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# Antibiotic-resistant *Citrobacter* spp. from duck eggs in poultry farms and selected tributaries in Laguna de Bay, Philippines: A cross-sectional study

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## Abstract

Antibiotic-resistant bacteria are not recently discovered issues but remain a prevailing and urgent worldwide concern. Recent reports have shown a significant rise in the number of antibiotic-resistant bacteria around the world, including *Citrobacter* spp. The prominent industry that contributes to this is the agriculture industry, especially the livestock-rearing sector. As an opportunistic pathogen, *Citrobacter* spp. poses a considerable health risk, especially to vulnerable populations. This study aims to determine the presence and antibiotic susceptibility pattern of *Citrobacter* spp. in the 36 duck eggshells collected from the three poultry farms in Victoria, Laguna, and 36 water samples from the selected tributaries in Laguna de Bay, Philippines. Presumed *Citrobacter* spp. was confirmed by using VITEK, and specific antibiotic resistance was determined. The VITEK results showed 8 water samples and 2 eggshell samples tested positive for *Citrobacter*. Among these isolates, 3 were sensitive to Ampicillin, 1 to Azithromycin, and 7 to Tetracycline. Additionally, 2 isolates showed intermediate resistance to Ampicillin, while 6 were resistant to Ampicillin, 10 to Azithromycin, and 4 to Tetracycline. In conclusion, the study discovers antibiotic resistant *Citrobacter* spp. in Laguna de Bay tributaries and poultry farms in Victoria, Laguna, Philippines. Thus, emphasizing the need of battling antibiotic resistance, as the bacteria have varying susceptibility to antibiotics. The findings have serious implications to consumers' health and food safety, as antibiotic-resistant bacteria in tributaries and duck eggs pose a substantial threat to human health and the environment.

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## Introduction

*Citrobacter*, a Gram-negative bacillus within the Enterobacteriaceae family, is a commensal pathogen typically found in the intestinal tracts of humans and animals. They can also be detected in various environmental sources like water, sewage, and soil (Liu *et al*., 2018). As opportunistic pathogens, it poses a considerable risk to people's health, especially to infants and immunocompromised individuals. It is known to cause many diseases, including urinary tract infections (UTIs), gastroenteritis, wound infections, pneumonia, brain abscesses, septicemias, meningitis, and endocarditis (Ahmed, 2023). *C. freundii* is typically transmitted through the fecal-oral route. Eggs, raw or undercooked poultry meat, and carcasses are all potential sources of this zoonotic bacteria (Hasan, 2019).

Antimicrobial Resistance (AMR) among highly pathogenic microorganisms is becoming a critical global concern, posing significant threats to the health and safety of humans, animals, and the environment (Nair *et al*., 2018). A study by Liu *et al.* (2018) revealed that the increase in resistance of *Citrobacter freundii* is due to the widespread use of broad-spectrum antibiotics. The combination of widespread antibiotic usage in poultry farms and the low virulence of *C. freundii* has led to the accumulation of genes that code for resistance to various antimicrobial classes, resulting in the emergence of multidrug-resistant *Citrobacter* spp. (Ahmed, 2023).

A rapid increase in the emergence of antibiotic-resistant bacteria is being observed worldwide, posing a significant threat to the community's health and well-being (WHO, 2020). The agricultural sector stands out as a significant contributor to antibiotic resistance due to its extensive use of antibiotics in food production, most notably for livestock rearing. A study by Kasimanickam *et al.* (2021) showed that to promote weight gain in livestock and enhance production efficiency, many farms rely on antibiotics. Correspondingly, a study by Arsene *et al.* (2022) states that over 80% of animals in food production consume and are being treated with antibiotics. This substantial use of antibiotics in raising livestock has resulted in the emergence and colonization of resistant bacteria, not only in animals but also in humans that consume them (Manyi-Loh *et al.*, 2018). Given the nature of these farms, their proximity to bodies of water may lead to transmission of antibiotic-resistant pathogens into the surrounding environment. Most duck-raising industries are along the shoreline of Laguna de Bay because of the abundant fresh water supply and freshwater snails that serve as food for the ducks. This can lead to significant environmental contamination with antibiotic-resistant pathogens due to the potential runoff of fecal matter and other farm waste into the water bodies. Hence, a thorough investigation of the cross-contamination between the poultry industry and tributaries alongside the vicinity of Laguna de Bay will aid in the dissemination of awareness to the public with regards to that must be taken in relation with the emergence of *Citrobacter* spp. within the vicinity.

