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Effect of physiochemical factor induced Reactive Oxygen Species (ROS) on Marine Microalgal population in South East Coast of India - A Spatial Temporal approach

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Original Research Article

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Abstract: Microalgae represent the main source of biomass production in an aquatic ecosystem which also possess system for generation and elimination of reactive oxygen species (ROS). Marine water receives increased number of anthropogenic chemicals from point and non-point sources. ROS concentration is dynamic in nature, that enhanced by the chemical sources in the marine environment and later it induces the oxidative stress in the marine organisms such as microalgae. In order to understand ROS changes with respect to seasonal fluctuation of marine pollutants and their impact on microalgal communities, two regions of Southeast coast of India were selected i.e., Tuticorin – a pollution impact site and Rameshwaram – the reference site. In this study microalgae distribution during three seasons i.e., summer, monsoon and winter were analysed. Quantification of physiochemical and heavy metal ions were analysed. ROS such as superoxide radical, hydrogen peroxide and hydroxyl radicals were measured using Electron Spin Resonance. Species richness, dominance and cell density of microalgae were calculated. All season data analysis showed significant reduction of species distribution and diversity in both sites. The species richness i.e Shannon- wiener diversity was high in the reference site, where as the dominance simpson diversity was high in impact size showed that the pollutants decreased the richness increase the dominance of some microalgal species. Correlation between the chemical parameter and microalgal distribution showed that the physiochemical parameters like Alkalinity, Phosphorus, Nitrogen compounds, BOD, DO were determined the microalgal density, species richness and dominance in both sites. Dynamic changes of heavy metals ions such as Hg, Cd, Cr, Fe, Zn and Al with the other marine in an chemicals impact site during different season's sites enhance the ROS production. It was found that the micro algal communities were highly affected by chemicals in polluted marine water and that could enhance the oxidative stress.

Keywords: Physicochemical properties, water quality, ROS, Microalgae, temporal variation

INTRODUCTION

Environmentally-induced stresses frequently activate the production of reactive oxygen species (ROS) not only in living systems of terrestrial ecosystem but aquatic. ROS are reduced oxygen intermediates formed by the reduction of molecular oxygen, O_2 that include the superoxide radical (O_2^-), the hydroxyl radical (OH•), and the non-radical species hydrogen peroxide (H_2O_2). The superoxide anion is formed directly from the one-electron reduction of molecular oxygen [1]. Hydrogen peroxide is then formed from the disproportionation of the superoxide anion. The reduction of hydrogen peroxide yields the hydroxyl radical, $H_2O_2 \leftrightarrow 2OH^{\bullet}$, which can then get reduced to the hydroxyl ion and water [2]. Different kinds of environmental stresses [3], [4] industrial pollutants [5], ionizing or ultraviolet radiation, radiolysis and photolysis of water molecules, cellular respiration of animals, plants, and some bacteria [6], presence of elevated levels of transition metals such as copper and zinc; and large range of man-made xenobiotics, polycyclic aromatic hydrocarbons, dioxins, and toxic metals are reported to trigger and release ROS in coastal seawater [7].

Coastal marine pollution is a serious menace, not only to marine biodiversity of India but the entire globe. The Indian coastal waters are under considerable stress from the effluents discharged by the industries and municipalities. Most dreaded are the municipal wastes, sewage sludge and dredged spill dumping, oil spills and leakages. These effluents contain a wide range of hazardous elements such as petroleum hydrocarbons, chlorinated hydrocarbons and heavy metals [8]. In particular, the coastal water of Tuticorin, southeast coast of Tamil Nadu India is prone to different types of pollution. Major coastal activities responsible for coastal/ marine pollution in Tuticorin are discharge and disposal of treated/untreated sewage and industrial wastes; discharge on industrial coolant waters, harbour activities such as dredging, cargo handling, dumping of ship wastes, spilling of cargoes such as chemicals and metal ores, oil transport, fishing activities such as mechanized fishing vessels movements, draining of waste oil, painting of fishing vessels, scrapping of metal lining of fishing vessels, dumping of wastes and trash fishes, salt production etc. Obviously these contaminants are expected to stimulate ROS production in costal seawater via a number of direct and indirect mechanisms. Earlier studies have established that ROS has the potential to harm aquatic organisms and ecosystem structure [9, 10] and, in fact, it has been implicated as the cause of massive death of fish, bacteria, and protists [11-13]. Over-production of ROS leads to oxidative damage of proteins, lipids, and DNA [14, 15]. As the conventional practice of correlating biodiversity loss to its prevailing physicochemical parameters in polluted sea water fails to estimate the impact *in too*, measures of ROS in seawater, an emerging new concept, can be implicated as an indicator of pollution levels for a population living in a contaminated habitat. Amongst marine organisms, the worst sufferers to pollution are microalgae. Microalgae being the major contributors of primary production in the ocean, the bio-regulators of the carbon dioxide, the base of the marine food web and respond to environmental stressors rapidly; they are used as candidate organism to evaluate the impact of ROS in this study. Comparing the concentration of ROS in a polluted site like coastal seawater of Tuticorin (impact site) and unpolluted site (reference site) like the Gulf of Mannar, Southeast coast of Tamil Nadu, India shall reveal the impact of ROS on microalgal biodiversity. The objective of this study is to understand the ecological health of marine microalgae from the correlation between the harmful reactive oxygen species and their impact on natural organisms in the Gulf of Mannar Biosphere Reserve and coastal areas of Tuticorin. Such information has not been documented for natural environments in India.

MATERIALS AND METHODS

Collection of seawater sample

Water samples were collected from the coastal sea of Rameshwaram (reference site) and Tuticorin (impact site) in Gulf of Mannar, Southeast coast of Tamil Nadu, India (Fig. 1) during 2013 – 2014 in summer (March - June), monsoon (July - October) and winter (November - February) for the analysis of reaction oxygen species (ROS), physicochemical parameters and microalgae. Seawater samples were collected in clean, sterilized airtight brown bottles.

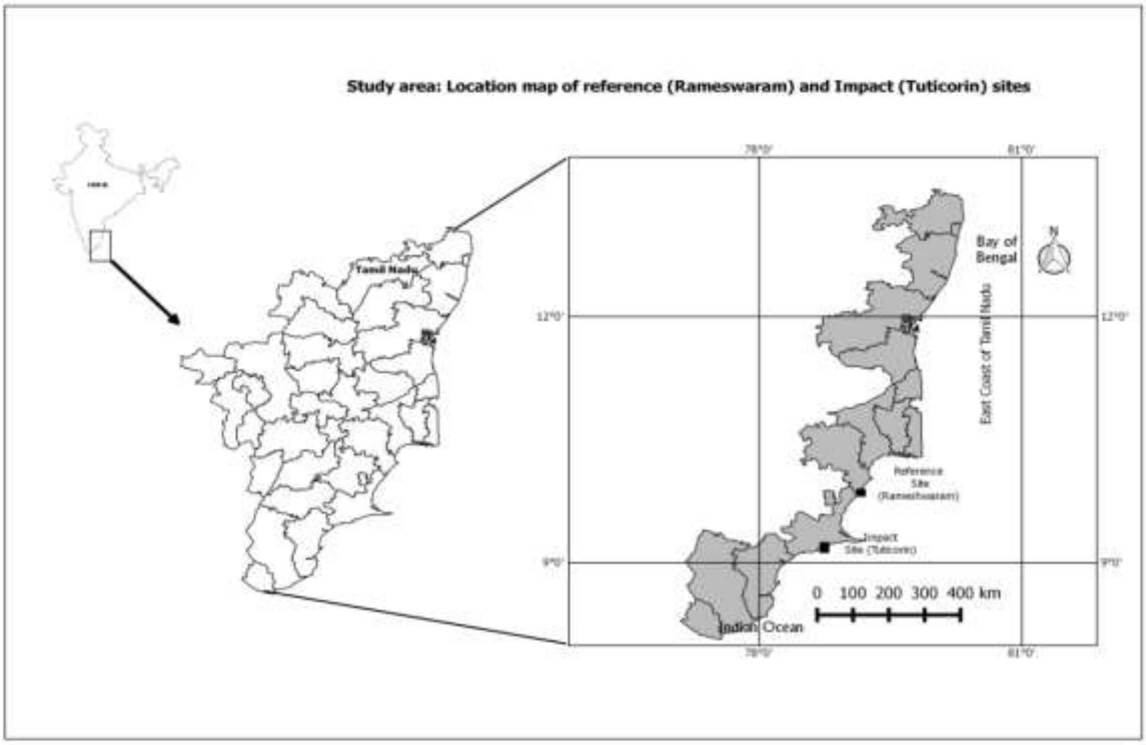

Fig-1: Study area Location map.

