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Investigating bio-remediation capabilities of a constructed wetland through spatial successional study of the sediment microbiome

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Constructed wetlands (CWs) are engineered environments designed to utilise natural processes to treat urban or industrial wastewater, with the core driver of the bioremediation process provided by the microorganisms present within. This study isolated 32 bacterial strains from sediment across the Sardar Bherry CW to find candidates with remediation properties and to understand how the physiochemical gradient from wastewater input influences the functional properties of the bacteria present. Bacterial isolates recovered closer to the wastewater effluence were more likely to be pathogenic, with increased haemolytic activity, causing high rates of fish mortality. In contrast, isolates recovered further from the wastewater source were observed to be non-pathogenic and have increased inhibitory effect against pathogenic strains. Extracellular proteins extracted from non-pathogenic isolates also appeared to be effective at inhibiting the growth of pathogenic bacteria, including multidrug resistant strains. Non-pathogenic isolates recovered across all sampling sites displayed the ability to reduce high levels of ammonia in solution during laboratory testing. Antibiogram assays of the recovered isolates showed a relatively high rate of multidrug resistant or marginally resistant bacteria across all sampling sites, highlighting a potential limitation within the CW bioremediation process in mitigating antibiotic resistant strains. This isolate based study provided an avenue to understand the influence of spatial succession from wastewater effluence on bacterial characteristics, as well as obtain candidates that can be further investigated for optimisation in bioremediation efforts. The cultured isolates can supplement future environmental sequencing studies by providing wet lab specimens to compare (meta)genomic information discovered within the CW ecosystem.

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INTRODUCTION

Wastewater pollution is a threat to both people and aquatic life and contributes to the largest percentage of water-based pollution worldwide. Globally, an estimated 80% of wastewater, which includes domestic and industrial effluents, is discharged into the environment without treatment, releasing an array of harmful contaminants and causing direct harm to aquatic ecosystems[1–3]. Most treatment measures are often limited by economic constraints and difficulty in obtaining professional expertise, making them infeasible in developing countries[4]. Constructed wetlands (CW) are organic wastewater treatment systems that mimic the ecological functions of natural wetlands and provide an economical, efficient and sustainable way of treating wastewater[5,6]. Moreover, due to the advantages of low energy requirements, easy operation and maintenance, CW technology has been adopted worldwide as a green technology for environmental wastewater pollution treatment[7].

In CW systems, microorganisms play key roles in pollutant removal, such as the degradation of organic pollutants and the removal of excess nutrients[8]. Moreover, the ability to regulate chemical influx within CWs is found to be strongly linked with microbial diversity and community composition[9]. For instance, Lee et al.[10] reported that bacterial community structure and the specific bacterial consortia within wetlands determine the denitrification potential in CWs. Interestingly, the soil matrix of CWs contains different microbial fauna, viz., aerobic zones are rich in microbial diversity which assists in metal oxidation (bacteria fixes inorganic compounds by reacting only with its organic content) while the latter anaerobic zones are rich in sulfate-reducing bacteria[11,12]. Apart from decomposition and biodegradation, microorganisms in CWs can also remove organic compounds from wastewater through biosorption, bioaccumulation and speciation transformation[13]. In addition, microorganisms can improve the remedial capabilities of CWs to pollutants by enhancing phytoremediation[14,15]. As such, to understand how to further optimise CWs, it is necessary to investigate the microorganisms associated with remedial properties.

To address some of these knowledge gaps, we undertook a study in Sardar Bherry, situated in the East Kolkata floodplain wetland, India, as the model ecosystem to investigate the functionality of a CW receiving wastewater. The East Kolkata wetlands are classified as a site of community importance and included in the list of wetlands of international importance ("Ramsar List"), under the Ramsar Convention, 2002[16,17]. These wetlands are one of the most important engineered ecological systems in India that serve as natural wastewater treatment plants to reuse/treat urban wastewater flowing out daily from Kolkata, India. Hence, our goals were: (1) to determine the spatial succession of the microbiome across wetland from the input site (sewage) to the output site (natural waterways) through the isolation of bacterial specimens; (2) explore the environmental

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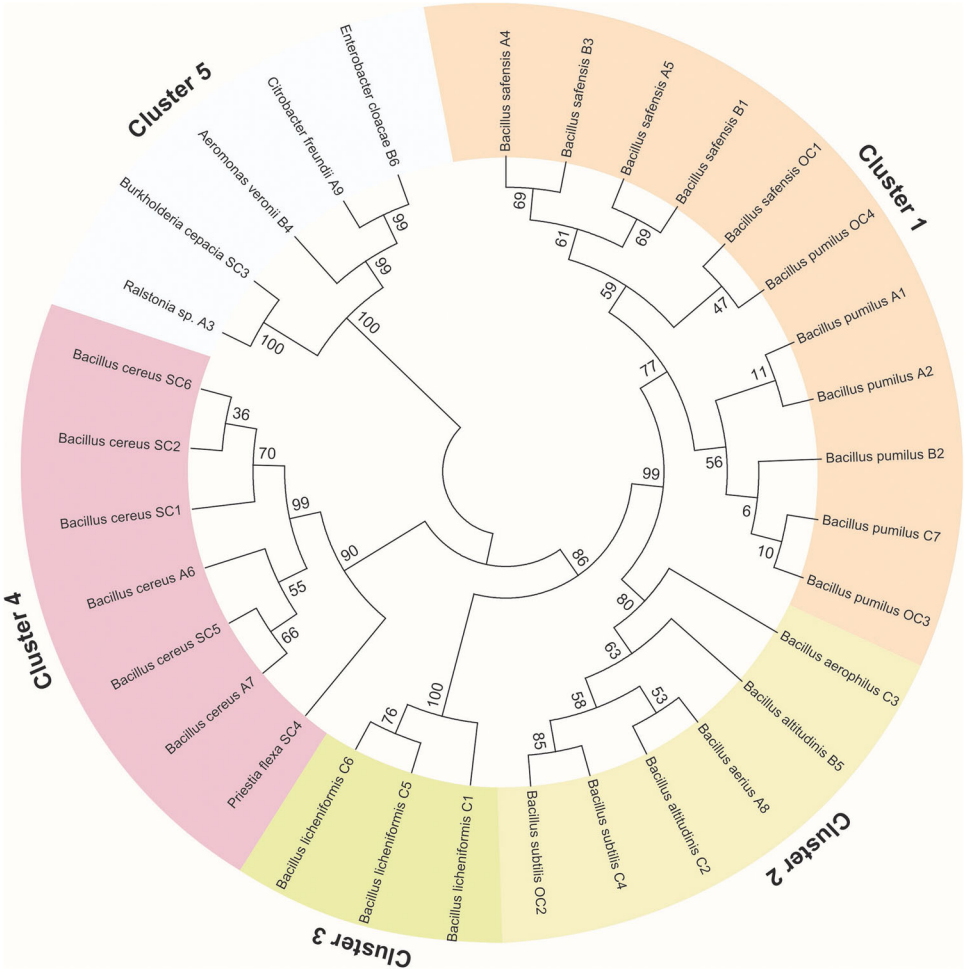


Fig. 1 Isolation and phylogenetic comparison of bacterial isolates. Phylogenetic tree of 32 bacterial isolates based on 16S rRNA nucleotide sequences following maximum composite likelihood method by the MEGA11 software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. Bootstrap values above 50% are shown at the nodes. The isolates were categorised into 5 clusters indicated by colour shading.

factors that drive variations in bacterial biochemical characteristics; (3) investigate the bacterial contributions to the health of the waterways and aquatic life forms within; (4) evaluate the presence of pathogens, and how the constructed wetland non-pathogenic microbes exhibit inhibitory activity against them; and (5) investigation on the presence of multiple antibiotic resistance (MAR) bacteria in the constructed wetland.

We employed a straightforward cultivation procedure along with unique morphology selection to gain further insights into diversity-driven biology. This 'deep-cultivation' approach focused on selectively obtaining pure cultures from all observed unique morphology present within the bacterial isolates. Our approach also investigated the relationship of bacterial isolates with host wellbeing, as well as their involvement within the environmental processes. We unearthed unexpected bacterial traits and demonstrated that there is far more 'out there' in terms of biogeochemical cycling, aquatic animal health and immunity, antimicrobial activity and potential bacterial pathogens.

RESULTS

Cultivation and characterisation of 32 bacterial isolates previously undescribed from constructed wetland system

We based our sampling strategy on the hypothesis that uniqueness in morphology, colour and size of bacterial colony are likely to be representative of a divergent bacterial group. A carefully developed culture strategy was employed to target beneficial bacteria, viz., Bacillaceae, Enterobacteriaceae and Aeromonadaceae inhabiting the sampled sediments of the constructed wetland. Using this method developed for selection, we were able to obtain a total of 32 strains in axenic cultures from the constructed Sardar Bherry wetland (Supplementary Figs. 1 and 2). The phylogenetic diversity of the recovered isolates based on 16S rRNA amplicon sequencing data showed that the isolates were from Bacillaceae, Burkholderiaceae, Enterobacteriaceae and Aeromonadaceae. The bacterial isolates include 26 *Bacillus* species, and one each of *Ralstonia* sp., *Citrobacter freundii*, *Aeromonas veronii*, *Enterobacter cloacae*, *Burkholderia cepacia* and *Priestia flexa*. Most isolates cluster within Bacillaceae, a clade known to confer positive impact on environment and have health benefits. Interestingly, we also found several members of Burkholderiaceae (*Ralstonia* sp. and *Burkholderia cepacia*), Enterobacteriaceae (*Citrobacter freundii* and *Enterobacter cloacae*) and Aeromonadaceae (*Aeromonas veronii*) from our sediment samples of wetland (Fig. 1, Supplementary Fig. 3 and Supplementary Table 1). The 32 bacterial isolates were grouped into five categories based on the origin of sediment samples, *viz*., sewage canal (SC) - 7 isolates, site A - 9 isolates, site B - 6 isolates and site C - 6 isolates and outlet canal (OC)- 4 isolates (Table 1).

