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DIVULGING PIGMENTED MICROBIAL COMMUNITY FROM HYPERALKALINE LONAR LAKE.

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ABSTRACT:

Lonar lake, the only basaltic lake in India 19°58' N & 76°31' E from Lonar, Buldhana district of Maharashtra India, with alkaline and saline water. The diameter of the lake is 1.8 km and 137m deep which is found to be changing due to environmental changes and human intervention. This observation gave path to study the physicochemical parameters which ultimately might affect the uniqueness and biodiversity of the lake. In the present study isolation of pigmented bacteria (pigments being versatile bioactive secondary metabolites hence emphasized) and physicochemical parameters of ground water sample analysis and was carried out. Out of all microbial isolates, 14 pigment producing bacteria were identified at molecular level, with pH 9.4 the lake water was highly alkaline-(11845 mg/L) and characterised by high concentrations of chlorides (7450 mg/L), nitrates (138 mg/L) and sulphates (117 mg/L). The pigmented microbial community and physicochemical parameters were compared with the previously recorded data which significantly concludes that there is a need to conserve the world's only one meteorite impact crater in India with its uniqueness.

KEY WORDS: Lonar Crater, Pigmented bacteria, Physicochemical parameters.

I INTRODUCTION

About 190 craters have been created on Earth due to the Asteroid impacts. Many of which have been seen in the form of lakes. India has three meteorite impact craters, Lonar Crater in Buldana district of Maharashtra, Dhala crater in Shivpuri district of Madhya Pradesh and Ramgarh crater in Ramgarh Baran district of Rajasthan. Lonar crater[1,2] is the result of a massive basaltic rock impact around 50,000 years ago. Amongst the three meteorite impact craters of India, Lonar crater has been seen in the form of lake positioning third largest natural salt water lake and fourth largest craters (2.0 km. wide) in the world[3]. The mysterious lake with its saline and alkaline water harbours unique bowl of biodiversity.

Recent researches have tended to support the view that the Lonar structure is probably a hypervelocity impact crater of Quarternary age. And thus grabbed the attention of geologist, archaeologist, ecologists, naturalists and microbiologist to explore more about the lake. Lonar lake is circular and closed without the intervention of inlet for the source of water. Water is dark green in colour due to the presence of algal blooms in it. Lonar lake is unique in itself, naturally existing hypersaline water bodies on the earth with the presence of large amounts of Na salts. This lake contains depleted amounts of Ca and Mg salts, as these cations are insoluble in alkaline conditions and thus harbours a diverse group of microbes as halophilic bacteria and archaea [4]. Saline Soda lakes represent extreme environments characterized by moderate to high concentrations of salts (as Na2CO3/NaHCO3) and alkalinity with pH (9–10.5). The abundance of sodium carbonates leads to a highly buffered alkalinity[5]. The organisms living in lake are in general obligately haloalkaliphilic, requiring the presence of Na+ and at least pH 9 for growth. Their diversity and biology have been extensively reviewed [1,2]. The quantity and variety of chemicals present in the lake water decides the type of aquatic life of the lake, and in turn decides its sustainability[6] .

The occurrence of diverse functional groups of microbes, such as haloalkaliphilic bacteria and Archaea, methanogens, methanotrophs, phototrophs, denitrifiers, sulfur oxidizers, sulfate reducers and syntrophs in soda lakes, suggests that these habitats harbor complex microbial food webs that

(a) interconnect various biological cycles via redox coupling and

(b) impact on the production and consumption of greenhouse gases [7].

Soda lake microorganisms produce several biotechnologically relevant enzymes and biomolecules (for example, cellulases, amylases, ectoine) and there is the need to augment bioprospecting efforts in soda lake environments with new integrated approaches.Many studies regarding microbial diversities and physicochemical analysis of lonar lake water are been carried out. Rotifers- zooplanktons are widely distributed in the lake which contributes to the eutrofication of the lake. Research relating to pigmented bacteria and their biological activity is still to be explored from Lonar lake water. Fluctuating results of aquatic flora fauna may be the results of variations in the chemical content of water, seasonal variation and vice versa. Importantly, some saline and alkaline lake ecosystems like Lonar lake around the world need to be protected from anthropogenic pressures that threaten their long-term existence[8]. Keeping this in view a study is been carried out to know the physicochemical parameters of the lonar lake water.

