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Isolation of pathogens from leaf, root and rhizosphere water samples of aquatic weeds, *Eichhornia crassipes* and *Pistia stratiotes* present in the backwaters of YMCA canal, Alappuzha, Kerala, India

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Abstract
The presence of pathogenic microorganisms in backwaters is of a great concern and the unsafe water is responsible for 1.2 million deaths each year. In the present study, water samples beneath the aquatic weeds, leaf and root samples of *E. crassipes* and *P. stratiotes* were collected from YMCA canal, Alappuzha, Kerala state, India, and subjected to physicochemical and microbiological analysis. The pH of the water samples were in the range of 6.6 to 6.9, salinity was nil, electrical conductivity was 5.327 mS for *Eichhornia crassipes* sample and 882.2 mS for *Pistia stratiotes rhizosphere* water sample. Total dissolved solids (TDS) were 2.777 ppm and 448 ppm for *E. crassipes* and *P. stratiotes* respectively. Heterophilic plate count of *E. crassipes* is 11 x 10^3 and 3 x 10^2 CFU/ml for bacteria and fungi respectively. Similarly the rhizosphere water sample of *P. stratiotes* showed a bacterial count of 75 × 10^3 CFU/ml and the fungal count was 8 x 10^2 CFU/ml. Human pathogens such as *Klebsiella. pneumoniae, Salmonella typhi, Enterococcus faecalis, E. coli, Vibrio cholerae, Salmonella enteritidis, Enterobacter aerogenes, Vibrio vulnificus* etc. were isolated from leaf, root and rhizosphere water samples. Antibiotic sensitivity studies revealed that *Klebsiella pneumoniae* was resistant to antibiotics like Azithromycin, Co-Trimoxazole, Cefuroxime and Cefalexin. Most of the pathogens were resistant to Cefalexin.

Keywords: Back waters**,** *E. coli, Klebsiella pneumoniae,* pathogens, *Vibrio cholerae*

Introduction

Water has been of great importance to human beings and other organisms of the environment for sustenance of life and maintaining the balance of the nature, hence water is called as the "life blood of the earth" (Ram *et al*., 2009) [10]. Dead leaf, woody debris, animal remains etc. constitute the main sources of organic matter in backwater environment. Alappuzha, known as the Venice of the East, boasts of shimmering waterways and a rich biodiversity. A common sight in Alappuzha is the mesmerizing houseboats. But because of the lack of proper regulation and hygiene they give adverse effects to the backwaters. Oil spillages from the boats, CO_2 emissions, discharge of untreated wastes, disposal of organic waste, fertilizer residues and run offs from agricultural fields and accumulation of aquatic weeds play a major role in backwater pollution. As a result of this water bodies have become a congenial breeding ground for water borne vectors like mosquitoes and pathogenic microorganisms (Chandran 2014) [8]. Aquatic weeds are unwanted and undesirable plants that are floating on the surface of water and form a dense mat there by reducing the penetration of sunlight into the water and reduce the photosynthetic efficiency of microscopic algae and results in the depletion of dissolved oxygen. Reduced levels of dissolved oxygen seriously affect the aquatic life and also alter the equilibrium of physicochemical properties of water. The microbial quality of water have an important influence on public health. Poor microbiological quality is likely to lead water borne disease outbreaks. Opportunistic pathogens are naturally present in the environment and normally present no risk to human health. They are able to cause disease in people with impaired local or general immune defences (Sreedevi and Sebastian 2020) [21]. Anthropogenic activities result in a significant decrease in surface water quality of aquatic systems in watersheds (Massoud *et al*., 2006) [16]. River inflows contribute many pollutants, thereby tending to induce ecological and hygienic problems (Wang *et al*., 2007) [24]. Escalating water pollution causes not only the deterioration of water quality but it also compromises human wellbeing and the permanence of aquatic ecosystems, economic growth and community affluent (Milovanovic, 2007) [17].

