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OUTCOME OF NATURAL INDICATORS *Brassica oleracea* (RED CABBAGE), *Curcuma longa* (TURMERIC) EXTRACT AND CHEMICAL INDICATOR PHENOLPHTHALEIN ON FRESHWATER ZEBRAFISH (*Danio rerio*) OF MUMBAI SUBURBAN REGION

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ABSTRACT

The development of the chemical indicators could be considered as one of the contributing factors to the tremendous increase in water pollution over past 10 years. Phenolphthalein commonly used as a Chemical indicator of industrial effluents is directly discharged in riverine sources which pollute Mumbai and its surrounding areas during rainy seasons as it percolates in ground waters. Such chemical indicators are prepared in organic solvents which are insoluble in water and have deteriorating effects on the environment and aquatic life. To clean the polluted water with natural plant-based sources like utilization of *Brassica oleracea* (Red cabbage) and *Curcuma longa* (Turmeric) extract as Natural indicators, as they are readily soluble in water & this new method can be implemented without any side effects on aquatic life. During the investigation period, it was observed that the lipid content of *Danio rerio* treated with Phenolphthalein reduced from 4% to 2.23% as compared to 4.80% & 4.81% in the zebra fish treated with Red-cabbage, Turmeric extract respectively. The maximum carbohydrate content found in *Danio rerio* treated with Red cabbage and Turmeric extract was 3.22%, 3.31% which was significantly high from the Phenolphthalein treated *Danio rerio* with 1.98%. Phenolphthalein also showed undesirable and negative effects on water quality by raising Biological oxygen demand, Total hardness, Acidity and by lowering Dissolved oxygen, Alkalinity & pH respectively. Indicator analysis by HPLC & IR further identified 20 types of different Anthocyanins in Red cabbage; three Curcuminoids such as Curcumin, Demethoxy Curcumin & Bis demethoxy curcumin were also obtained. High amount of Phthalic anhydride, Phenol and Stannic chloride were detected from HPLC & IR. Thus, natural indicators which are eco-friendly, economical, low cost can be considered as a better option for safeguarding of water quality and also efficient to replace the conventional chemical indicators. The increased acceptability of natural indicators over chemical indicators has focused the attention for practicing it on larger scale. It is also helpful to rear a very popular model organism like zebra fish inhabiting in the polluted water of Mumbai and surrounding suburban region.

***Keywords: Brassica oleracea, Curcuma longa,* Phenolphthalein, *Danio rerio,* Water quality, Anthocyanin.**

1. INTRODUCTION

Water pollution is the defile of water bodies when pollutants like various chemicals from Pharmaceuticals, industrial wastage, and chemical indicators are discharged directly or indirectly into water bodies without adequate treatment to remove harmful compounds (Arif., *et al* 2020). If chemical waste is not handled or disposed of properly, both the environment, the water in which they are disposed and nearby individuals are put at risk by its potentially corrosive, toxic, flammable or explosive nature. Fish is one of our most valuable sources of biological macromolecules. World-wide, people obtain about 25% of their animal lipid and carbohydrate from fish. World Fish Centre and IFPRI2 global per capita fish death rate has doubled over the past 10 years due to domestic households, industrial and agricultural practices produce wastewater that can cause pollution of many lakes and rivers; dumping of organic materials in the fresh-waters can cause huge problems as some solvents like ethanol, ether are not soluble in water which forms a layer over water bodies blocking the oxygen from air. In these the effect is damaging not only to individual species and populations, but also to the natural biological communities. As the commercial industries utilize chemical indicators to test the acidic and basic nature of water & soils, for checking whether a liquid is either a strong acid or alkaline or a weak acid or weak base or it can be neutral or neither. They also check the presence of vitamins or calcium for testing vitamin C in foods and drinks. So Chemical indicator is a substance that gives a visible sign, usually by a color change by the presence or absence of a threshold concentration of a chemical such as an acid or an alkali in a solution. The color of an indicator alters when the acidity or the oxidizing strength of the solution, or the concentration of a certain chemical species, reaches a critical range of values. In general Phenolphthalein is used as an acid-base indicator, in making dyes, and formerly in medicine as a laxative. So it is a widely popular indicator in chemical industries even after having adverese effect it is extensively used. Excess consumption of Phenolphthalein causes indigestion, skin irritation after prolonged or repeated exposure may result in skin redness, skin rashes, swelling, scaling and thickening of skin in humans. It may cause discoloration, elevated acidity resulting in death in fishes (Boyd., *et al* 2011). The unmonitored released into water resources has leads to total loss of freshwater water life forms.

Thus to control water pollution *Brassica oleracea* (Red cabbage) and *Curcuma longa* (Turmeric) as a low cost & value added natural plant-based indicator serves as a biodegradable alternative to reduce the water pollution rate and maintain a better aquatic supporting ecosystem. It is observed that some important minerals and nutrients are present in Red cabbage which also helps in the growth of fish. Several investigations has showed that the fish grown in red cabbage juice exhibit remarkable growth in bone development, lipid, carbohydrate, protein content & also help in lowering of overall acidity of fish like humans. Turmeric is known to possess anti-oxidant properties, anti-inflammatory property due to its active component of Curcumin. It controls the hormone level, and also decelerates the spread of cancer. Turmeric voraciously speeds up the effect of paclitexal in reducing metastasis of cancer by (Saghatelyan., *et al* 2020). The cosmopolitan city like Mumbai & Thane is adversely affected by water pollution & leads diseases outbreaks. The important neighboring rivers flowing through the outer Mumbai region like Vaitarna, Ulhas, Tansa, Surya are important drinking water resources. Various aquatic lives including important fish species are found in these rivers. Zebra fish, *Danio rerio* is a small tropical freshwater model fish which is found abundantly in these rivers. Till date no study has been conducted to assess the effect of Phenolphthalein on water quality as well as on growth of Zebra fish. Thus, the primary aim of the investigation is to evaluate how Phenolphthalein, Red-cabbage & Turmeric influence the fish profile of *Danio rerio as* well as to assess the physicochemical changes in the water quality. Also, to identify various organic component of red-cabbage, Turmeric extract and Phenolphthalein using HPLC & IR.