Table 1. Sources of isolates with MAR indices of Sardar bherry wetland.

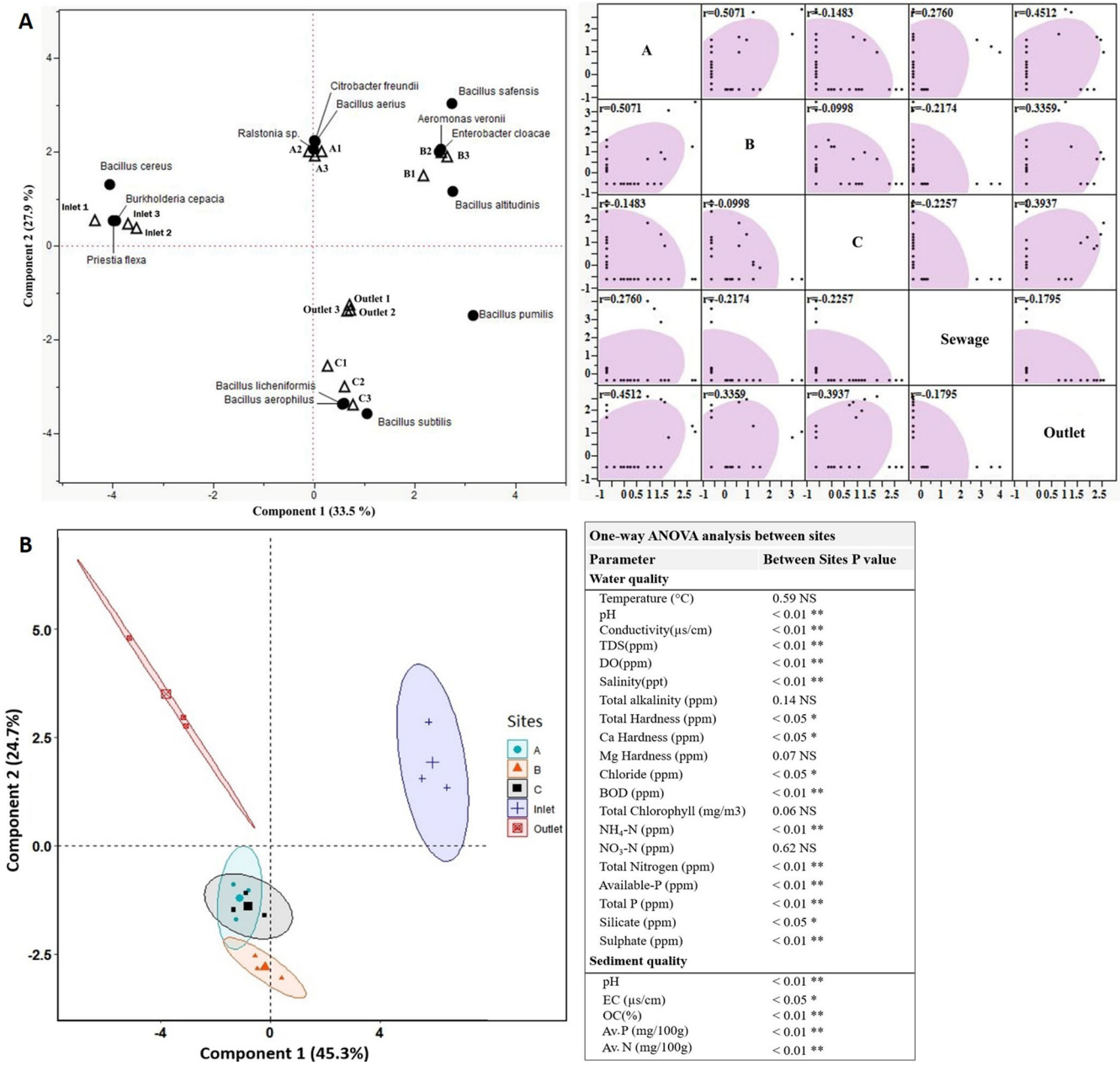
Bacterial species	Accession number	Taxon	Sampling location	Gram staining	Oxygen requirement for growth
Bacillus pumilus A1	OL654366	Bacillaceae	**Station A**	Gram-positive	Aerobic
Bacillus pumilus A2	OL533641	Bacillaceae		Gram-positive	Aerobic
***Ralstonia sp.* A3**	OL630947	Burkholderiaceae		Gram negative	Aerobic
Bacillus safensis A4	OL477593	Bacillaceae		Gram-positive	Aerobic
Bacillus safensis A5	OL477600	Bacillaceae		Gram-positive	Aerobic
***Bacillus cereus* A6**	OL477341	Bacillaceae		Gram-positive	Facultative anaerobic
***Bacillus cereus* A7**	OL477342	Bacillaceae		Gram-positive	Facultative anaerobic
Bacillus aerius A8	OL493030	Bacillaceae		Gram-positive	Facultative anaerobic
Citrobacter freundii A9	OL583978	Enterobacteriaceae		Gram-negative	Facultative anaerobic
Bacillus safensis B1	OK428851	Bacillaceae	**Station B**	Gram-positive	Aerobic
Bacillus pumilus B2	OL533628	Bacillaceae		Gram-positive	Aerobic
Bacillus safensis B3	OL477587	Bacillaceae		Gram-positive	Aerobic
Aeromonas veronii B4	OL519559	Aeromonadaceae		Gram-negative	Facultative anaerobic
Bacillus altitudinis B5	OL519154	Bacillaceae		Gram-positive	Aerobic
Enterobacter cloacae B6	OL519118	Enterobacteriaceae		Gram-negative	Facultative anaerobic
Bacillus licheniformis C1	OK428925	Bacillaceae	**Station C**	Gram-positive	Facultative anaerobic
Bacillus altitudinis C2	OL519130	Bacillaceae		Gram-positive	Aerobic
Bacillus aerophilus C3	OL629462	Bacillaceae		Gram-positive	Aerobic
Bacillus subtilis C4	OL629481	Bacillaceae		Gram-positive	Aerobic
Bacillus licheniformis C5	OL519183	Bacillaceae		Gram-positive	Facultative anaerobic
Bacillus licheniformis C6	OL519186	Bacillaceae		Gram-positive	Facultative anaerobic
Bacillus pumilus C7	OL533630	Bacillaceae		Gram-positive	Aerobic
***Bacillus cereus* SC1**	OL477339	Bacillaceae	**Sewage canal**	Gram-positive	Facultative anaerobic
***Bacillus cereus* SC2**	OL477343	Bacillaceae		Gram-positive	Facultative anaerobic
***Burkholderia cepacia* SC3**	OL504492	Burkholderiaceae		Gram-negative	Aerobic
Priestia flexa SC4	OL484881	Bacillaceae		Gram-positive	Facultative anaerobic
***Bacillus cereus* SC5**	OL477347	Bacillaceae		Gram-positive	Facultative anaerobic
***Bacillus cereus* SC6**	OL477349	Bacillaceae		Gram-positive	Facultative anaerobic
Bacillus safensis OC1	OL477589	Bacillaceae	**Outlet canal**	Gram-positive	Aerobic
Bacillus subtilis OC2	OL629484	Bacillaceae		Gram-positive	Aerobic
Bacillus pumilus OC3	OL654365	Bacillaceae		Gram-positive	Aerobic
Bacillus pumilus OC4	OL477638	Bacillaceae		Gram-positive	Aerobic

Bold entries indicate pathogenic isolates.

Environmental parameters define the distribution of bacterial species in sediments across constructed wetland

The PCA biplot with an ~60% variability in bacterial abundance was observed from the first two components (PC1, 33.5% and PC2, 27.9 %; Fig. 2A). The study also found that samples from wastewater inlet site (SC and site A) were observed to be strongly influenced by bacterial species *viz. Bacillus* sp., *Priestia flexa, Ralstonia* sp., *Citrobacter freundii* and *Burkholderia cepacia* belonging to Bacillaceae, Enterobacteriaceae and Burkholderiaceae taxon. Moreover, in sampling site B, C and OC *Bacillus* sp., was found to be more prominent along with *Enterobacter cloacae* and *Aeromonas veronii*. The Scatterplot matrix revealed that there was a positive correlation between site A and B ($r = 0.51$) with respect to bacterial abundance. We noted that bacterial abundance and diversity pattern of inlet site (SC) is positively correlated with site A but inversely related with B, C and outlet sampling site (OC), which indicates that the growth of certain groups of bacteria are gradually reduced spatially from the SC to OC sampling sites. The study also investigated possible correlations among sampling sites based on water and sediment quality parameters. The PCA plot revealed that the water and sediment quality of inlet (SC) and outlet (OC) were quite different and largely distanced from each other (Fig. 2B), whereas sampling sites A, B and C form overlapping clusters indicating some similarity in nature. The associated ANOVA results, however, show that physicochemical parameters are significantly different between the sampling sites, except temperature, total alkalinity, Mg, chlorophyll and NO_3^--N.