Eichhornia crassipes and *Pistia stratiotes* are the two major aquatic weeds found in the canals of Alappuzha. Water hyacinth (*E. crassipes*) is an aquatic plant which can flourish and reproduce floating freely on the surface of water or it can also be anchored in mud. It is a perennial weed, form dense rafts in the water and mud. Some of the principal problems are its interference with navigation, water flow, and the recreational use of aquatic systems, as well as the risk it poses of mechanical damage to hydroelectric systems. It is also responsible for drastic changes in the plant and animal communities of freshwater environments and acts as an agent for the spread of serious diseases in tropical countries (Chandran, 2011) [6]. Pistia is a genus of aquatic plant in the arum family, Araceae. The single species it comprises, *Pistia stratiotes*, is often called water cabbage, water lettuce, Nile cabbage, or shell flower. Microbial assemblage as a biofilm commonly occurs on the leaves of submerged plants, rhizosphere, especially on rhizoplane and on the solid surfaces of sediments.

Aquatic weeds are also responsible for lowering quantity as well as quality of water. These weeds cause taste and odour problems and also increases biological oxygen demand because of organic loading (Gopal and Sharma, 1979) [12]. In the present study aquatic weeds *E. crassipes* and *P. stratiotes* were collected from YMCA canal, Alappuzha, Kerala state, India and subjected to physicochemical and microbiological analysis. The human pathogenic microorganisms were also isolated using selective media and performed antibiotic sensitivity studies.

Materials and methods

Collection of samples

Water samples beneath the aquatic weeds of *E. crassipes* and *P. stratiotes* were collected from YMCA canal, Alappuzha were collected separately in sterile 250 ml Erlenmeyer flasks at normal atmospheric temperature and transported to the laboratory for further analysis (APHA 1998) [3]. Both the weeds as whole plants were also collected in buckets with water and brought to the laboratory for further studies.

Chemicals and media used

Glucose, tryptone yeast extract agar, potato dextrose agar, Thiosulphate Citrate Bile salt Sucrose (TCBS) agar, MacConkey agar, Eosin Methylene Blue (EMB) agar, antibiotic assay medium, Bismuth sulphate (BS) agar, KF streptococcal agar, Lauryl sulphate broth, antibiotic discs such as Co-Trimoxazole (25 mcg), Gatifloxacin (5 mcg), Cefuroxime (30 mcg), Cefalexin (30 mcg), Chloramphenicol (30 mcg), Doxycycline hydrochloride (30 mcg), Azithromycin (15 mcg), Ciprofloxacin (5 mcg) were procured from HiMedia Laboratories Private Limited, Mumbai, India.

Physicochemical parameters

Physical parameters

For physicochemical analysis water samples were collected from the rhizosphere regions of *E. crassipes* and *P. stratiotes.* The pH of water samples were checked using pH meter (Systronics 361, India). The temperature was measured using standard mercury filled centigrade thermometer. The electrical conductivity and Total Dissolved Solids (TDS) were measured using pre calibrated conductivity TDS meter (Systronics 308, India). Salinity and specific gravity of the water samples were estimated using a handheld refractometer (Erma, ERS10, Tokyo Japan).

Microbiological analysis

Hetrophilic plate count (HPC)

Water samples collected from YMCA canal, Alappuzha, Kerala state, India, were serially diluted and plated on Tryptone Glucose Extract Agar (tryptone 5g, yeast extract 3g, glucose 1 g, and agar 15 g and final pH (at 25°C) 7.0±0.2) and the plates were kept for 24 hours of incubation at 37 °C and the number of colonies were counted after incubation (APHA 1998) [3]. For fungal analysis, the serially diluted water samples were plated on potato dextrose agar and the plates were kept for incubation at room temperature for 48-72 hours. The fungal colonies developed were noted after incubation.

Isolation of microorganism from root, leaf and rhizosphere water sample

Rhizosphere water samples collected from *E. crassipes* and *P. stratiotes* were processed and were serially diluted and the dilution 10^{-2} was spread on sterile specific media such as TCBS agar (for *Vibrio cholerae*), Bismuth sulphite agar (for *Salmonella typhi*), Eosin Methylene Blue agar (for *E.coli*), Antibiotic assay medium D (for *Klebsiella pneumonia*), Mac Conkey agar (for *Enterobacter aerogenes*), KF streptoccocal agar (for *Enterococcus faecalis*) for the isolation of pathogenic microorganisms. Pathogenic microorganisms found in the root, leaf and in the water sample near the weeds were isolated by using spread plate method after enriching the samples in respective enrichment media.