1.1. EXPERIMENTAL DESIGN

The study was carried out in two phase 2 weeks for the acclimatization process & 2 week for analysis purposes. After acclimatization, the fishes were divided into 4 tanks- 1 control and other 3 experimental tanks. In control tank fish were added to fresh water, Experimental fish tanks consist of Phenolphthalein tank, Red-cabbage tank and Turmeric indicator tank. There were two broad parts of activities in this experiment. The first part of the activity concerned with the effect of various indicators on the fish profile. The second part of the experiment was performed to assess the effect of Red-cabbage, Turmeric extract & Phenolphthalein on the water quality of the experimental fish tanks.

1.2. MATERIALS & METHODS

Fish Rearing and Conditioning-

Experimental fish species *Danio rerio* of almost similar size were collected from Pet World aquarium, Thane west. The average weight of the fish was 1.5 gm and length was 1.2 inch. The fish were acclimatized in the lab for 2 weeks [14days] in the tanks of laboratory of dimensions 20x 12x12 (Length x Breadth x Height), with a capacity of 30 Liters water. All the fishes were fed twice a day to ad libitum with commercial pellets comprising 1-2% of the body weight. The water in the tanks was partially changed after 3 days.

Preparation of Indicator

Red-cabbage (*Brassica oleracea)* Indicator was collected from Mumbai suburban market. The leaves were boiled in distilled water for 30 minutes. Filter the extract using filter paper after the color leaches out. The filtrate containing anthocyanin is further stored for investigation. Turmeric indicator was prepared by adding 10 gm of Turmeric powder in 100 mL of distilledwater and was heated for 20 minutes. Phenolphthalein Indicator-0.5 gm of Phenolphthalein powder was added to 50% of ethanol which was prepared in distilled water. The indicator was allowed to dissolve thoroughly and was stored for further use.

Analysis of Acidity of fish

After treating the Zebra fish with *Brassica oleracea,* Turmeric extract and Phenolphthalein their weight was measured in electronic weighing balance by cutting the fish into equal pieces & placing them into oven at 70 degree Celsius for drying. Powder the dried fish and mix
it with 0.1 N HCl for titration against NaOH. Treated tanks water was also titrated for the assessment of change in acidity while investigation period. The pH was analyzed by putting pH strips inside the various tanks.

2.1. FISH PROFILE ANALYSIS

a) Estimation of carbohydrates by Anthrone Method (1944)

Total amount of carbohydrates was measured by Anthrone method in *Danio rerio,* Glucose in the fish gets dehydrate to hydroxyl methyl furfural which reacts with Anthrone reagent. Weigh 1g of fish sample of various fish tanks & add 50 mL of ethanol and heat it for 15 minutes. Cool the sample, add 10 mL of ethanol. Filter it, add 1mL of filtrate to 9 mL of Fehling solution and heat it in boiling water bath for 15 minutes. After cooling pour 10mL of Anthrone reagent measure the absorbance at 610 nm.

b) Extraction of Lipids by Bligh and Dyer's Method (1959)

Estimation of lipids in *Danio rerio* was done by Bligh and Dyer's method. Lipids were calculated by precipitating them by using chloroform: methanol (1:2) mixture. Weigh 2g of sample and homogenize the tissue using mortar and pestle in 5 mL distilled water. Add 15 mL of chloroform: methanol (1:2) in the homogenate. After mixing store it in dark overnight. Centrifuge at 3000 rpm for 15minutes at room temperature. Evaporate chloroform containing lipids on boiling water and measure the total lipid content.

2.2. WATER ANALYSIS OF FISH TANKS

Physiochemical parameter of fish tank water was assessed as per BIS 10500 (2012) for Dissolved oxygen, Biochemical Oxygen Demand, Total Hardness, Acidity, Alkalinity and pH.

2.3. INDICATOR ANALYSIS

a) Infrared Spectroscopy

The infrared spectrum of a sample is recorded by passing a beam of infrared light through the fish sample. Place sample in the sample holder and the spectrometer is purged for 5 minutes. Scan the wavelength range using a monochromatic for observing the total energy absorbed at each frequency. Alternatively, the whole wavelength range is measured at once using a Fourier transform instrument and then transmittance or absorbance spectrum is generated. Analysis of the position, shape and intensity of peaks in this spectrum in then revealed and the molecular structure of the sample is shown in the form of peaks. Spectra are then obtained from samples with IR active bonds and high levels of purity.

b) High Performance Liquid Chromatography (HPLC)

2uL of sample was collected into syringe and was inserted into the injection valve and introduce the desired volume into the 20uL injection loop. Sample was injected by switching the manual injection valve from the load

to inject position. Sample will be sent to the column, which will start the data acquisition. The display should change from "Waiting for Injection" to "Running". Check pump pressure, N2 pressure at the ELSD, and ELSD Voltage output to ensure correct values.

