

ABUNDANCE OF POLLUTION-INDICATOR BACTERIA IN CIMANUK WATERS, INDRAMAYU

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Received: May 14, 2024; Revised: October 08, 2024; Accepted: November 07, 2024

Abstract

Cimanuk waters are one of Indonesia's areas with abundant natural resource potential that have been threatened with contamination due to impulsive population growth and high human activities. Microbiological aspects are one of the parameters that can indicate environmental pollution. Therefore, pollution-indicator bacteria such as coliform and pathogenic bacteria have become one of the most important indicators of environmental conditions. This study conducted an analysis of pollution indicator bacteria at several sampling sites around the Cimanuk estuary to assess the environmental status. Surface seawater (± 30 cm) was taken using a Zobell water sampler with a sterile 300 ml bottle. Samples were subjected to microbiological analysis, including total coliform contaminating bacteria, *Escherichia coli*, heterotrophic bacteria, halotolerant, and pathogenic bacteria. Results showed severe fecal contamination of stations compared to the WHO standards, and total coliforms varied between stations in the range of 1500-3000 CFU/100mL. The presence of pathogenic bacteria such as *E. coli*, *Vibrio cholera*, *Aeromonas* spp., *Salmonella* spp., and other bacteria indicative of fecal contamination were reported. Coliform bacteria and *E. coli* were found at the highest density in coastal waters near the mainland, indicating waste input due to anthropogenic activities. This study shows that Cimanuk waters are not good for biota and recreational studies.

Keywords: Cimanuk Waters; Bacterial Density; Microbiological Parameter; Pollutant Indicator

Introduction

The Cimanuk River flows from the foothills of Mount Papandayan to the Cimanuk Delta. Natural resources abound in Cimanuk waters (Mulyani *et al.*, 2020). However, owing to human activities, some of them have changed their functions (Mulyani *et al.*, 2020; Solihuddin *et al.*, 2021). The Cimanuk River Valley is highly populated, whereas the downstream area receives much shipping due to

multiple ports (Wulandari *et al.*, 2019). Land conversion and the high degree of human activity in this area have led to anthropogenic contamination that can harm the environment.

Anthropogenic contamination caused by human activities can be detected using microbiological methods. The abundances of various microorganisms in the environment can be

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DOI: <https://doi.org/10.55766/sujst-2024-06-e04798>

Suranaree J. Sci. Technol. 31(6):030235(1-7)

used to predict waste pollution (Wen *et al.*, 2020). These characteristics include water pollution indicators, microorganisms, heterotrophic bacteria, halotolerant bacteria, and pathogenic bacteria. Organic molecules in water are decomposed by heterotrophic and halotolerant bacteria that break them down into simple chemicals (Roseline, 2019; Zhao *et al.*, 2020). These bacteria play an important role in maintaining the equilibrium of marine ecosystems and in providing nutrients for marine species. Halotolerant microorganisms exhibit salinity tolerance. In general, halotolerant bacteria are brought to the sea from the land and can adapt to living in high salinity, such as *Halomonas* and *Staphylococcus* genera (Passante *et al.*, 2022).

Pollutant indicator bacteria, such as coliforms and *E. coli* indicate contamination from home, garbage, and farm waste (Wen *et al.*, 2020; Kristanti *et al.*, 2022). Coliform bacteria have been detected in the digestive tracts of humans and warm-blooded animals that live in water (Bisimwa *et al.*, 2022). Coliform bacteria proliferate rapidly in wastewater from densely populated towns and villages (Bisimwa *et al.*, 2022). Coliform bacteria are rod-shaped Gram-Negative bacteria that do not form spores, can be motile or nonmotile, and can ferment lactose to create acid and gas when incubated at 35°C to 37°C (Li and Liu, 2019). They are often employed as water pollution indicators to assess whether water has been contaminated with pathogens (Li and Liu, 2019; Shen and Zhang, 2022).