Enrichment of water samples

Isolation of *E. coli*

For the enrichment of the *E. coli* strains, all samples were cultured in Lauryl sulphate broth overnight at 37 °C and subsequently were streaked onto Eosin Methylene Blue (EMB) agar plates and incubated at 37 °C for 24 h.

Isolation of *Vibrio* sp.

Water samples suspected to contain *Vibrio spp.* and *V. parahaemolyticus* were enriched by adding 100 ml water sample in 200 ml of double strength alkaline peptone water (pH 8.6) at 37 °C for 24 hours and swabs from the alkaline peptone water was streaked on to Thiosulphate Citrate Bile salt Sucrose (TCBS) agar and further incubated at 37 °C for 24 - 48 hours.

Isolation of *Salmonella typhi*

Salmonella typhi was isolated by inoculating one ml of water sample into 10 ml of selenite enrichment broth and incubated at 37 °C for 12-18 hours. Swabs from the selenite broth were streaked on to Bismuth Sulfite (BS) Agar plates, and further incubated at 37 °C for 24 - 48 hours.

Antibiotic sensitivity assay

The antibacterial sensitivity assay was carried out by disc diffusion method (Chandran 2013, 2015) [7, 9]. Antibiotic susceptibility of the isolates was tested according to National Committee for Clinical Laboratory Standards (NCCLS) by disc diffusion method with an inoculum of 10^8 cfu, and agar dilution method with 10^4 fu/spot (Deb Mandal *et al*, 2011) [10]. The test bacterial cultures were evenly spread over Mueller Hinton agar plates using a sterile cotton swab. The antibiotic discs (6 mm in diameter) were placed in the inoculated agar. The plates were then incubated at 37 °C for 24 to 48 hours. After incubation, the zone of inhibition thus developed were measured with the scale to the nearest in centimeters (CM). The antibiotics (content per disc) used in the study were Co-Trimoxazole (25 mcg), Gatifloxacin (5 mcg), Cefuroxime (30 mcg), Cefalexin (30 mcg), Chloramphenicol (30 mcg), Doxycycline hydrochloride (30 mcg), Azithromycin (15 mcg), Ciprofloxacin (5 mcg).

Results and Discussion

Physicochemical parameters

The physicochemical parameters of the rhizosphere water sample collected from the YMCA canal were analysed and the results are given in table 1. The pH of the water samples ranged from 6.5 to 6.9. Temperature ranged from 32 to 32.3 ºC, TDS ranged from 2.777 to 448 ppm and electrical conductivity ranged from 5.327 to 882.2 ms. Salinity and specific gravity is nil for the rhizosphere water sample. The pH (6.6 and 6.9) and temperature (32 ºC) also favoured the bacterial growth. The variables such as pH, temperature, salinity, nutrient availability, and geographic locations influence the growth of microorganisms in the natural habitat (Amend *et al*., 2013) [1]. Over 1.6 million people directly or indirectly depend on water for various purposes such as agriculture, fishing, transportation and recreation. So there is a possibility of spread of waterborne diseases from these aquatic bodies will pose a great threat.

Table 1: Physicochemical parameters of rhizosphere water samples

Aquatic weeds	pH	Salinity	Electrical conductivity (mS)	TDS (ppm)	Temperature (ºC)	Specific gravity
Eichhornia crassipes	6.6	Nil	5.327	2.777	32.3	0
Pistia stratiotes	6.9	Nil	882.2	448.0	32	0