The physicochemical analysis of water is important not only from aesthetic point of view but also highlights about the aquatic flora and fauna which in turn reflects the quality of water body. Also these studies are significant from ecological, anthropological point of view. Such studies of natural water bodies' serve valuable source of data to meet the need based measures to be taken to sustain the uniqueness. Under this backdrop the current study is carried out

Total solids

This term implies the suspended particles and soluble particles in the water. Dissolved solids from the water are the particles which can pass through the membrane of around 2 µ on filtration like calcium, chlorides, nitrate, phosphorus, iron, sulphur, and other ions. While suspended particles are the one which remain in the filtrate like silt and clay particles, plankton, algae, fine organic debris, and other particulate matter. Many factors can contribute to the total solids in water. Soil erosion, agricultural runoff containing fertilizers and suspended soil particles, industrial wastes, effluent from water treatment plants, and urban runoff from parking lots, roads, and rooftops. The death and decay of the aquatic life also contributes to the organic water deposition adding to dissolved solids of water. Thus dissolved solids and suspended solids both reduce water clarity, make the water aesthetically unpleasant, unpalatable for drinking and human usage, decrease photosynthetic rate, increase water temperature and can be harmful and unfavourable for the growth of aquatic life. The Environmental Protection Agency's National Water Quality Inventory has concluded that siltation, one of the primary contributors to total solids, is the most common pollutant of streams and rivers they sampled. The standard and acceptable values of total solids must be below 20 mg/L -50 mg/L[9]. Sum of organic and inorganic substances dissolved in the water contributes to total dissolved solids in water which includes Calcium, magnesium, sodium, potassium, bicarbonate, sulphate, chloride, nitrate, and silica.

Hardness

Hardness of water is commonly and naturally found in the environment due to the dissolved compounds of calcium, magnesium and sometimes other divalent and trivalent metallic elements as iron, manganese, strontium, etc. The total hardness of the water by collective concentration of carbonates and bicarbonates of calcium and magnesium results in the temporary hardening of water. While sulphates and chlorides causes permanent hardness. Water hardness cannot be overlapped with salinity of water. According to one German degree of harness is 10mg/L of calcium and magnesium oxide or 17.8ppm of CaCO3 in the USA. Low saline water can be hard with rich calcium and magnesium content. On the other hand, fresh water can be rich in sodium chloride with very low hardness [10].

Nitrates

Natural unpolluted waters contain minute quantities of nitrates which are the most oxidized forms of nitrogen and the end product of the aerobic decomposition of organic nitrogenous matter. The most significant sources of nitrates are fertilizers, animal waste drainage from livestock feeds, as well as domestic and industrial sources. The extensive growth of algae in the water body increases the nitrate content resulting in eutrophication. Looking to the source of nitrates in water bodies indicates the contamination through various undesirable sources making water not suitable for human usage. Nitrates in drinking water (4-9 mg/L acceptable level) can be of serious issue as it causes methemoglobinemia, a temporary blood disorder in infants also known as blue baby syndrome [11].

Sulphates

Presence of sulphates in water imparts hardness to it along with other cations. They are naturally present in water bodies with high salt content. Water bodies polluted with domestic sewage, industrial disposal and also biological oxidation by microbes to hydrogen sulphide[12] adds up the sulphates to water body. 250mg/L concentration is maximum level of contamination. Sulphur content in water can have laxative effect leading to dehydration in infants.