3. RESULTS AND DISCUSSION

3.1. Red-cabbage pH indicator colors-

Red cabbage indicator was added to different concentrations of HCl and NaOH solutions and pH was checked using pH strips. Bright red color to extreme left is acidic and yellow color to extreme right is basic.

3.2. Analysis of acidity by using fish powder-

a) Titrating HCI against NaOH control, using indicators; Red-cabbage indicator, Turmeric extract, Phenolphthalein and with and without using dry fish powder.

Indicator	Dry fish powder	Titration value (mL)	End point
Control with Phenolphthalein indicator	-	4-5	Colorless To Pink
Control with Red-cabbage indicator	-	4-5	Pink To Purplish Blue.
Control with Turmeric indicator	-	4-5	Yellow To Red
Control:Fresh water containing Phenolphthalein indicator	+	5-6	Colorless To Pink
Control: Fresh water containing Red-cabbage indicator	+	4-5	Pink To Purplish Blue.
Control: Fresh water containing Turmeric indicator	+	4-5	Yellow To Red.

Table.1 Analysis of acidity by using fish powder in control fish tank-The acidity value for the Control Fresh water containing Phenolphthalein indicator was found to be higher as compared to the other control value containing fish powder which implies that the acidity of fish having pH 6 has been mixed up with the fish tank water.

b) Dry Fish powder is titrated with HCI against NaOH of experimental tanks, using indicators; Red-cabbage, Turmeric indicator and Phenolphthalein.

Indicator	Titration value (mL)	End point
Fish treated with Phenolphthalein indicator	7-8	Colorless To Pink
Fish treated with Red-cabbage indicator	4-5	Pink To Purplish Blue.
Fish treated with Turmeric indicator	4-5	Yellow To Red

Table. 2 Analysis of acidity of Zebrafish reared in fish tank containing different indicators- The value of Fish treated with Phenolphthalein indicator was significantly high from the other fishes reared in Red cabbage and Turmeric indicator due to the fact that the acidity of fish as well as the fish tank water has increased after the addition of Phenolphthalein

c) Weight of *Danio rerio*:

The fish treated with Phenolphthalein indicator shows a decrease in weight by 0.44g when compared with the fish kept in fresh water. Whereas the fish treated with Red-cabbage indicator shows a difference of 0.005g when compared with the fish kept in fresh water and the fish treated with Turmeric indicator shows a difference of 0.014g compared to water.

Indicator	Wt. of Petri-plate [with lid] (g)	Wt. of Petri-plate[with lid] + fish (g)	Wt. of fish (g)	Difference between the wet weights of the fish comparing with the original weight of the fish (g)
Control	90.117	91.435	91.435- 90.117 = 1.318	1.318-1.318 = 0
Phenolphthalein	90.117	90.995	90.995-90.117=0.8789	1.318-0.878 = 0.440
Red-cabbage	90.117	91.430	91.430-90.117=1.3139	1.318-1.313= 0.005
Turmeric	90.117	91.421	91.430 - 90.117= 1.304	1.318-1.304= 0.014

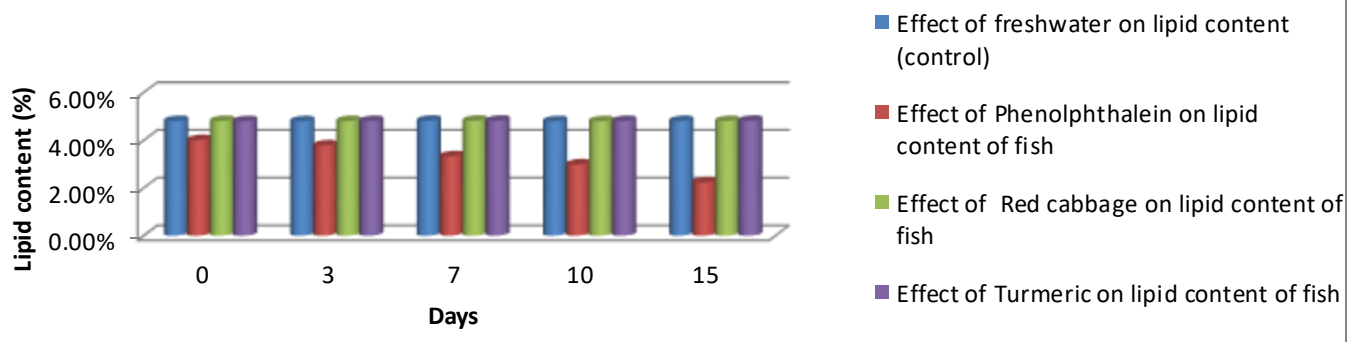
Table. 3. Weight of Zebrafish

4. Analysis of fish profiles-

Days	Effect of fresh water on lipid content (control)	Effect of Phenolphthalein on lipid content of fish	Effect of Red cabbage on lipid content of fish	Effect of Turmeric on lipid content of fish
0	4.80%	4%	4.80%	4.79%
3	4.79%	3.78%	4.80%	4.80%
7	4.81%	3.32%	4.81%	4.81%
10	4.79%	2.98%	4.79%	4.80%
15	4.80%	2.23%	4.80%	4.81%