Microbiological analysis

Heterophilic plate count

The bacteriological examination of water has an important relevance in pollution studies and it can provide a direct measure of deleterious effect of pollution on human health. HPC of rhizosphere water sample of *E. crassipes*, i.e. 11 x 10^3 and 3 x 10^2 CFU/ml for bacteria and fungi respectively. The rhizosphere water sample of *P. stratiotes* showed a bacterial count of 75 × 10^3 CFU/ml and the fungal count was 8 x 10^2 CFU/ml (Table 2). In the present study the heterophilic bacterial population was high in the rhozsphere region of *E. crassipes* and *P. stratiotes.* The heavy heterophilic bacterial count in the rhizosphere region was mainly due to the chemotaxis of microbes towards rhizosphere region of aquatic weeds. This chemotaxis was attributed to the secretion of different amino acids found in the root exudates secreted by *E. crassipes* (Chandran 2014) [8]. The abundance of pathogen in water depends on actors such as the contamination level, pathogen persistence in water bodies, biological reservoirs including aquatic plants and sediments (Dechense *et al*., 2006) [11]. Sediments and submerged aquatic vegetation are the important reservoirs of microorganisms (Badgley *et al*., 2010) [5].

Table 2: HPC of rhizosphere water sample

Microorganisms	Culture media	*Eichhornia crassipes*	*Pistia stratiotes*
Bacteria	Tryptone glucose extract agar	11 × 10^3	75 × 10^3
Fungi	Potato dextrose agar	3 x 10^2	8 x 10^2

Isolation of pathogens

Human pathogens isolated form root, leaf and rhizosphere water samples of *E. crassipes*, such as *V. cholerae* and *V. vulnificus* were cultured using TCBS agar medium. Enterobacter species such as *Enterobacter aerogenes* and *Enterococcus faecalis* were cultured using Mac Conkey agar medium produced pink and pale pink colonies. The increased number of coliform and faecal coliforms in water bodies is mainly due to the higher sewage contamination. In the EMB agar medium, *E. coli* formed purple with black centered colonies and *K. pneumoniae* formed white colonies in antibiotic assay medium. Species of *S. enteritidis* and *S. typhi* formed brown and black (with metallic sheen) colonies in BS agar medium. Maroon colonies of *E. faecalis* were observed in KF streptococcal agar medium. The presence or absence of microorganisms found in the root, leaf and rhizosphere water samples of aquatic weeds are given in table 3 and 4.

Faecal contamination of water introduces a variety of pathogens into water ways, including bacteria, viruses, protozoa and parasitic worms. *E. coli*, *Salmonella* and *Vibrio* spp. forms the most important pathogen that spread through water. *E. coli* is the only member of the total coli form group that is found exclusively in faeces, other members of the group are found naturally in water, soil, and vegetation, as well as in faeces. *S. typhi* is one of the major causes of food and water borne gastroenteritis in human (Tsen *et al*., 2000) [23] and remains an important health problem worldwide (Athira *et al*., 2019) [4]. The presence of *E. coli*, *Klebsiella,* and *Enterobacter* species in water is a likely indicator of the presence of pathogenic organisms such as *Clostridium perfringens*, *Salmonella,* and Protozoa (Anyamene and Ojiagu 2014) [2]. These pathogens cause diarrhea, giardiasis, dysentery, and gastroenteritis, which are common among the rural dwellers of developing nations (Thliza *et al*., 2015, Oludairo and Aiyedun 2016, Isikwue and Chikezie 2014) [22, 18, 13].

Table 3: Microorganisms found in different parts of *E. crassipes*

Pathogens	Medium	Colony colour	Presence (+) or absence (-) of pathogens		
			Root surface	Leaf	Rhizosphere water
Vibrio cholerae	TCBS agar medium	Yellow Greenish yellow	+	+	+
Vibrio vulnificus			+	+	-
Enterobacter aerogenes	Mac Conkey agar medium	Pink	_	+	+
E. coli	EMB agar medium	Purple with black center	+	+	+
Klebsiella pneumoniae	Antibiotic assay medium	White	+	+	+
Salmonella typhi	Bismuth sulphate agar medium	Black	+	+	+
Enterococcus faecalis	KF streptococcal agar medium	Maroon	+	+	+

Table 4: Microorganisms found in *P. stratiotes*

Pathogens	Medium	Colony colour	Presence (+) or absence (-) of pathogens in		
			Root surface	Leaf	Water
Vibrio cholera Vibrio vulnificus	TCBS agar medium	Yellow Green	+	-	+
			+	-	+
Enterobacter aerogenes	Mac Conkey agar medium	Pink	+	+	+
E. coli	EMB agar medium	Blue purple	+	+	+
Enterobacter aerogenes		Pink without sheen	+	+	+
Klebsiella pneumoniae	Antibiotic assay medium	White	+	+	+
Salmonella enteritidis	Bismuth sulphate agar	Black with metallic sheen	+	+	+
Salmonella typhi		Brown grey	+	+	+
Eterococcus faecalis	KF streptococcal agar medium	Maroon	+	+	-