Quantification of reactive oxygen species (ROS) in seawater samples

Free radicals such as superoxide [16] and hydroxyl radical [17] were measured by electron spin resonance method. Hydrogen peroxide was estimated spectrophotometrically [18].

Analysis of environmental parameters

Physicochemical parameters such as pH, dissolved oxygen, total alkalinity, Phenolphthalein alkalinity, biological oxygen demand, chloride, nitrate, nitrite, ammonia, inorganic phosphorus, sulfate, sulfide, calcium and magnesium for seawater samples were analysed following standard protocols [19]. Heavy metal analysis for chromium, titanium, silicon, cadmium, iron, zinc, nickel, aluminium, mercury and lead were made in inductively coupled plasma optical emission spectrometry [20].

Identification and quantification of microalgae

Samples of microalgae were collected by towing plankton net (mesh size 30/µm, mouth diameter 45cm) in seawater, concentrated and preserved the samples in polythene bottles with Lugol's iodine solution (100:1), identified the microalgae using bright field microscope (Model number: SDC- 313BPD) following standard monographs [21-23] quantified using haemocytometer [24] and determined their cell density using the formula cited below.

$$\text{Cells mL}^{-1} = \frac{Total\ No.of\ cells\ counted\ in\ 4\ squares}{4} \times 10{,}000$$

Statistical analysis and Microalgal diversity indices

Diversity indices namely Shannon-Wiener diversity index (H') = -Σpi log pi [25] and Simpson diversity index (cd) = Σpi^2 [26] were done in PAST (PAleontological Statistics) software, where 'pi' is the abundance of species 'i', calculated as the proportion of individuals of a given species to the total number of individuals in the community.

Correlation analysis

Correlation matrix in terms of correlogram was done using R (Version 3.4.1) for physiochemical variables ROS, microalgae diversity and density. Correlogram is a graphical representation of cells of a matrix of correlations used to compute a matrix of distances between environmental variables during summer, winter and monsoon seasons of impact and reference sites. The correlogram is representative coloured cells of the matrix and shows the correlation values *i.e.*, green denotes positive correlation and red denotes negative correlation. The coefficient ranges (r) varies from +1 to -1, where +1 and -1 indicates the perfect positive and perfect negative relationships.

Multivariate analysis

Multivariate analysis namely Principal Component Analysis (PCA) was done using STATISTICA 10 software (Math works, Australia). PCA allows identifying the components of dataset that maximizes variance in the selected dataset. It identifies the patterns to reduce the dimension of the dataset and transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables.

RESULTS AND DISCUSSION

Seasonal analysis showed variation in levels of ROS, chemical parameters and microalgal diversity in the reference and impact site.

Reactive Oxygen Species

Superoxide radical concentration ranged between 2.4 to 2.8 µg/L in reference site and 6.2 to 8.8 µg/L. in impact site. A maximum concentration of 8.8 µg/L in impact site during summer and a minimum of 2.4 µg/L in reference site during monsoon season were recorded (Fig 2-4). Hydrogen peroxide concentration ranged between 0.0115 to 0.0133 µg/L in reference site and 0.0356 to 0.0643 µg/L. in impact site with a maximum concentration of 0.0643 µg/L in impact site during summer and a minimum of 0.0115 µg/L in reference site during winter season. Values of hydroxyl radical concentration ranged between 1.11 to 1.82 µg/L in reference site and 3.43 to 5.79 µg/L. in impact site and the maximum of 5.79 µg/L in impact site during summer season and a minimum of 1.11 µg/L in reference site during winter season (Fig 2, 3 and 4). Aquatic organisms also possess systems for generation and degradation of free radicals [27], but changes in the chemical composition enhance the ROS production which induces the oxidative stress within these organisms. In the impact site there was the increase level of the marine chemicals and heavy metals, which showed the increased level of ROS in these regions. Chemicals and metal ions such as iron, copper, chromium and mercury are well known inducers of oxidative stress. They can stimulate ROS production via different mechanisms [28].

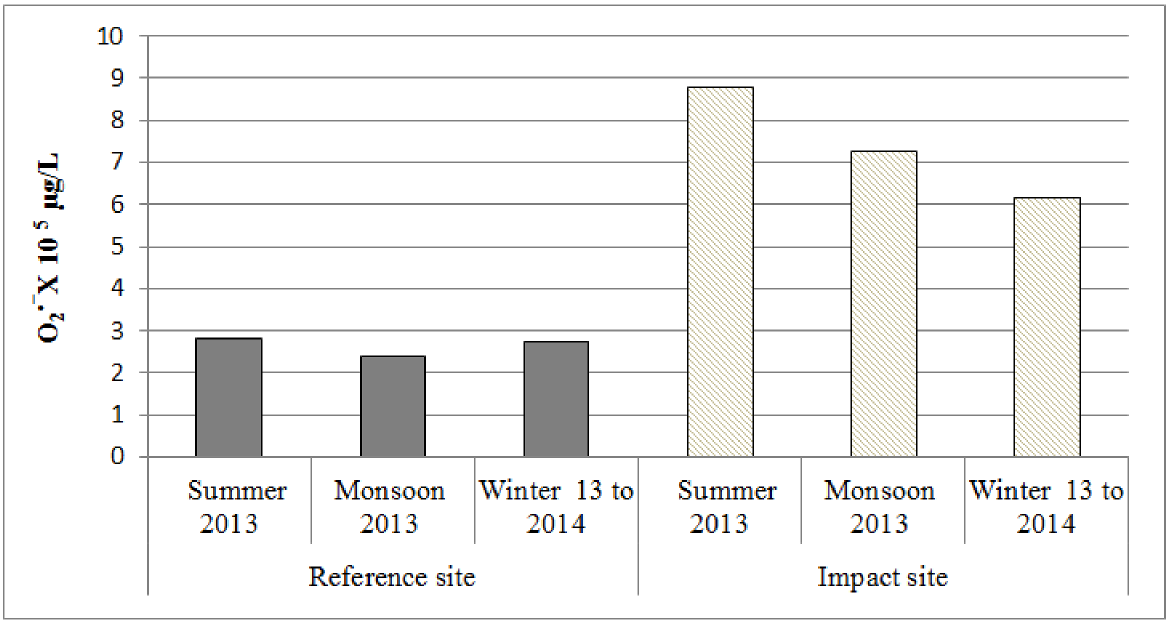

Fig-2: Superoxide radical concentration in reference and impact sites of Southeast coast of Tamil Nadu during the year 2013 - 2014