Table. 4.1. Lipid Content in fish when treated with 3 different indicators

Graph 1.Lipid Content in fish when treated with 3 different indicators-

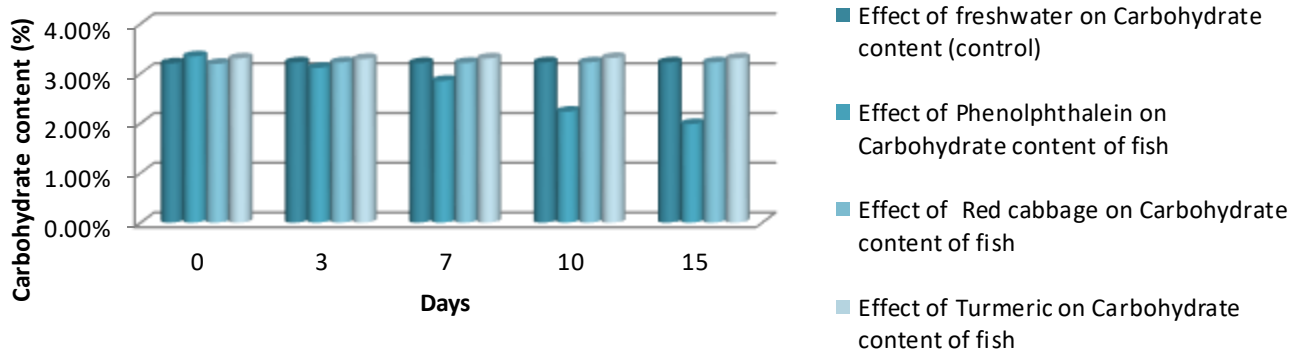


During the investigation period it was observed that the total lipid content in Red-cabbage, Turmeric treated fish was constant with value 4.80%,4.81 as compared with 4.80%value of control but a significant drop of 2% was seen in lipid content of Phenolphthalein treated fish. This result was in accordance with Lovell (1979) who has also obtained lower lipid level in *Carassius auratus, Danio feegradei* when treated with Phenolphthalein giving 2.08%, 1.89% respectively and 5.46%, 4.92% when treated with Turmeric.(Graph 1 & Table.4.1)

Days	Effect of freshwater on Carbohydrate content (control)	Effect of Phenolphthalein on Carbohydrate content of fish	Effect of Red cabbage on Carbohydrate content of fish	Effect of Turmeric on Carbohydrate content of fish
0	3.20%	3.34%	3.19%	3.30%
3	3.22 %	3.11%	3.22%	3.29%
7	3.21 %	2.85%	3.21%	3.30%
10	3.22%	2.23%	3.22%	3.31%
15	3.22%	1.98%	3.22%	3.30%

Table. 4.2. Carbohydrate content in fish when treated with 3 different indicators

Graph 2. Carbohydrate content in fish when treated with 3 different indicators-

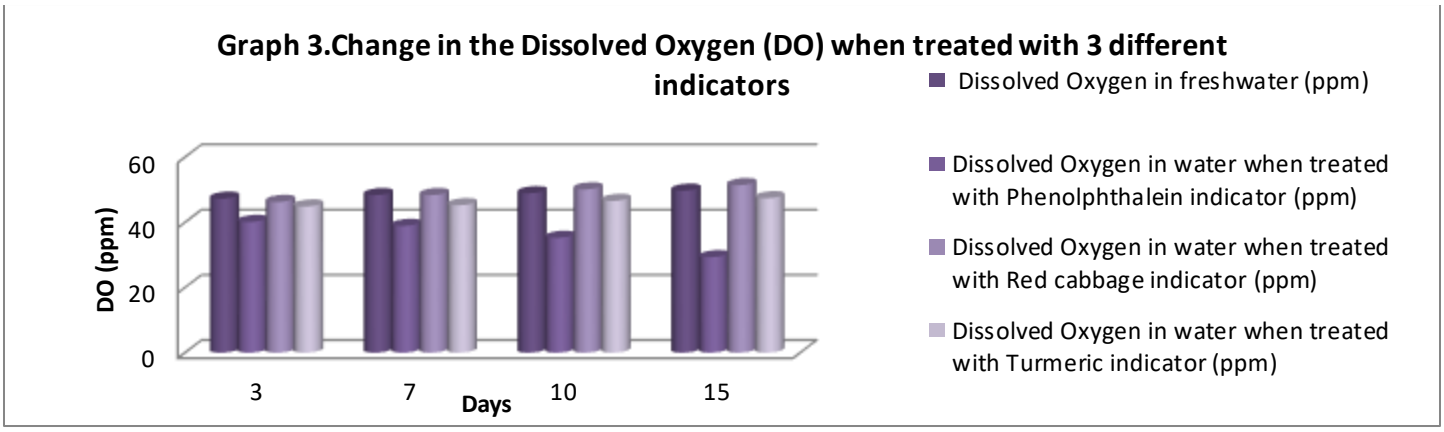


The total carbohydrate content in Red cabbage, Turmeric treated fish was observed in the range from 3.19%-3.22%, 3.30%-3.30% which is in constant range as compared to control but a drop of 1.24% was detected in carbohydrate content of fish reared in Phenolphthalein. Aiyelari *et al.*, (2020) showed the maximum carbohydrate content in *Danio kyathit* was 4.2% when fed with Red cabbage juice for 30 days. (Graph 2 & Table.4.2)