Canonical Correspondence Analysis (CCA) is generally preferred to relate the abundance of bacteria to environmental variables, with a CCA plot prepared comparing bacteria isolates with water and sediment quality present in the sampling wetland (Fig. 3A, B). The results revealed that most bacteria found in inlet SC site favours water quality high in TDS, conductivity, NH_4, Ca, phosphorus, sulfate and BOD, but low in pH and chlorophyll. Bacterial isolates mainly found in Site A appear to prefer high DO and low nitrogen levels in water, while bacteria in site B showed better growth in high concentrations of nitrogen with low DO levels. In comparison, OC site bacteria appear not to be strongly influenced by any water quality parameters. In the case of sediment parameters, both SC and OC site bacterial isolates were observed not to be influenced by the sediment quality parameters. In contrast, bacterial isolates from site B and C,



One-way ANOVA analysis between sites	
Parameter	**Between Sites P value**
Water quality	
Temperature (°C)	0.59 NS
pH	< 0.01 **
Conductivity(µs/cm)	< 0.01 **
TDS(ppm)	< 0.01 **
DO(ppm)	< 0.01 **
Salinity(ppt)	< 0.01 **
Total alkalinity (ppm)	0.14 NS
Total Hardness (ppm)	< 0.05 *
Ca Hardness (ppm)	< 0.05 *
Mg Hardness (ppm)	0.07 NS
Chloride (ppm)	< 0.05 *
BOD (ppm)	< 0.01 **
Total Chlorophyll (mg/m3)	0.06 NS
NH_4-N (ppm)	< 0.01 **
NO_3-N (ppm)	0.62 NS
Total Nitrogen (ppm)	< 0.01 **
Available-P (ppm)	< 0.01 **
Total P (ppm)	< 0.01 **
Silicate (ppm)	< 0.05 *
Sulphate (ppm)	< 0.01 **
Sediment quality	
pH	< 0.01 **
EC (µs/cm)	< 0.05 *
OC(%)	< 0.01 **
Av.P (mg/100g)	< 0.01 **
Av. N (mg/100g)	< 0.01 **

Fig. 2 Principal component analysis (PCA) for bacterial abundance. A PCA biplot and scatterplot matrix of the bacterial communities found at different sampling sites in Sardar Bherry. **B** PCA plot showing the differences among sampling sites based on water and sediment quality parameters. One-way ANOVA analysis between the sampling sites. [NS denotes non-significant, *<0.05 and **<0.01].

appear to have preferences for high of pH in sediment, with site A bacteria favoring sediment rich in N and P.

Constructed wetland treatment system modulates the phenotype of microbiome

The bacterial strains isolated from the sediment samples were mostly identified as Gram-positive bacteria. Additionally, the strains were observed to contain unique irregular, filamentous and rhizoid morphology on TSA plates. The biochemical test showed that bacterial isolates from sampling site A-C and OC were mostly positive for ONPG, lysine, ornithine, urease, citrate, Malonate utilisation, Esculin hydrolysis, trehalose, glucose and oxidase activity, whereas the bacterial isolates from SC sampling sites were mostly positive for urease, citrate, esculin hydrolysis, trehalose and glucose activity. Moreover, the 32 isolated bacterial strains displayed negative results for Phenylalanine Deamination, H_2S, Voges Proskauer's, Methyl red, indole, Adonitol, Rhamnose, Melibiose, Raffinose and lactose activity (Supplementary Table 2). These results show high similarities with previously reported biochemical characteristics of *Bacillus* spp., *Ralstonia* spp., *Citrobacter freundii*, *Aeromonas veronii*, *Enterobacter cloacae*, *Burkholderia cepacia* and *Priestia flexa*.

Our findings of the antibiotic susceptibility of bacterial strains isolated from SC to OC site showed that 83.33% of the bacterial isolates from Inlet of wetland (SC) had MAR index of ≥0.2 followed by site A (55.56%), site B (50%), site C (42.86%), with none in the outflow (OC) site ≥0.2 (Supplementary Table 3 and Supplementary Fig. 4). The isolates from OC site of the Sardar Bherry have the lowest range of MAR index and ranged from 0.11 to 0.19 (Table 2). The MAR index results suggest that

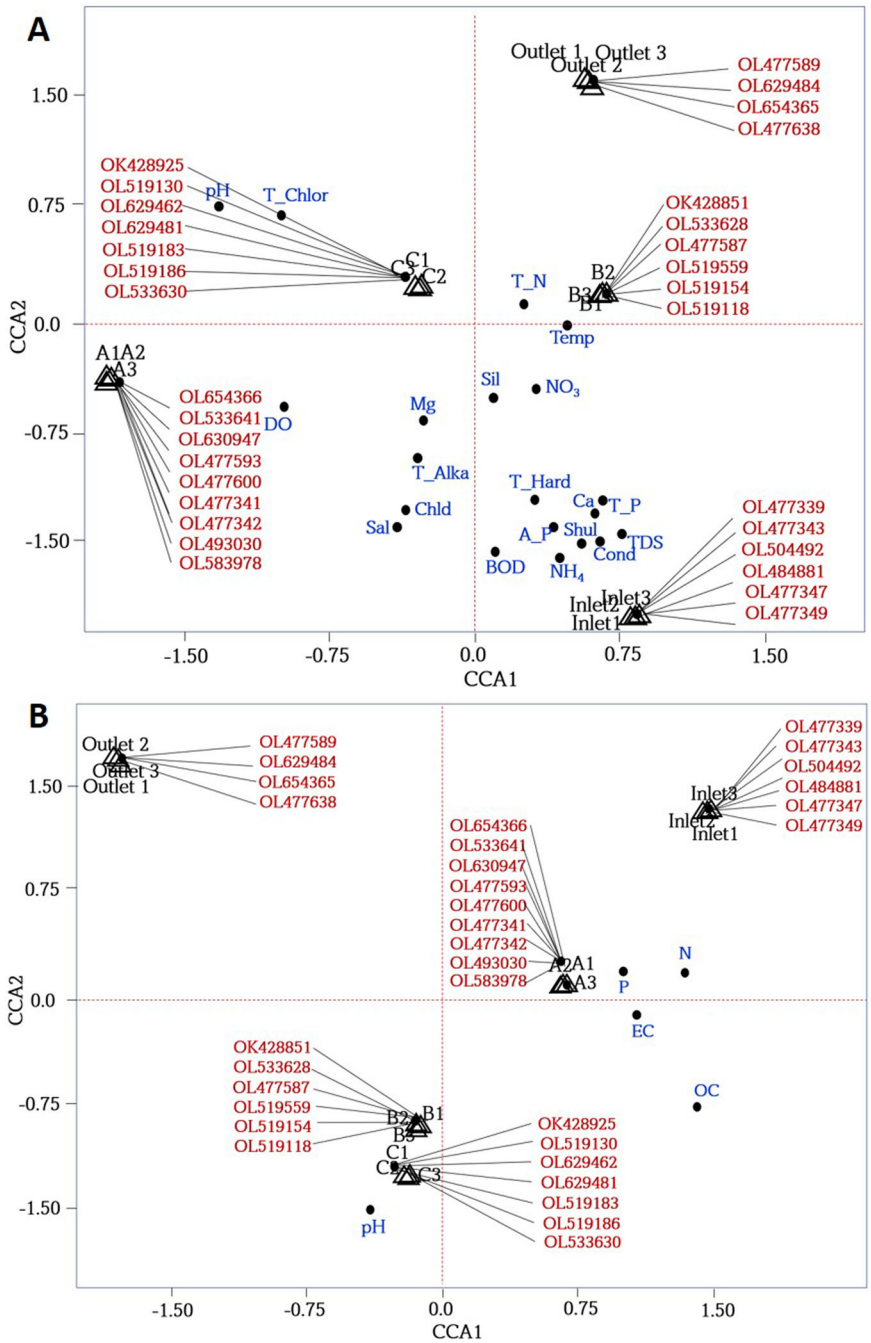


Fig. 3 Canonical correlation analysis (CCA). A Bacterial isolates, water quality parameters and sampling sites; and **B** bacterial isolates, sediment quality parameters and sampling sites.

multidrug resistant bacteria were able to propagate far from the wastewater inlet, with some OC site isolates showing borderline multidrug resistance.

Spatial succession regulates the pathogenicity of bacterial isolates

The study discovered that bacterial isolates including *Bacillus cereus* (2 strains from A and 4 from SC site), *Ralstonia* sp. (1 strain from site A) and *Burkholderia cepacia* (1 strain from site SC) induced significantly high mortality in fish fingerlings (*Labeo rohita*) (Fig. 4 and Table 3). The bacterial strains isolated from SC sampling site have the highest number of pathogenic isolates followed by sampling site A. In contrast, no mortality was observed across 24–120 h time point in *L. rohita* treatment group challenged with bacterial strains isolated from sampling sites B, C and OC. In parallel with the survival assay, results showed that isolates from SC and sampling site A exhibited significantly higher haemolytic activity (Fig. 5A), whereas the sampling site B, C and OC isolates had no effect on in blood agar (Fig. 5B).

Non-pathogenic bacterial isolates exhibit antimicrobial activity

We then sought to determine the antimicrobial activity of the recovered non-pathogenic isolates including 20 *Bacillus* species, *Citrobacter freundii* A9, *Aeromonas veronii* B4, *Enterobacter cloacea* B6 and *Pristia flexa* SC4 against virulent bacteria. Non-pathogenic

Table 2. Isolation and phylogenetic comparison of bacterial isolates.