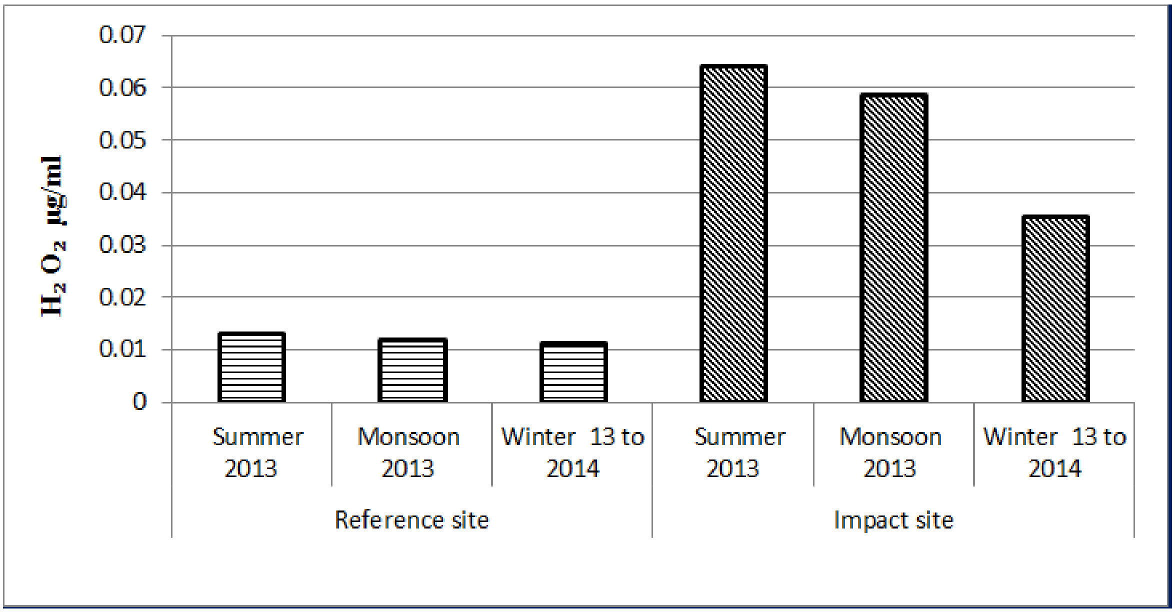

Fig-3: Hydrogen peroxide concentration in reference and impact sites of Southeast coast of Tamil Nadu during the year 2013 - 2014

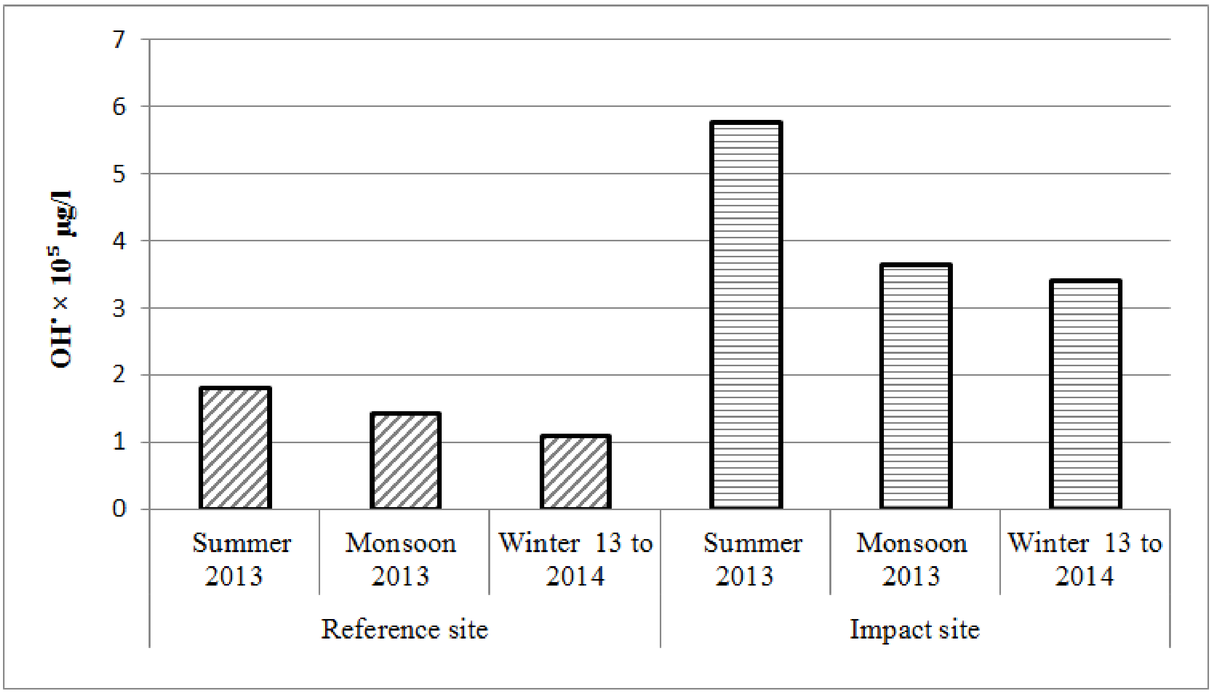

Fig-4: Hydroxyl radical concentration in reference and impact sites of Southeast coast of Tamil Nadu during the year 2013 - 2014

Physicochemical parameters

Physicochemical parameters on reference site and impact site showed in Table 1.The pH of seawater of both sites remained alkaline throughout the study period. Dissolved oxygen (DO) values ranged between 4.3 to 4.71 mg/L in reference site and 1.96 to 4.86 mg/L. in impact site. Dissolved oxygen plays an important role in life processes in aquatic environment [30]. Low dissolved oxygen concentration would bring about a decrease in the number of species thriving in the environment and it can be attributed to eutrophication [31]. BOD values ranged between 7.9 to 11.24 mg/L in reference site and 3.13 to 18.4 mg/L in impact site. Increased concentration of DO in reference site showed the availability of oxygen potential to survive microalgae. Increased BOD level would exploit the ability of microorganisms to oxidize organic material into CO_2 and water using molecular oxygen [32]. In the reference site phosphorous (IP and TP) was more during summer because it could be due to evaporation of seawater [33] during summer. The NO_3^{1-} values ranged between 66.12 to 116.79µM/L in reference site and 136.67 to 144.27 µM/L in impact site. The NO_2^- values ranged between 69.12 to 88.12µM/L in reference site and 92.61 to114. 37 µM/L in impact site. The NH_3 values ranged between 73.66 to 90.14 µM/L in reference site and 110.93 to 139.4 µM/L in impact site. Nitrogen cycle involves elementary dissolved nitrogen oxides such as NO_3, and NO_2 for reference sites. NH_3 play a significant role in sustaining the quality of aquatic life in marine environment. Elevated levels of nitrate, nitrite and ammonia in the polluted site are the indicators of water pollution and represented the highest oxidized form of nitrogen. The most important source of nitrogen is the biological oxidation of organic nitrogenous substances derived from sewage and industrial wastes or produced indigenously in the water [34]. [35] Observed that variation in nitrate and its reduced inorganic compounds were predominantly the result of biologically activated reactions. Quick assimilation by phytoplankton and enhancement by surface run-off results in large scale spatio-temporal variation of nitrate in the coastal regions. Increasing trend of the following chemical parameters namely total alkalinity, phenolphthalein alkalinity, chloride, nitrate, nitrite, ammonia, total phosphorus, inorganic phosphorus, sulphate, sulphide, calcium and magnesium were observed in impact site than the reference site. Ca++ and Mg++ have vital importance in plants, which photosynthesis in the aquatic environment, Mg++ is in the structure of chlorophyll, and the concentration of Mg++ has a great effect on algae [36]. [37] Reported that the inorganic constituents such as sodium, calcium, potassium, magnesium, chlorine, sulphur, phosphate, ammonium salts increased the level of pollutants from different sources from towns, chemical manufacturing industries and leachates from solid waste disposal sites. Values of heavy metals such as Cu, Fe, Ni, Ti, Zn, Al, Cd, Si, Pb and Hg showed that there was minor variation between impact and reference site The minor variation of heavy metals in aquatic ecosystem showed a great tendency to accumulate metals in the bodies of planktons [38] and its high tolerance to heavy metal [39]. But the species diversity and cell density of microalgae was varying in both sites, which could be due to heavy metal pollution (Table 1). In this study six species of *Phormidium* were reported and among them *P. valderianum* was reported only in the impact site.

[29] Explained the some genera like Phormidium was often abundant in alkaline waters polluted by heavy metals.