5. Water analysis –

Days	Dissolved Oxygen in freshwater (ppm)	Dissolved Oxygen in water when treated with Phenolphthalein indicator (ppm)	Dissolved Oxygen in water when treated with Red cabbage indicator (ppm)	Dissolved Oxygen in water when treated with Turmeric indicator (ppm)
3	47.25	40.099	46.25	44.85
7	48.35	38.901	48.35	45.234
10	48.90	35.234	50.107	46.566
15	49.70	29.293	51.394	47.384

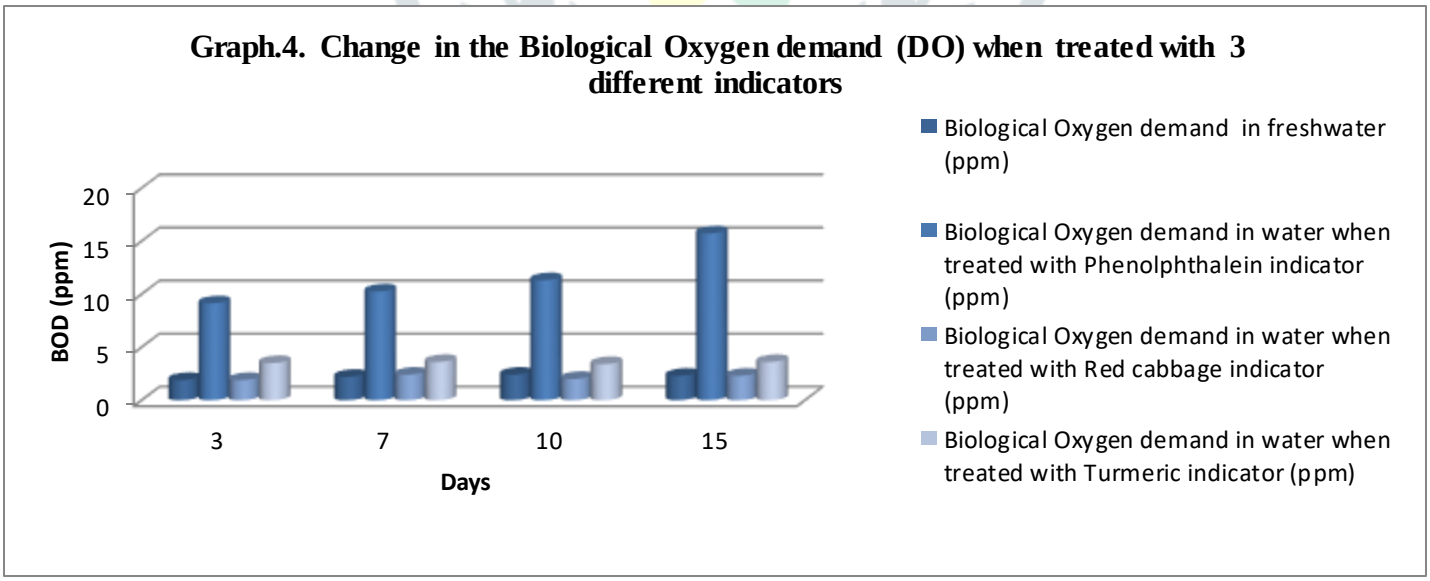
a. Table .5.1. Change in the Dissolved Oxygen (DO) when treated with 3 different indicators



The dissolved oxygen content of Phenolphthalein treated *Danio rerio* was notably low as compared to control which was in contrast with the DO value of 51.394 ppm Red cabbage, 47.384ppm Turmeric respectively. Raj *et al.*, (2015) found in the study that the DO of water containing Phenolphthalein was decreased from 42.989ppm to 20.880ppm during the spam of 30 days which was directly comparable with the present study investigated DO value. (Graph 3 & Table.5.1)

Days	Biological Oxygen demand in freshwater (ppm)	Biological Oxygen demand in water when treated with Phenolphthalein indicator (ppm)	Biological Oxygen demand in water when treated with Red cabbage indicator (ppm)	Biological Oxygen demand in water when treated with Turmeric indicator (ppm)
3	1.9	9.13	1.9	3.5
7	2.2	10.25	2.4	3.6
10	2.4	11.31	2.0	3.4
15	2.3	15.72	2.3	3.6

b.Table.5.2. Change in Biological Oxygen demand treated with 3 different indicators

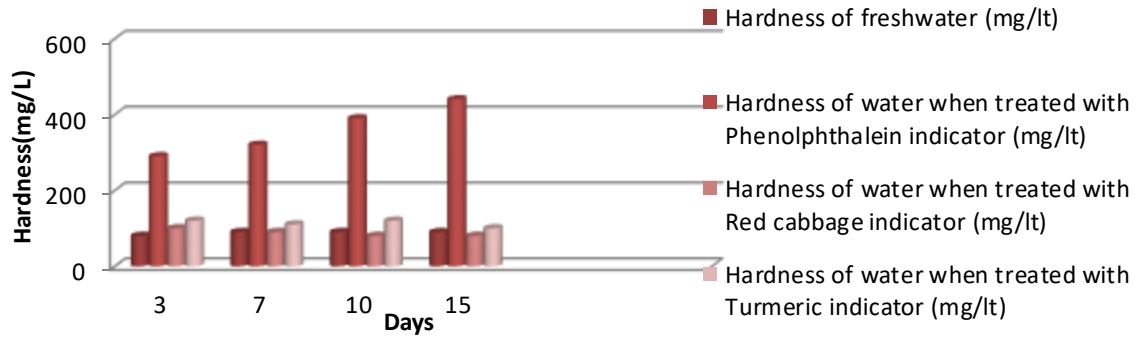


The present study has showed that the BOD level of water containing Phenolphthalein is remarkably high which is in accordance with Raj *et al.*, (2015) who observed that the BOD level of Phenolphthalein treated water was increased from 8.21ppm to 18.21ppm in 30 days. (Graph 4 & Table.5.2)