Chlorides

Chlorides are salts formed by the combination of the gas chlorine with a metal. sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) are the most common chlorides. Chlorine alone as Cl_2 is highly toxic but in combination with a metal such as sodium is significant and essential need for life. Salt depositions in the form of (Cl^-) ion contribute the chloride content of water. Also contamination with sewage, industrial wastes, intrusion of seawater or other saline water adds up to the chloride s in water. Chlorides above 400 mg/L can be toxic to the aquatic life and 250mg/L considered safe for drinking water[13].

Fluorides

Fluoride, apatite and cryolite are the major minerals of fluoride found in hard rock. Hydrogeological conditions lead to mobilization of fluoride in ground water and alkalinity of ground water solubilise the fluoride minerals. Ground water rich in fluorides have more Mg than Ca. Fluoride is such a compound having dual significance. High concentration causes dental fluorosis and lower concentration (<0.8 mg/L) causes dental caries. Considering this dual role 1mg/L is recommended in drinking water. Water contaminated with industrial waste water coke, glass and ceramic, electronics, pesticide and fertiliser manufacturing, steel and aluminium processing and electroplating industries often results in adding the load of fluorides [14].

Heavy metals

Elements of high atomic number are the referred to as heavy metals which makes them significantly important to be used in industries like paper, automobiles. They are often found as ores in earth. These metals getting segregated from ores are toxic pollutants in environment. Their occurrence in water is the result of sewage pollution from industrial effluents and runoff. Through the usage of water, they get deposited in food web at different levels. Lead, copper, nickel, chromium, and iron are some among the heavy metals. Iron is an abundant element in the earth's crust, but exists generally in minor concentrations in natural water systems. Iron is found in the +2 *(ferrous)* and +3 (*ferric)* states depending on the oxidation-reduction potentials of the water. The ferric state of iron imparts orange strain to any settling surfaces, including laundry articles, cooking and eating utensils, and plumbing fixtures.

II Material and Methods

Collection of water sample

Water sample for the isolation of pigment producing bacteria was collected from four locations of Lonar lake, Lonar, district Buldhana, Maharashtra during September (S1), October (S2), November (S3) and December (S4) month as considered ideal season for sample collection. Temperature and pH of water sample was analysed on site with the help of simple thermometer and pH paper respectively at the time of collection. Water was collected in sterile bottle by standard protocol and brought to laboratory for biochemical analysis.

Isolation of bacteria

The water was dark green in colour with musty odour. The chemical analysis of water sample which included alkalinity, total solids, hardness, nitrates, sulphates, chlorides, fluoride and iron was carried out followed by microbial analysis. As the pH of water was found to be alkaline, the isolation of microbes was carried out on Zobell marine agar (Himedia M385) by spread plate method. The inoculated plates were incubated at 37^{o} C for 24 hours for the visible growth of colonies but the plates were monitored for the period of 2-10 days for the production of pigmented colonies. The coloured colonies were picked up and sub cultured for further studies.

Purification and selection of pigment producing bacterial isolates

All the pigment producing colonies that appeared on the agar plates were picked and successively streaked on Zobel marine agar plate. The isolated colonies were purified by three successive streaking on Zobel marine agar medium. Potential isolates were selected based on the difference in the colour of colonies. For this, isolates were streaked on Zobel marine agar medium and incubated at 37^{o} C for 48 h and observed for the pigment production. The isolates that showed bright pigmentation were selected. Preliminary identification of bacterial isolates was carried out by Gram's staining followed by molecular Detection of bacterial isolate at 16s rRNA level for complete identification at NCMR- National Centre for Cell Science, Pune. The partial 16S rRNA gene sequences of these isolated strains were compared with those in the public databases including GenBank, DDBJ, EMBL, ENA, to search for the nearest phylogenetic neighbour. Species level identification was determined by the 16S rRNA sequence similarity of 99% with that of the prototype sequence available in GenBank. Sequence alignment and comparison was performed using the multiple sequence alignment program ClustalW(Thompson et al., 2003).

Total solids

A known volume of the well-mixed sample (50ml) is measured into a pre-weighed dish and evaporated to dryness at 103^{o} C on a steam bath. The evaporated sample is dried in an oven for about an hour at 103-105^{o} C and cooled in a desiccator and recorded for constant weight.