Table-1: Levels of physicochemical parameters in the seawater samples of reference site and impact site in Southeast coast of Tamil Nadu, India in the year 2013 - 2014

S. No.	Chemical Parameters	Reference site			Impact site		
		Summer	Monsoon	Winter	Summer	Monsoon	Winter
1	pH	7.6	7.6	7.7	7.5	7.8	7.7
2	TA mg/L	113.1	117.5	110.2	124.5	143.1	121
3	PA mg/L	111.7	119.3	115	131.2	136.7	119
4	DO mg/L	4.3	4.47	4.71	4.86	1.96	3.29
5	BOD mg/L	7.9	11.24	8.56	18.4	3.13	3.78
6	Cl g L	19.58	20.71	19.67	22.42	20.22	21.95
7	NO_3-µM/L	86.12	116.79	66.12	136.67	139.36	144.27
8	NO_2- µM/L	69.12	78.76	88.12	92.61	106.27	114.37
9	NH_3 µM/L	73.66	90.14	79.7	110.93	117.06	139.4
10	TP µM/L	0.35	0.26	0.37	0.66	0.76	0.55
11	IP µM/L	0.29	0.26	0.23	0.93	0.58	0.61
12	SO_4^{2-} mg/L	178.7	195.45	241.44	286.83	295.82	288.48
13	S^{2-} mg/L	165.66	158.13	221.62	315.82	278.59	289.3
14	Ca mg/L	408.34	419.73	298.34	513.6	532.24	494.57
15	Mg mg/L	122.61	124.11	130.61	176.4	179.33	144.33
16	Cr mg/L	0.0010	0.0014	0.0011	0.0009	0.0010	0.0012
17	Cu mg/L	0.0019	0.0018	0.00193	0.0029	0.0039	0.0042
18	Fe mg/L	0.0298	0.0268	0.0267	0.0426	0.0476	0.0516
19	Ni mg/L	0.0015	0.0013	0.0015	0.0026	0.0029	0.0031
20.	Ti µg/L	0.0022	0.002	0.005	0.00003	0.00007	0.00008
21.	Zn mg/L	0.00697	0.00627	0.00617	0.0054	0.0057	0.006
22.	Al µg/L	0.0315	0.0295	0.03153	0.0467	0.04967	0.05367
23.	Cd mg/L	0	0	0	0.00013	0.00013	0.00012
24.	Si µg/L	0.01	0.09	0.1	0.08	0.013	0.09
25.	Pb µg/L	0.001	0.0013	0.0019	0.0022	0.0031	0.0045
26.	Hg µg/L	0.002	0.0019	0.0029	0.002	0.0039	0.0051

Microalgae Species Diversity

Microalgae representing four different families viz. Chlorophyceae, Bacillariophyceae, Dinophyceae and Cyanophyceae were recorded from reference and impact sites (Table 2). *Chroococcus minutus* and *Surirella nervatus* were recorded in both the sites in all seasons. *Actinoptychus splendens, Bacillaria paradoxa, Biddulphia rhombus, Odontella mobiliensis, Paralia sulcata, Trachynesis antillarus, Triceratium robertianum, Oscillatoria foreaui, Oscillatoria minnesotensis, Cosmarium subtumidium, Pediastrum tetras* and *Scenedesmus armatus* were observed only in reference site in all season while *Auliscus sculptus, Nitzschia vitrea, Triceratium dubium* and *Pediastrum simplex* occurred only in impact site in all seasons. Nevertheless these species were reported as potential bio indicators [40-43] and it is a well suited tool for understanding water pollution [44]. *Amphora lineata* was recorded from the reference and impact sites only during winter season. [45] Observed similar distribution of *Amphora sp.* during winter in the Belgian coastal zone. In the present study, *Cyclotella meneghiniana* recorded only in reference site during all seasons but not in impact site indicated that *C. meneghiniana and Scenedesmus bijugatus* were sensitive to pollution and significantly replaced by *Pleurosigma angulatum, Bacteriastrum varians, Microcoleus acculismus* and *Cosmarium subtumidium. Actinoptychus splendens, Diploneis bombus, Mastogloia lanceolata, Navicula granulata, Coscinodiscus gigas, Thalassionema* *nitzschioide* and *Triceratium robertianum* reported only from reference site but not in the impact site could be due to toxic waste disposal from thermal power stations and other industries in that area.

Table-2: Presence of phytoplankton species (✓) during different seasons at the reference and impact sites during the year 2013 - 2014

Species	Reference site			Impact site		
	Summer 2013	Monsoon 2013	Winter 13/2014	Summer 2013	Monsoon 2013	Winter 13/ 2014
Bacillariophyceae						
Actinoptychus splendens	✓	✓	✓			
Actinoptychus undulatus	✓		✓	✓	✓	
Amphora coffeiformis		✓	✓			✓
Amphora lineata	✓	✓	✓	✓	✓	
Amphora ovalis	✓	✓		✓	✓	✓
Amphora proteus	✓	✓	✓	✓	✓	✓
Amphora sp.	✓	✓	✓	✓		
Asteromphalus flabellatus	✓				✓	✓
Auliscus caelatus	✓	✓	✓	✓		
Auliscus sculptus				✓	✓	✓
Bacillaria paradoxa	✓	✓	✓			
Biddulphia rhombus	✓	✓	✓			
Campylodiscus intermedius	✓			✓	✓	✓
Chaetoceros affinis		✓	✓	✓	✓	
Climacosphenia elongata	✓		✓			✓
Coscinodiscus asteromphalus	✓	✓				
Coscinodiscus centralis	✓		✓	✓	✓	
Coscinodiscus kuetzingii		✓	✓			✓
Cyclotella meneghiniana	✓	✓	✓	✓	✓	
Cyclotella stylorum	✓	✓		✓	✓	✓
Navicula sp.	✓	✓	✓	✓	✓	✓
Nitzschia acicularis	✓	✓	✓	✓		
Nitzschia plana	✓				✓	✓
Nitzschia sigma	✓	✓	✓	✓		
Nitzschia vitrea				✓	✓	✓
Odontella mobiliensis	✓	✓	✓			
Paralia sulcata	✓	✓	✓			
Pinnularia acrosphaeria	✓			✓	✓	✓
Planktoniella sol		✓	✓	✓	✓	
Pleurosigma angulatum	✓		✓			✓
Pleurosigma staurophorum	✓	✓			✓	
Pseudo-eunotia doliolus	✓		✓	✓	✓	
Rhabdonema minutum		✓	✓			✓
Surirella armoricana	✓	✓	✓	✓	✓	
Surirella fastuosa	✓	✓		✓	✓	✓
Surirella nervatus	✓	✓	✓	✓	✓	✓
Thalassiosira lineata	✓	✓	✓	✓		
Trachynesis antillarus	✓				✓	✓
Trachynesis aspera	✓	✓	✓	✓		
Triceratium dubium				✓	✓	✓
Triceratium favus	✓	✓	✓			
Triceratium robertianum	✓	✓	✓			
Triceratium reticulam	✓			✓	✓	✓
Thalassionema oestrpii		✓	✓	✓	✓	
Dinophyceae						

Ceratium fusus	✓	✓	✓	✓		
Ceratium lineatum	✓		✓	✓	✓	
Ceratium massiliense		✓	✓			✓
Cyanophyceae						
Chroococcus gomontii	✓	✓		✓	✓	✓
Chroococcus minutus	✓	✓	✓	✓	✓	✓
Chroococcus sp	✓	✓	✓	✓		
Gloeocapsa polydermatica	✓				✓	✓
Lyngbya burgertii	✓	✓	✓	✓		
Lyngbya sp.				✓	✓	✓
Oscillatoria foreaui	✓	✓	✓			
Oscillatoria minnesotensis	✓	✓	✓			
P.valderianum	✓			✓	✓	✓
Phormidium sp.		✓	✓	✓	✓	
Phormidium foveolarum	✓	✓	✓			✓
P .fragile	✓	✓				
P. tenue	✓		✓	✓	✓	
Phormidium mole		✓	✓			✓
Phormidium uncinatum	✓	✓	✓	✓	✓	
Spirulina subsalsa	✓	✓		✓	✓	✓
Chlorophyceae						
Cosmarium obsoletum	✓	✓	✓	✓		
Cosmarium subtumidium	✓				✓	✓
Pediastrum boryanum	✓	✓	✓	✓		
Pediastrum simplex				✓	✓	✓
Pediastrum tetras	✓	✓	✓			
Scenedesmus armatus	✓	✓	✓			
Scenedesmus bijugatus	✓			✓	✓	✓

Microalgal cell density

Density of microalgae varied between 5, 40 and 78,000 cells L^{-1} and it represented the maximum during summer and minimum in winter season from both site (Fig. 5). Among the microalgae reported, bacillariophyceae was the dominant group representing 71.42% of the total microalgae population studied. In reference site maximum density of bacillariophyceae was reported during summer season (78,000 cells L^{-1}) followed by monsoon season (64,000 cells L^{-1}), while minimum density was seen in winter season (42,000 cells L^{-1}). In impact site maximum level of density occurred during summer season (53,000 cells L^{-1}) followed by monsoon season (32,000 cells L^{-1}), while minimum occurrence was in winter season (23,000 cells L^{-1}). Maximum density of cyanophyceae was recorded during summer (33,000 cells L^{-1}) at reference site followed by monsoon (27,000 cells L^{-1}), while minimum cell density was reported in winter (21,000 cells L^{-1}) and maximum (22,000 cells L^{-1}) during summer season followed by monsoon season (18,000 cells L^{-1}). In impact site the minimum occurrence was recorded during the winter season (13,000 cells L^{-1}). Chlorophyceae members were the third dominant group with 7% of the total population in microalgae in the study areas. In reference site maximum density was recorded during summer (7,000 cells L^{-1}) followed by monsoon (6,000 cells L^{-1}) and winter (3,000 cells L^{-1}) whereas in impact site maximum density reported during the monsoon (4,000 cells L^{-1}) followed by summer (5,000 cells L^{-1}) and winter (2,000 cells L^{-1}).