Days	Hardness of freshwater (mg/L)	Hardness of water when treated with Phenolphthalein indicator (mg/L)	Hardness of water when treated with Red cabbage indicator (mg/L)	Hardness of water when treated with Turmeric indicator (mg/L)
3	80	290	100	120
7	90	320	90	110
10	90	390	80	120
15	90	440	80	100

c.Table.5.3. Change in Hardness of water when treated with 3 different indicators

Graph. 5 Change in the Hardness when treated with 3 different indicators



The hardness of water containing Phenolphthalein was significantly higher than the control and the duration of 15 days as compared to water containing Red cabbage, Turmeric was observed to be constant with the control value. (Graph 5 & Table.5.3)

Days	Acidity of freshwater (gm/L)	Acidity of water when treated with Phenolphthalein indicator (gm/L)	Acidity of water when treated with Red cabbage indicator (gm/L)	Acidity of in water when treated with Turmeric indicator (gm/L)
3	0.344	0.598	0.339	0.363
7	0.340	0.608	0.338	0.362
10	0.347	0.640	0.336	0.361
15	0.347	0.688	0.336	0.360

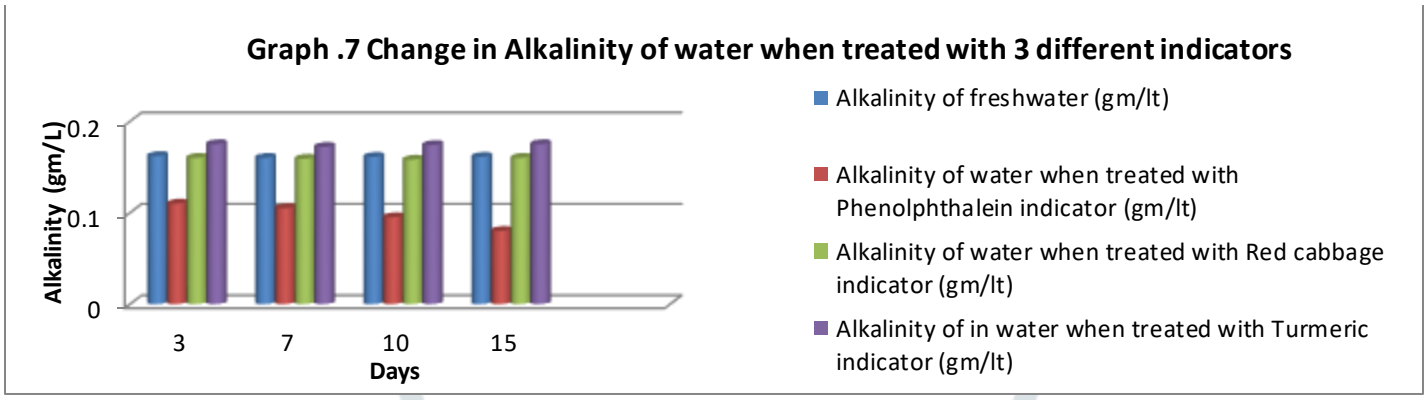
d.Table.5.4 Change in Acidity of water when treated with 3 different indicators

Graph .6 Change in the Acidity of water when treated with 3 different indicators

The estimated acidity value was found to be 0.336, 0.360 for Red cabbage, Turmeric treated water which was lowered to control acidity value. Phenolphthalein treated water has notably higher value of 0.688 as compared to the control. (Graph 6 & Table.5.4)

Days	Alkalinity of freshwater (gm/L)	Alkalinity of water when treated with Phenolphthalein indicator (gm/L)	Alkalinity of water when treated with Red cabbage indicator (gm/L)	Alkalinity of in water when treated with Turmeric indicator (gm/L)
3	0.162	0.110	0.160	0.175
7	0.160	0.105	0.159	0.172
10	0.161	0.095	0.158	0.174
15	0.161	0.080	0.160	0.175

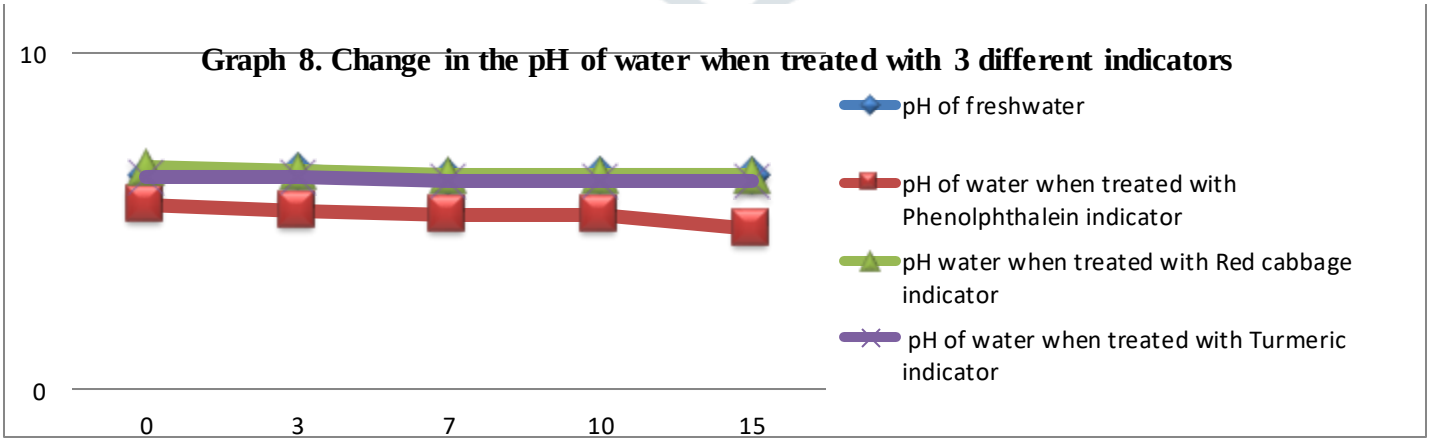
e.Table. 5.5 Change in Alkalinity of water when treated with 3 different indicators