Calculation $Total\ solids = \frac{\text{W1}-\text{W2}}{\text{Sample volume(ml)}}$

W1 = Weight of dried residue + dish
W2 = Weight of empty dish

Total dissolved solids The difference in the weights of Total Solids (W1) and Total Suspended Solids (W2) expressed in the same units gives Total Dissolved Solids (TDS).

Calculation: $Total\ dissolved\ solids\ (mg/L)\ = \frac{\text{W1}-\text{W2}}{\text{Sample volume}}$

W1 = Weight of total solids + dish
W2 = Weight of total suspended solids

Total hardness Exactly 50ml of the well-mixed sample is pipetted into a conical flask, to which 1ml of ammonium buffer and 2-3 drops of Eriochrome black -T indicator is added. The mixture is titrated against standard 0.01M EDTA until the wine red colour of the solution turns pale blue at the end point.

Calculation $Total\ hardness(\frac{mg}{L}) = \frac{\text{T X 1000}}{\text{V}}$

Where, T = Volume of titrant
V = Volume of sample

Chlorides

A known volume of filtered sample (50ml) is taken in a conical flask, to which about 0.5ml of potassium chromate indicator is added and titrated against standard silver nitrate till silver dichromate (AgCrO4) starts precipitating.

Calculation $Chlorides = \frac{A-B\ X\ N\ X\ 35.45}{\text{sample taken in ml}}$

Where,
A - Volume of silver nitrate consumed by the sample
B - Volume of silver nitrate consumed by the blank
N - Normality of silver nitrate (Standard methods, APHA, 16th edn, pp 286-88)

Fluoride
A standard graph is prepared by using fluoride concentrations ranging from 0.005 mg/L to 0.150 mg/L at 570nm. A reference solution is prepared by adding 4ml of acid zirconyl-SPADNS reagent to 21ml of distilled water. A known volume of filtered sample (21ml) is taken in a test tube, 4ml of acid zirconyl-SPADNS reagent is added to the sample along with a reference solution. The mixture is left for about 30 min for complete colour development and the optical density is read at 570nm.

Calculation

$$Fluoride(mg/L) = \frac{\text{O. D sample X Conc. of std X 1000)}}{\text{O. D Standard X sample taken}}$$

Nitrates A known volume (50ml) of the sample is pipetted into a porcelain dish and evaporated to dryness on a hot water bath. 2ml of phenol disulphonic acid is added to dissolve the residue by constant stirring with a glass rod. Concentrated solution of sodium hydroxide or conc. ammonium hydroxide and distilled water is added with stirring to make it alkaline. This is filtered into a Nessler's tube and made up to 50ml with distilled water. The absorbance is read at 410nm using a spectrophotometer after the development of colour. The standard graph is plotted by taking concentration along X-axis and the spectrophotometric readings (absorbance) along Y-axis. The value of nitrate is found by comparing absorbance of sample with the standard curve and expressed in mg/L.

Calculation $Nitrates\ (mg/L) = \frac{\text{Absorbance of sample X Conc.of Std X 1000}}{\text{Absorbance of Std.X Sample taken}}$

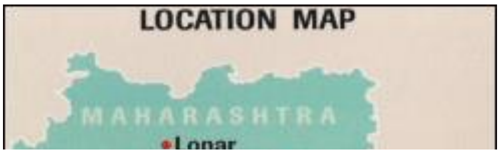
Sulphates
100ml of the sample is filtered into a Nessler's tube containing 5ml of conditioning reagent. About 0.2g of barium chloride crystals is added with continued stirring. A working standard is prepared by taking 1ml of the standard, 5ml of conditioning reagent and made up to 100ml, to give 100 NTU. The turbidity developed by the sample and the standards are measured using a Nephelometer and the results are tabulated.

