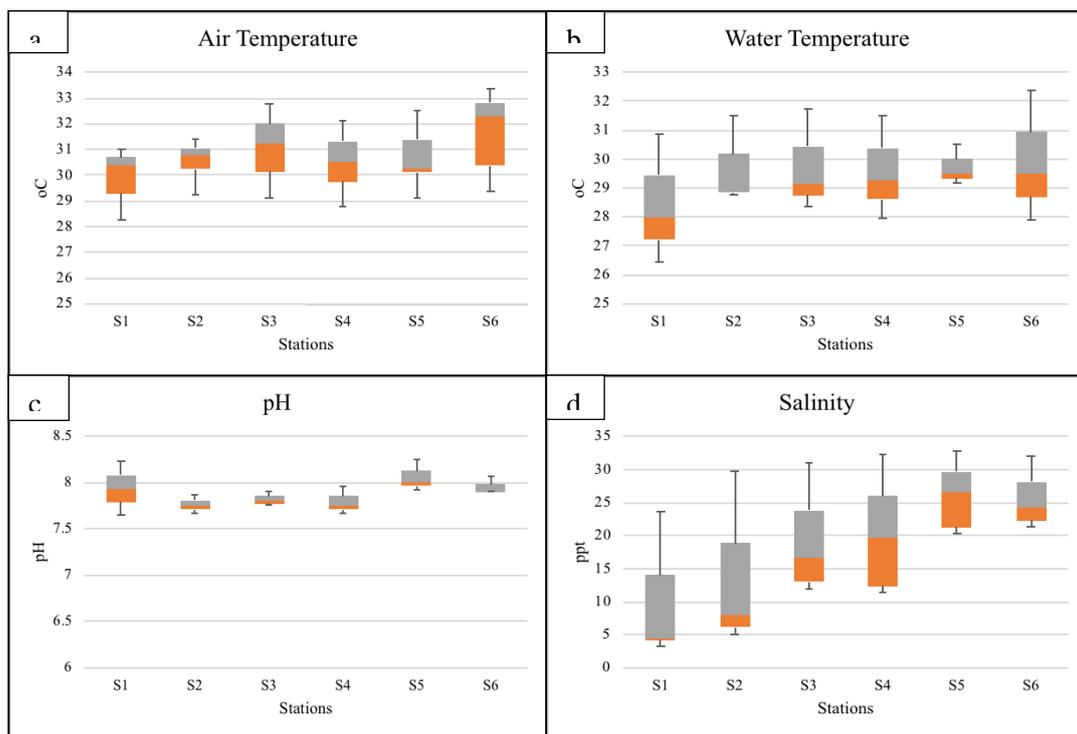

Figure 4: Variation in Physico-Chemical Parameters across the Sampling Stations during pre-post and monsoon Seasons (4.a-d)
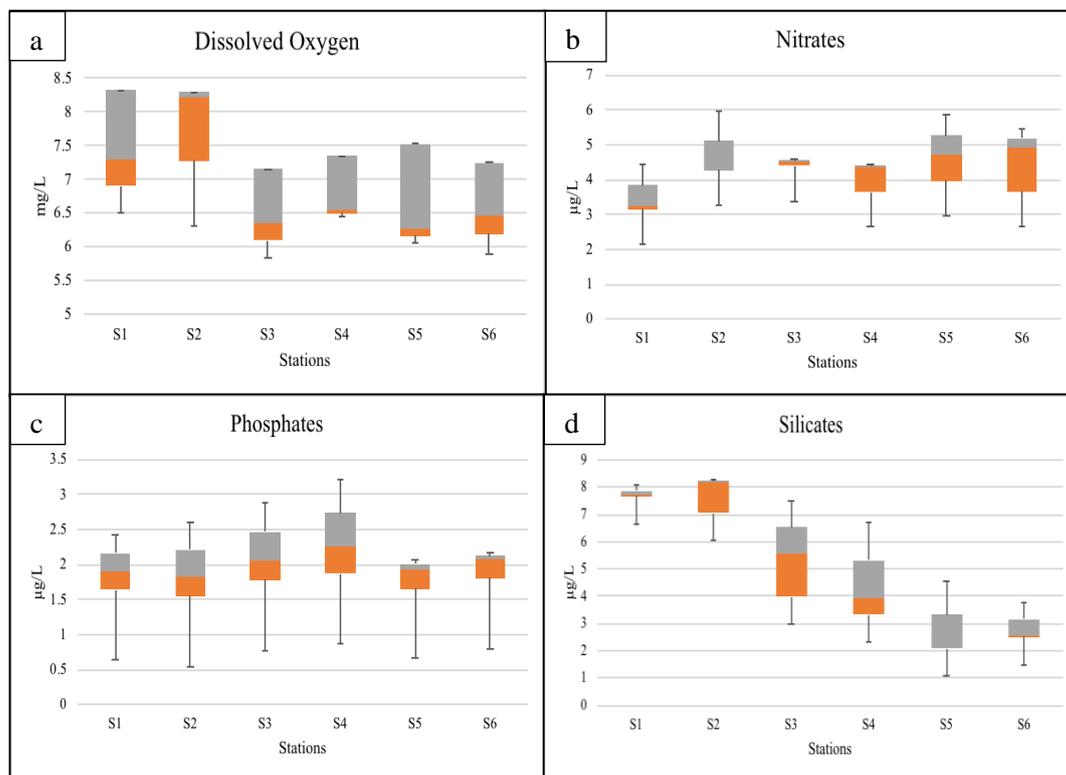

Figure 5.a-d reflect variations in DO and nutrient levels. DO was lower (6.33 ± 0.26 mg/L) during pre-monsoon (6.33 ± 0.26 mg/L) and post-monsoon (6.72 ± 0.88 mg/L) compared to monsoonal DO (8.44 ± 0.52 mg/L).

Higher DO levels during monsoons could be attributed to the influx of rainwater and subsequent riverine catchment run-offs reaching the estuary. The temporal variation of DO among the stations during monsoon was higher, evident from Figure 5.a. DO levels observed in this region were relatively higher compared to Cochin backwaters (Madhu et al., 2007).

Effect of hydrological parameters on Species Richness

Two-way ANOVA analysis revealed a significant variation in species richness with salinity, DO, and nutrients (nitrates, phosphates, and silicates) across stations (P=0.015) and seasons (P<0.05%).