Water containing Red cabbage, Turmeric solution has a constant alkalinity value in accordance with the control observed value in the span of 15 days, which is in contrast with the Phenolphthalein containing water having a lower alkalinity value as compared to the constant. (Graph 7 & Table.5.5)

Days	pH of freshwater	pH of water when treated with Phenolphthalein indicator	pH water when treated with Red cabbage indicator	pH of water when treated with Turmeric indicator
0	6.4	5.5	6.6	6.3
3	6.5	5.3	6.5	6.3
7	6.3	5.2	6.4	6.2
10	6.4	5.2	6.4	6.2
15	6.4	4.8	6.4	6.2

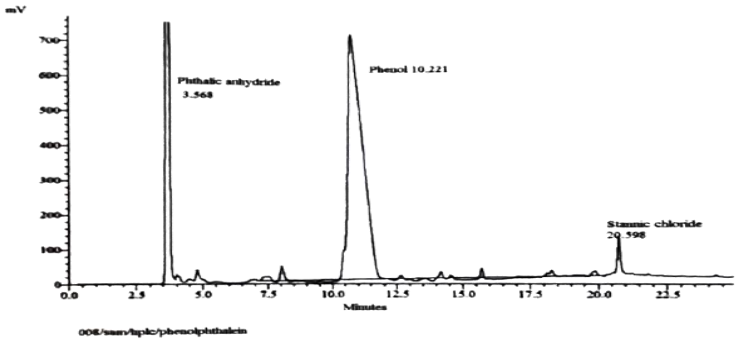
f. Table. 5.6 Change in the pH of water when treated with 3 different indicators



Phenolphthalein decreases the pH level of water from 5.5 to 4.8 during the investigation period which suggest that the water has become more acidic as compared to the control value of 6.4 pH. Zahangir, *et al.*,(2015) showed that the biometric index of zebra fish was significantly affected by the Acidity which was having a low pH then 4.2. (Graph 8 & Table.5.6)

6. Indicator analysis by High Performance Liquid Chromatography (HPLC) and Infrared Spectroscopy (IR)

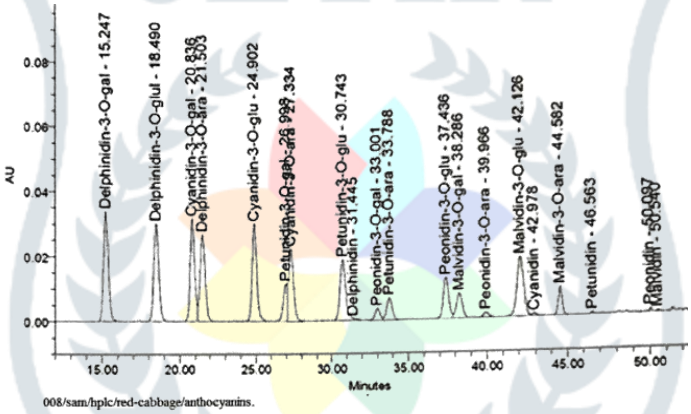
a) HPLC of phenolphthalein indicator –



Graph 9. HPLC of Phenolphthalein indicator

In the present study 3 different types of compounds were found i.e. Phthalic anhydride, Phenol and Stannic chloride. Most non-polar was Phthalic anhydride showing it was insoluble in water. (Graph 9)

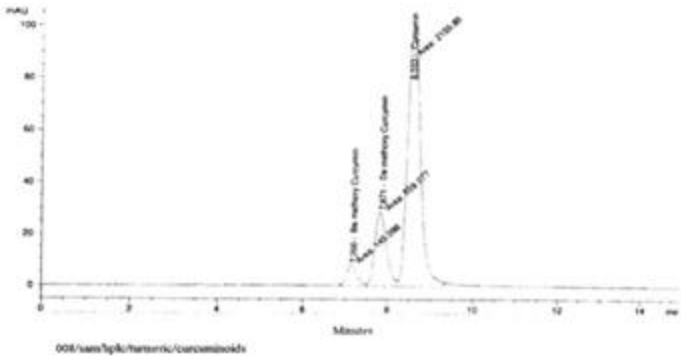
b) HPLC of Red-cabbage indicator –



Graph 10- HPLC of Red- cabbage indicator

HPLC of Red-cabbage indicator showed that it contained about 15-20 anthocyanin which were highly stable such as Delphindin-3-o-gal-15.247, Cyanidin-3-o-glu-24.902, Petunidin-46.563, Malvidin-50.540 etc., which were of polar nature.(Graph 10)