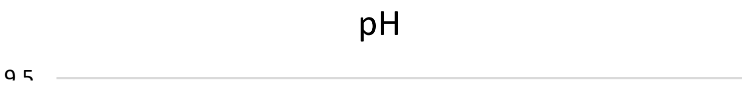
Calculation $Sulphate\ \left(\frac{mg}{L}\right) = Nephlometric\ readingX\ 0.4\ X\ Dilution\ Factor$

Results and Discussion
Total 29 pigment producing bacterial colonies were isolated from the Lonar lake water sample on Zobel Marine agar. However not all the bacterial isolates were identified in the study. As upon subsequent sub culturing there was no consistent pigment formation by the isolates. Hence the study includes the identification of 14 bacterial isolates.
Amongst the identified bacteria, 86.6% of isolates belonged to class bacilli of phylum Firmicutes and 13.3% belong to class Gammaproteobacteria of phylum Proteobacteria. Molecular characterization of the bacterial isolates at 16 s rRNA level revealed them at their species level identification. The obtained sequence was subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/) for the identification of their phylogenetic origin through homology. Further the sequence data was submitted to NCBI Genbank for public access. with accession number and % similarity of all the isolates are as shown in the table. which is shown in the table 1.

The physicochemical parameters like pH, total dissolved solids, total hardness, alkalinity, chlroride, fluoride, nitrate, sulphate and iron content of the water collected during the period from September to December 2019. The values of these parameters from September to December are depicted in (Table 2,3,4 and 5) and also the mean values are mentioned in (Fig 3,4 and 5). The data revealed that there was a considerable variation in the physicochemical parameters.

The colour of the lake water is pale to dark green due to extensive growth of algae *Spirulina(Fig 1,2)*. The odour of water was murky and was very offensive from far distance even. The pH of Lonar lake water was in the range of 8.9- 9.1 with high alkalinity which was comparable with other studies reported. Natural waters are never pure due to abundance of salts, acids and bases which in turn influences the H+ and OH^- ion concentrations of water bodies, thus fluctuation the pH of water. Also conversion of sulphates to carbonates results in alkalinity of water[15]. The total alkalinity of Lonar lake water reported in the year 2003 was 1493.6 mg/L, in the year 2012- 4796 mg/L. Also the metabolic activities of the aquatic life influence the pH in turn. Thus the high pH of Lake itself selects the growth of extremophiles [16].





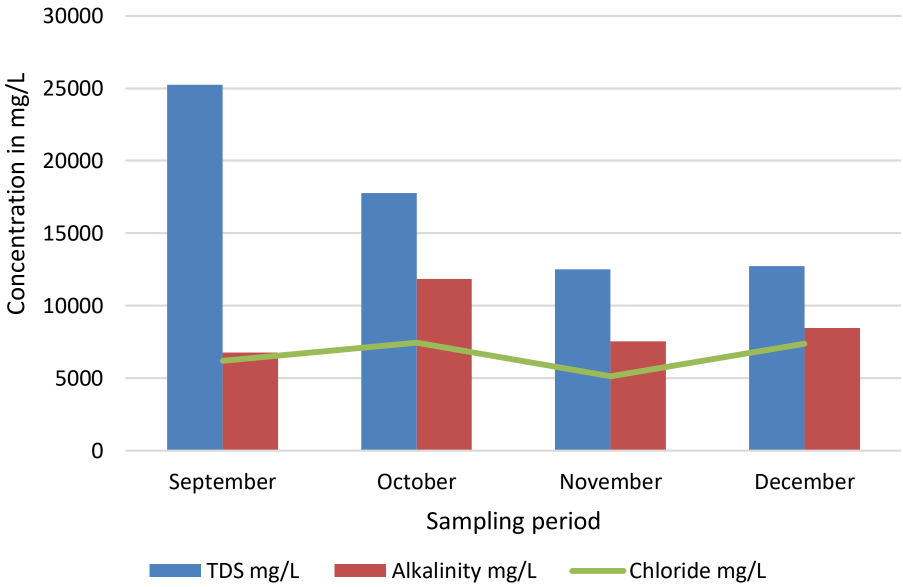


Fig 4 Mean values of total dissolved solids, alkalinity and chlorides from the Lonar lake water at four different sampling periods