Diatom Diversity, Dominance, and Species Composition

An estuary is a dynamic aquatic eco-system that resonates with spatial and temporal changes. A total of 85 different species (Appendix 1) of diatoms were recorded with similar species occurring at different stations in one season. Station wise overall species diversity indices (Shannon-wiener Diversity) and Simpson's dominance indices were estimated and are given in Fig. 6. The Shannon's diatom diversity index (H') was maximum for the mesohaline, upper reach station (S2) of the estuary with H' = 3.19 and the least diversity was observed at the polyhaline station (S5) in the lower reaches of the estuary with H' = 1.73, while all other 4 stations had intermediate H' values. Simpson's dominance index (D) was the highest for S1 and S6 with values D = 0.9 and 0.86 respectively due to the outnumbering dominance of *Coscinodiscus subtilis* and *Navicula lanceolata* in station S1, *Melosira* sp. and *Navicula* spp. in station S6 over other species.
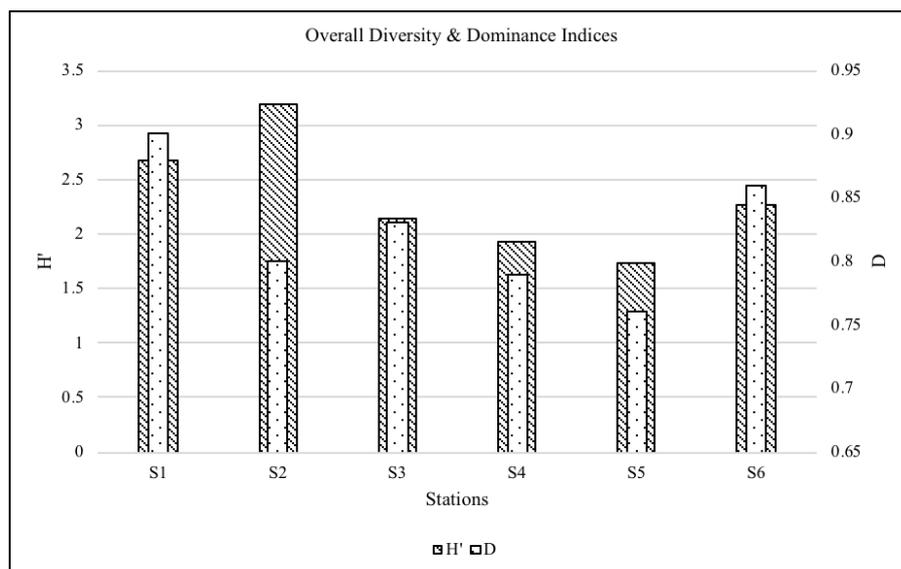

Figure 6: Diversity & Dominance Indices of the Sampling Stations

Substrata wise Species Richness and Abundance

The data recorded substrata wise for a period of one year were consolidated to understand the role of substrata in species assemblage (Figure 7). The results demonstrated the highest species abundance (36%) at S2 followed by S3 (31%). Station S6 showed the least species abundance (4%). Stations S4 and Station S5 contributed to 11% and 5% of species abundance respectively. Among the different substrata considered, sediments had maximum epipelic diatom species richness of 40, 36, and 28 respectively at stations S2, S3, and S4. Higher species richness in sediments was followed by stones/rocks (Epilithons) at almost all the stations. Epiphytons attached to plant substrata had the third-highest species richness in S2, S3, and S4 stations with species richness of 35, 21, and 23 respectively, which is similar to the earlier report (Townsend and Gell, 2005). Lower species richness and poor community structure are due to the grazing pressure. Episammons (diatoms growing on sand) had the least species richness in polyhaline lower reaches stations S5 and S6 with S = 12 and S = 18, due to persistent wave action, washing away the community leading to lower diatom affinity in that substrata.

Species Tolerance

The presence of a species, its abundance as well as its distribution is largely determined by their level of tolerance to abiotic environmental factors. Tolerance is the ability or endurance of a species to survive in hostile environmental conditions. When a species is exposed to prolonged unfavorable conditions, the species tends to build up resilience to withstand harsh environmental conditions and in turn evolve as a tolerant species having higher ability to survive under wider ranges of fluctuating conditions, which is observed in certain diatom species with the consistent presence in fluctuating environmental conditions (seasonal variability). These tolerant species abundances w.r.t different seasons at each station is illustrated in the form of a radar plot in Figure 8. The species present during all the three seasons with their relative abundances greater than 15% were considered as "tolerant species" capable of surviving fluctuating environmental conditions and their season-wise relative abundances were plotted, which illustrates the site-specific abundances of tolerant species in premonsoon, post-monsoon and monsoon. The graph was generated by clubbing the tolerant species to the genus level.
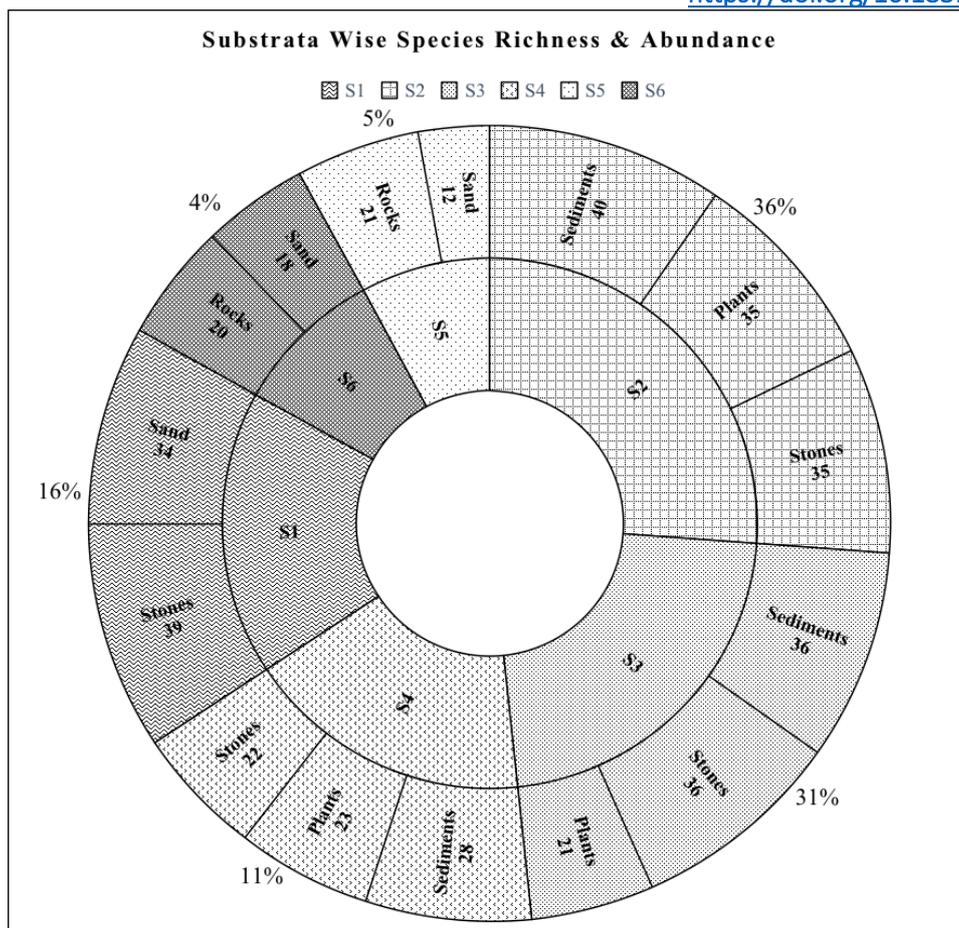
Figure 7: Pie chart representing SR and SA w.r.t Substrata

Overall tolerant species' abundance was highest during pre-monsoon, followed by post-monsoon and the least abundance in monsoon season. During premonsoon, the species abundance of *Cyclotella meneghiniana* at station S2 was the highest (74.5%) followed by consortia of *Navicula sp.* (*Navicula lanceolata*, *Navicula bicapitata*, *Navicula forcipata*, *Navicula mutica* and *Navicula amphisbaena*) with 68.9% at station S3. Polyhaline (S5) station showed a dominance of *Melosira* spp. with corresponding species abundance of 72.7% during the premonsoon season. Though the abundance of these species showed a marked peak during pre-monsoon season of the year, the presence of those species was recorded at other seasons as well with varying abundance levels, proving the species to be tolerant with evolved ecological adaptations to season-based dynamism prevailing in real-time estuarine systems.

