# MATERIALS AND METHODS

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## 3.1 Location of the study area

Haridwar district is located in the south-western part of India's Uttarakhand state, encompassing around 2360 $km^2$. About 314 meters above sea level, Haridwar is situated between the north and northeast are the Siwalik hills, while in the south is the Ganges River. Both of these geographic features can be seen from the city. It is the largest city in the district and the second largest city in the state. The location of the city falls within the latitude of $29.58^0$ north and the longitude of $78.13^0$ east. The Haridwar district has a climate that is usually hot and humid during summers and during winters temperature ranges between 10°C to 30°C, with an average annual rainfall of roughly 2136.7mm [130]. The water sources selected for Haridwar region were based on the guidance and technical inputs given by the state's maintenance and drinking water supply agency i.e. Uttarakhand Jal Sansthan (UJS) and samples were collected quarterly.

## 3.2 Socio-economy and major drinking water practices

In Haridwar, a city located in the northern state of Uttarakhand, India, the socio-economy is intricately linked to its status as a major pilgrimage site and tourist destination. The city's economy is primarily driven by tourism, religious activities, and related industries, making it a significant hub for both domestic and international visitors.

The socio-economic landscape of Haridwar is shaped by the presence of numerous temples, ashrams, and ghats along the banks of the sacred Ganges River. These religious sites attract millions of pilgrims and tourists each year, contributing

significantly to the local economy through the sale of religious offerings, accommodation, and other services. Additionally, the city's proximity to the Himalayas and its natural beauty makes it a popular destination for eco-tourism and adventure sports, further boosting its economic prospects.

Despite its economic vibrancy, Haridwar faces challenges in ensuring access to clean and safe drinking water for its residents and visitors. The city primarily relies on surface water sources, with the Ganges River being the main source of drinking water. However, the quality of water in the Ganges is often compromised due to pollution from industrial effluents, sewage discharge, and religious rituals. This poses a significant health risk to the population, as contaminated water can lead to waterborne diseases and other health issues.

To address these challenges, the government and local authorities in Haridwar have implemented various measures to improve the quality and availability of drinking water. These include the construction of water treatment plants, the implementation of water conservation initiatives, and the promotion of sustainable water management practices. Additionally, efforts are being made to raise awareness about the importance of clean drinking water and the need for collective action to protect water sources.

In conclusion, the socio-economy of Haridwar is closely intertwined with its religious and tourism sectors, which drive economic growth and development in the city. However, ensuring access to clean drinking water remains a critical issue that requires concerted efforts from all stakeholders. By implementing sustainable water management practices and raising awareness about water conservation, Haridwar can safeguard its water sources and promote the well-being of its residents and visitor for

generations to come [131-132].

**Table 3- Location of all sources of Haridwar for sample collection**

| S.No. | Name of source | Longitude | Latitude | Elevation |
|---|---|---|---|---|
| **Haridwar 1st zone** | | | | |
| 1 | Bhopatwala 1 New | 29°58'16.5" | 78°04'21.3" | 305 |
| 2 | Bhopatwala 2 New | 29°58'20.7" | 78°03'50.1" | 313 |
| 3 | Bhopatwala 3 New | 29°58'26.2" | 78°03'45.6" | 314 |
| 4 | Bhopatwala 26 | 29°58'17.0" | 78°10'49.1" | 299 |
| 5 | Bhopatwala 16 | 29°58'14.8" | 78°10'44.0" | 286 |
| 6 | Saptsarover | 29°59'12.7" | 78°11'45.2" | 287 |
| 7 | Parmarth Ashram | 29o58'58.1" | 78o11'30.7" | 295 |
| 8 | Bheemgoda 50 | 29o57'58.6" | 78o10'18.3" | 288 |
| 9 | Pantdeep 1 | 29o57'36.6" | 78o10'24.0" | 283 |
| 10 | Saptsarover | 29o59'12.7" | 78o11'45.2" | 287 |
| 11 | Parmarth Ashram | 29o58'58.1" | 78o11'30.7" | 295 |
| 12 | Roribelwala 24 | 29o57'02.0" | 78o10'13.1" | 284 |
| 13 | Roribelwala 25 | 29o57'04.4" | 78o10'16.0" | 285 |
| 14 | Van Samadhi 42 | 29o56'57.0" | 78o10'08.5" | 280 |
| 15 | Van Samadhi 43 | 29o56'58.2" | 78o10'09.3" | 295 |
| 16 | Bhopatwala 4 | 29o58'55.8" | 78o11'28.2" | 294 |
| 17 | Vishnu Ghat 44 | 29o57'02.2" | 78o10'02.1" | 279 |
| 18 | Laltaro Pul 17 | 29°56'49.8" | 78°09'47.7" | 284 |