Coliform bacteria pose a significant risk to public health, particularly for infants, young children, and immunocompromised individuals who are vulnerable to waterborne diseases, such as gastroenteritis, dysentery, diarrhea, and viral hepatitis, even at low infectious doses (Some *et al.*, 2021). The State Minister of the Environment, Indonesia, through Environmental Ministerial Decree No. 51/2004, advises that the total coliform count for marine recreation water should be less than 1000 colony/mL, without the presence of *E. coli*, Enterococci, or *Pseudomonas aeruginosa* (MOE, 2004). Coliform bacteria, including the genera *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia*, and *Yersinia*, are members of the Enterobacteriaceae family. The prevalence of coliform bacteria is also linked to the presence and number of pathogenic bacterial species such as *Shigella* spp., *Salmonella* spp., and *Vibrio cholerae* (Curutiu *et al.*, 2019). Coliform bacteria can synthesize ethionine, a compound with carcinogenic properties (Dziubańska-Kusibab, 2020). Furthermore, spoilage bacteria can produce diverse toxins, including indole and skatole, which

may lead to diseases when present in excessive quantities in the human body (Ma *et al.*, 2021). According to Murray *et al.* (2009), this bacterium serves as an indicator of other harmful bacteria responsible for causing diseases. This study examined the presence of coliform, pathogenic, heterotrophic, and halotolerant bacteria as potential bioindicators of pollution at many water sampling stations in the Cimanuk Waters of Indramayu.

Materials and Methods

Sampling Site

Seawater was sampled from 18 locations in Cimanuk, Indramayu, Indonesia, during the rainy season in March, as shown in Figure 1. Physicochemical parameters, including temperature, salinity, and concentrations of ammonia, nitrite, nitrate, and phosphate, were analyzed using a Horiba multi-parameter water quality meter U-53-10 (Horiba Ltd, Japan). Surface seawater (± 30 cm) was taken using a Zobell water sampler with a sterile 300 ml bottle. Samples were stored in an icebox for further microbiological analysis, including analysis of coliform-contaminating bacteria, *E. coli*, heterotrophic bacteria, and halotolerant and pathogenic bacteria (*Vibrio* and *Salmonella*).

Microbiological Parameter Analysis

Water pollutant indicator bacteria were analysed using 3M Petrifilm EC coliform count plates. A 45 μ m-pore polycarbonate filter was used to filter two quantities of seawater samples, 5 and 10 mL. The filter containing microbial cells made three repetition of the 3M Petrifilm with media specific for coliform bacteria. The samples were incubated at 35°C for 24 h. The formation of blue and purple bacterial colonies suggested the presence of *E. coli* and total coliform bacteria, respectively. The number of bacteria was counted in colonies per 100 mL.

Using the pour plate method, 1 mL of the sample to 10^{-3} and inoculated twice on two different media to identify heterotrophic and halotolerant bacteria. Two types of media were used for bacterial detection. The heterotrophic media consisted of Bacto agar (15 g), yeast extract (3 g), Bacto peptone (5 g), 750 mL of filtered seawater, and 250 mL of distilled water. In comparison, the halotolerant medium consisted of Bacto agar (15 g), yeast extract (3 g), Bacto peptone (5 g), and 1000 mL distilled water. The number of bacteria was assessed using the total plant count technique after ten days of incubation at 25°C.

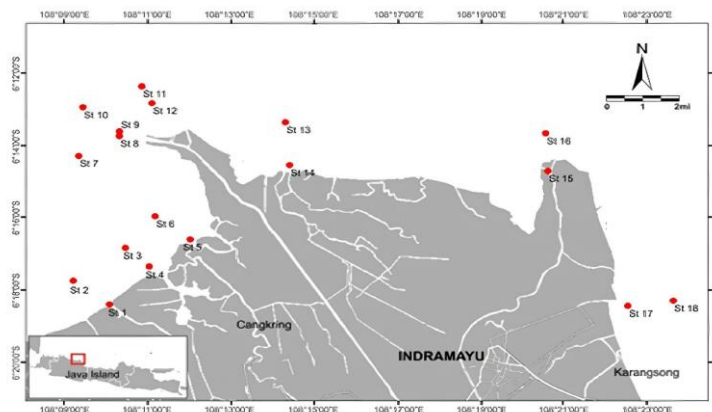


Figure 1. Location of sampling stations in Cimanuk Estuary, Indramayu

A thiosulfate-citrate-bile salt-sucrose (TCBS) agar medium was used to cultivate viable pathogenic bacteria of the Vibrionaceae family. Three replicates of the 0.1 mL water sample were inoculated onto a selective agar medium using the spread plate method. The samples were incubated at 35°C for 24 h. The presence of bacterial colonies was meticulously documented. After re-inoculation on new media, the dominant bacteria were identified using biochemical assays (Barrow and Miller, 1976). The tests included Triple Sugar Iron (TSI), Lysine decarboxylase (LDB), Methyl Red/Voges-Proskauer (MR-VP), and 8% NaCl.