Season wise analyses of species' relative abundances reveal higher relative abundance during pre-monsoon with >15% of tolerant species followed by post-monsoon and the least relative abundances were observed during monsoon season (due to influx of freshwater). As per the tolerance results primarily determined by their presence in almost all the seasons with their corresponding species-wise relative abundances to be >15%, the diatoms belonging to the genus of *Achnanthes* spp., *Nitzschia* spp., *Navicula* spp., *Cyclotella* spp., *Melosira* spp. were found to be tolerant with consistent availability at different stations as well as during different seasons.

Among the pennate ones, the genus belonging to *Amphora* and *Nitzschia* are known to have pronounced heterotrophic capabilities (Linkins, 1973; Werner, 1977). Most of the *Nitzschia sp.* is known to be obligate heterotrophs with their habitat preferences in strong heterotrophic environments like decaying piles of seaweeds, with little or nil light penetration. *Cyclotella meneghiniana* was also known to have strong heterotrophic capabilities (Horner and Alexander, 1972; Lewin and Hellebust, 1976; Lylis and Trainor, 1973). Another dominant taxon observed very frequently in lower reaches (S5 and S6) was known to be a taxon frequently encountered in higher insolation regions, especially in tidal pools and upper inter-tidal regions. This taxon was proven to be relatively tolerant of inter-tidal exposure to desiccation (Castenholz, 1963; Stevenson et al., 1996) which could have given *Achnanthes sp.* the ability to survive at higher insolation over large boulders subjected to prolonged intertidal exposure. *Melosira sp.* could dominate in varying pH conditions and warmer waters with higher light intensity. It is found that *Navicula sp.* which occurs mostly over the sediment surfaces and on other aquatic plants are facultative heterotrophs that utilize organic matter found in the substrate (Werner, 1977)., which justifies dominance of certain species across different seasons in more than one station.
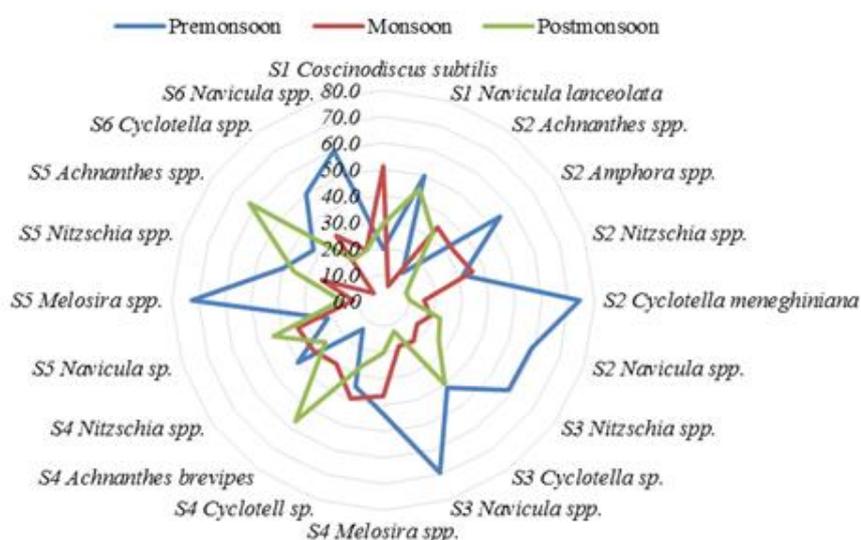
Figure 8: Relative abundance of tolerant species w.r.t seasons

*spp. represents species composition with more than one diatom species at respective stations from S1 to S6
S2 Achnanthes spp. – *Achnanthes brevipes*, *Achnanthes longipes*
S2 Amphora spp. – *Amphora salina*, *Amphora ovalis*
S2 Nitzschia spp. – *Nitzschia obtusa*, *Nitzschia sigma*, *Nitzschia apiculata*, *Nitzschia fasciculata*, *Nitzschia panduriformis*
S2 Navicula spp. – *Navicula forcipata*, *Navicula amphisbaena*, *Navicula permagna*
S3 Nitzschia spp. – *Nitzschia obtusa*, *Nitzschia sigma*, *Nitzschia fasciculata*, *Nitzschia acicularis*, *Nitzschia panduriformis*
S3 Navicula spp. – *Navicula lanceolata*, *Navicula bicapitata*, *Navicula forcipata*, *Navicula mutica*, *Navicula amphisbaena*
S4 Melosira spp. – *Melosira varians*, *Melosira jurgensii*
S4 Nitzschia spp. – *Nitzschia obtusa*, *Nitzschia fasciculata*, *Nitzschia sigma*, *Nitzschia acicularis*
S5 Melosira spp. – *Melosira lineatus*, *Melosira jurgensii*
S5 Nitzschia spp. – *Nitzschia obtusa*, *Nitzschia sigma*, *Nitzschia apiculata*, *Nitzschia fasciculata*
S5 Achnanthes spp. – *Achnanthes brevipes*, *Achnanthes longipes*
S6 Cyclotella spp. – *Cyclotella meneghiniana*, *Cyclotella operculata*
S6 Navicula spp. – *Navicula lanceolata*, *Navicula granulata*, *Navicula mutica*
S6 *Cyclotella* spp. includes *Cyclotella meneghiniana*, *Cyclotella operculata*

Clustering based on Species Abundance

Month-wise species composition along with its relative abundance of diatoms was organized season-wise and subjected to agglomerative hierarchical clustering (Figure 9) to understand the species predominance and season sensitivity. Cluster analysis resulted in three distinct clusters based on their species abundance across different seasons. Cluster 1 formed the major group of diatom species that are sensitive and predominantly either freshwater species or euryhaline species.

The presence of which is observed only in specific sites at a particular season with its relative abundance <2%. Cluster 1 includes freshwater species such as *Cocconeis placentula*, *Aulacoseira granulata* and salinity tolerant euryhaline species such as *Amphiprora alata*, *Eunotia flexuosa*, *Spermatogonia sp.*, *Pleurosigma marcum* etc., with least abundance recorded in one of the three seasons. Cluster 2 formed the second major group with a moderate species abundance of >5%. Cluster 3 formed the dominant groups with a higher relative species abundance of 8% - 15%. Out of 86 different species of diatoms, more than 80% of the population were either recorded only in upstream (less saline) regions during monsoon periods or only at downstream regions during pre/post-monsoon seasons forming cluster 1. Those species could be collectively termed as sensitive species, the presence or absence of which is influenced by one or more of the environmental parameters and even the slightest change in those parameters could have led to the species turnover by tolerant species. The species recorded in the upstream regions were found to exhibit predominant freshwater habitat characteristics. The species that exhibited such characteristics include *Synedra ulna*, *Aulacoseira granulata*, *Bacillaria paradoxa*, *Cocconeis sp. Gomphonema sp.*, *Cymbella* sp., *Pinnularia* sp., and *Diploneis* sp. (Appendix 1). The presence of freshwater species was confined to upstream and middle estuarine portions in monsoon season. On the other hand, species like *Biddulphia laevis*, *Licomophora* sp., *Amphiprora alata*, *Epithema argus* were found only in downstream (S5 and S6) stations of the estuary. Hence these species could be considered as salinity loving/euryhaline species and their absence during monsoon and post monsoon could be attributed to overall low salinity of the estuary during those two seasons.
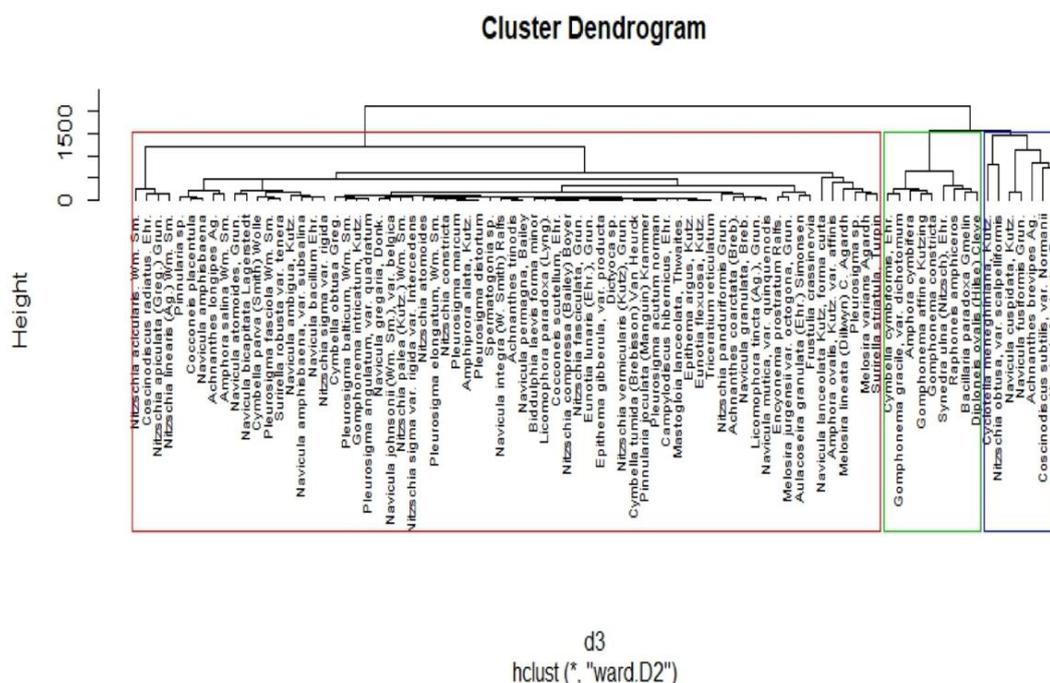
Figure 9: Agglomerative Hierarchical Clustering of Species w.r.t Species Abundance