| | | | | |
|---|---|---|---|---|
| 19 | Pantdeep 40 | 29°57'45.4" | 78°10'26.9" | 283 |
| 20 | Khad khadi | 29°58'15.3" | 78°10'24.7" | 294 |
| 21 | C.C.R. 07 | 29°57'12.1" | 78°10'16.5" | 285 |
| 22 | Satyam Vihar | 29°59'15.0" | 78°10'58.5" | 299 |
| 23 | Pawan Dham | 29°58'27.0" | 78°10'51.2" | 286 |
| 24 | G.D. Puram | 29°59'21.9" | 78°11'04.1" | 296 |
| 25 | Booster pump Brahampuri | 29°57'03.7" | 78°09'35.0" | 275 |
| 26 | Pump No. 18 | 29°57'39.9" | 78°10'33.6" | 284 |
| | **Haridwar 2nd zone** | | | |
| 27 | Sharvan Nath | 29°56'46.2" | 78°09'21.6" | 270 |
| 28 | Mayapur T.W. No- 4 | 29°56'33.0" | 78°08'55.4" | 286 |
| 29 | Mayapur T.W. No- 5 | 29°56'29.0" | 78°09'07.1" | 270 |
| 30 | Bhalla College T. W. – 6 | 29°56'23.1" | 78°09'01.0" | 278 |
| 31 | Bhalla College T. W. No – 68 | 29°56'29.8" | 78°09'01.6" | 272 |
| 32 | Devpura T. W. No -.38 | 29°56'26.6" | 78°08'49.0" | 284 |
| 33 | Devpura T. W. No - 57 | 29°56'33.0" | 78°08'55.4" | 286 |
| 34 | Arihant Vihar | 29°55'06.3" | 78°07'48.1" | 273 |
| 35 | Indra Basti | 29°55'00.9" | 78°08'34.5" | 262 |
| 36 | T.W. I.T.I. | 29°55'04.5" | 78°08'17.5" | 283 |
| 37 | Rajpoot Dharamshala | 29°55'46.6" | 78°08'45.6" | 279 |
| 38 | Vishnu Garden | 29°55'21.9" | 78°07'53.6" | 315 |
| 39 | Beragi Camp, T.W. 29 | 29°56'18.1" | 78°09'27.8" | 319 |

| | | | | |
|---|---|---|---|---|
| 40 | Beragi Camp, T.W. 49 | 29°56'24.5" | 78°09'29.2" | 276 |
| 41 | T.W.28, Mahila Milan | 29°56'21.2" | 78°09'22.7" | 270 |
| 42 | Turist Bangla, T.W.21 | 29°56'38.5" | 78°09'37.3" | 284 |
| 43 | Gyanlok | 29°55'50.5" | 78°08'37.2" | 277 |
| 44 | Govind Puri | 29°56'02.4" | 78°08'12.8" | 288 |
| 45 | Bhgwant Purum | 29°55'34.3" | 78°08'11.6" | 274 |
| | **HARIDWAR 3RD ZONE** | | | |
| 46 | Pandeywali New | 29°5533.0" | 78°06'10.9" | 283 |
| 47 | Pandeywali 35 | 29°5535.8" | 78°06'11.9" | 285 |
| 48 | B.H.E.L Purniwas | 29°55'57.2" | 78°06'17.1" | 283 |
| 49 | Ahebab Nagar | 29°55'49.2" | 78°06'30.6" | 332 |
| 50 | Shastri Nagar | 29°5543.1" | 78°0658.4" | 333 |
| 51 | Chaklan T.W No-34 | 29°55'35.2" | 78°06'17.5" | 294 |
| 52 | Sharda Nagar | 29°55'23.9" | 78°06'50.3" | 282 |
| 53 | Shanti Vihar | 29°55'21.5" | 78°07'05.4" | 284 |
| 54 | Nand Vihar | 29°55'43.1" | 78°07'57.2" | 285 |
| 55 | Jain Mandir | 29°55'23.1" | 78°06'26.6" | 287 |
| 56 | Shastri Nagar | 29°5543.1" | 78°0658.4" | 333 |
| 57 | Arya Nagar | 29°55'25.0 | 78°07'18.2" | 301 |
| 58 | Model Colony | 29°58'00.4 | 78°07'58.7" | 283 |
| 59 | Ram Nagar | 29°55'37.9" | 78°07'46.4" | 284 |
| 60 | Tibri T.W No-23 | 29°56'16.1" | 78°07'51.0" | 289 |
| 61 | Tibri T.W No-58 | 29°56'26.1" | 78°07'55.6" | 283 |