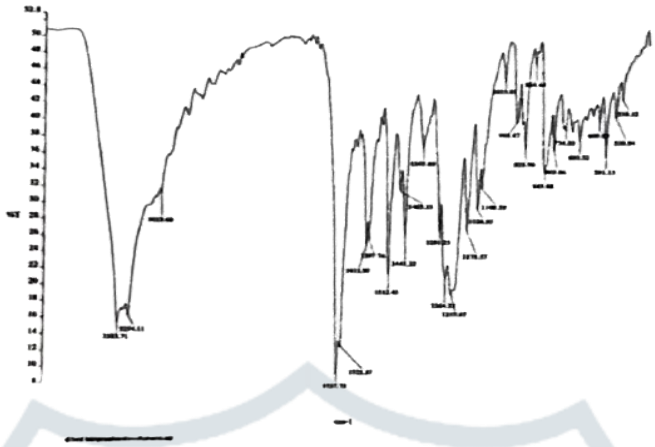
c) HPLC of Turmeric indicator –



Graph 11. HPLC of Turmeric indicator.

HPLC of Turmeric indicator detected 3 peaks comprising of Bis methoxy Curcumin, De methoxy Curcumin and with highest peak of Curcumin. (Graph 11)

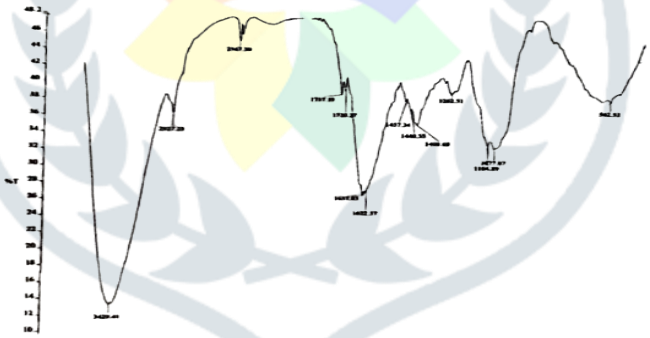
d) IR of Phenolphthalein indicator-



Graph 12. IR of Phenolphthalein indicator

Phenolphthalein is a chemical indicator made from Phthalic anhydride, Phenol and Sulphuric acid or Stannic chloride. The components are dangerous for the environment when exposed. It doesn't have useful property like the natural indicators. (Graph 12)

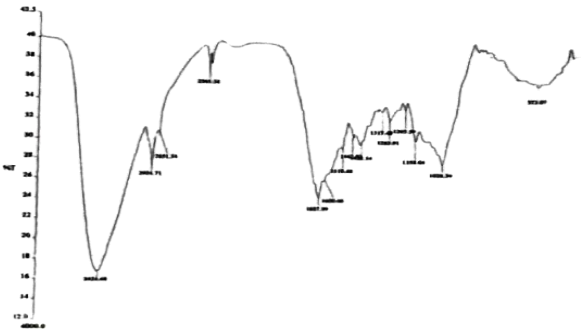
e) IR of Red-cabbage indicator –



Graph 13. IR of Red-cabbage indicator.

IR of Red-cabbage indicator showed the absorption of UV-visible light by organic pigments causes excitation of their electrons, a transition that is accessible in the absorbing pigments because they have an extensive system of conjugated π - electrons Pigments in plants absorb the energy required for photosynthesis. The pigments in red cabbage belong to a group of compounds called Anthocyanins, which are valued by nutritionists for the antioxidant behavior of their phenolic functional groups. Anthocyanins are part of a larger group of structures called flavonoids, many of which arse important antioxidants.(Graph 13)

f) IR of Turmeric indicator -



Graph 14. IR of Turmeric indicator

Curcuma longa, commonly known as Turmeric is also widely used as coloring agent and known for its medicinal properties. Many research studies have been conducted on *Curcuma longa,* its secondary metabolites are sources of antioxidant. (Graph 14)

4. CONCLUSION

It is almost confirm that polluted water is one of the major causes of environmental pollutions. It is observed that chemical indicators used in the chemical factories, pharmaceutical industries etc. are made from organic solvents like ethanol, acetone, etc. which are insoluble in water & are directly released in riverine sources those led to heavy pollution. Thus present study showed that after 15 days of treatment with phenolphthalein as an indicator in fish tank have disadvantageous and negative effects on both fish and water. It also deteriorates the water quality of fish tank by increasing the BOD, Hardness, and Acidity reduces the DO, pH and Alkalinity. Whereas Red cabbage, turmeric reduces the acidity, maintain constant alkalinity, BOD, total hardness and increases the DO of fish tanks. Phenolphthalein decreases the overall lipid and carbohydrate content of Zebra fish as compared to Red cabbage & Turmeric treated fish 0.014gm was seen with turmeric indicator and 0.005gm was seen with Red-cabbage indicator. The purity of the indicators was shown by High Pressure Liquid Chromatography (HPLC). Almost 20 Anthocyanins were found and 3 Curcuminoids were detected. Components of phenolphthalein such as Phthalic anhydride, Phenol and Stannic chloride were also observed. Hence it was shown that natural indicators are better indicators than chemical indicators. In the view of the polluting effects on the environment, due to the use of chemical indicators, the Lay person and corporation & government of particular region should emphasize on the use of natural indicators rather than chemical indicators. As by using natural indicators, on large scale will lower the toxicity level of the fishes and the quality fresh water will be available in those proximity. The scientific application of natural indicators give accurate results, hence, it is a step towards environment friendly GREEN chemistry.

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