Antibiotic sensitivity assay

The antibiotic sensitivity assay revealed that all the organisms isolated from *E. crassipes* except *V. cholerae* were found to be resistant to antibiotic Cefalexin. *V. cholerae* showed a zone of inhibition towards the antibiotic, Celalexin with a zone of inhibition of 1.1 cm. All the five organisms *K. pneumoniae, S. typhi, E. faecalis, E. coli* and *V. cholerae* showed a zone of inhibition towards the antibiotics Ciprofloxacin, Chloramphenicol, Doxycycline hydrochloride and Gatifloxacin. The overall result of the antibiotic sensitivity test is given in table 5. Based on the antibiotic sensitivity assay, it was found that all the pathogens present in *P. stratiotes* were also found to be resistant to the antibiotic Cefalexin. All the five organisms (*Klebsiella pneumoniae, S. typhi, E. faecalis, E. coli* and *V. cholerae* showed a zone of inhibition towards the antibiotics Ciprofloxacin, Chloramphenicol, Doxycycline hydrochloride and Gatifloxacin. The overall results of the antibiotic sensitivity assay are given in table 6. Development and spread of antimicrobial resistance have become a global public health problem, impacted by both human and nonhuman antimicrobial usage (Rigos *et al*., 2010) [20]. Aquatic environments, rivers and streams are considered as an ideal reservoir for antibiotic resistance dissemination because antimicrobials and antimicrobial resistant bacteria are often directly released in the environment (Lupo *et al*., 2012) [15].

Table 5: Antibiogram of pathogens isolated from *E. crassipes*

Pathogens isolated from *Eichhornia crassipes*	Antibiotics/ Diameter of zone of inhibition in cm							
	Ciprofloxacin	Azithromycin	Chloramphenicol	Co-Trimoxazole	Cefuroxime	Cefalexin	Doxycycline hydrochloride	Gatifloxacin
Klebsiella pneumoniae	1.5	_	0.7	_	_	_	1	1.5
Salmonella typhi	1.5	_	1	0.5	0.7	_	1.1	1.6
Enterobacter faecalis	1.2	1	0.9	0.3	0.4	_	0.7	1.3
E. coli	1.5	_	0.4	0.4	0.4	_	0.8	1.5
Vibrio cholerae	1.4	0.6	1.4	_	0.4	1.1	1.5	1.2

Table 6: Antibiogram of pathogens isolated from *P. stratiotes*

Pathogens isolated from *Pistia stratiotes*	Antibiotics/ diameter of zone of inhibition in cm							
	Ciprofloxacin	Azithromycin	Chloramphenicol	Co-Trimoxazole	Cefuroxime	Cefalexin	Doxycycline hydrochloride	Gatifloxacin
Klebsiella pneumoniae	1.1	0.7	0.4	0.4	_	_	1	1
Salmonella typhi	1.6	1.7	1.6	0.5	0.4	_	1.1	1.7
Enterobacter faecalis	1.2	1	0.5	0.5	0.5	_	0.7	1.2
E. coli	1.7	_	0.3	1	0.5	_	0.8	1.7
Vibrio cholerae	1.6	1.3	0.8	_	0.3	_	1.5	1.6

Conclusion

The aquatic weeds harbor the growth of human pathogenic microorganisms, even some of them are antibiotic resistant and the water bodies can act as a reservoir of pathogens which pose a threat to human population. The isolation of human pathogenic microorganisms such as *K. pneumoniae, S. typhi, E. faecalis, E. coli, V. cholerae, S. enteritidis, E. aerogenes, V. vulnificus etc.* clearly indicates the mixing of sewage and other pollutants with the water body. Regular monitoring and controlling the mixing of sewage with water body has to be prevented. Removal of aquatic weeds from the backwaters should also be given priority.

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