The pathogenic Salmonella bacteria were isolated by adding 1 mL of water sample into the enrichment media and then incubating the mixture at 30°C for 24 h. One millilitre of enrichment culture was added to the selenite medium and incubated at 30°C for 24 h. The bacteria were inoculated onto Xylose Lysine Deoxycholate (XLD) agar medium and incubated at 30°C for 24 h. Bacterial isolates grown on XLD agar were evaluated for bacterial strains using several test media, including TSI, Sulfur Indole Motility (SIM), LDB, Sulfite, and Urea.

Results and Discussion

Physicochemical Parameters of Seawater

The environment in which saltwater exists encourages bacterial growth Table 1 lists the environmental features of the 18 sampling locations. The results showed that the depth of Cimanuk waters ranges from 0.5-12 m, with salinity between 17-31‰; seawater temperature is

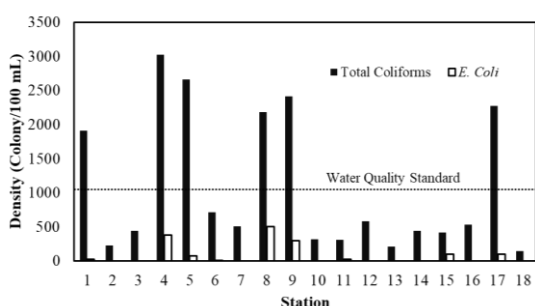
relatively high, around 29.1 - 31.9°C, and sediments generally consist of mud. The concentration of $\text{NH}_4^+\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$ and PO_4^{3-}P were in the range of 0.002-0.084, 0.001-0.015, 0.005-0.161, and 0.005-0.015 mg/L, respectively. The highest $\text{NH}_4^+\text{-N}$ and $\text{NO}_2\text{-N}$ concentrations were observed at stations 4 and 17, respectively, whereas station 9 had the highest $\text{NO}_3\text{-N}$ and PO_4^{3-}P concentrations. These three stations were located at the mouth of the estuary, which is most affected by river outflow and oceanic water (Nascimento *et al.*, 2021). In addition, sediment transport in estuaries through water bedload and suspended load may result in variability in nutrient levels and water quality components (Rahaman *et al.*, 2013). Station 4 and 17 have similar salinity and temperature, whereas station 9 has the lowest salinity, about 17‰. Temperature and salinity in water affect the growth of pollution indicator bacteria, including other pathogenic bacteria.

Coliform Bacteria in Cimanuk Waters

Figure 2 shows the densities of coliforms and *E. coli* at 18 sampling stations. At sampling stations 1, 4, 5, 8, 9, and 17, the coliform bacterial density was 1500 - 3000 colony/100 ml, which exceeded the water quality standard. According to the World Health Organization (WHO, 2021), coliform bacteria for marine biota cultivation and tourism should be less than 1000 colony/100mL and 200 colony/100 mL, respectively. In Indonesia, the Ministry of Environment has set coliform under 1000 colony/100 mL (MOE, 2004, Megantara *et al.*, 2023).

Table 1. Physicochemical data in Cimanuk Estuary

Station	Longitude	Latitude	Salinity (psu)	Temperature (°C)	NH ₄ ⁺ -N (mg NL ⁻¹)	NO ₂ ⁻ -N (mg NL ⁻¹)	NO ₃ ⁻ -N (mg NL ⁻¹)	PO ₄ ³⁻ -P (mg PL ⁻¹)
1	108°10'5.45"E	6°18'24.13"S	31.2	31.9	0.020	0.001	0.004	0.005
2	108°9'11.79"E	6°17'44.96"S	31.1	30	0.005	0.003	0.022	0.007
3	108°10'27.15"E	6°16'50.25"S	31	30	0.002	0.003	0.007	0.011
4	108°11'1.44"E	6°17'21.00"S	28.3	31.3	0.084	0.003	0.019	0.010
5	108°12'1.18"E	6°16'35.95"S	29.5	31.6	0.035	0.002	0.008	0.007
6	108°11'10.93"E	6°15'57.71"S	31	29.9	0.008	0.003	0.010	0.010
7	108°9'15.39"E	6°14'16.17"S	31.7	29.8	0.008	0.002	0.022	0.010
8	108°10'18.61"E	6°13'42.13"S	17	31.8	0.055	0.003	0.006	0.008
9	108°10'20.14"E	6°13'36.64"S	17	31.8	0.058	0.008	0.161	0.015
10	108°9'25.49"E	6°12'55.28"S	30.3	29.1	0.005	0.007	0.137	0.011
11	108°10'50.66"E	6°12'22.62"S	31.1	30.4	0.001	0.001	0.004	0.010
12	108°11'7.02"E	6°12'49.92"S	31.4	30.1	0.021	0.001	0.005	0.010
13	108°14'20.00"E	6°13'21.03"S	31.7	30.4	0.028	0.001	0.005	0.008
14	108°14'24.03"E	6°14'31.83"S	31.5	30.5	0.026	0.001	0.005	0.007
15	108°20'38.16"E	6°14'42.44"S	31.1	29.6	0.026	0.007	0.023	0.005
16	108°20'33.18"E	6°13'38.83"S	31.3	30.2	0.005	0.002	0.009	0.008
17	108°22'32.01"E	6°18'24.73"S	29.8	29.6	0.024	0.015	0.053	0.007
18	108°23'38.38"E	6°18'16.87"S	30.1	29.9	0.024	0.002	0.020	0.008