Dinophyceae were the fourth dominant group that was represented by 3 species accounting for 4% of the total microalgae population. During summer seasons maximum density of cells was recorded (2,000 cells L^{-1}) in reference site followed monsoon (1,500 cells L^{-1}) and winter (1, 200 cells L^{-1}). In impact site the maximum density was recorded during summer (840 cells L^{-1}) followed by monsoon (730 cells L^{1}) and winter (540 cells L^{-1}). It was reported that the cell density of cyanophyceae, bacillariophyceae, chlorophyceae and dinophyceae were higher in all seasons in reference site when compared to Impact site. This was due to the effect of pollutants from various point and non-point sources in the impact site. [46] reported that the water pollution changes the algal density. Analysis showed that the most of the chemical parameters were negatively correlated with the cell density as discussed later in this article.

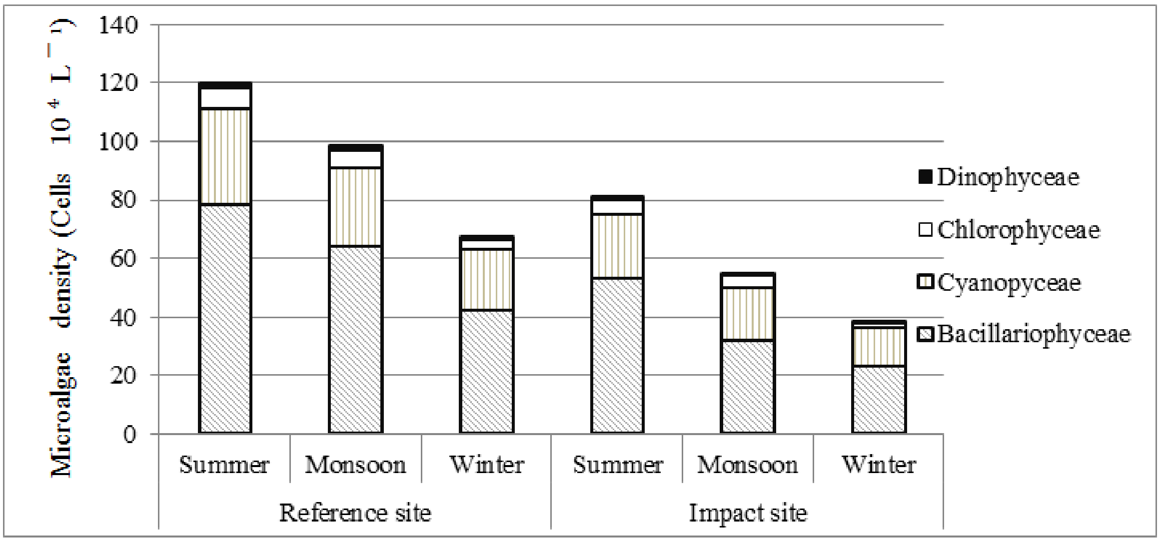

Fig-5: Microalgae cell density during three different seasons in reference and impact sites

Simpson Diversity index of the Reference and impact site

Shannon-Weiner diversity index for reference and impact site was 3.9542 and 3.6911 during summer followed by monsoon (3.8194, 3.5517) and winter (3.4664, 3.4049). The value of Simpson diversity index was 0.0229 and 0.0312 during monsoon followed by winter (0.0228, 0.0360) and summer (0.0203, 0.0264) in reference and impact sites respectively (Fig-6 & 7). Shannon- Weiner diversity index *i.e.*, species richness, that was low in Impact site than reference site in all season. Contradictory the Simpson diversity index was high in impact site which showed that the increase of dominance of some microalgal species due to the pollutants that reduce the diversity of microalgae in this site, which supported by low diversity leads to dominance of a tolerant species [47-49].