Season-wise Variation in Species Composition

Non-metric multidimensional scaling analysis was performed to understand the dynamics of the diatom community structure among stations by considering season-wise species abundance across each station (Figure 10). NMDS is a rank-based approach in which the ordination is achieved through an iterative process based on pairwise dissimilarity in a low dimensional space. Here NMDS is plotted considering species abundance summed up season-wise to understand the commonness in species across stations. The plot shows ordination hulls formed with respect to pre- post- and monsoon seasons across stations (S1 PRM – S6 PRM), (S1 M – S6 M), and (S1 POM – S6 POM). The ordination hulls generated season-wise, which demarcates tolerant species that are present throughout the year from station-specific sensitive species. For example, diatom species like *Melosira lineatus* (MLI), *Nitzschia obtusa* (NIOB), *Gomphonema gracile* (GG), *Cyclotella meneghiniana* (CM), *Coscinodiscus subtilis* (CS) were present in the overlapping intersections of all three ordination hulls indicating its presence during all three seasons at different stations. Whereas species like *Achnanthes brevipes* (AB), *Achnanthes longipes* (AL), *Amphora salina* (AS), *Navicula amphisbaena* (NAM), *Melosira jurgensii* (MJ) were observed only during monsoon and pre-monsoon seasons at more than one study locations. Whereas *Pleurosigma angulatum* (PAN), *Cocconeis scutellum* (COS), *Navicula ambigua* (NAG), *Coscinodiscus radiatus* (CR) were present during pre- and post-monsoon seasons in one or two study locations. Whereas species like *Frustulia crassinervia* (FS), *Aulacoseira granulata* (AUG), *Licomophora paradoxa* (LP), *Nitzschia sigma var. intercedens* (NISV), *Pleurosigma acutum* (PAC), *Gomphonema intricatum* (GI), *Cymbella parva* (CP) were confined to one particular station with insignificant relative abundances and observed during only one of the three seasons.

Tolerant Species for Bioremediation and Biofuel

Recent research on third-generation biofuel production is increasingly concentrating on using wastewater as a source of nutrients for the growth of microalgae. If a biofuel industry is to be set up based on open/raceway ponds using wastewater as a source of nutrients, running the industry at consistent productivities throughout the year proves challenging as contamination control becomes quite difficult in such open systems. The introduction of monoculture strains or sensitive species further complicates the scenario as the pond is continuously exposed to both diurnal and seasonal variations in weather as well as water quality used for biomass enrichment. Hence, phyco-prospecting consortia of tolerant species with year-round availability irrespective of microclimate/environmental conditions would be a better choice in averting the risks of contamination or crash encountered in monoculture sensitive strain-based cultivation systems. Thus, from the results of the present study, it is apt to consider tolerant diatom or consortia of tolerant diatoms capable of exhibiting robust growth despite fluctuations and variations in the environmental factors that greatly influence its growth. The species having potential for phyco-prospecting evident from the results of the present study are *Amphora sp.*, *Nitzschia sp.*, *Navicula sp.*, *Achnanthes sp.*, *Melosira sp.*, *Coscinodiscus sp.*, and *Cyclotella sp*. The above-mentioned species when chosen as a candidate strain or as consortia of strains for phyco-prospecting has better chances of survival in open pond/raceway pond systems exhibiting enhanced biomass productivities.
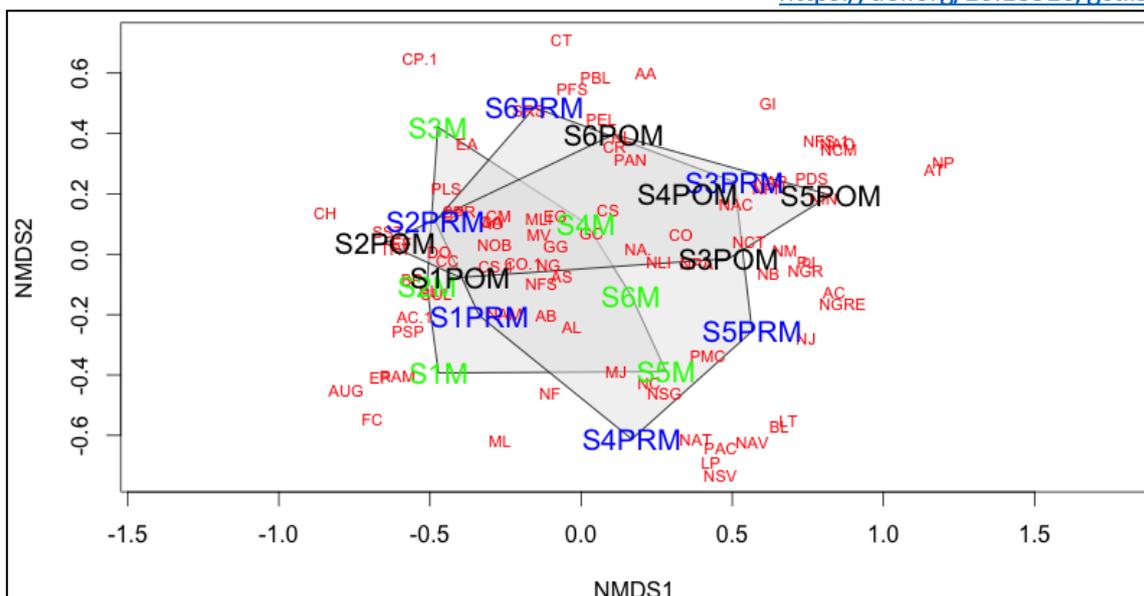
Figure 10: nMDS Plot based on Site-wise and Season-wise Species Abundance