**Figure 2. Location of sampling stations in Cimanuk Estuary, Indramayu**

Among 18 sampling station, six stations were at mouth of estuary. Similar results were obtained in the Sayung Estuary, Demak and Cimandiri Estuary, and Pelabuhan Ratu (Safitri *et al.*, 2018; Afianti *et al.*, 2020). Residential waste is responsible for the coliform bacteria that contaminate water biologically. However, the concentration decreases in proximity to the sea, which has a high salinity level. Elevated salinity results in a diminished presence of coliform bacteria (Li *et al.*, 2021). However, the overall coliform levels in the Cimanuk Estuary exceeded the maximum permissible limit for the marine ecosystem.

Heterotrophic and Halotolerant Bacteria in Cimanuk Waters

The density of heterotrophic and halotolerant bacteria in Cimanuk waters were $(0.71\text{--}13.1) \times 10^4$ CFU/ml and $(0.3\text{--}6.3) \times 10^4$ CFU/ml, respectively (Figure 3). The highest number of heterotrophic

bacteria was observed at station 9. Halotolerant microorganisms predominate in locations near river flows at stations 4 and 15. The prevalence of heterotrophic bacteria could be attributed to the mud and sand in the area; waters closer to the mainland contained more organic matter than stations positioned further from the mainland. The abundance of heterotrophic bacteria shows that coastal waters contain nutrients in organic matter, which are converted into inorganic matter to maintain the balance of organic and inorganic material sources in the sea. The inorganic material produced is needed for nutrition by other biota such as phytoplankton, the basic pyramid of the food chain system, and marine plants, which require inorganic compounds, thereby ensuring the sustainability of marine life (Follett *et al.*, 2022; Nair *et al.*, 2022).

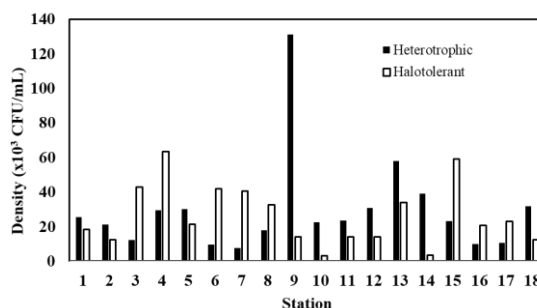
**Figure 3. The density of heterotrophic and halotolerant bacteria in the waters of Cimanuk, Indramayu**

Table 2. The number of pathogenic bacteria at each station in the Cimanuk Waters

Pathogenic Bacteria	Station																		Number of contaminated stations
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Vibrio group																			
<i>V. parahaemolyticus</i>	√			√			√	√				√	√	√		√		√	9
<i>V. alginolyticus</i>				√	√	√	√		√			√	√						7
<i>V. cholera</i>			√							√				√	√		√	√	6
Others <i>Vibrio</i>	√										√						√		3
<i>Proteus spp.</i>				√	√	√			√								√		5
<i>Aeromonas spp.</i>					√						√			√					3
Salmonella group																			
<i>Salmonella spp.</i>				√								√	√						3
<i>Yersinia spp.</i>	√	√								√	√							√	5
<i>Citrobacter spp.</i>	√	√	√						√				√	√		√			7
<i>Hernia spp.</i>								√											1
<i>Shigella spp.</i>															√				1
<i>Pseudomonas spp.</i>						√	√								√				3

Pathogenic Bacteria in Cimanuk Waters

Table 2 shows the presence of pathogenic *Vibrio* and *Salmonella* groups in Cimanuk waters. Among the *Vibrio* spp., *V. parahaemolyticus* was present at nine stations, whereas for the *Salmonella* group, *Citrobacter* spp. were found at seven sampling stations. Stations 4, 5, 9, 14, and 17 were detected to have three species of *Vibrio* groups. It was less than that of the *Salmonella* group. Among 18 stations, pathogenic bacteria were observed at stations 4, 5, and 17.