| | | | | |
|---|---|---|---|---|
| 62 | Tibri T.W No-01 | 29°56'16.6" | 78°07'55.6" | 282 |
| 63 | Tibri T.W No-02 | 29°56'17.8" | 78°08'11.0" | 280 |
| 64 | Tibri T.W No-03 | 29°56'13.3" | 78°08'16.1" | 277 |
| 65 | Durga Ghat | 29°55'18.4" | 78°06'43.0" | 286 |
| 66 | Mandi ka kua | 29°5540.2" | 78°0635.2" | 284 |
| 67 | Trimurti Nagar | 29°55'18.0" | 78°05'23.6" | 278 |
| 68 | Jain mandir | 29°55'23.1" | 78°06'26.6" | 287 |
| 69 | Chowk Bajar T.W No-15 | 29°55'25.8" | 78°06'24.2" | 288 |
| 70 | Dheerwali | 29°55'52.3" | 78°06'09.7" | 285 |
| 71 | Tibri, Near railway phatak | 29°56'17.8" | 78°08'11.0" | 280 |
| 72 | Tibri T.W No-12 | 29°56'19.7" | 78°08'05.6" | 281 |
| 73 | Tibri Mini T.W,Sanjay nagar | 29°56'26.1" | 78°07'55.6" | 283 |
| 74 | Chaklan | 29°55'35.2" | 78°06'17.5" | 294 |
| 75 | Kadcha | 29°5549.6" | 78°0639.7" | 289 |
| 76 | Ambedkar Murti | 28°55'49.4" | 78°06'37.6" | 294 |
| 77 | Raj Nagar | 29°55'33.0" | 78°07'14.0" | 291 |
| 78 | Tehsil T.W No-47 | 29°55'41.6" | 78°07'34.2" | 323 |
| | **SHIVALIK NAGAR** | | | |
| 79 | Shivalik Nagar T.W. 1 | 29°56'07.5" | 78°04'23.1" | 286 |
| 80 | Shivalik Nagar Phase 3rd | 29°55'54.7" | 78°04'30.3" | 279 |
| 81 | Shivalik Nagar, Phase 3rd, T.W. 3 | 29°55'51.2" | 78°04'37.6" | 279 |

| | | | | |
|---|---|---|---|---|
| 82 | Shivalik Nagar, Phase 3rd, T.W. 3 | 29°55'53.5" | 78°04'09.3" | 296 |
| 83 | Shivalik Nagar, Phase 2nd, T.W. 2 | 29°56'07.0" | 78°04'04.9" | 289 |
| | **LAKSAR** | | | |
| 84 | Laksar T.W. 3 | 29°45'17.8" | 78°01'39.1" | 225 |
| 85 | Laksar T.W. 4 | 29°45'20.3" | 78°01'52.1" | 222 |
| 86 | Laksar T.W. 2 | 29°44'57.7" | 78°01'04.0" | 224 |
| 87 | Laksar T.W. 5 | 29°44'54.4" | 78°01'02.7" | 221 |
| 88 | Mangalore T.W. 2 | 29°47'36.3" | 77°52'47.1" | 260 |
| 89 | Manglore T.W. 1 | 29°47'35.2" | 77°52'44.0" | 262 |
| 90 | Landhora T.W. 2 | 29°48'14.9" | 77°56'09.5" | 258 |
| 91 | Landhora T.W. 1 | 29°48'32.6" | 77°55'54.5" | 262 |
| 92 | Landhora T.W. 3 | 29°46'14.4" | 77°55'39.6" | 255 |
| 93 | Jhabrera T.W.1 | 29°46'50.5" | 77°46'34.0" | 260 |
| 94 | Jhabrera T.W.2 | 29°49'02.3" | 77°46'24.4" | 258 |
| 95 | Jhabreda T.W. – 3 | 29°48'43.0" | 77°46'35.6" | 239 |
| | **ROORKEE** | | | |
| 96 | Sati Mohalla, Roorkee | 29°52'50.9" | 77°53'22.6" | 255 |
| 97 | Sot Mohalla, Roorkee | 29°52'49.6" | 77°53'13.5" | 247 |
| 98 | Nagar Palika T.W. | 29°52'27.4" | 77°53'23.4" | 239 |
| 99 | Gandhi Vatika, T.W. | 29°52'24.1" | 77°53'19.8" | 254 |
| 100 | Civil Line, T.W. | 29°52'28.1" | 77°53'44.7" | 245 |

## 3.3 Isolation of iron oxidizing bacteria (IOB)

### 3.3.1 Starter media

For preparation, 0.2 gm of $(NH_4)_2SO_4$, 0.2 gm of $CaCO_3$, 0.04 gm of $MgSO_4$, 0.002 gm of $KH_2PO_4$, and 0.8 gm of $KHC_8H_4O_4$ were added to 100 ml of distilled water and autoclaved to create the starter medium. To the salt solution, add 100 ml of 18% $FeSO_4$. It was sterilized separately. In order to achieve the desired final concentration of 9% $FeSO_4.7H_2O$, $7H_2O$ was added to the medium. The pH of the medium was brought upto 4.1 using 10 N $H_2SO_4$, which was used. This resulted in a medium that was more transparent and had a yellowish colour. There was less precipitate in the media, and itwas simple to filter it out. At 37ºC, the broths were incubated. After 72 hours of incubation, the medium began to become turbid, and after one week, it was dark brownwith some residue at the flask's bottom. After one week, one loop of the enriched medium B was streaked onto the same media solidified with agar and incubated at $37^0$C. On the plates, dark yellow powdery growth was detected after three days. The culture was sub-cultured further for identifying purposes [133].