*The acronyms of species names used in NMDS graph are in Appendix 1

Lipid Potential of Select Tolerant Diatom Strains

Microalgae are widely considered as biodiesel feedstocks due to its better lipid productivity and shorter cycling period with quick multiplication rates when compared to conventional terrestrial oil seeds. Many kinds of research around the globe had focussed on lab-scale cultivation of monoculture strains to estimate lipid productivity. Studies so far on *Achnanthes sp.* under lab conditions showed a lipid content that varies from (19.6 – 27.7) % based on dry cell weight (Zhao et al., 2016). Lipid content studies on *Nitzschia* spp. showed values ranging between (37.5 – 46) % (Sheehan et al., 1998; Zhao et al., 2016). *Melosira* sp. exhibited lipid content varying from (10.52 – 33.01) % in two different studies (Chen et al., 2012; Renaud et al., 1994). *Amphora sp.* had shown the highest lipid content reported so far on diatoms with 49.95% (De La Peña, 2007). Different species of *Navicula*, experimented under varied conditions showed lipid content with 17.3 – 39.84% based on dry cell weight (Fields and Kociolek, 2015). These results corroborate the prospects of select tolerant species as potential biofuel strains.
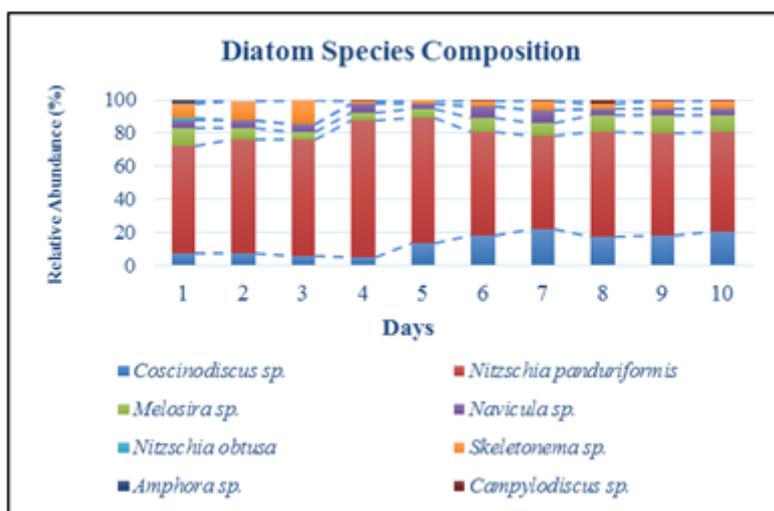

Figure 11: Laboratory Experiments on Growth Rate and Species Composition

Laboratory Experiments to Elucidate the Lipid Potential of Indigenous Diatom strains

Benthic diatoms were collected from rocks from one of the study sites (S4) and immediately inoculated in F/2 media under sterile conditions. The nutrient enrichment provided by F/2 media facilitated a mixed consortium of diatoms to grow in the culture with a working volume of 250 mL. The light intensity was maintained at 90 µmol m^{-2} s^{-1} and the temperature was maintained at 29 ± 2°C. The salinity of the cultures was maintained at 28 psu. The nutrient-enriched mixed cultures were then analyzed for species composition variations throughout the growth period as well as its lipid content once the cell reached its stationary phase. The consortium consisted of *Coscinodiscus sp.*, *Nitzschia panduriformis*, *Melosira* sp., *Navicula* sp., *Skeletonema* sp., *Amphora* sp., *Camphylodiscus* sp., and *Nitzschia obtusa*. Figure 11 represents the changes

in the relative abundance of each species in the consortium during the complete growth period of ten days. After ten days, the diatoms were harvested and subjected to lipid extraction using a modified Folch method.

The culture consortium was further sub-cultured as aliquots under various treatments such as nitrogen and silicon starvation to understand the percentage increase of lipids as dry cell weights with respect to no treatment (control) sample. Results on varying treatments revealed that nitrogen starvation had exhibited the highest lipid content (17 - 25% based in terms of dry cell weight). The biomass yield was found to be 80 – 160 mg/L. The nitrogen starved algal biomass extracted lipids were further subjected to acid catalysed transesterification to obtain FAMEs. The FAME thus obtained were analysed using GC-MS to understand the types of fatty acids produced by the diatom consortia.
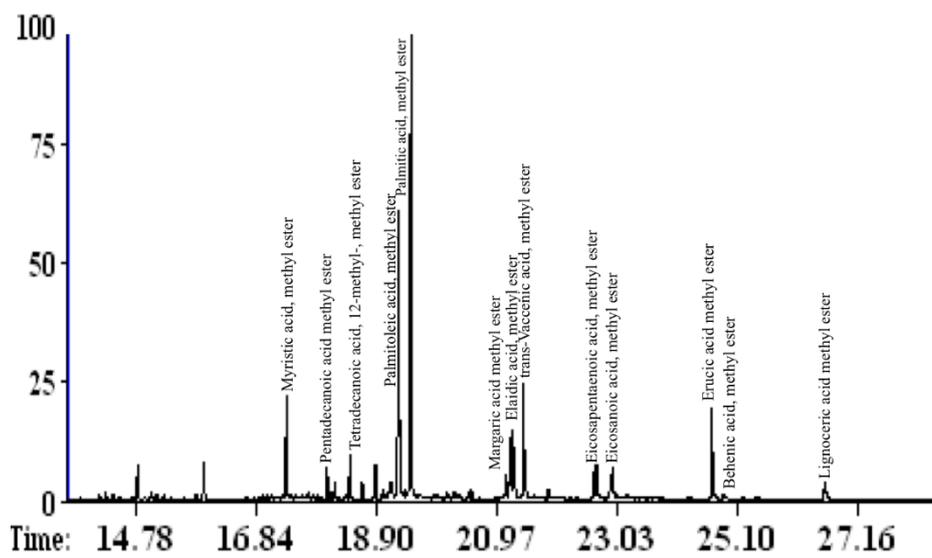

Figure 12: Fatty acid Methyl Ester Profiles of harvested algal biomass

Figure 12 represents the peaks showing variations (%) in diatom derived FAMEs. Palmitic acid methyl ester was the dominant fatty acid recorded followed by palmitoleic acid, myristic acid, and trans-vaccenic acid methyl ester. These results elucidate the potential of indigenous diatom strains consortium in the production of third-generation biofuels.

CONCLUSIONS

Understanding ecological characteristics and habitat preferences of microalgae are crucial to avoid contamination issues in large scale microalgal production systems, especially while using wastewater as a source of nutrient for algal growth. , Season tolerant strains would ensure year-round microalgal biomass productivity and reduce the effort as well as additional operating costs involved in eliminating species turn over. This research has provided valuable insights pertaining to the seasonal dynamics of diatoms along an estuarine shoreline of Indian west coast in-order to screen tolerant diatoms species over sensitive ones. *Amphora sp.*, *Nitzschia* sp., *Navicula* spp., *Achnanthes* sp., *Melosira* sp., *Coscinodiscus* sp., and *Cyclotella* sp. have better tolerance to seasonal variations in water quality with potential to phyco-prospecting these indigenous strains.

RECOMMENDATION

Screening a diatom or consortia of dominant diatom species capable of surviving in rugged environmental conditions, could become ideal candidates for third-generation biofuel cultivation system on a regional basis, especially suitable for tropical countries like India. Lab-scale results on indigenously isolated mixed consortia of diatoms reinstate its potential and prospects of diatoms becoming valuable feedstock for third generation biofuel production as well as value-added products.