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The total dissolved solids were in the range of 11790- 12717.5 mg/L which was very high and so the total hardness of the water was also on the higher values of 105-225 mg/L. Due to total dissolved and suspended contents of the water the composition of the Lonar Lake water was Na_2HCO_3Cl type [17] (Babar et al. 2010). The physical and chemical variation of the lake water may be due to the microbial fluctuation [16,21].
The chloride concentration of the lake varies from 5117- 7450 mg/L, which indicates organic pollution of lake water. Total alkalinity is the measure of capacity of water to neutralise the acids and this is imparted by salts of carbonates, bicarbonates, nitrites, phosphates together with hydroxyl ions in free state. The high alkalinity of lake water indicates the impressive interactions between the salts and long-time stagnant nature of the lake water. Also conversion of sulphates to carbonates results in alkalinity of water. Natural water bodies are rich in sulphate contents due to natural process of withering of rocks. The sulphates content of the lake water was in the range of 105-117 mg/L. However, human activities like domestic and industrial effluents also add up the sulphate[17] (Babar et al. 2006). Considerable amount of Nitrates shows the presence of degradation of organic matter. The nitrate was in the range of 117-138 mg/L and the fluoride content of lake water was in the range of 1.56-1.63 mg/L. Similar reports from the studies carried out from Lake Naivasha Nitrates ranged between 0.10 mg/l – 0.30 mg/l[18]. The physicochemical analysis

of water from Obutu Lake, Ndikelionwu, Anambra, State, Nigeria concluded that the physical parameters like pH, nitrate, phosphates and alkalinity was temperature dependent [19].

Physicochemical studies from Lake Solai, Nakuru County, Kenya revealed that all the parameters were at higher levels and also varied as compared to previous years. Limnological studies from shallow lake of western Himalayas, and found that there was a positive correlation between temperature and pH, depth of water and chloride content [20]. Also there was many folds' depletion in the concentrations of magnesium, calcium, nitrates and alkalinity compared to last four decades.

Sl.No	Isolate code	Scientific Name of the Isolate	Accession number	Colony Colour
	LL-1	*Pseudomonas stutzeri* strain JMBKUDGK2	OL823113.1	Blue
	LL-2	*Brevibacillus borstelensis* strain JMBKUDGK7	OL823129.1	Red brown
	LL-3	*Bacillus licheniformis* strain JBTKUDGKM11	OL823139.1	Red
	LL-4	*Lysinibacillus macrolides* strain JBTKUDGKZ13	OL823144.1	Red
	LL-5	*Lysinibacillus fusiformis* strain JBTKUDGKV12	OL823141..1	Light orange
	LL-6	*Bacillus thermophiles* strain JMBKUDGK14	OL823143.1	Pale yellow
	LL-7	*Brevibacillus parabrevis* strain JMBKUDGK1	OL672499.1	Red
	LL-8	*Bacillus subtilis* strain JMBKUDGK3	OL823117.1	Brown
	LL-9	*Bacillus thuringiensis* strain JMBKUDGK4	OL823120.1	Red
.	LL-10	*Bacillus paramycoides* strain JMBKUDGK6	OL825725.1	Yellow
.	LL-11	*Enterobacter cloacae* strain JMBKUDGK8	OL823131.1	Yellow
.	LL-12	*Bacillus haynesii* strain JMBKUDGK9	OL823133.1	White
.	LL-13	*Lysinibacillus sp.* strain JBTKUDGKV10	OL823132.1	Light orange
.	LL-14	*Bacillus cereus* strain JMBKUDGK5	OL823124	Pink-orange

Table 1. Molecular characterization of pigmented bacterial isolates from Lonar lake water sampl