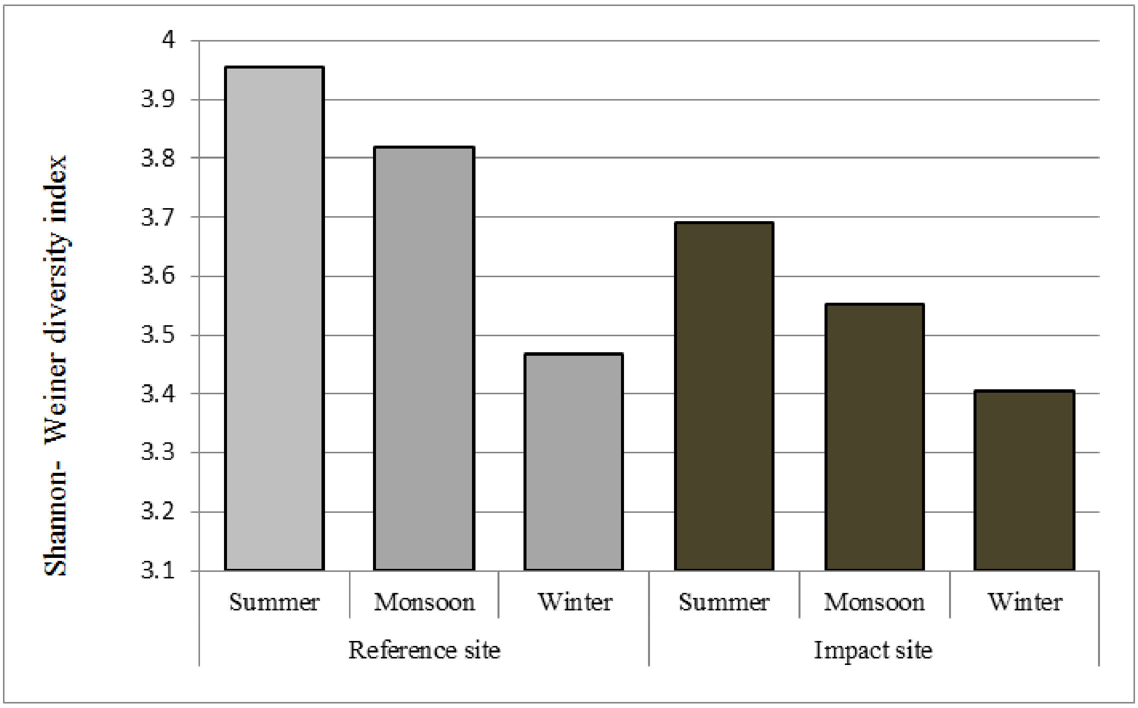

Fig-6: Shannon-Weiner Diversity index of the Reference and impact site

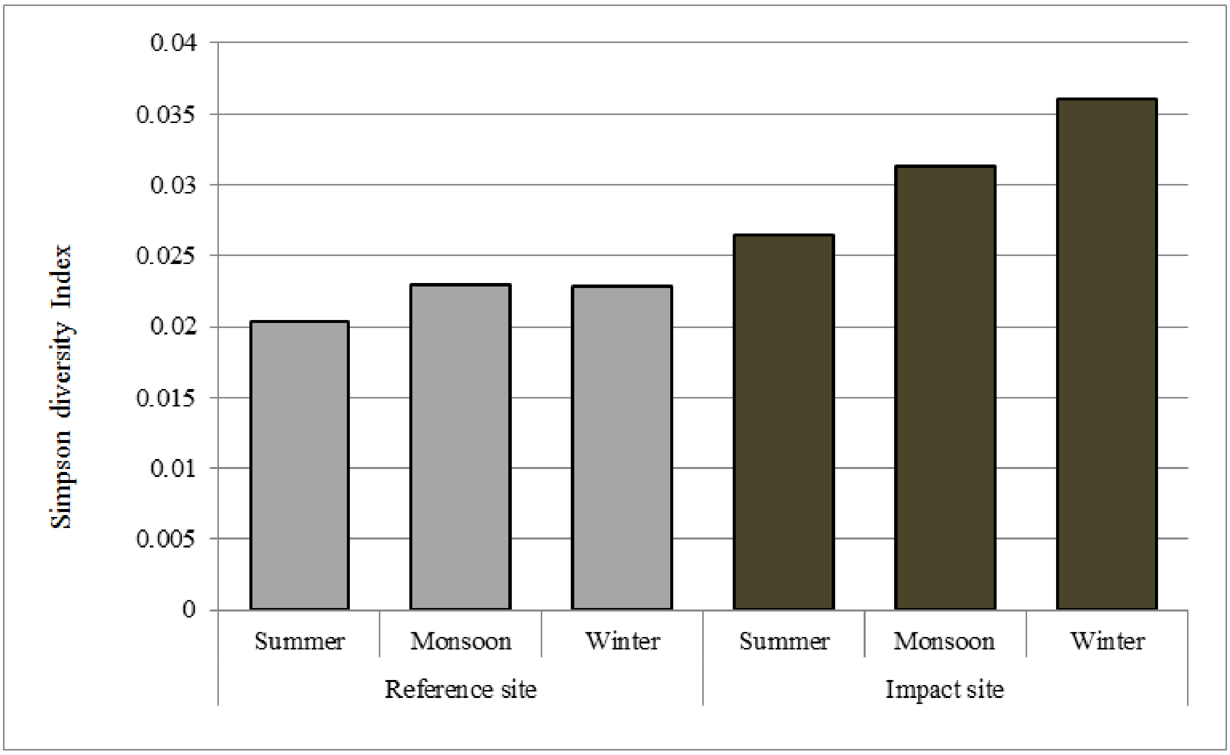

Fig-7: Simpson Diversity index of the Reference and impact site

Correlogram analysis

The statistical relationships between the microalgal density, among the species diversity (Shannon-Weiner, simpson diversity index) and marine chemicals environment variable during different seasons were analysed using the correlogram R statistical environment. The correlogram during summer season Microalgae density were positively correlated with Zn, Ni, Fe, S_3^-, IP, BOD, Cl at reference site and negatively correlation with Hg, Si, Al, Ti, Cr, Ca, S^{2-}, TP, NO_2^-, NH_3 and NO_3. Where as in impact site Microalgae density was strongly positively correlation Pb, Si, Cu, NH_3, Cr, IP and Al strongly negative correlated with Cd, Zn, Ni, Fe, S_4^{2-}, S^{2-}, NO_3, Cl, DO, BOD, Mg, Ti, Ca, TA and TP. During monsoon at reference site microalgal density was strongly positively correlation with Pb, Si, Cu, NH_3, Cr, IP, PA and Al, it strongly negative correlation with Cd, Zn, Ni, Fe, S_4^{2-}, S^{2-}, NO_3, Cl, DO, BOD, Mg, Ti, Ca, TA and TP. In impact site microalgal density was in positive correlation with by P, Si strongly negative correlation by BOD and Pb. During winter in the reference site microalgae was strongly positive correlation with Pb, Ti, Ni, S^{2-}, PA and NH_3, strongly negative correlation with Si, Zn, Fe, Cu, Cr, Mg, Ca, S_4^{2-}, S^{2-} , TP, NO_3, NO_2, BOD, TA, DO and in impact site it was strongly positively Pb, Si, Al, Fe, Cu, Mg, S^{2-}, NO_2^-, BOD, PA, DO, Hg with strongly negatively correlation in Cd, Zn, Ni, S_4^{2-}, IP, TA, TP, Ti,

It was reported that the microalgal density is negatively correlated with free radicals in all seasons except OH radicals during winter, that moderately positive correlation with microalgal density. The correlogram microalgae density microalgae density were in Zn, Ni, Fe, S_4^{2-}, IP, BOD, Cl. in strongly positively correlation from summer at reference site where in Hg, Si, Al, Ti, Cr, Ca, S_2^-, TP, NO_2^-, NH_3, NO_3^-, $OH^\bullet$ and strongly negatively correlation from summer at reference site. In impact site during summer microalgae density was strongly positively correlation Pb, Si, Cu, NH_3, Cr, IP, Al and strongly negative correlation Cd, Zn, Ni, Fe, S_4^{2-}, S^{2-}, NO_3, Cl, DO, BOD, Mg, Ti, Ca, TA, TP, H_2O_2, $OH^\bullet$ and $O_2^{\bullet-}$. This reveals that the Heavy metals where enhanced of the ROS production that affect the microalgal density. In reference site during monsoon microalgae density was strongly positively correlation with Pb, Si, Cu, NH_3, Cr, IP, PA and Al and it strongly negative correlation with Cd, Zn, Ni, Fe, S_4^{2-}, S^{2-}, NO_3^-, Cl, DO, BOD, Mg, Ti, Ca, TA, TP, H_2O_2, $OH^\bullet$ and $O_2^{\bullet -}$ Where as in the impact site microalgae was in strangely positive by TP, Si and $O_2^{\cdot -}$ Strongly negative correlation by BOD and Pb. In monsoon was impact site. In reference site during winter microalgae density was strongly positive correlation with Pb, Ti, Ni, S^{2-}, PA, NH_3 and strongly negative correlation with Si, Zn, Fe, Cu, Cr, Mg, Ca, S_4^{2-}, S^{2-}, TP, NO_3, NO_2, BOD, TA and DO by winter was reference site. The phytoplankton abundance and diversity vary with season and attributed to change in nutrient availability, light and temperature [50]. Nitrate the highest oxidized form of nitrogen, is considered as one of the pollution indicators in the water quality and its distribution is influenced by the season. The positive correlation indicated the preference of these species to nitrite rich water and negative correlation indicated the inadaptability to survive in the adverse condition. Dissolved oxygen plays an important role in life processes in aquatic environment. [51]. Low dissolved oxygen concentration would bring about a decrease in the number of species thriving in the environment. Low dissolved oxygen can be attributed to eutrophication [52]. The correlogram microalgae density were in Hg, Si, Al, Ti, Cr, Ca, S_2^-, P, NO_2, NH_3 and NO_3^- in strongly negatively correlation from summer at reference site. Microalgae densities were in Zn, Ni, Fe, S_4^{2-}, IP, BOD and Cl. in strongly positively correlation from summer at reference site**.** Microalgae densities were in Fe. In strongly positively correlation from summer at reference site which indicate the fact that the iron uptake by marine microalgae species belonging to different phyla reported [53].