## 3.4 Gradient isolation and enrichment technique

We performed our enrichments in glass tubes measuring 60 mm x 15 mm that contained contrasting oxygen and Fe gradients using a modified Wolfe's mineral medium [134]. Semisolid mineral salt-bicarbonate-buffering medium and an air-filled headspace were enclosed within the tubes, with the plugs made of FeS or $FeCO_3$ [135]. A combination of FeS or $FeCO_3$ precipitates and modified Wolfe's mineral medium was poured into a flask, adding 1% (wt./vol) agarose (Pharmacia) to the mixture, forming MWMM. In a separate flask, agarose was included in the MWMM medium

at a weight-to-volume ratio of 0.15%. So that it could be used as an overlayer, each set went through an autoclave cycle for 20 minutes at a temperature of 121 degrees Celsius. A volume of 0.75ml of the molten FeS was transferred to a tube that had been pre-sterilized. The agarose then solidified, resulting in the formation of a plug. The overlayer was combined with filter-sterilized vitamins at 1 ml per litre and sodium bicarbonate at a final concentration of 5 mM. The sodium bicarbonate was prepared as a sterile stock solution at a concentration of 1 M before being added to the mixture. The resulting mixture was subsequently used. After that, 3.75 cc of this solution was brushed onto the FeS plug. After exposure to filter-sterilized system $CO_2$, the overlayer's ultimate pH ranged between 6.2 and 6.4. This exposure was achieved by delivering the $CO_2$ through a cannula at 1.8 ml/s over 4 seconds while the overlayer remained liquid. The tubes were hermetically sealed using covers or plugs made of butyl rubber. They were subsequently left undisturbed for a period ranging from 6 to 24 hours before the introduction of the inoculant. During the inoculation process, the tube cap was removed, and pipette tip containing 10 to 15 ml of the inoculant was cautiously inserted near the FeS plug, and expelling the contents as the tip was removed. In the dark the tubes were incubated at 21°C (61°F) in an incubator with a constant temperature. Gradient tubes were employed to assess additional substrates with growth potential. In the case of manganese, the FeS compound was replaced with $MnCl_2$ at concentrations of 0.5, 5, or 10 mM. Similarly, for acetate, glucose, and pyruvate, the respective substrates were introduced at 1,5, or 10mM concentrations. These substrates were added to the agarose plug at the bottom of the tube or, in specific instances, to the overlayer. A concentration of 2.5 mM of thiosulfate was introduced into the agarose plug, following the described procedure to generate sulphide gradients

[136]. Each big vial was injected into the gel at five evenly spaced locations growth research. On various substrates the cell growth rates were quantified by utilizing acridine orange direct counts. Several gradient tubes were simultaneously infected with equal amounts of cell material. To homogenize the growth band the entire semisolid overlayers from the same test tubes were removed and vortexed for 30 seconds at daily intervals (or as indicated). For cell counting, two 10-ml aliquots were spread out in specified circles on an agar-coated microscope slide, and then the slide was allowed to air-dry. Two separate slides were prepared for each growth tube. When conducting cell counts, a solution containing 0.0005% acridine orange was added, and an epifluorescence microscope equipped with a magnification setting of 31,000 was used to count all of the cells in 15 microscope fields. In order to calculate the overall quantity of iron that has been accumulated over the course of development, a 1 ml aliquot of the initial mixed sample was transferred to an eppendorf tube, where it was then frosted at 220 degrees celsius for subsequent analysis. The growth bands from multiple growth tubes per isolate were extracted and centrifuged at one point in $PO_4$ buffer in order to determine whether or not the isolates were able to reduce the iron oxides that they produced. As previously described, the sample was deposited in vials that contain an anaerobic medium & incubated. The total Fe concentration was measured as previously stated. A specified quantity of Iron oxides was diluted in stated volume of a 0.25 M hydrochloric acid (HCl) and 0.25 M hydroxylamine solution. Following an incubation period of 1.5-2 hours at a temperature of 30°C, accompanied by moderate agitation, a portion of the reduced oxides was diluted in ferrozine. Subsequently, the absorbance of this sub-sample was measured at a wavelength of 562 nm [137-138].

## 3.5 Preparation of stock solutions

The research used various chemicals and equipment, including 8-hydroxyquinoline, often known as sulphuric acid, ferric ammonium sulphate, oxine, distilled water, and chloroform. Additionally, analytical grade aluminium sulphate was used. The apparatus involved in the study included a UV spectrophotometer, test tubes, a measuring cylinder, pipettes, a beaker, and volumetric flasks.

### 3.5.1 Preparation of ferric ammonium sulfate solution

To prepare the ferric ammonium sulfate solution, 8.635 gm of ferric ammonium sulfate is dissolved in 3 ml of concentrated $H_2SO_4$ (sulfuric acid), and the resulting solution is diluted to 1 liter with distilled water. This stock solution is used as a standard for subsequent experiments. The stock solution was diluted with dilute sulphuric acid (0.005 M) to create working solutions.The pH of the acidified water used to dissolve the 1.0 g of oxine ranged from 1-2 [139].

### 3.5.2 Preparation of 8-hydroxyquinoline solution

To prepare the 8-hydroxyquinoline solution, a specified amount of 8-hydroxyquinoline is dissolved in chloroform to obtain the desired concentration. This solution is utilized in the analysis of metal ions.