Sampling sites	Isolates	MAR value
A	*Bacillus pumilus* (A1)	0.30
	Bacillus pumilus (A2)	0.11
	Ralstonia sp. (A3)	0.42
	Bacillus safensis (A4)	0.11
	Bacillus safensis (A5)	0.11
	Bacillus cereus (A6)	0.42
	Bacillus cereus (A7)	0.38
	Bacillus aerius (A8)	0.23
	Citrobacter freundii (A9)	0.19
B	*Bacillus safensis* (B1)	0.11
	Bacillus pumilus (B2)	0.23
	Bacillus safensis (B3)	0.11
	Aeromonas veronii (B4)	0.26
	Bacillus altitudinis (B5)	0.26
	Enterobacter cloacae (B6)	0.19
C	*Bacillus licheniformis* (C1)	0.46
	Bacillus altitudinis (C2)	0.15
	Bacillus aerophilus (C3)	0.19
	Bacillus subtilis (C4)	0.11
	Bacillus licheniformis (C5)	0.23
	Bacillus licheniformis (C6)	0.30
	Bacillus pumilus (C7)	0.15
SC	*Bacillus cereus* (SC1)	0.35
	Bacillus cereus (SC2)	0.35
	Burkholderia cepacia (SC3)	0.53
	Priestia flexa (SC4)	0.15
	Bacillus cereus (SC5)	0.38
	Bacillus cereus (SC6)	0.38
OC	*Bacillus safensis* (OC1)	0.11
	Bacillus subtilis (OC2)	0.11
	Bacillus pumilus (OC3)	0.19
	Bacillus pumilus (OC4)	0.19

Bacterial isolates from sediment samples of Sardar bherry wetland.

Bacillus species isolated from sampling site A-C and OC mostly exhibited antimicrobial activity against virulent *Ralstonia* sp. A3, *Bacillus cereus* A6, *B. cereus* A7, *B. cereus* SC1, *B. cereus* SC2, *Burkholderia cepacian* SC3, *B. cereus* SC5, *B. cereus* SC6 and against two model fish pathogens, *viz*., *Vibrio parhaemolyticus* and *Pesudomonas aeruginosa*. Moreover, *Bacillus subtilis* C4, *B. safensis* OC1 and *B. subtilis* OC2 displayed highest inhibition diameter zones against pathogenic tested bacteria. We also discovered that non-Bacillaceae group isolates recovered in this study including *A. veronii* B4, *E. cloacea* B6 and *P. flexa* SC4 also exhibited antimicrobial activity (Fig. 6A, Supplementary Table 4 and Supplementary Fig. 5), with *C. freundii* A9 displaying a comparatively better inhibitory effect, similar to our observation of *Bacillus* species against pathogenic strains.

Bacterial ECPs were tested against the growth of pathogenic strains to determine their potential antibacterial activity. ECPs produced from four non-pathogenic bacterial isolates that showed inhibitory activity against 8 or more pathogenic strains were selected for antibacterial assay. The results demonstrated that among the four tested isolates, ECPs extracted from *Citrobacter freundii* A9 showed the best inhibitory effect, followed by *Bacillus subtilis* OC2, *B. subtilis* C4 and *B. safensis* OC1. The ECPs

Fig. 4 Survival assay of *Labeo rohita* challenged with bacterial strains isolated from sediment samples of wetland. The experimental fish were injected with 0.2 ml @ 10^6 CFU/ml of bacterial suspension. The control group of fish injected with 0.2 ml of sterile saline solution displayed no clinical signs or mortality throughout the experiment. The strains inducing significantly high mortality post injection exhibited clinical signs like haemorrhagic patches on the body surfaces.

obtained from the isolates also exhibited strong inhibitory effect against multi-drug resistant fish pathogens *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* (Supplementary Table 5). SDS-Page analysis showed that *C. freundii* cells secrete proteins in the 20–100 kDa range, while *B. subtilis* OC2, *B. subtilis* C4 and *B. safensis* OC1 cells secrete prominent protein from ~50–100 kDa, along with other proteins that are differentially secreted by both cell types (Fig. 6B).

Capability of bacterial isolates to remediate high ammonia levels

Among 24 non-pathogenic isolates, 1 strain from SC (*Priestia flexa* SC4), 4 strains from site A (*Bacillus safensis* A4, *B. safensis* A5, *B. aerius* A8, *Citrobacter freundii* A9), 3 strains from site B (*B. safensis* B3, *Aeromonas veronii* B4, *Enterobacter cloacae* B6), 4 strains from site C (*B. aerophilus* C3, *B. subtilis* C4, *B. licheniformis* C6, *B. pumilus* C7) and 4 strains from OC site (*B. safensis* OC1, *B. subtilis* OC2, *B. pumilus* OC3, *B. pumilus* OC4) exhibited ammonia-oxidising activity (Fig. 7). In most cases, the NH_4^+ -N concentration was reduced by >90%, from 26 mg/L to ~1 mg/L, within 24 h of growth. The

Table 3. Survival assay of *Labeo rohita* challenged with bacterial strains isolated from sediment samples of wetland.

Sampling sites	Bacterial isolates	Survival % (mean ± S.E.)				
		24 h	48 h	72 h	96 h	120 h
A	*Bacillus pumilus* A1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus pumilus A2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	***Ralstonia sp.* A3**	**28.3 ± 3.3**	**21.2 ± 2.0**	**20 ± 2.9**	**0 ± 0**	**0 ± 0**
	Bacillus safensis A4	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus safensis A5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	***Bacillus cereus* A6**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**
	***Bacillus cereus* A7**	**10 ± 1.2**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**
	Bacillus aerius A8	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Citrobacter freundii A9	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
B	*Bacillus safensis* B1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus pumilus B2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus safensis B3	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Aeromonas veronii B4	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus altitudinis B5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Enterobacter cloacae B6	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
C	*Bacillus licheniformis* C1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus altitudinis C2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus aerophilus C3	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus subtilis C4	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus licheniformis C5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus licheniformis C6	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus pumilus C7	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
SC	***Bacillus cereus* SC1**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**
	***Bacillus cereus* SC2**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**
	***Burkholderia cepacia* SC3**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**
	Priestia flexa SC4	100	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	***Bacillus cereus* SC5**	**66.6 ± 3.3**	**60 ± 2.8**	**20 ± 1.2**	**18 ± 1.2**	**0 ± 0**
	***Bacillus cereus* SC6**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**	**0 ± 0**
OC	*Bacillus safensis* OC1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus subtilis OC2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus pumilus OC3	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	Bacillus pumilus OC4	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

The experimental fish were injected with 0.2 ml @ 10^6 CFU/ml of bacterial suspension. The control group of fish injected with 0.2 ml of sterile saline solution displayed no clinical signs or mortality throughout the experiment.
Bold entries indicate pathogenic isolates.

Nitrification products, NO_2^- -N and NO_3^- -N were detected during the removal process within the 72 h of incubation. In contrast, 8 bacterial isolates including *B. pumilus* A1, *B. pumilus* A2 (site A), *B. safensis* B1, *B. pumilus* B2, *B. altitudinis* B5 (site B), *B. licheniformis* C1, *B. altitudinis* C2 and *B. licheniformis* C5 (site C) displayed ammonia producing activity. The ammonia concentration increased ~2 folds in the bacteria supplemented group as compared to the control.

DISCUSSION

Constructed wetlands offer an economic, self-maintained and cost-effective alternative for the conventional treatment of different types of wastewaters. Therefore, an in-depth analysis of the community structure and diversity of microorganisms within CWs, especially for beneficial candidates, is important to understand its performance patterns and explore optimised strategies. The Sardar Bherry, a constructed wetland that receives large volumes of wastewater, presented a model ecosystem to analyse bacterial biodiversity, wastewater nutrient supply, and potential ecological interactions. Based on a straightforward deep-cultivation procedure coupled with unique morphology selection methods and 16S rRNA gene amplification and phylogenetic tree analysis, we obtained 32 pure cultures of bacterial isolates from sediment samples of wetland, viz., sewage canal (SC) - 7 isolates, site A - 9 isolates, site B - 6 isolates, site C - 6 isolates and outlet canal (OC)- 4 isolates. Our results from the recovered isolates indicate that wastewater nutrient supply, physicochemical parameters and ecological interactions are potential drivers of greater bacterial community structure in the sediments of the Sardar Bherry wetland ecosystem.

Nutrient-rich environments favours bacterial growth that can potentially cause an intensive modification of the environment through the release of harmful metabolites that inhibit the growth of competitors in the community[18]. The "selfishness" of the winning species impedes species co-existence and thus reduces the levels of biodiversity. Active growth may also lead to competition for essential nutrients, counterbalancing the biotic