ACKNOWLEDGEMENTS

AUTHORS CONTRIBUTION

Saranya G: design and performed experiments, data analyses, manuscript writing.

Ramachandra T V: conceptualization and design of experiments, validation of experiment, data analysis and interpretation of data, article revision, and final editing.

REFERENCES

1. Armbrust, E.V. (2009). "The life of diatoms in the world's oceans:" Nature, Nature Publishing Group Vol. 459, p. 185. https://doi.org/10.1038/nature08057, PMid:19444204

2. Azadi, P., Malina, R., Barrett, S.R.H. and Kraft, M. (2017). "The evolution of the biofuel science:" Renewable and Sustainable Energy Reviews,Pergamon Vol. 76, pp. 1479-1484. https://doi.org/10.1016/j.rser.2016.11.181

3. Bere, T. (2016). "Challenges of diatom-based biological monitoring and assessment of streams in developing countries:" Environmental Science and Pollution Research, Springer Berlin Heidelberg Vol. 23, No. 6, pp. 5477-5486.https://doi.org/10.1007/s11356-015-5790-y, PMid:26573306

4. Castenholz, R.W. (1963). "An Experimental Study Of The Vertical Distribution Of Littoral Marine Diatoms1:" Limnology and Oceanography,Wiley-Blackwell Vol. 8, No. 4, pp. 450-462. https://doi.org/10.4319/lo.1963.8.4.0450

5. Chen, L., Liu, T., Zhang, W., Chen, X. and Wang, J. (2012). "Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion:" Bioresource Technology, Elsevier Vol. 111, pp. 208-214. https://doi.org/10.1016/j.biortech.2012.02.033, PMid:22401712

6. Dalu, T. and Froneman, P.W. (2016). "Diatom-based water quality monitoring in southern Africa: challenges and future prospects:" Water SA, Water Research Commission (WRC) Vol. 42, No. 4, p. 551. https://doi.org/10.4314/wsa.v42i4.05

7. Dixit, S.S., Smol, J.P., Kingston, J.C. and Charles, D.F. (1992). "Diatoms: powerful indicators of environmental change:" Environmental Science & Technology, American Chemical Society Vol. 26, No. 1, pp. 22-33. https://doi.org/10.1021/es00025a002

8. Fatema, K., Wan Maznah, W.O. and Isa, M.M. (2014). "Spatial and Temporal Variation of Physico-chemical Parameters in the Merbok Estuary, Kedah, Malaysia.:" Tropical life sciences research, School of Medical Sciences, Universiti Sains Malaysia Vol. 25, No. 2, pp. 1-19.

9. Fields, F.J. and Kociolek, J.P. (2015). "An evolutionary perspective on selecting high-lipid-content diatoms (Bacillariophyta):" Journal of Applied Phycology, Vol. 27, No. 6, pp. 2209-2220. https://doi.org/10.1007/s10811-014-0505-1

10. Hering, D., Johnson, R.K., Kramm, S., Schmutz, S., Szoszkiewicz, K. and Verdonschot, P.F.M. (2006). "Assessment of European streams with diatoms, macrophytes, macroinvertebrates and fish: A comparative metric-based analysis of organism response to stress:" Freshwater Biology, Wiley/Blackwell (10.1111) Vol. 51, No. 9, pp. 1757-1785. https://doi.org/10.1111/j.1365-2427.2006.01610.x

11. Van Heurck, H. Van Heurck 1896 - A Treatise on the Diatomaceae. Translated by W.E. Baxter. | Citations - Diatoms of North America, Available at: https://diatoms.org/citations/van_heurck_h-1896-a_treatise_on_the_diatomaceae_translated_by_we_baxter (Accessed: August 18, 2018).

12. Hildebrand, M., Davis, A.K., Smith, S.R., Traller, J.C. and Abbriano, R. (2012). "The place of diatoms in the biofuels industry:" Biofuels, Vol. 3, No. 2, pp. 221-240. https://doi.org/10.4155/bfs.11.157

13. Horner, R. and Alexander, V. (1972). "Algal Populations in Arctic Sea Ice: An Investigation of Heterotrophy:" Limnology and Oceanography, Wiley-Blackwell Vol. 17, No. 3, pp. 454-458. https://doi.org/10.4319/lo.1972.17.3.0454

14. Jeffryes, C., Campbell, J., Li, H., Jiao, J. and Rorrer, G. (2011). "The potential of diatom nanobiotechnology for applications in solar cells, batteries, and electroluminescent devices:" Energy & Environmental Science, Vol. 4, No. 10, p. 3930. https://doi.org/10.1039/c0ee00306a

15. Karthick, B., Hamilton, P.B. (Paul B., Kociolek, J.P. and Ashoka Trust for Research in Ecology and the Environment. (2013). An illustrated guide to common diatoms of peninsular India, Gubbi Labs.

16. Kelly, M.G., Cazaubon, A., Coring, E., Dell'Uomo, A., Ector, L., Goldsmith, B., Guasch, H., Hürlimann, J., Jarlman, A., Kawecka, B., Kwandrans, J., Laugaste, R., Lindstrøm, E.-A., Leitao, M., Marvan, P., et al. (1998). "Recommendations for the routine sampling of diatoms for water quality assessments in Europe:" Journal of Applied Phycology, Vol. 10, No. 2, p. 215. https://doi.org/10.1023/A:1008033201227

17. Kennish, M.J. (2002). "Environmental threats and environmental future of estuaries:" Environmental Conservation, Vol. 29, No. 1, pp. 78-107.https://doi.org/10.1017/S0376892902000061

18. Krammer K and Bertolet H (1986). Krammer, K. and Lange-Bertalot, H. (1986) Bacillariophyceae. 1. Teil: Naviculaceae In: Ettl, H., J. Gerloff, H. Heynig and D. Mollenhauer (eds.) Süsswasserflora von Mitteleuropa, Band 2/1.

19. De La Peña, M.R. (2007). Cell growth and nutritive value of the tropical benthic diatom, Amphora sp., at varying levels of nutrients and light intensity, and different culture locations. Journal of Applied Phycology, Springer Verlag, pp. 647-655.https://doi.org/10.1007/s10811-007-9189-0

20. Leblanc, K., Quéguiner, B., Diaz, F., Cornet, V., Michel-Rodriguez, M., Durrieu De Madron, X., Bowler, C., Malviya, S., Thyssen, M., Grégori, G., Rembauville, M., Grosso, O., Poulain, J., De Vargas, C., Pujo-Pay, M., et al. (2018). "Nanoplanktonic diatoms are globally overlooked but play a role in spring blooms and carbon

export:" Nature Communications, Vol. 9, No. 1, p. 953.https://doi.org/10.1038/s41467-018-03376-9, PMid:29507291, MCid:PMC5838239

21. Levitan, O., Dinamarca, J., Hochman, G. and Falkowski, P.G. (2014). "Diatoms: A fossil fuel of the future:" Trends in Biotechnology, Vol. 32, No. 3, pp. 117-124.https://doi.org/10.1016/j.tibtech.2014.01.004, PMid:24529448

22. Lewin, J. and Hellebust, J.A. (1976). "Heterotrophic nutrition of the marine pennate diatom Nitzschia angularis var. affinis:" Marine Biology,Springer-Verlag Vol. 36, No. 4, pp. 313-320. https://doi.org/10.1007/BF00389192

23. Linkins, A.E. (1973). Uptake and Utilization of Glucose and Acetate by a Marine Chemoorganotrophic Diatom Nitzschia alba Clone Link 001, University of Massachusetts, Amherst.