In reference site during summer Shannon-Weiner (H') diversity index was positively correlated with Hg, Si, Al, Ti, Cr, Ca, S^{2-}, TP, NH_3, NO_2^-, NO_3^- and Simpson diversity negatively correlated with Hg, Si, Al, Ti, Cr, Ca, S^{2-}, NH_3, NO_2^-, NO_3^-, Ca and Mg. Where as in impact site H' positively correlated with PA and Al negatively correlated with all other parameters. Simpson diversity index positively correlated with $OH^{\bullet}$, Cd, Zn, Ni, Fe, S_4^{2-}, S^{2-}, NO_3^-, Cl, DO and BOD negatively correlated with Si, Cu, Cr, NH_3 and IP. During monsoon in reference site H' positively correlated with $OH^{\bullet}$, O_2•- and NH_3 and Simpson negatively correlated with Microbial density. In impact site H' positively correlated with BOD and Pb and with Simpson positively correlated with H_2O_2, pH and $OH^{\bullet}$. During winter at reference site H' negatively correlated with $O_2^{\bullet-}$, Pb, (Microalgal density), Ti, Ni, S^{2-}, PA, NH_3, and positively with IP, Cl, Al, DO, TA, BOD, NO_2^-, NO_3^-, TP, S_4^{2-},Ca, Mg, Cr, Cu, Fe, Zn and Si whereas Simpson positively correlated with IP, Cl, Al, DO, TA, BOD, NO_2^-, NO_3^- , TP, S_4^{2-}, Ca, Mg, Cr, Cu, Fe, Zn and Si negatively with $O_2^{\bullet-}$, Pb, (Microalgal density), Ti, Ni, S^{2-} , PA, and NH_3,. In impact site H' negatively correlated with Si, NH_3, PA, S^{2-}, Ni, Ti, Microalgal density, Pb, $OH^{\bullet}$, $O_2^{\bullet-}$ and pH positively with Hg. The Simpson diversity in this season it negatively correlated with $OH^{\bullet}$, $O_2^{\bullet-}$ and pH positively with H_2O_2, and Hg. Diversity indices are applied in water pollution research to evaluate the effects of pollution on species composition [54]. The climate and water quality parameters effect on the biodiversity [55]. The positive correlation of some water properties with phytoplankton density may be due to playing a pivotal role in regulation various biological activities and growth [56] (Fig-8: a-f).

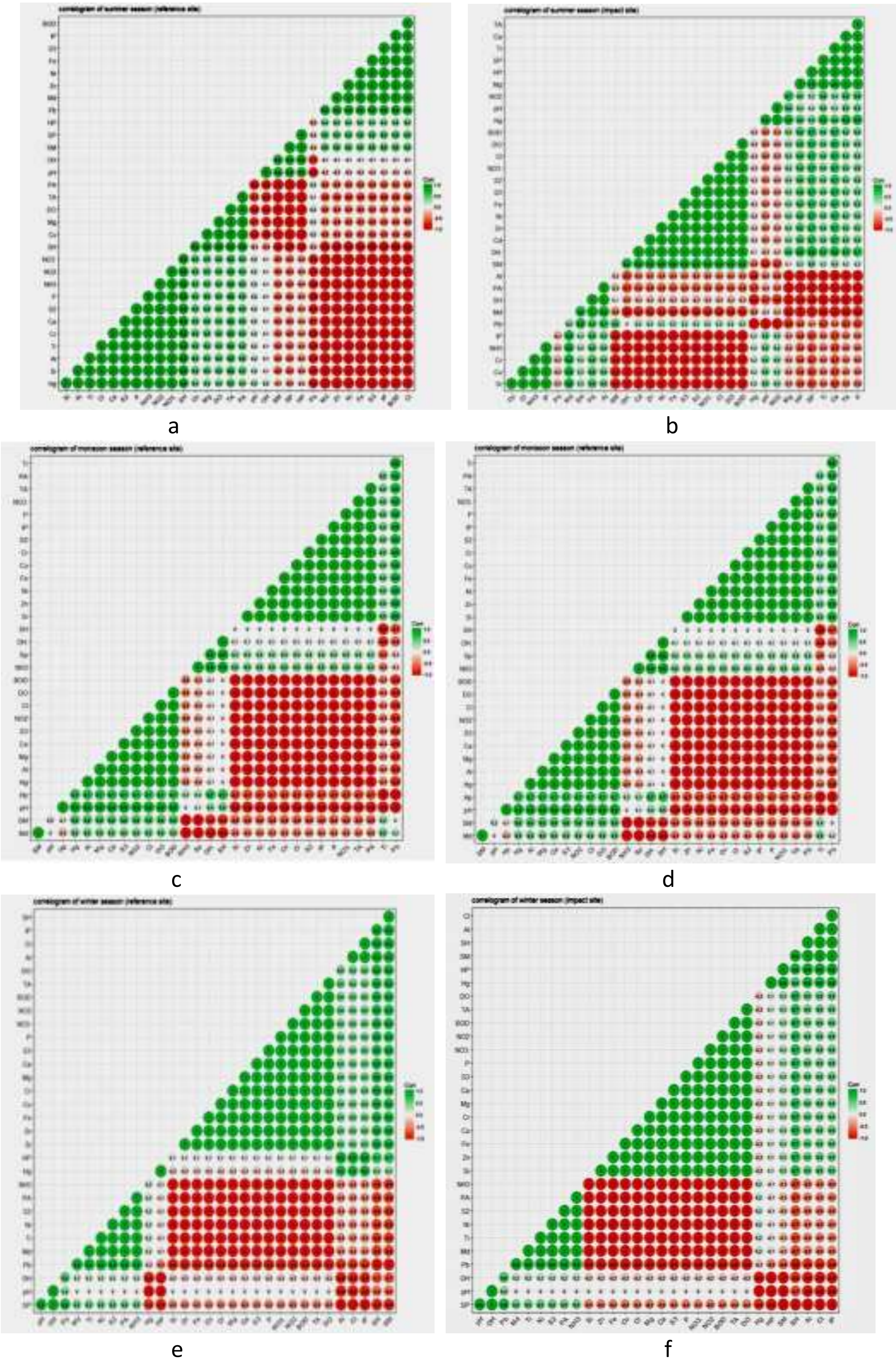

Fig-8: Correlation analysis on physiochemical parameters of reference and impact sites, a) Summer season (reference site), b) Summer season (impact site), c) Monsoon season (reference site), d) Monsoon season (impact site), e) Winter season (reference site), f) Winter season (impact site)

Principal Component Analysis (PCA)

Principal component analysis (PCA) of reactive oxygen species (ROS), chemical parameters and microalgal density variables developed two principal components (PCs). A PCA biplot of the reference site clearly indicates the relationship between variables and explain the overall variability in the environmental data.

In the reference site the 79.84 % of the total variability that be explained by PC-1 (44.21%) associated with NO_3^-, TA, Cl, BOD, Cr, Ca, Microalgal density, NH_3, PA and PC2 (35.62%). Mostly associated with NO_2^-, DO, S_4^{2-}, Mg, PA, H_2O_2, NH_3, Ti, S^{2-}, Cr, BOD and Cl .

Where as in the impact site the 83.76 % of the total variability that explained by PC1 (46.59%) NO_2^-, Fe, Cu, Al, NO_3^- , Cr, Ni, NH_3, Zn, pH and PC2 (37.16%) associated with TA, Ti, S_4^{2-}, TP, Ca, $OH^{\bullet}$, PA, Mg. Positive loading denotes the role of parameters in explaining the variation in PCA and reduce the values of variables have small role, in explaining the variation were given in Table-3.

Table-3: Principal component analysis (PCA) of physico-chemical parameters, reactive oxygen species and microalgae density in the reference site and impact site during year 2013 to 2014.

Variable	Reference site		Impact site	
	Component 1	Component 2	Component 1	Component 2
pH	0.046586	0.191753	0.508935	0.410991
TA	0.983321	-0.032567	0.001981	0.997741
PA	0.697695	0.693741	-0.605917	0.787903
DO	-0.378096	0.914313	-0.638624	-0.769807
BOD	0.850162	0.517934	-0.903777	-0.429485
Cl	0.911836	0.401638	-0.358940	-0.927228
NO_3^{1-}	0.997670	-0.054703	0.958272	-0.278717
NO_2-	-0.357543	0.933865	0.998922	0.034756
NH_3	0.753110	0.649267	0.893454	-0.441178
TP	-0.976345	-0.192525	-0.380974	0.919754
IP	0.340829	-0.925793	-0.876828	-0.482548
S_4^{2-}	-0.582537	0.815871	0.254268	0.963082
S^{2-}	-0.831615	0.559469	-0.770890	-0.638072
Ca	0.819311	-0.577412	-0.391548	0.916475
Mg	-0.619825	0.784195	-0.746772	0.659961
Cr	0.821340	0.558834	0.953806	0.044265
Cd	0.000000	0.000000	-0.68432	-0.42343
Cu	-0.949503	-0.305321	0.982542	0.184633
Fe	-0.210416	-0.973803	0.997377	-0.055856
Ni	-0.838530	-0.317694	0.922451	-0.229445
Ti	-0.711428	0.623960	0.175957	0.982263
Zn	-0.062493	-0.996159	0.825319	-0.389885
Al	-0.834859	-0.462281	0.977855	-0.200716
Si	-0.581946	0.009574	-0.632149	-0.417786
Pb	-0.135116	-0.257938	0.003517	-0.989687
Hg	-0.059005	-0.290483	-0.268531	-0.065006
$O_2\bullet^-$	0.019133	-0.007652	-0.415342	0.071565
H_2O_2	0.331081	0.670961	-0.054853	0.043061
$OH^{\bullet}$	0.011880	0.050376	0.339265	0.847757
Microalgal density	0.807825	-0.590035	0.094476	0.044534
% Total variance	44.21625	35.62605	46.59950	37.16519