A 100 ml volumetric flask was used to dissolve 1.0 g of 8-hydroxyquinoline in chloroform, and the rest of the volume was brought up to the correct level using chloroform. To prevent loss due to evaporation, the flask was quickly sealed. Maximal absorption wavelengths of metal-oxide complexes determined using a separating funnel, we added 10 ml of oxine-chloroform to a solution containing 10 ppm of iron standard and properly shook the mixture for 3–5 minutes. Separation of the aqueous

layer from the denser chloroform oxine-metal complex layer was enabled by a distinct boundary between the two layers. After it had been isolated, a test tube was used to store the oxine-metal complex. 10 ml of chloroform were mixed with 10ml of distilled water in a separating funnel to create a blank. After 3–5 minutes of vigorous shaking, the two layers were collected in individual test tubes. The maximum absorbance (Amax) of a 10 ppm oxine-metal complex was determined by scanning its absorbance at various wavelengths in order to establish the maximum absorbance (Amax). Standard solution preparation - to serially dilute the stock solution, 0.005 M sulphuric acid was used. Absorbances were taken after adding 10 ml of chloroform oxine reagent to each standard solution (1-10 ppm) to extract the metal oxide complex. We accurately weighed 100 g of oxine and dissolved it in 100 ml of acidified water (pH 1 to 2). This preparation was done in deionized water [140].

### 3.5.3 Preparation of other reagents

Other reagents such as aluminum sulfate and other necessary chemicals are prepared by dissolving appropriate amounts in distilled water to achieve the required concentrations for experimental use [141].

### 3.5.4 Preparation of calibration curve

Iron (III) standard solutions ranging in concentration from 1 to 14 ppm were made. The oxine solution was diluted to 0.5 ml and added to 10 ml of the stock solution. The degree to which they absorbed was determined. Interference studies using an acidified water solution and oxine chloroform reagent. To a solution containing l0 ppm of iron (III) standard and l0 μg/ml of $Al^{3+}$ was added; the solution was made up to 10ml. The extraction was done in the same way as was explained before. In a volumetric flask,

solution containing 10ppm of iron (III) standard and 10 μg/ml of $Al^{3+}$ were added to create an acidified water solution and the solution was made up to 10ml. A green metal oxine complex was formed after adding 0.5 ml of an acidified oxine reagent [142].

### 3.5.5 Determination of iron (III) in water samples

Each water sample was 10 ml in volume, and a single drop of sulphuric acid was added. In the end, it was treated same as the standard. For comparison, the iron concentration in the water sample were also measured using atomic absorption spectroscopy.

### 3.5.6 Recovery and stability studies of the proposed method

An aliquot of 10 ml water sample was spiked with three ppm of Fe (III) standard solution. Extraction was performed in the manner previously described. The level of absorbance was quantified. Over about 72 hours, the absorbance was continuously monitored to examine the standard concentrations. Absorbance at the concentration standard was plotted versus time. Standard addition recovery studies on 10ppm to 10ml of water samples, a 10 ppm Fe (III) standard was added [142]. The process for developing the colour was identical to what was previously described.

Percent Recovery = Covered Amount/Spike Amount x 100 / 1

## 3.6 Identification and characterization of iron oxidizing bacterial isolates

The identification and characterization of iron oxidizing bacteria (IOB) are critical for understanding the diversity and potential of these organisms in bioremediation processes. This section details the isolation, identification, and characterization of IOB through morphological, biochemical methods [143,144].

### 3.6.1. Isolation of iron oxidizing bacteria

**1. Sample collection:**

- Water and soil samples were collected from various iron-rich environments in the Haridwar district, including hand pumps and Uttaranchal Koops.

**2. Culturing:**

- Samples were cultured on selective media designed for iron oxidizing bacteria, such as gradient tubes containing FeS or FeCO3. The cultures were incubated under microaerophilic conditions to promote the growth of IOB.

**3. Morphological characterization:**

- The colony shape of the isolates was documented after 72 hours of growth on media at 37ºC, dependent on growth. The general form, size, opacity, elevation, margin, andtexture were noted. After Gram staining, the morphology of cells was studied throughan oil immersion lens of a microscope.

### 3.6.2. Biochemical characterization

**1. Biochemical tests:**

- A series of biochemical tests were conducted to characterize the metabolic capabilities of the isolates. These tests included:
- Oxidase test
- Catalase test
- Nitrate reduction test
- Fermentation of various sugars

**2. Iron oxidation activity:**

- The ability of each isolate to oxidize iron was confirmed by measuring the decrease in $Fe^{2+}$ concentration and the formation of $Fe^{3+}$ precipitates.

## 3.7 Characterization and formulation of soil media/ carriers for the treatment of raw water

In the bioremediation process, carriers play a critical role in supporting the growth and activity of iron oxidizing bacteria (IOB) and enhancing the removal efficiency of iron from water. This section provides a detailed analysis and characterization of the carriers used in this study, including gravel, sand, coarse sand, bentonite, and lignite [145,146].