24. Lohrenz, S.E., Dagg, M.J. and Whitledge, T.E. (1990). "Enhanced primary production at the plume/oceanic interface of the Mississippi River:" Continental Shelf Research,Pergamon Vol. 10, No. 7, pp. 639-664. https://doi.org/10.1016/0278-4343(90)90043-L

25. Lylis, J.C. and Trainor, F.R. (1973). "THE HETEROTROPHIC CAPABILITIES OF CYCLOTELLA MENEGHINIANA:" Journal of Phycology,Wiley/Blackwell (10.1111) Vol. 9, No. 4, pp. 365-369. https://doi.org/10.1111/j.1529-8817.1973.tb04109.x

26. Madhu, N. V., Jyothibabu, R., Balachandran, K.K., Honey, U.K., Martin, G.D., Vijay, J.G., Shiyas, C.A., Gupta, G.V.M. and Achuthankutty, C.T. (2007). "Monsoonal impact on planktonic standing stock and abundance in a tropical estuary (Cochin backwaters - India):" Estuarine, Coastal and Shelf Science,Academic Press Vol. 73, No. 1-2, pp. 54-64.https://doi.org/10.1016/j.ecss.2006.12.009

27. Martin, G.D., Vijay, J.G., Laluraj, C.M., Madhu, N.V., Joseph, T., Nair, M., Gupta, G.V.M. and Balachandran, K.K. (2008). "Fresh water influence on nutrient stoichiometry in a tropical estuary, Southwest coast of India:" Corvinus University of Budapest.

28. Nelson, D.M., Tréguer, P., Brzezinski, M.A., Leynaert, A. and Quéguiner, B. (1995). "Production and dissolution of biogenic silica in the ocean: Revised global estimates, comparison with regional data and relationship to biogenic sedimentation:" Global Biogeochemical Cycles,Wiley-Blackwell Vol. 9, No. 3, pp. 359-372. https://doi.org/10.1029/95GB01070

29. Patrick, R.M. and Reimer, C.W. (1966). "The diatoms of the United States exclusive of Alaska and Hawaii. Volume 1. Fragilariaceae, Eunotoniaceae, Achnanthaceae, Naviculaceae. Monographs of the Academy of Natural Sciences of Philadelphia., Vol. 13:" Philadelphia, Patrick R. & Reimer C.W, Vol. 13, p. 688 pp., 64 pls.

30. Prestegard, S.K., Oftedal, L., Coyne, R.T., Nygaard, G., Skjærven, K.H., Knutsen, G., Døskeland, S.O. and Herfindal, L. (2009). "Marine Benthic Diatoms Contain Compounds Able to Induce Leukemia Cell Death and Modulate Blood Platelet Activity:" Marine Drugs,Molecular Diversity Preservation International Vol. 7, No. 4, pp. 605-623. https://doi.org/10.3390/md7040605, PMid:20098602 PMCid:PMC2810217

31. Qiu, D., Huang, L., Zhang, J. and Lin, S. (2010). "Phytoplankton dynamics in and near the highly eutrophic Pearl River Estuary, South China Sea:" Continental Shelf Research,Pergamon Vol. 30, No. 2, pp. 177-186. https://doi.org/10.1016/j.csr.2009.10.015

32. Ramachandra, T. V and Hegde, G. (2015). "Energy Trajectory in India: Challenges and Opportunities for Innovation:" Journal of Resources, Energy and Development,IOS Press Vol. 12, No. 1-2, pp. 1-24. https://doi.org/10.3233/RED-120115

33. Ramachandra, T.V., Jain, R. and Krishnadas, G. (2011). "Hotspots of solar potential in India:" Renewable and Sustainable Energy Reviews,Pergamon Vol. 15, No. 6, pp. 3178-3186. https://doi.org/10.1016/j.rser.2011.04.007

34. Ramachandra, T. V., Mahapatra, D.M., B, K. and Gordon, R. (2009). "Milking Diatoms for Sustainable Energy: Biochemical Engineering versus Gasoline-Secreting Diatom Solar Panels:" Industrial & Engineering Chemistry Research, American Chemical Society Vol. 48, No. 19, pp. 8769-8788. https://doi.org/10.1021/ie900044j

35. Ramachandra, T.V. and Shwetmala (2009). "Emissions from India's transport sector: Statewise synthesis": Atmospheric Environment,Pergamon Vol. 43, No. 34, pp. 5510-5517. https://doi.org/10.1016/j.atmosenv.2009.07.015

36. Ramachandra, T. V. and Shwetmala (2012). "Decentralised carbon footprint analysis for opting climate change mitigation strategies in India:" Renewable and Sustainable Energy Reviews,Pergamon Vol. 16, No. 8, pp. 5820-5833.https://doi.org/10.1016/j.rser.2012.05.035

37. Reid, M. (2005). "Diatom-based models for reconstructing past water quality and productivity in New Zealand lakes:" Journal of Paleolimnology, Vol. 33, No. 1, pp. 13-38. https://doi.org/10.1007/s10933-004-0424-2

38. Reid, M.A., Tibby, J.C., Penny, D. and Gell, P.A. (1995). "The use of diatoms to assess past and present water quality:" Australian Journal of Ecology,Wiley/Blackwell (10.1111) Vol. 20, No. 1, pp. 57-64. https://doi.org/10.1111/j.1442-9993.1995.tb00522.x

39. Renaud, S.M., Parry, D.L. and Thinh, L.-V. (1994). "Microalgae for use in tropical aquaculture I: Gross chemical and fatty acid composition of twelve species of microalgae from the Northern Territory, Australia:" Journal of Applied Phycology, Vol. 6, No. 3, pp. 337-345. https://doi.org/10.1007/BF02181948

40. Saranya, G., Subashchandran, M.D., Mesta, P. and Ramachandra, T. V. (2018). "Prioritization of prospective third-generation biofuel diatom strains:" Energy, Ecology and Environment, Vol. 3, No. 6, pp. 338-354. https://doi.org/10.1007/s40974-018-0105-z

41. Schoeman, F.R. (1979). "DIATOMS AS INDICATORS OF WATER QUALITY IN THE UPPER HENNOPS RIVER (Transvaal, South Africa):" Journal of the Limnological Society of Southern Africa,Taylor & Francis Vol. 5, No. 2, pp. 73-78. https://doi.org/10.1080/03779688.1979.9633188

42. Seguro, I., García, C.M., Papaspyrou, S., Gálvez, J.A., García-Robledo, E., Navarro, G., Soria-Píriz, S., Aguilar, V., Lizano, O.G., Morales-Ramírez, A. and Corzo, A. (2015). "Seasonal changes of the microplankton community along a tropical estuary:" Regional Studies in Marine Science,Elsevier Vol. 2, pp. 189-202. https://doi.org/10.1016/j.rsma.2015.10.006

43. Sheehan, J., Dunahay, T., Benemann, J. and Roessler, P. (1998). Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae; Close-Out Report: https://doi.org/10.2172/15003040

44. Srinivas, K., Revichandran, C., Maheswaran, P.A., Asharaf, T.T.M. and Murukesh, N. (2003). "Propagation of tides in the Cochin estuarine system, southwest coast of India:" Indian Journal of Marine Sciences,Council of Scientific & Industrial Research Vol. 32, No. 1, pp. 14-24.

45. Stevenson, R.J., Bothwell, M.L. (Max L.. and Lowe, R.L. (1996). Algal ecology : freshwater benthic eco-systems, Academic Press.