Table-4: Percentage of variance in PCA analysis of microalgal diversity indices and physicochemical properties of seawater in reference and impact sites

Sites	Component	Eigenvalues	% Total variance	Cumulative eigenvalue	Cumulative %
Reference site	1	14.14920	44.21625	14.14920	44.21625
	2	11.40034	35.62605	25.54953	79.84229
Impact site	1	15.37784	46.59950	15.97784	46.59950
	2	12.26451	37.16519	27.64235	83.76469

In reference site Superoxide, Hydroxyl radical and Hydrogen perioxide radical is closely related to TA, PA, NH_3, Cr, BOD, pH and Cl during monsoon. There was negative impact on microalgal density was reported. In the impact site during monsoon Mg, PA, Ca TP, Hg, Pb were closely correlated with $O_2^{\bullet-}$, which was closely correlated with microalgal density and TA, Ti, S^{2-}, pH, Cu, NO_2^-, Fe were closely correlated with the H_2O_2 and $OH^•$ radical.

The PCA plot showed that axis I was highly and positively correlated with summer season at reference site of microalgae density Ca, NO_3^-, IP whereas axis II strongly correlated with winter season on reference site S_4^{2-}, NO_2^-, DO, Mg, Ti, S^{2-} with axis II also Fe, Zn to be negatively correlated with axis I, and TP, Cu, Ni, Al, Pb, Hg. The difference in the relative size of axis I and axis II was small (Eigen values of 14.14 and 11.40 respectively) (Table 3 and 4) (Fig. 9 & 10). The variable lines were obtained from the factor loadings of the original variables. They stand for the contribution of the variables to the samples. More closely the two variable lines, the stronger is the mutual correlation [57]. Positive loading of NO_3-, total N, alkalinity, free CO_2, Ca^{2+}, and Na^+ indicated organic matter accumulation from domestic wastewater [58]. Marine chemical the analysis of present study revealed that marine chemicals and heavy metals enhance by various pollutants in impact site then the reference site. There increase level of pollutants affects diversity, density of micro algae enhance level of ROS production which induced the oxidative stress in marine microalgae. Which proved that by the micro algal diversity, density is reduced some tolerant species are increased that which shows the microalgae has antioxidant defence mechanism.

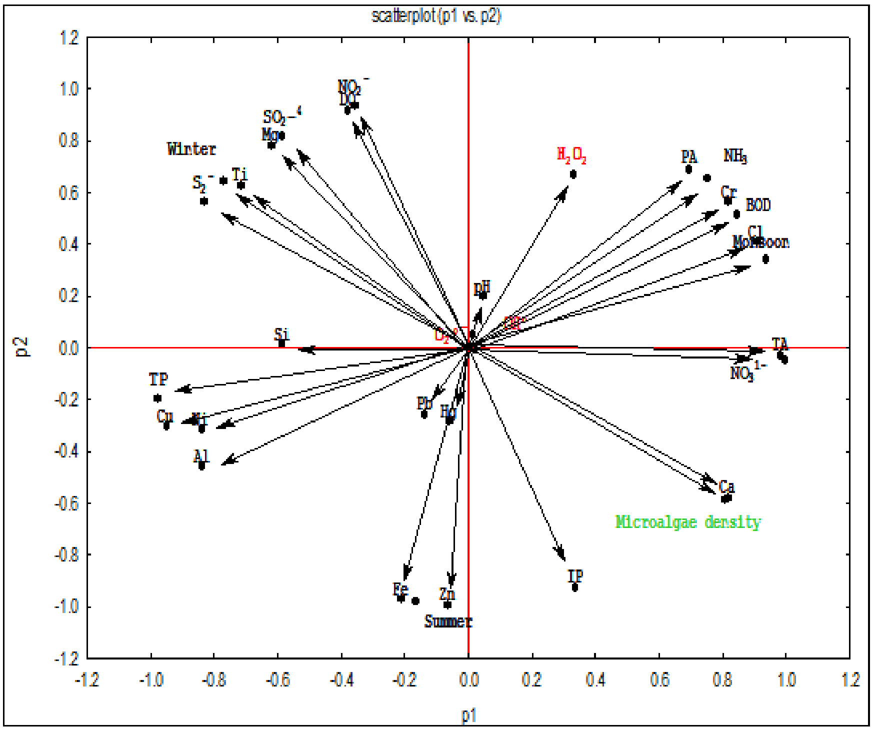

Fig-9: Biplot diagram of PCA analysis summarizing the contribution of dominant physicochemical factors in different season at reference site

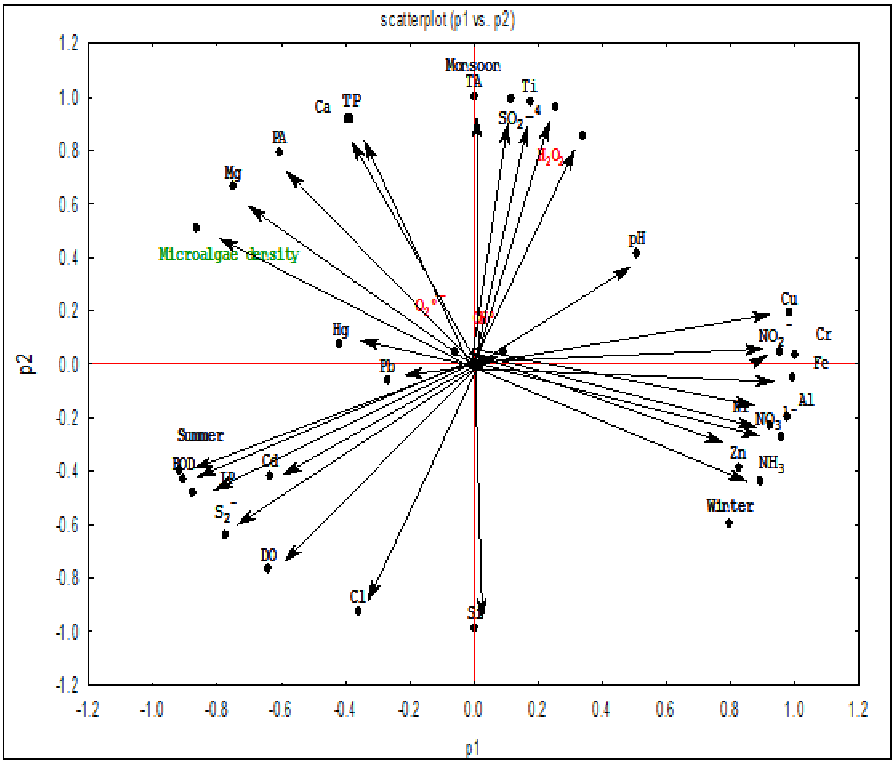

Fig-10: Biplot diagram of PCA analysis summarizing the contribution of dominant physicochemical factors in different season at impact site.

CONCLUSION

Relationship between spatial and temporal changes of microalgae diversity with reference to physicochemical parameters and accumulation of ROS in marine water was assessed using correlation and multivariate analysis in impact and reference sites. The correlation analysis showed that the chemicals and metals ions influenced the microalgal distribution, which was low in reference affecting the species richness and high in impact site affecting the dominance of microalgal species. From multivariate analysis it could be concluded that the micro algal diversity and distribution was affected by the quality of physicochemical properties of seawater from point to non-point sources. The dynamics of Reactive Oxygen Species were significantly enhanced by the chemical and metals ions present in the polluted site and which increased the oxidative stress (ROS) in the microalgal species.

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