### 3.7.1 Gravel

Gravel is a deposit of approximately spherical rock fragments that are smaller in size compared to sand, with a diameter exceeding 2 mm (0.08 inch). Certain geographical areas contain gravel beds that consist of dense metallic ore minerals, such as cassiterite (the primary source of tin), as well as native metals like gold, which can be found in

the form of small flakes or nuggets. Gravels are a standard construction material. Gravel fragments range in size from cobbles ([2.52–10.08 inches] 64–256 mm) to pebbles ([0.16–2.52 inches] 4–64 mm in diameter) to boulders (256 mm in diameter [10.08 inches]) (larger than 256 mm). Gravel becomes rounded due to abrasion during conveyance by either washing by the sea or by streams. Gravel deposits form in areas like beaches and stream channels where the water passes too quickly to allow sand to reside. Due to fluctuating environmental circumstances, gravel depositions are typically more restricted and varied in roughness, configuration and thickness as compared to sand or clay deposition. Along the inner zone of spilling waves on an otherwise sandy beach, persistent aggregation of pebble beds or gravel may occur. Pebble and cobble (shingle beaches) are frequently formed at the base of stony cliffs.

**Physical characteristics:** Gravel used in this study had a size range of 2-5 cm. The surface area, porosity, and bulk density were measured using standard methods. **Chemical composition:** The elemental composition of the gravel was determined using X-ray fluorescence (XRF) analysis. Key elements such as silicon (Si), aluminum (Al), and iron (Fe) were quantified.

### 3.7.2 Sand

Sand is a granulated substance made up of mineral particles and finely separated rock. The makeup of sand varies, but its grain size determines it. Sand grains are coarser than gravel grains and smaller than silt grains. Sand is also the term for a textural category of soil or a specific type of soil; more precisely, the term "sandy soil" denotes soil with a weight composition of over 85 per cent sand-sized particles. $SiO_2$, also known as silicon dioxide or silica, commonly exists as quartz and is the primary

constituent of sand found in continental interiors and non-tropical coastlines. The conformation of sand can differ depending on the conditions and local rock sources. Fine sand particle size ranges from 0.075 to 0.425 millimeters, and medium sand particle size is from 0.425 to 2 millimeters.

**Physical characteristics:** The sand had a particle size range of 0.1-0.5 mm. The specific surface area was measured using the BET (Brunauer-Emmett-Teller) method, and the porosity was determined using mercury intrusion porosimetry.

**Chemical composition:** The sand's composition was analyzed using XRF, highlighting the presence of silicon dioxide ($SiO_2$) and trace amounts of other minerals.

### 3.7.3 Coarse sand

The particle size range of coarse sand is between 2 mm and 4.75 mm. It is significantly coarser than fine sand; hence, it can be used as a carrier.

**Physical characteristics:** Coarse sand particles ranged from 0.5-2 mm in size. surface morphology was examined using scanning electron microscopy (SEM).

**Chemical composition:** XRF analysis was performed to identify the primary and secondary components.

### 3.7.4 Bentonite

An absorbent swelling clay '*bentonite'* is composed of montmorillonite in the majority. Typically, it is produced in seawater by the weathering of volcanic ash, which converts the volcanic glass present in the ash into clay minerals. These provide the clay with a massive total surface area, which makes bentonite an effective absorbent.

**Physical characteristics:** Bentonite is a clay with high cation exchange capacity

(CEC). Its swelling index and specific surface area were measured to evaluate its adsorption potential.

**Chemical composition:** X-ray diffraction (XRD) analysis was used to determine the mineralogical composition, focusing on montmorillonite content.

### 3.7.5 Lignite

Brown coal, also known as lignite, is a type of combustible sedimentary rock with a brown colour and is quite soft. They are composed of peat that has been naturally compressed. Globally mined lignite is almost exclusively utilized as a fuel for steam-electric power generation.

**Physical characteristics:** Lignite particles were characterized by their particle size distribution, porosity, and specific surface area.

**Chemical composition:** The organic content and elemental composition of lignite were determined using thermogravimetric analysis (TGA) and XRF.

## 3.7.6 Characterization techniques

Various characterization techniques are as follows [147-150] –

**1. X-ray Fluorescence (XRF):**

- Used to determine the elemental composition of the carriers. This technique involves exposing the carriers to X-rays and measuring the secondary (fluorescent) X-rays emitted by the elements within the sample.

**2. Scanning Electron Microscopy (SEM):**

- Used to observe the surface morphology and structure of the carriers. SEM provides high-resolution images to examine the texture and porosity of the materials.

**3. Brunauer-Emmett-Teller (BET) Method:**

- Used to measure the specific surface area of the carriers by nitrogen gas adsorption. This method helps in understanding the adsorption capacity of the carriers.

**4. Mercury Intrusion Porosimetry:**

- Used to determine the porosity and pore size distribution of the carriers. This technique involves forcing mercury into the pores of the material under controlled pressure.

**5. X-ray Diffraction (XRD):**

- Used to identify the mineralogical composition of the carriers, particularly for clay materials like bentonite. XRD patterns help in determining the crystalline phases present.

**6.Thermogravimetric Analysis (TGA):**

- Used to measure the changes in physical and chemical properties of materials as a function of temperature. This technique is particularly useful for analyzing the organic content of lignite.