Sl.No	Sites	pH	TDS mg/L	Total Hardness mg/L	Alkalinity mg/L	Chloride mg/L	Fluoride mg/L	Nitrate mg/L	Sulphate mg/L
1.	S 1	9.1	29347	100	11560	10900	1.83	100	100
2.	S 2	8.9	34021	80	6440	5340	1.53	134	126
3.	S 3	8.8	15369	100	2440	2570	1.18	122	130
4.	S 4	9.1	22165	140	6700	6050	1.81	126	112

Table 2 Physicochemical parameters of Lonar Lake water from the month of September 2019

Sl.No	Sites	pH	TDS mg/L	Total Hardness mg/L	Alkalinity mg/L	Chloride mg/L	Fluoride mg/L	Nitrate mg/L	Sulphate mg/L
	S 1	9.5	18520	100	12020	11200	1.92	92	90
	S 2	9.5	18530	240	12100	10210	1.62	124	116
	S 3	9.4	18500	280	11440	7250	1.02	146	120
	S 4	9.4	15610	180	11820	11440	1.96	108	102

Table3 Physicochemical parameters of Lonar Lake water from the month of October 2019

Sl.No	Sites	pH	TDS mg/L	Total Hardness mg/L	Alkalinity mg/L	Chloride mg/L	Fluoride mg/L	Nitrate mg/L	Sulphate mg/L
	S 1	9.3	12500	320	7400	5800	1.90	110	85
	S 2	9.0	12480	160	7640	5220	1.60	142	126
	S 3	8.9	12530	260	7260	3550	1.10	156	123
	S 4	9.2	12460	160	7900	5900	1.90	120	100

Table 4 Physicochemical parameters of Lonar Lake water from the month of November 2019

Sl.No	Sites	pH	TDS mg/L	Total Hardness mg/L	Alkalinity mg/L	Chloride mg/L	Fluoride mg/L	Nitrate mg/L	Sulphate mg/L
	S 1	9.2	12930	220	7820	8860	1.80	118	78
	S 2	9.1	12830	300	8880	4980	1.50	149	128
	S 3	8.9	12590	220	9280	6540	1.20	160	120
	S 4	9.2	12520	120	7840	9100	1.90	126	96

Table 5 Physicochemical parameters of Lonar Lake water from the month of December 2019

Conclusion

Lonar lake is unique in its existence as the only hypersaline basaltic meteorite crater lake. The mysterious existence of biodiversity of the lake with aquatic flora, fauna, animals, migratory birds and microbes makes it an ecological wonder. Pigmented bacteria are unique and mysterious in their functions in various therapeutic areas. The results revealed considerable number of such miraculous microbes from the lake. Further the Water loss from the lake is only because of natural evaporation as the Lake does not have any out flow and is lined with basaltic rock all around, so no seepage of water to ground water table. The physicochemical parameters of Lonar Lake water like pH, total hardness, total dissolved solids, alkalinity and other salts contributing to salinity are found to be at higher levels. However, the salinity, pH and alkalinity goes on decreasing as per time. The hydrological studies reported the devastating change in physiochemical, flora, fauna and microbes has deteriorated the uniqueness of the lake. During lockdown 2020 (due to Covid -19 pandemic) the lake water was observed to turn pink overnight, which surprised Scientists and Ecologists. Studies carried out by Agharkar Research Institute Pune revealed the possible reason for the pink colouration of water that, the absence of rain, less human interference (owing to lockdown) and high temperature resulted in the evaporation of water which increased its salinity and pH. The increased salinity and pH facilitated the growth of halophilic microbes, mainly *Haloarchae*, pink pigment producing bacteria. The pink colour gradually disappeared due to settling of biomass at the bottom and thus water body regained its colour (after rains and growth of algae). Owing to this surprising and change in the colour of lake water for the first time, have given a ray to scientists, ecologists, archaeologist to make an effort towards the conservation of lake and preserve the World heritage. Thus the conservation of Lonar lake in turn develops conducive environment for the diverse life forms of the lake makes it natural splendour.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Principal author has performed the designing of experiment carried out assay and wrote the manuscript. Provision of laboratory facility, equipment's was provided by second author. Corresponding author supervised the work and helped in evaluation of manuscript.

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