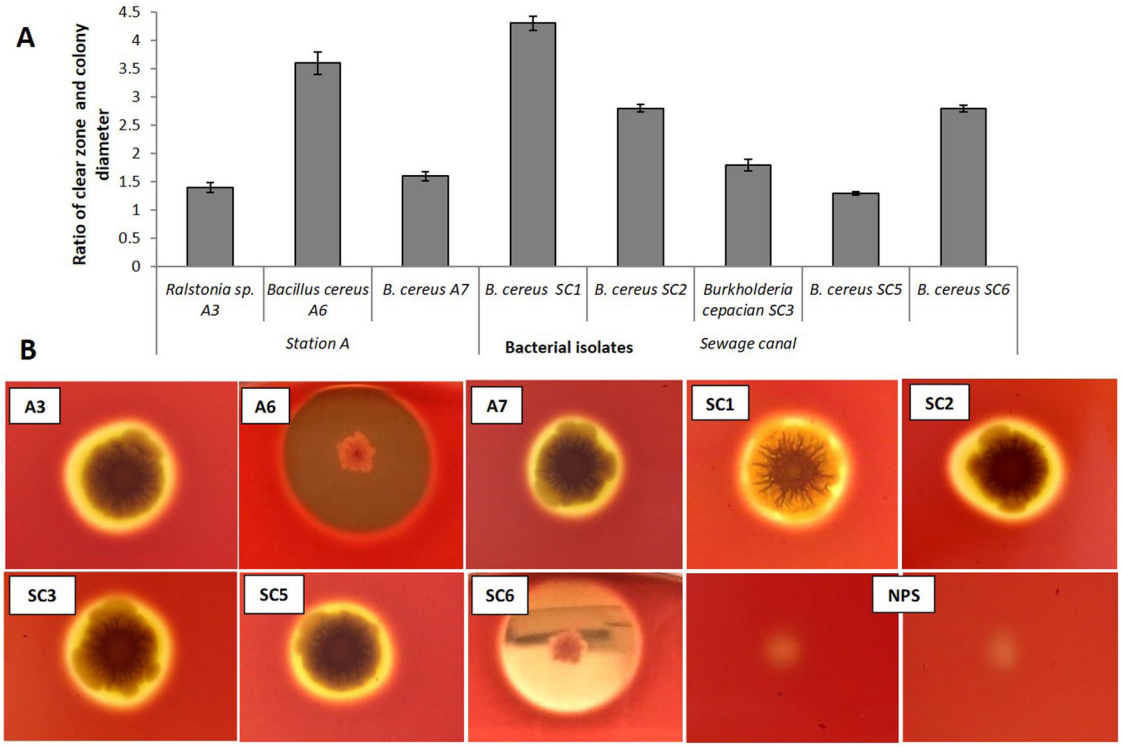


Fig. 5 Haemolysin assay of bacterial species isolated from sediment samples. Among the 32 isolated bacterial species, 8 isolates exhibited haemolytic activity on Tryptone soya broth (TSB) supplemented with 5% defibrinated sheep blood. **A** Ratio of clear zone and colony diameter was calculated from bacterial strains displayed haemolytic activity (results are expressed as mean ± SE). **B** 8 bacterial isolates (A3, A6, A7, SC1-3, SC5, and SC6) exhibiting haemolytic activity; Non-pathogenic strains (NPS): example of remaining strains which had no activity on TSB plates supplemented with sheep blood.

harshness produced by strong competitors[19,20]. Additionally, the water and sediment quality parameters have a strong influence on the microbial community structures and their efficiency to remove contaminants[21]; hence, assessing the relationship of microbial ecology and environmental parameters would possibly lead to identify biomarkers responsible for succession of bacterial communities in CWs. Here we observed that water and sediments in a nutrient rich environment (sewage canal) due to wastewater with high TDS, conductivity, NH_4, Ca, phosphorus, sulfate and BOD, but low in pH and chlorophyll, contains lower diversity with higher abundances of *Bacillus cereus*, *Priestia flexa* and *Burkholderia cepacia*.

The effect of nutrient selection appeared to be reduced with the effect dilution in the constructed wetland. The higher values of organic carbon, pH, DO and chlorophyll support the growth of wider bacterial populations likely leading to the increase in community diversity observed in site A, B, C and outlet canal of the wetland[22]. Similar observations were found by Dai et al.[19] who proposed a hunger game hypothesis and reported that along with nutrient availability, biotic and abiotic factors affect the natural microbial communities and their co-existence in aquatic environment. Lew et al.[23] and Zheng et al.[24] demonstrated that physicochemical parameters of wetlands, in general, chemical oxygen demand, organic carbon, nitrogen and phosphorus levels have distinct effects on bacterial community structure and interactions. As such, the nutrient supply from wastewater likely pushes the wheel of community succession through the course of the CW in this study. The effect of nutrient availability and physicochemical parameters on community stability is uncertain, however, as lower biodiversity often reduces stability, but the higher negative network association by nutrient amendment observed in microbial communities might instead enhance stability[25,26].

Biochemical reactions can reveal the vital information necessary for accurately identifying the various bacteria genera within a sample. By their nature, bacteria produce large volumes of enzymes, and it is these enzymes that allow for their identification via biochemical methods[27,28]. Hence, the type of enzymes produced by a bacterium can usually be used to classify its species given that bacteria have distinct enzymatic profiles. The recovered isolates from sites A-C and OC were mostly positive for ONPG, lysine, ornithine, urease, citrate, Malonate utilisation, Esculin hydrolysis, trehalose, glucose and oxidase activity; whereas, the bacterial isolates from SC sampling sites were mostly positive for urease, citrate, esculin hydrolysis, trehalose and glucose activity. Zhao et al. highlighted the importance of biochemical characterisation in bacterial identification. In the study, a total of 36 bacterial isolates recovered from sewerage samples collected at Nanchang City, Jiangxi Province, China, were identified through specific enzymatic activity[29]. Syed et al.[30] demonstrated that a newly isolated strain can be identified as *Enterobacter* sp. based on variable biochemical reactions. Our results suggest that the bacteria present in Sardar Bherry are efficient at utilising various substrates from wastewater for energy and growth, however, the substrate of utilisation varies from inlet to outlet canal of CW and is reflected in the differentiation of species recovered from successive locations.

Aquatic environments are key niches for the emergence, evolution and dissemination of antimicrobial resistance[31]. There is evidence that suggests that urban wastewater can contain various chemicals with antimicrobial properties, and these incidental antimicrobial substances might alter the phenotype of the microbes, potentially causing them to develop resistance to chemotherapy[32–34]. Growing resistance to antibiotics in bacteria has been documented for several decades[35,36], with wastewater sites recently being considered as one of the main hotspots for

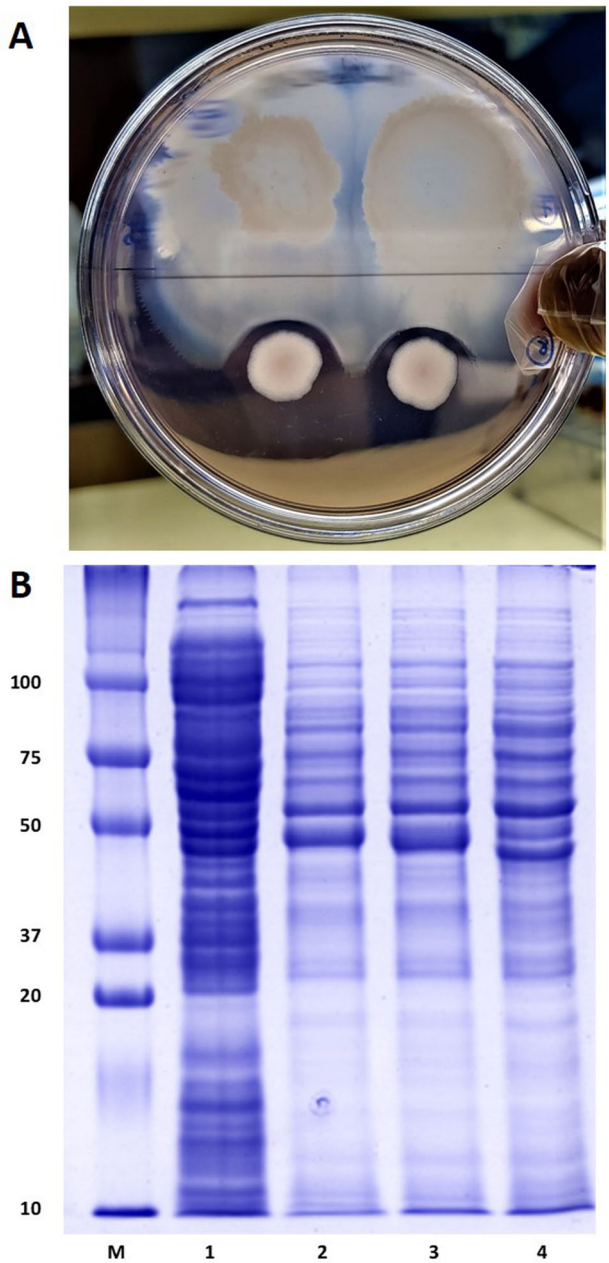


Fig. 6 Characterisation of antibacterial assay of non-pathogenic bacterial isolates. A Antibacterial assay of non-pathogenic bacteria isolates and extracellular proteins (ECPs) against pathogenic bacteria. The non-pathogenic bacteria suspension/ ECP were cultured on TSA plates. After 24–48 h incubation at 37 °C, the diameters of inhibition halos surrounding the non-pathogenic bacteria suspension/ ECP were measured and expressed in millimetres. **B** SDS-PAGE analysis of extracellular proteins (ECPs) concentrated from bacterial cell free supernatant filtered with a 3 kDa cut-off size amicon filter. Molecular mass standards (M) in kilodaltons (Protein ladder) are shown on the left. Lane 1 - *Citrobacter freundii* A9 ECP3, Lane 2 - *Bacillus subtilis* C4 ECP3, Lane 3 - *B. subtilis* OC2 ECP3 and Lane 4 - *B. safensis* OC1 ECP3.

the spread of antibiotic resistance. The main reasons suggested for this occurrence are that wastewaters have been observed to contain high prevalence of subclinical levels of antibiotics, heavy metal ions, and other bactericidal factors present in low concentrations that potentially increase the selection of resistant strains in this environment[37,38].