## 3.8 Utilization of carrier for absorption of iron in water samples

Due to their notable advantages, biosorption techniques are progressively gaining recognition as a highly effective and economically viable approach for eliminating diverse metallic compounds from aqueous solutions. (The low-cost treatment of heavy metals, the relatively close revivification of residual heavy metals from dissipated biosorbents, the minimizing of ensuing sludge, the easy operation andthe excellent performance, etc.). Biosorbents for removing heavy metals have been investigated using various biomasses, including fungi, yeast, algae, peat, and various wastes from

agricultural production. These biosorbents have been evaluated under a variety of different experimental circumstances [138,151-161].

Gravel, sand, coarse sand, bentonite, and lignite were evaluated in terms of

a) initial solution pH and

c) Contact time,

b) biosorbent dose for the biosorption processes of heavy metals.

d) beginning temperature and heavy metals concentration. Intra-particle diffusion kinetic models, pseudo-first-order models, pseudo-second-order models, and the Freundlich kinetic equation were used to simulate experimental results and identify optimal conditions, also Dubinin-Radushkevich and Langmuir, isotherm models, in order to emphasize the biosorption mechanism's most crucial aspects. Also computed were the biosorption process' thermodynamic characteristics.

## 3.9 Utilization of carrier along with microbes for absorption of iron in water samples

The research aimed to determine the percentage of iron that could be removed by utilizing carriers in combination with microbial consortia consisting of iron-oxidizing bacteria (IOBs). In order to formulate the consortia, the individual strains of iron-oxidizing bacteria were first evaluated for their compatibility with one another and then bound with the most appropriate and effective carrier.

## 3.10 Compatibility screening of iron oxidizing bacteria

The compatibility screening of iron oxidizing bacteria (IOB) is a crucial step in the development of an effective bioremediation process for the removal of iron from water. This screening ensures that the selected bacterial strains can work synergistically,

enhancing their collective iron removal efficiency. The following section details the methodology used for compatibility screening and the significance of the results obtained [143-144].

**1. Preparation of bacterial broth cultures:**

- Each bacterial isolate (IOB-1 to IOB-6) was inoculated separately into sterile nutrient broth. The cultures were incubated at 30°C for 48 hours to ensure sufficient bacterial growth [133].

**2. Centrifugation and collection of supernatants:**

- After the 48-hour incubation period, the broth cultures were centrifuged at 5000 revolutions per minute (rpm) for 10 minutes. This step was performed to separate the bacterial cells from the broth.

- The supernatants, which contain the metabolites and excreted products of the bacteria, were carefully collected from each culture and stored in sterile containers.

**3. Well diffusion technique for compatibility testing:**

- Fresh nutrient agar plates were prepared and allowed to solidify.

- Using a sterile corkborer, wells were created in the agar plates. Each well was approximately 6 mm in diameter and spaced adequately to prevent overlap of diffusion zones.

- Each well was filled with 100 µL of the supernatant from the centrifuged broth cultures.

- A separate set of nutrient agar plates was inoculated with each bacterial isolate (IOB-1 to IOB-6) by spreading a thin layer of the bacterial culture across the surface of the agar.

**4. Application of supernatants:**

- The supernatants from each bacterial strain were tested against all other strains. For instance, the supernatant of IOB-1 was added to wells on agar plates inoculated with IOB-2 to IOB-6, and this procedure was repeated for all other strains.

- The plates were then incubated at 30°C for 24 hours to allow diffusion of the supernatants and interaction with the bacterial strains on the agar.

**5. Observation and interpretation of results:**

- After the incubation period, the plates were examined for zones of clearance around the wells. A clear zone around a well indicated that the supernatant of one strain inhibited the growth of another strain, signifying incompatibility.

- If no clear zone was observed, it indicated that the bacterial strains were compatible and could coexist without inhibiting each other's growth [142].

**6. Criteria for compatibility**

- Bacterial pairs that did not produce a zone of clearance were considered compatible. These pairs could be used together in subsequent applications, as they would not inhibit each other's activity.

- Bacterial pairs that produced a zone of clearance were deemed incompatible and would not be used together to avoid negative interactions.

By using this detailed methodology, the compatibility screening of the iron-oxidizing bacteria was conducted systematically, allowing for the identification of bacterial pairs that could be co-cultured effectively without inhibitory effects. This process ensures

that only compatible bacterial strains are selected for further experiments and practical applications in iron oxidation processes.

## 3.11 Preparation of consortia

Isolates of iron-oxidizing bacteria were placed in a well-defined and sterilized nutrient medium (liquid broth). The temperature ranged from 35-37$^0$C, and the pH ranged from 4.5-5.5. The broth was then placed in an orbital shaker for 48 hours. It was determined that spores count in the broth culture that contained the consortium.

## 3.12 Preparation of formulation using the suitable carrier

Coarser sand was chosen as the solid carrier for the adsorption of microbial consortia because it was found to be more effective in the absorption of iron and the removal of iron than any of the other carriers tested, including gravel, sand, coarser sand, bentonite, and lignite. The coarser sand particles were given an appropriate washing before being covered with microbial consortiums and a small amount of liquid agar dispersion. The coarser sand coated with microorganisms was then placed in bags that had been sterilized before being used as a slurry in a fixed-bed bioreactor.