46. Sun, C., Wang, B., Li, Z. and Qin, Z. (2015). "[Progress on Diatom Test in Drowning Cases].:" Fa yi xue za zhi, Vol. 31, No. 6, pp. 462-465.

47. Tan, X., Zhang, Q., Burford, M.A., Sheldon, F. and Bunn, S.E. (2017). "Benthic Diatom Based Indices for Water Quality Assessment in Two Subtropical Streams:" Frontiers in Microbiology,Frontiers Vol. 8, p. 601. https://doi.org/10.3389/fmicb.2017.00601

48. Taylor, J.C., Harding, W.R. and Archibald, C.G.M. (2007). A methods manual for the collection, preparation and analysis of diatom samples.

49. Townsend, S.A. and Gell, P.A. (2005). "The Role of Substrate Type on Benthic Diatom Assemblages in the Daly and Roper Rivers of the Australian Wet/Dry Tropics:" Hydrobiologia, Vol. 548, No. 1, pp. 101-115. https://doi.org/10.1007/s10750-005-0828-7

50. Tréguer, P., Bowler, C., Moriceau, B., Dutkiewicz, S., Gehlen, M., Aumont, O., Bittner, L., Dugdale, R., Finkel, Z., Iudicone, D., Jahn, O., Guidi, L., Lasbleiz, M., Leblanc, K., Levy, M., et al. (2018). "Influence of diatom diversity on the ocean biological carbon pump:" Nature Geoscience, Vol. 11, No. 1, pp. 27-37. https://doi.org/10.1038/s41561-017-0028-x

51. Werner, D. (1977). The Biology of diatoms, University of California Press.

52. Zhao, F. in the T.L.C. and B.C. of D.S. for P.B.P., Liang, J., Gao, Y., Luo, Q., Yu, Y., Chen, C. and Sun, L. (2016). "Variations in the Total Lipid Content and Biological Characteristics of Diatom Species for Potential Biodiesel Production:" Fundamentals of Renewable Energy and Applications, Vol. 6, No. 1, pp. 22-26.

53. Zhu, L., Wang, Z., Shu, Q., Takala, J., Hiltunen, E., Feng, P. and Yuan, Z. (2013). "Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment:" Water Research,Pergamon Vol. 47, No. 13, pp. 4294-4302. https://doi.org/10.1016/j.watres.2013.05.004, PMid:23764580

Appendix 1

Species	Acronyms	S1	S2	S3	S4	S5	S6
Achnanthes brevipes Ag.	AB	+				+	+
Achnanthes coarctata (Breb).	AC						+
Achnanthes longipes Ag.	AL	+	+				+
Achnanthes trinodis	AT					+	
Amphiprora alata, Kutz.	AA			+	+		
Amphora cymbifera	AC1						
Amphora ovalis, Kutz. var. affinis	AO	+	+		+	+	+
Amphora salina Wm. Sm.	AS		+			+	
Aulacoseira granulata (Ehr.) Simonsen	AUG	+	+				
Bacillaria paradoxa Gmelin	BP	+	+	+			
Biddulphia laevis forma minor	BL					+	
Campylodiscus hibernicus, Ehr.	CH			+	+		
Cocconeis scutellum, Ehr.	COS	+				+	

Species	Code						
Cocconeis placentula	COP	+	+				
Coscinodiscus radiatus. Ehr.	CR				+	+	+
Coscinodiscus subtilis, var. Normanii	CS		+				
Cyclotella operculata	CO					+	+
Cyclotella meneghiniana, Kutz.	CM	+	+	+	+	+	+
Cymbella cymbiformis, Ehr.	CC		+				
Cymbella obtusa Greg.	CO1				+		
Cymbella parva (Smith) Wolle	CP			+			
Cymbella tumida (Brebisson) Van Heurck	CT		+				
Dictyoca sp.	DT				+		
Diploneis ovalis (Hilse) Cleve	DO	+	+				
Encyonema prostratum Ralfs.	EP			+	+		
Epithema argus, Kutz.	EA			+	+		
Epithema gibberula, var. producta	EG			+	+		
Eunotia flexuosa, Kutz.	EF			+			
Eunotia lunaris (Ehr.), Grun.	EL			+			
Gomphonema affine Kutzing	GA	+	+				
Gomphonema constricta	GC	+				+	
Gomphonema gracile, var. dichotomum	GG		+				
Gomphonema intricatum, Kutz.	GI		+				
Licomophora paradoxa (Lyng).	LP					+	
Licomophora tincta (Ag.), Grun.	LT					+	
Mastogloia lanceolata, Thwaites.	ML		+				
Melosira jurgensii var. octogona, Grun.	MJ	+			+	+	+
Melosira lineata (Dillwyn) C. Agardh	MLI		+				
Melosira varians Agardh	MVR			+			
Navicula ambigua, Kutz.	NAG				+	+	
Navicula amphisbaena	NAM			+			
Navicula amphisbaena, var. subsalina	NAV				+		
Navicula atomoides, Grun.	NAT		+				
Navicula bacillum Ehr.	NBC		+				
Navicula bicapitata Lagerstedt	NBI	+					
Navicula cuspidata, Kutz.	NCU		+				
Navicula forcipata, Grev.	NFO	+	+	+	+	+	+
Navicula fusiformis Grun.	NFU			+			
Navicula granulata, Breb.	NGN						+
Navicula gregaria, Donk.	NGR	+					
Navicula integra (W. Smith) Ralfs	NIN	+	+				
Navicula johnsonii (Wm. Sm.), var. belgica	NJO					+	
Navicula lanceolata Kutz, forma curta	NLN		+				
Navicula mutica, var. quinquenodis	NMU			+			+
Navicula permagna, Bailey	NPM	+	+				
Nitzschia acicularis. Wm. Sm.	NIAC	+	+				
Nitzschia apiculata (Greg.) Grun.	NIAP	+	+				+
Nitzschia atomoides	NIAT	+					

Species	Code						
Nitzschia compressa (Bailey) Boyer	NICM	+	+				
Nitzschia constricta	NICT	+	+	+			
Nitzschia fasciculata, Grun.	NIFS	+	+				
Nitzschia linearis (Ag.) Wm. Sm.	NILI		+				+
Nitzschia obtusa, var. scalpelliformis	NIOB	+	+	+	+	+	+
Nitzschia palea (Kutz.) Wm. Sm.	NIPA		+				
Nitzschia panduriformis Grun.	NIPN			+	+		
Nitzschia sigma var. rigida	NISG	+	+				+
Nitzschia sigma var. rigida var. Intercedens	NISV	+					
Nitzschia vermicularis (Kutz), Grun.	NIVR	+	+				
Pinnularia joculata (Manguin) Krammer	PJ	+	+				
Pinnularia sp.	PSP	+	+				
Pleurosigma acutum norman	PAC	+					
Pleurosigma angulatum, var. quadratum	PAN	+	+				
Pleurosigma balticum, Wm. Sm.	PBL		+				
Pleurosigma distortum	PDS		+				
Pleurosigma elongatum, Wm. Sm.	PEL					+	
Pleurosigma fasciola Wm. Sm.	PFS			+			
Pleurosigma sp.	PSP.1					+	+
Raphoneis amphiceros	RAM		+		+		
Spermatogonia sp.	SPR			+	+		
Surirella robusta var. tenera	SUR			+	+		
Surirella striatula, Turpin	SST	+		+	+		
Synedra ulna (Nitzsch), Ehr.	SUL	+	+				
Triceratium reticulatum	TR		+				