The presence of pathogenic bacteria belonging to the *Vibrio* group in coastal waters indicates interactions with industrial and domestic effluents such as human waste or other food residues, which provide an environment conducive to the growth and development of these bacteria. These interactions subsequently impact aquatic biota and human health. Bacteria belonging to *Vibrio* species are known to act as direct pathogens that can kill marine biota that live in water, and bacteria transported by marine biota, such as fish, can be ingested by humans, resulting in the development of diseases (Valente and Wan, 2021). When in contact with the human body, opportunistic pathogenic bacteria in freshwater or estuaries can induce infection, potentially leading to disease (Stec *et al.*, 2022). Some of them are *Acinetobacter calcoaceticus*, *Aeromonas hydrophila*, *Clostridium botulinum*, *K. pneumoniae*, *P. aeruginosa*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Stec *et al.*, 2022).

V. parahaemolyticus is an enteric pathogen that generally lives along the coast and is found in fish caught in tropical and temperate regions. It causes acute gastroenteritis with symptoms,

including cramping in the abdominal region, nausea, vomiting, headache, fever, and bloody diarrhea (Letchumanan *et al.*, 2014). *Vibrio* can cause food poisoning, generally associated with eating uncooked seafood and other clinically important human pathogens (Jung, 2018; Valente and Wan, 2021)

Several types of pathogenic *Vibrio* bacteria produce virulence factors such as enterotoxins, hemolysins, cytotoxins, proteases, lipases, phospholipases, siderophores, binding factors, and hemagglutinins (Zhang and Austin, 2005; Mekasha and Linke, 2021). The virulence of *V. parahaemolyticus* strains usually involves high levels of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) produced by the *tdh* and *trh* genes, respectively. The *tdh* and *trh* genes can serve as markers for finding pathogenic strains (Flynn *et al.*, 2019). *V. parahaemolyticus* is distributed globally. However, the reported densities of this species in the environment and seafood are highly variable, depending on the season, location, sample type, and analytical methodology used (Khouadja *et al.*, 2013; Letchumanan *et al.*, 2014).

Citrobacter spp. were the most abundant species in the *Salmonella* group at the 18 sampling stations. *Citrobacter* spp. are opportunistic pathogens that can lead to invasive diseases, including infections of the urinary tract, respiratory tract, CNS, skin, and soft tissue. *Yersinia* spp. found in cimanuk water can cause arthritis and enteritis in humans. If left untreated, *Citrobacter* spp. infections can be dangerous and fatal. Invasive infections have a high mortality rate of 33-48% (Antonara and Ardura, 2018). The presence of *Salmonella* in

seawater is attributable to the domestic organic waste industry and recreational places (springs, lakes, and beaches), as observed in this study.

The current study shows that water resources are contaminated, which can have detrimental consequences. Because enteric pathogens typically cannot grow in water, water is not a means of human infection (WHO, 2021). Nonetheless, in individuals with compromised natural defenses, locally or generally, the presence of *Vibrio* and *Salmonella* groups would be sufficient to cause infection at low levels. Individuals receiving immunosuppressive therapy, as well as very young and old, are likely to be at risk. There is also a risk for other immunocompromised AIDS patients. Additionally, if bacteria-contaminated water is allowed to contaminate food, it will cause the pathogens to multiply to extremely high concentrations.

Conclusions

Polluting bacteria are found in Cimanuk waters, Indramayu, and surrounding areas. Coliforms and *E. coli* have the highest densities in the coastal waters near the mainland. However, the concentrations of the pollution indicator bacteria remained below the established quality limits. A high density of heterotrophic and halotolerant bacteria was detected at sampling station 9, indicating that Cimanuk waters are rich in inorganic materials beneficial for aquatic biota, such as fish. However, since pathogenic bacteria were found at all sampling stations, appropriate regulations, procedures, monitoring programs, and methodologies to control waterborne pathogens must be considered in environmental management strategies in Cimanuk waters

Acknowledgement

The COREMAP-CTI program under the Research Center for Oceanography (RCO - LIPI) funded this research through the Priority Research scheme. Nur Fitriah Afianti contributed to the methodology, investigation, data collection, data interpretation, analysis, original draft preparation, writing, and editing of the manuscript. Lies Indah Sutiknowati performed methodology development, data collection, investigation, original draft preparation, writing, and editing. Risky Ayu Kristanti worked on the conceptualization, data interpretation, validation, visualization, writing (original draft, review, and editing), supervision, and correspondence.

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