The exposure of bacteria to environmental chemical and/or pollutants may promote diversity of resistance genes in various forms (resistant bacteria able to conjugation, free plasmids/DNA, and phage particles), enabling a high probability of gene transfer within the bacterial community[39,40]. The antibiotic resistance assay has shown the role of wastewater systems in the proliferation of antibiotic resistance in the constructed wetland that receives urban wastewater. Our study found high levels of environmental antibiotic resistance indicators within the bacteria that thrive in the wastewater system, with multi-resistance patterns to antibiotics being common among the isolates. The significantly high MAR indices indicate that the sediments of Sardar Bherry, especially at SC site, potentially harbours multidrug resistant bacteria. There are several reports which indicate that wastewater effluents has significant effect on development of antimicrobial-resistant bacteria[41–43]. For instance, Gessew et al.[44] reported that river site receiving wastewaters have high abundance of multiple drug resistant bacteria (28%), mainly *Providencia alcalifaciens*, *Kluyvera cryocrescens* and *Citrobacter freundii*. The possibility of antibiotic resistance spread from wastewater to CWs is also a major threat that impacts the food chain, with potential downstream ramifications during human consumption[45]. These observations suggest that CW systems are not able to effectively filter out multiple drug resistant bacteria, and the treatment of wastewater for chemical pollutants prior to discharge into CWs may be required to prevent the propagation of resistant strains carrying into the natural waterways.

The abundance profiles are subject to various interspecies interactions that affect individual organism growth and loss rates[46,47]. Bacterial communities constitute "social networks" in which the members interact with each other in various ways, including competition for nutrients, cooperation by cross-feeding, communication via secretion, and detection of extracellular substances[48]. In addition, organisms may also indirectly affect other community members by modifying their environment, termed "niche construction theory"[49]. For example, excretion of secondary metabolites by actively growing species could change environmental conditions, influencing the growth of other organisms and shifting relative abundance levels[50,51]. Theoretically, biotic factors (e.g., bacterial strain and ecological interactions) and abiotic factors (e.g., nutrient supplies) intertwine to affect individual species abundance, which determines community composition.

To better understand how ecological factors affect interspecies interaction, we investigated how proximity to wastewater effluence correlated with bacterial pathogenicity in fish species. Haemolysin activity is a target virulence factor that is used to determine the pathogenicity of bacterial specimens and was the criteria used to classify potentially pathogenic strains[52,53]. We observed that CW areas receiving nutrient rich wastewater, SC and Site A, both contained a higher abundance of pathogenic microbes, resulting in 100% mortality in freshwater model fish species (*Labeo rohita*), as well as higher haemolytic activity. Urban wastewater in other regions has also been reported to carry potentially pathogenic microbes, which would be detrimental to aquatic animal health and the ecology of the environment if the wastewater effluence were directed into natural waterways without treatment[54,55]. Moreover, the two sites closest to the wastewater effluence (SC and Site A) had a lower abundance of recovered bacterial isolates that were non-pathogenic with antimicrobial activity against tested pathogenic bacterial strains. This suggests that the ecological conditions at the sites that receive the most wastewater tend to favour the proliferation of pathogenic bacteria, as well as suppress the growth of beneficial/ remedial bacteria.

The spatial succession of Sardar Bherry demonstrated the potential remedial properties of a CW ecosystem toward the influx of pathogenic strains. There appeared to be a higher abundance

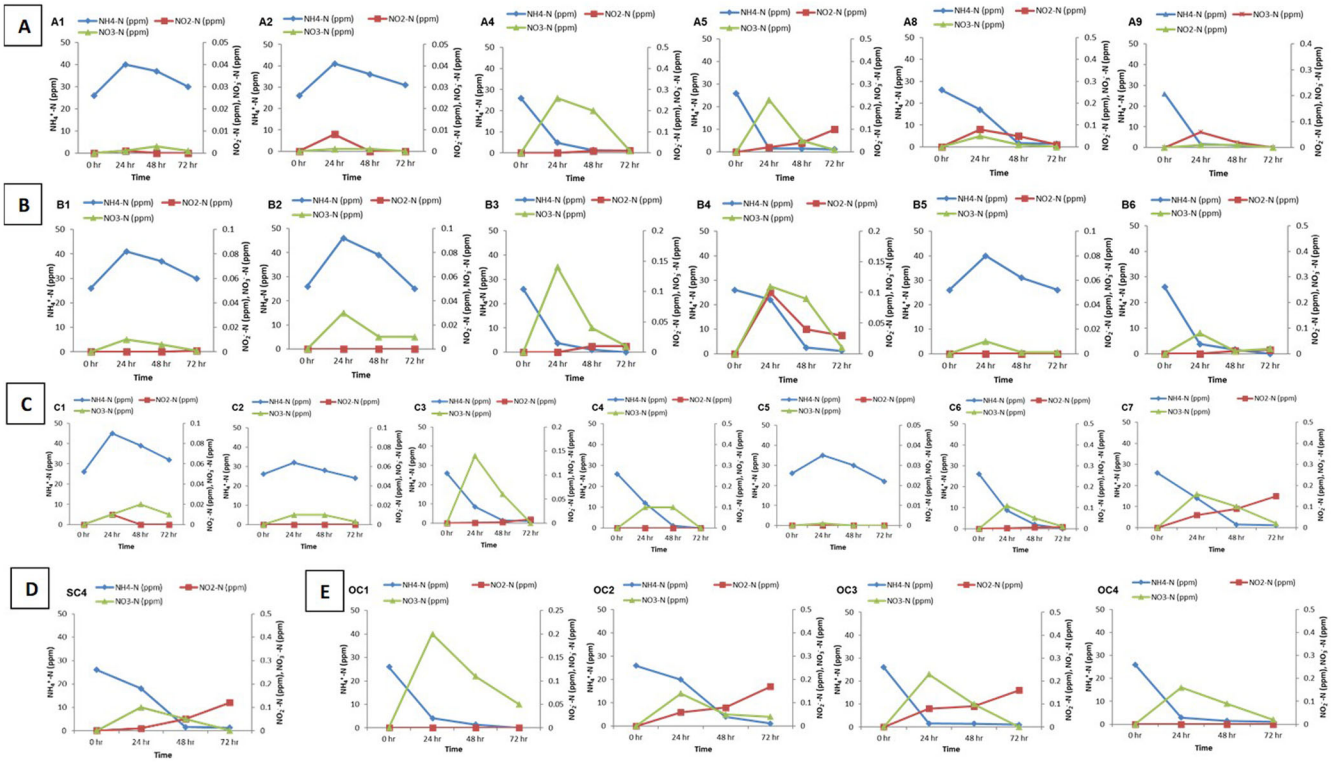


Fig. 7 Ammonia removal assay of bacterial isolates recovered from Sardar bherry wetland. Cultivation of non-pathogenic isolates at C/N mass ratio 10 and initial concentration of NH^{4+} -N of 26 ppm. The suspension was incubated at 37 °C under constant agitation (120 rpm) and samples were collected at 24, 48, and 72 h post bacterial addition for ammonia, nitrate, and nitrite estimation. The production of each intermediate nitrification product, NO_2^- -N and NO_3^- -N were shown for each non-pathogenic strain isolated from 5 sampling sites A (**A**), B (**B**), C (**C**), SC (**D**), and OC (**E**). All the assays were performed with freshly prepared media in three replicates.

of non-pathogenic bacteria, with comparatively better inhibitory effect against pathogenic microbes in areas further (Sites B, C and OC) from the location of the wastewater effluence. The wetland sediments with low effluent load contained more bacterial isolates (e.g., *Bacillus* sp.) showing inhibitory activity against pathogenic microbes (*Corynebacterium fimi* and *Listeria* sp.)[56,57]. Hence, the disparate interaction of bacterial strains in either nutrient rich or scarce sediment samples give rise to predominant bacterial communities, which are likely due to secretion of secondary metabolites, forming an equilibrium of stable co-existence and niche partitioning of dominant species arising from competitive exclusion. By conducting this spatial succession study, the study highlights those biotic factors and nutrient availability associated with proximity to wastewater effluence is what likely shapes the bacterial species composition and abundance, whilst demonstrating the filtering process performed by the endemic microbial communities on effluent-introduced pathogenic strains across the CW ecosystem.

Recovery of non-pathogenic bacterial isolates with inhibitory effects on the growth of pathogen strains from Sardar Bherry demonstrates the potential of CW ecosystems as a target for the discovery of new anti-bacterial agents. The antibacterial properties of the non-pathogenic isolates and their ECPs provide a potential source of probiotics or bacteriocins that can be investigated for use in aquaculture (Pereira et al.[69]). The ECPs tested in this study were able to inhibit the growth of multidrug resistant pathogenic bacteria, making them prime research targets as potential bacteriocins for further development as anti-bacterial agents (Benítez-Chao et al.[70]; Simons et al.[71]). Ecosystem structure and microbiome diversity have been reported to be key drivers in the production of ECPs (Garcia-Garcera & Rocha[72]). The observation of inhibitory ECPs at Sardar Bherry suggests that CWs or other similar sites that receive regular influxes of multidrug resistant bacteria from wastewater (Gessew et al.[44]) could foster the potential development of ECPs effective against resistant strains due to inter-species interactions with the endemic microbial population. Further investigation is needed to ascertain the therapeutical potential of CW microorganisms for applications in healthcare and aquaculture.

The discovery of isolates that can reduce ammonia levels substantially in solution and the consistent pattern of ammonia oxidising bacteria across the Sardar Bherry CW provides an indicator of the ecosystem's capability to effectively remediate ammonia. Wastewater has been shown to accelerate the build-up of ammonia and other nitrogenous compounds in the aquatic ecosystem, which causes serious ecological problems such as toxicity in fish, as well as in other aquatic animals[58,59]. Ammonia in particular can pose issues for the health of fish, increasing physiological stress, resulting in tissue damage and even death (Soler et al.[73]). Along with the pathogenicity study, this indicates that a better understanding of non-pathogenic CW microorganisms is important for elucidating the key taxa involved in preserving the health of aquatic lifeforms.