## 3.13 Preparation of pilot scale reactor with biological filtration

The initial experiments were carried out in burette bioreactor followed by an indigenously designed bioreactor which was connected to a water suction pump through a side arm containing sterilized glass wool to circulate air into the unit at the rate of 2 l/min. Treatment was carried out using the batch culture method. The removal efficiency of the strain was monitored. The suction of the sterilized air from the bottom of the reactor also provided an automatic stirring and mixing of the immobilized cells

with the medium. It is a single chamber bioreactor. An inlet was provided at the top of the reactor and outlet at the base.

Besides, these two ports were given in the design of the bioreactor for the exchange of gases across the fixed bed i.e., circulation of the sterilized air and removal of the foul gases. The unit was housed in a temperature-controlled chamber at 37ºC.The biofiltration system's initial filter, the Fe filter, is intended to remove iron (II). The filter column had four layers of filter media, starting from the top and working their way down to the bottom: granular anthracite, fine sand, coarse sand, and gravel. In order to aerate the groundwater, between the iron filters, an aeration tank was set up, complete with an air compressor.

A biofiltration experiment was conducted for a full six months, beginning in February 2022 and continuing through August 2022. This allowed for the inclusion of both the winter and the wet seasons. Table 3.1 provides anoverview of the biofiltration unit's operational parameters. Hydrologic data about the pollutants in the biofilter, specifically the initial iron (II), were acquired on-site during biofiltration. These concentrations were determined through AAS spectrophotometricanalysis, along with measurements of temperature and pH. These measurements aimed to ascertain the concentrations of Fe (II) in the effluent. The effluent and influentcharacteristics were evaluated to determine the filter's filtration capacity.

**Table 3.1: Evaluation of a number of different influent ground water parameters, as well as the operational parameters of a bio- filtration unit on a pilot scale (2-3L)**.

| **Parameters of operation** | | | | |
|---|---|---|---|---|
| Velocity of flow (m/h). | 4.56 | | | |
| Number of washes | 10 | | | |
| **Depth of Filter (cm)** | | | | |
| Head space | 5 | | | |
| Gravel | 3 | | | |
| Sand | 3 | | | |
| Coarse sand | 3 | | | |
| Bentonite | 3 | | | |
| Lignite | 3 | | | |
| **Ground water chemistry (Initial)** | | | | |
| **Sample** | **pH** | **Temperature ($^0$C)** | **Initial Fe (mg/L)** | |
| Intake water | 7.1 | $32^0$C | 4.67 | |

## 3.14 Removal of iron via filtration

To assess microbial iron elimination, a test was designed. It was based on filter material from a functioning water treatment plant (the Islevbro water treatment plant in Rdovre, Denmark) was incubated for a short period of time, and the results showed a gradual decrease in Fe concentrations. In order to get rid of the free iron precipitates, 1L of treated water was used for every 100 grammes (wet weight) of filter material that went through a sieve. The experiment involved the incubation of 10 grammes (wet weight) of post-filter material with 400 ml of water obtained from the same water works. This was done in a closed system using a 500ml infusion bottle. Iron (II) was added to the mixture, and the oxidation and adsorption processes were monitored throughout the duration of the experiment. Syringe-drawn samples of 10 mL were immediately acidified with HCl to get the pH down to less than 2.

## 3.15. Statistical analysis

The SPSS 13.0 software package was utilized throughout the process of statistical analysis. Following the completion of one-way and two-way analysis of variance (ANOVA), the statistical significance of the observed differences among the treatments was evaluated. In every experiment, the significance level, or P value, was determined to be less than 0.05.

### 1. Data normalization

Data normalization is an essential step in preparing data for analysis, particularly when dealing with variables measured on different scales. Normalization ensures that each variable contributes equally to the analysis. Min-Max Normalization: This method scales the data to a fixed range, typically [0, 1].

The formula used is:

$$X' = X - Xmin Xmax - Xmin X' = \text{Xmax} - \text{XminX} - \text{Xmin}$$

where $X$X is the original value, $Xmin$Xmin and $Xmax$Xmax are the minimum and maximum values of the feature, respectively, and $X'$X′ is the normalized value.

**2. Dependent and independent variables**

- Dependent Variable: The main dependent variable in this study is the iron removal efficiency, measured as the percentage of iron removed from the water.
- Independent Variables: These include various factors such as the type of carrier (gravel, sand, coarse sand, bentonite, lignite), contact time, initial iron concentration, pH, and temperature.

**3. ANOVA (Analysis of Variance)**

ANOVA is a statistical method used to compare means across different groups to determine if there are any statistically significant differences between them. One-way analysis of variance (ANOVA) were conducted to evaluate the statistical significance of the observed differences among the treatments. The significance level (P value) was determined to be less than 0.05 in every experiment, indicating statistically significant differences.

F-statistic: ANOVA produces an F-statistic, which is used to determine the p-value. If the p-value is less than the significance level (typically 0.05), the null hypothesis is rejected, indicating that at least one group mean is different.