The recovery of non-pathogenic bacterial isolates with high ammonia removal capabilities provides a potential source of bioremediating candidates for use in water treatment studies. Ammonia is a common pollutant in wastewater and biological approaches using living organisms have been explored as an inexpensive and efficient form of water treatment (Karri et al.[74]; Royan et al.[75]). Aside from aquatic health, the ammonia removal capabilities of CW isolates could be utilised in green biotechnology solutions, such as combining the CW with a microbial fuel cell system for the generation of bioelectricity (Liu et al.[76]). The Sardar Bherry demonstrates that CW microbiomes are not just efficient treatment systems, but also present a potential bioprospecting trove for microorganisms for use in sustainable solutions.

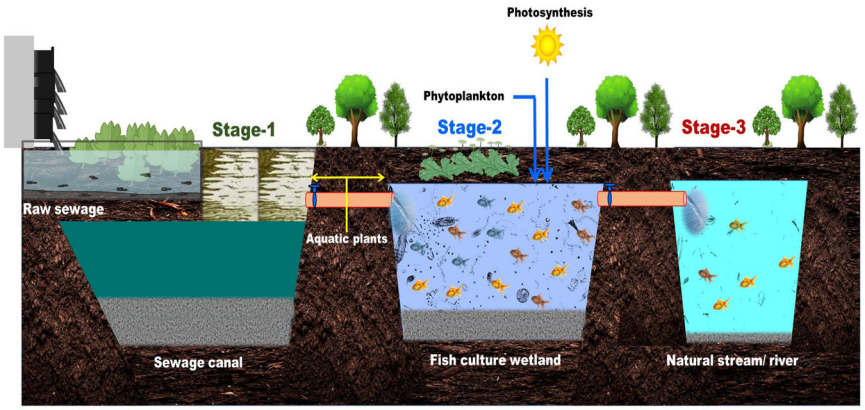


Fig. 8 Pictorial representation of wastewater treatment by constructed wetlands in East Kolkata, West Bengal, India. The raw sewage water from different sewage canals (stage 1) enters wetlands (stage 2) utilised for aquaculture activities and then goes into natural streams/ rivers (stage 3). The wetlands in stage 2 are purposed as natural treatment systems of urban wastewater. In the study, Sardar Bherry was selected as the model system to investigate the role of microbiome-based bio-treatment and reuse systems of urban wastewater in constructed wetland treatment systems.

This study provided insights into the ecological roles of bacteria within the Sardar Bherry CW sediment microbiome via the diversity-driven cultivation and functional characterisation of 32 bacterial strains. By summarising the main functional microorganisms in the CW, we found that the family Bacillaceae is the dominant one, containing microorganisms with a wide range of functions. In addition, the family Enterobacteriaceae and Aeromonadaceae are also frequently detected in CW. Regarding the effects of different pollutants on microbial diversity, we found that different microorganisms respond in different ways. The CW inlets containing high nitrogen and phosphorus levels, microorganisms with high pathogenic potential become dominant in the system. However, once the wastewater enters the CW, there was high abundance of genera of functional microorganisms, which can remove pollutants from CW and have health benefits to aquatic life. While nutrient availability appears to have fundamental coupling with bacterial community diversity, interaction between bacterial isolates potentially contributes, in part, to the assembly of distinct microbial communities in the constructed wetlands. We have shown that the culturing of highly diverse bacteria is indeed possible when their ecological niche is sufficiently well mimicked, and time is allowed for slow-growing strains to propagate.

The application of similar cultivation strategies to other understudied bacterial species will increase the number and diversity of axenic cultures from constructed wetland systems in the future. This discovery-driven culture and identification based on 16S rRNA amplicon sequencing and biochemical method provide valuable preliminary information which can be used to guide further study. Questions remain, however, on whether the available axenic cultures of bacterial isolates reflect bacterial diversity sufficiently, as ~99% of environmental bacteria are uncultivable with countless more hitherto undiscovered microorganisms remaining in the microbial 'dark matter'. Hence, metagenomics or single-cell techniques are needed to gain additional insights required for expanding the understanding of the bacterial communities present within the constructed wetland treatment system. Nevertheless, the study highlighted that the CW microbiome plays a critical role by providing the microbial-based bioremediation processes for the removal of pollutants and pathogenic bacteria. Further work in this area can aid researchers in finding links between remedial microorganisms and the physicochemical parameters of CWs, and can help develop optimisation strategies by facilitating the discovery of suitable and beneficial microbial candidates.

METHODS

Study area and sample collection

The East Kolkata floodplain wetland is situated in the lower Bhagirathi basin bounded by latitude 22˚25′N to 22˚40′N and longitudes 88˚20′E to 88˚35′E, and lies between two rivers, Hooghly River in the northwest and Bidyadhari in the east. These wetlands act as a natural wastewater treatment plant and reuse/ treat about 800 million liters of wastewater flowing out daily from Kolkata (Fig. 8). Among the East Kolkata floodplain wetlands, Sardar Bherry, consisting of an area of roughly 140 hectares, receives significant amounts of wastewater and is considered one of the polluted wetlands in the region (based on our previous water quality analysis). Due to these properties, Sardar Bherry was selected as the model CW ecosystem for study and sample collections.

To analyse the impact of anthropogenic pressure generated from urban wastewater dilution on the bacterial community, the sediment samples from 17 sampling sites were collected from sewage canals (SC), wetland sites A, B, C and outlet canals (OC) located in Sardar Bherry (Supplementary methods). Sites SC1-SC3, were located in sewage canal inlet sites (numbered from north to south), receiving wastewater from various drainages of the city. Site A1-A4, B1-B4 and C1-C4 were in the constructed wetlands (numbered from north to south). Site C1-C2 is located in the outlet canal site, discharging water to natural Kestopur canal/river. The direction of the water flow is from sites SC to OC (Fig. 9).

Measurement of environmental parameters

The water and sediment samples were quantified for physiochemical parameters. To prepare for the sediment physiochemical quality assessment, the sediment samples were first dried in the shade at room temperature and then ground with a wooden hammer, followed by sieving through a No. 10 mesh sieve (2 mm) and stored in plastic packets. Details of the physicochemical testing procedures for the water and sediment samples can be found in the supplementary methods.

Isolation of bacterial isolates from sediment samples

The sediment samples were used to determine the abundance of total cultivable bacteria following the protocol developed by Guan et al.[77] with slight modifications (Supplementary methods). Following the testing for cultivable bacteria, single colonies from Tryptone soya agar (TSA) plates used in the previous step were selected based on uniqueness in

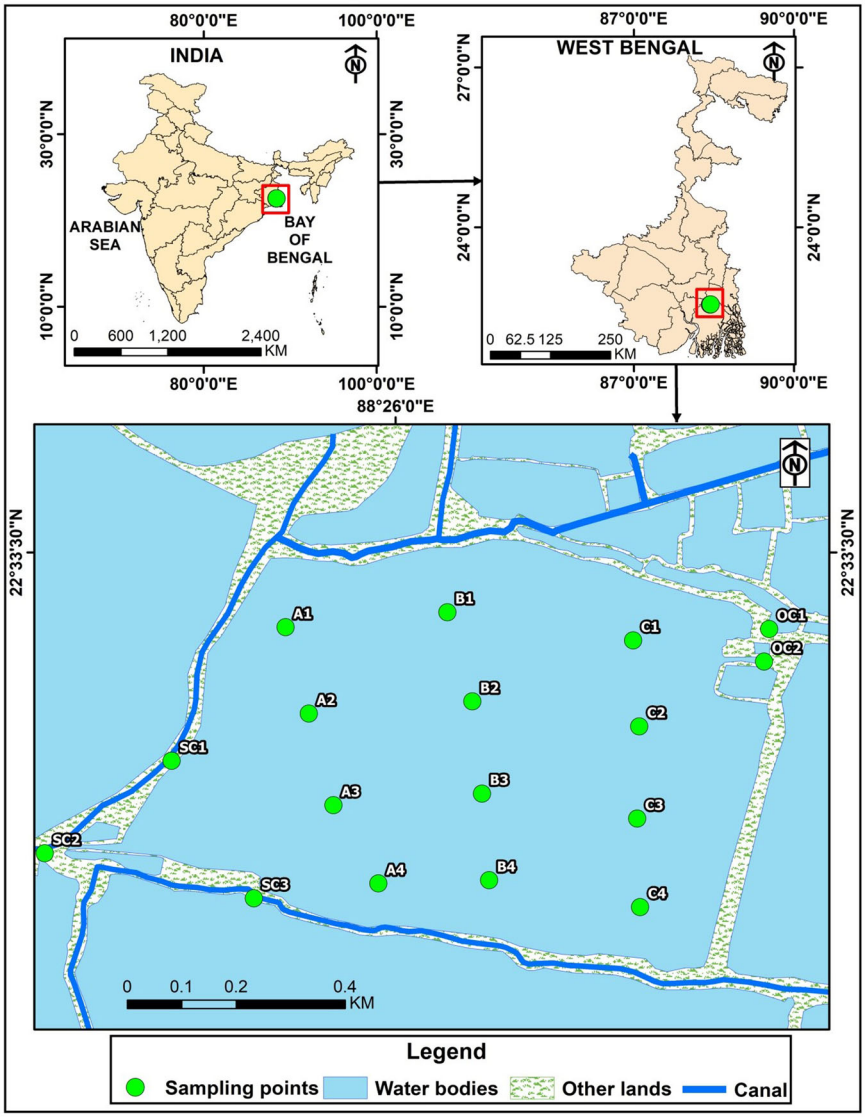


Fig. 9 GIS based map of study area and sampling locations. The selected location is Sardar Bherry, a constructed wetland receiving urban wastewater located in the Eastern part of Kolkata city, India, spanning *a* ~ 140 ha area. Sampling was done from sewage canals (SC1-SC3), wetland sites **A** (**A**1–**A**4), **B** (**B**1–**B**4), **C** (**C**1–**C**4) and outlet canals (OC1-OC2) located within the wetland.

morphology, size, and colour, and cultured overnight in Tryptone soya broth (TSB) at 37 °C with shaking at 120 rpm. The TSB culture was streaked into TSA media plates to check the purity of the isolates (based on the growth of colonies). Furthermore, a single colony was inoculated into TSB media, incubated overnight at 37 °C under constant agitation (120 rpm), with stock culture prepared containing 40% glycerol for storage at −80 °C until further use. A total of 32 stock cultures of bacterial isolates were produced from this process.

Identification and phylogenetic comparison of bacterial isolates

The bacterial isolates were identified based on 16S rRNA PCR amplicon sequencing methods. The methods for preparing the isolate cultures for PCR amplification, such as DNA extraction, PCR cycling protocols, as well as the primers used, can be found in the Supplementary methods and Supplementary Table 6, respectively. The amplified gene products were sequenced in forward and reverse direction using the ABI 373xl capillary sequencer (Applied Biosystem, Foster City, CA). Assembly of the 16 S rRNA sequences was performed by aligning forward and reverse sequences using DNA baser 7.0.0. The assembled bacterial 16 S rRNA sequences were aligned and compared with available sequences in the NCBI GenBank using BLAST (http://blast.ncbi.nlm.nih.gov). Afterward, the sequences were submitted to GenBank for the preparation of a phylogenetic tree.

Evolutionary analyses were conducted in MEGA11[60] using the Neighbor-Joining method, to create the evolutionary history of recovered bacterial strains with the optimal tree[61]. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[62–65] and the units represent the number of base substitutions per site, with the analysis performed on 32 nucleotide sequences. Codon positions included were $1^{st} + 2^{nd} + 3^{rd} +$ non-coding, with ambiguous positions removed for each sequence pair using the pairwise deletion option, resulting in a total of 1444 positions in the final dataset.

Biochemical characterisation and antibiogram assay of bacterial isolates

The 32 bacterial isolates were distinguished using the Gram-staining method with biochemical characterisation performed following standard procedures, viz.; ONPG (β-galactosidase), lysine utilisation, ornithine utilisation, urease, phenylalanine utilisation, nitrate reduction, H2S production, citrate utilisation, Voges Proskauer's (VP), methyl red, indole, malonate utilisation, esculin hydrolysis, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, lactose, and oxidase (KB003, Hi-media)[63,64]. Antibiotic resistance testing of the 32 bacterial isolates was done according to the methods given by Batoni et al. and Zidour et al., with details in the supplementary methods. Bacterial sensitivity towards antibiotics was interpreted as sensitive, intermediate or resistant according to the guidelines of the Clinical and Laboratory Standards Institute[33,34], with the antibiotic discs used in the antibiogram assay listed in Supplementary Table 7. The antibiotic resistance assay was performed in quintuplicate and is representative of two independent experiments. Multiple antibiotic resistance (MAR) index of each isolate were estimated according to the method developed by Krumperman[65].

Acclimatisation of experimental fish and challenge assay

Organization for Economic Cooperation and Development (OECD) guidelines were followed for the handling and care of experimental animals. The animal utilisation protocol was approved by Institutional Animal Ethics Committee, ICAR-Central Inland Fisheries Research Institute, Kolkata, India, (IAEC/2021/04) for the experimental setup.

Healthy *Labeo rohita* (Length = 132.8 ± 6.1 mm, Weight = 28.87 ± 2.4 g) were procured from a local fish hatchery. The fish were inspected to ensure that there were no external clinical symptoms like haemorrhage, ulcer, discoloration, descaling, or redness in the body of the fish. Additionally, before the challenge assay, fish were randomly selected and screened for the presence of infectious microbes following a standard protocol (Nickum et al.[78]; Johansen et al.[79]). The fish were acclimatised in 200L FRP tanks for 2 weeks, supplied with proper aeration, and fed a diet of commercial fish pellets (containing 35% crude protein and 10% crude fat) weighing ~2% of total body weight twice a day. The bacterial challenge assay was prepared from the 32 bacterial isolates recovered from the sediment samples. The 32 bacterial isolates were sub-cultured in 20 ml of sterile TSB in 50 ml Erlenmeyer flasks (Himedia, India) at 37 °C for 24 h. Bacterial cells were collected by centrifuging at 5000 rpm for 5 min and washed thrice with a sterile saline solution. Afterward, the pellets were resuspended in normal saline and the number of cells/ml was estimated through the spread plate method. Intraperitoneal injection of the experimental fish (20 numbers) was done with 0.2 ml @ 10^6 CFU/ml of bacterial suspension. The control fish were injected with 0.2 ml of saline solution. Afterward, the fish were kept in an FRP tank and observed every 24 h for a total of 120 h. To confirm Koch's postulate, the bacteria were reisolated and identified from the liver, kidney and blood of the moribund fish[66].

Haemolytic activity

The haemolytic activities of the 32 bacterial isolates were conducted according to a standard protocol with slight modifications[67]. Briefly, the TSA plates were prepared by supplementing 5% defibrinated sheep blood. The pure stock cultures of the 32 bacterial isolates were grown overnight in TSB at 37 °C under constant agitation. Later, the overnight culture was diluted to an OD600 of 0.5, and 2 µl of the diluted culture was spotted in the middle of the haemolysin test plates. The plates were incubated at 37 °C for 48 h, after which the diameters of the clearing zones were measured. The assays were done using freshly prepared media in four replicates.

Antibacterial assay of non-pathogenic bacterial isolates and extracellular proteins

In total, two separate tests were performed to determine the potential antibacterial role of non-pathogenic bacterial isolates (classified based on fish survival and haemolysin assay results) against isolated pathogenic strains (*Ralstonia* sp. A3, *Bacillus cereus* A6, *B. cereus* A7, *B. cereus* SC1, *B. cereus* SC2, *Burkholderia cepacian* SC3, *B. cereus* SC5 and *B. cereus* SC6) and two model fish pathogens (*Vibrio parhaemolyticus* and *Pseudomonas aeruginosa*). The pathogenic bacteria suspensions (100 µl @10^7 cells/ml) were spread onto TSA plates using a sterile spreader, and five different non-pathogenic bacteria suspensions (10 µl @10^5 cells/ml) were placed on agar plates with a micropipette. Later, the agar plates were covered with parafilm tape and were incubated for 24–48 h at 37 °C. Diameters of inhibition halos surrounding the non-pathogenic bacteria colony were measured and expressed in millimetres. Subsequently, the non-pathogenic bacterial isolates showing antibacterial activity were grown separately from pathogenic strains to reconfirm the activity. Testing and quantification of bacterial extracellular proteins (ECPs) were performed on non-pathogenic bacterial isolates that displayed inhibitory effects against 8 or more pathogenic strains to determine their antibacterial activity (Supplementary methods).

Ammonia removal assay

Synthetic wastewater was prepared for use as the basic medium for storage, cultivation and for subsequent studies on nitrogen removal (at C/N mass ratio 10)[68]. The synthetic wastewater mixture was prepared using (g/L): $Na_2HPO_4{\cdot}12H_2O$ 21.5, KH_2PO_4 0.91, NaCl 4, glucose (anhydrous) 2, NH_4Cl 0.08 g and 30 mL of each trace solution (pH maintained between 7–7.5). The trace solution was prepared using the following components (g/L): $MgSO_4$ 6.42, $MnSO_4$ 0.63, and H_3BO_3 3.36 g. Each solution was distributed evenly using 250 mL shaking flasks and inoculated for experimental analysis. For the assay, non-pathogenic bacterial isolates (based on fish survival, haemolysin and antibacterial assay results) were used to determine the potential nitrogen remediating properties (supplementary methods).

Statistical analysis

The data were arcsine transformed to satisfy normality and homoscedasticity requirements, then subjected to one-way analysis of variances (ANOVA) followed by Duncan's multiple range test using statistical software statistical package for the social sciences version 24.0 (P value ≤ 0.001). Statistical analyses were also performed to investigate the correlation between water/sediment quality with the sampling sites and bacterial isolates recovered from the wetland, with details of the multivariate data analyses performed in the supplementary methods.

DATA AVAILABILITY

OL654366, OL533641, OL630947, OL477593, OL477600, OL477341, OL477342, OL493030, OL583978, OK428851, OL533628, OL477587, OL519559, OL519154, OL519118, OK428925, OL519130, OL629462, OL629481, OL519183, OL519186, OL533630, OL477339, OL477343, OL504492, OL484881, OL477347, OL477349, OL477589, OL629484, OL654365, OL477638

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AUTHOR CONTRIBUTIONS

V.K., B.K.D. and B.K.B. conceived and designed the experiment. V.K., T.B., S.R., M.S.D., C.J., P.K.P., P.K., P.V., D.J.S. and M.S.D. performed the experiments and drafted the figures and manuscript. V.K., N.S., A.K.J. and A.K.R. made the laboratory analysis, statistics, and interpreted data. The manuscript is reviewed and edited by V.K., B.K.D., P.K., P.V. and B.K.B. All the authors approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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