## Material and methods

*Study design and setting*

The research employed a cross-sectional research design to comprehensively evaluate the presence of *Citrobacter* in ducks from poultry farms and adjacent tributaries. This design facilitated the simultaneous collection of data from multiple sources, offering an overarching view of *Citrobacter* prevalence in the specified areas. The study was conducted in Victoria Laguna, Philippines, utilizing three poultry farms as primary sources of eggshell samples, and water samples were obtained from three tributaries of Laguna de Bay: Pagsanjan River, Pila River, and Sta. Cruz River. These tributaries were strategically chosen based on their geographical proximity to the selected farms.

*Sampling procedure*

A sample size of 72 was obtained, consisting of 36 duck eggshells from three selected poultry farms and 36 water samples from three selected tributaries will be collected. Systematic Random Sampling was used to ensure an unbiased collection of duck eggs and water samples from the different sources and guaranty the representativeness of the collected samples by providing each duck egg from the three poultry farms and each location in the selected tributaries equal chances of being included in the study.

Eggshell samples were obtained from three different farms located in Victoria, Laguna, Philippines. Twelve (12) duck eggshells were randomly selected from each of the three farms, for a total of thirty-six (36) samples. A random starting point was selected among the identified duck coops within each farm, and 2 duck eggs were collected from every third duck coop. The collected samples were then placed in sterilized containers labeled in correspondence to their farm of origin. The samples were then transported in an ice box maintained at 4°C.

Water samples were also collected from three (3) chosen tributaries of Laguna de Bay, Pagsanjan River, Pila River, and Sta. Cruz River. A random starting point was selected in each tributary. From each starting point, twelve (12) water samples were collected from different locations at 20-meter intervals, for a total of thirty-six (36) samples. Samples were collected from both surface and below the surface waters (Acheamfour *et al.*, 2021). For each sample, 250 mL of water was collected and placed in sterile containers to prevent contamination and was labeled with the name of the tributary, date of collection, and sample location. The collected samples were then placed in an ice box maintained at 4°C and immediately transported to the laboratory.

*Sample processing*

Upon arrival at the laboratory, egg and water samples were processed immediately to help grow bacterial pathogens. Using a sterile cotton swab moistened with a solution of 0.85% Normal Saline Solution (NSS) and 0.1% peptone (Anukampa *et al.*, 2017), the researchers gently swabbed the surface of the eggshells and ensured that the swab covered the sample portion of the shell's surface. Swabbing must be thorough and not too aggressive to avoid breaking the eggshells and contaminating the samples. Swabbed samples were then immersed in a sterile tube containing 9 mL of non-selective buffered peptone water and incubated for 24 hours at 37°C to enhance the recovery of *Citrobacter* present on the surface of the eggshells (Hawal *et al.*, 2023).

Similarly, water samples were also processed immediately upon arrival at the laboratory. In a glass tube, 100 mL of water from each sample were centrifuged at 8000 g speed at 22°C for 10 minutes to concentrate the samples and facilitate the separation of the sediments (Boujnuoni *et al.*, 2022). After centrifugation, samples were decanted and remaining sediments were placed in sterilized tubes containing 9 mL of non-selective buffered peptone water agitated with a vortex mixer to displace the organisms in the sediment, and then incubated for 24 hours at 37°C (Tarazi *et al.*, 2021).

*Screening of Citrobacter in Salmonella-Shigella agar*

After pre-enrichment of samples, bacterial growths present in the buffered peptone were inoculated onto *Salmonella-Shigella* agar for screening. Isolates from the broth was inoculated onto plates containing *Salmonella-Shigella* agar and incubated at ambient air at 33 to 37°C for 18 to 24 hours (Aryal, 2022). *Citrobacter* spp. are *lactose* fermenters and will result in pale colonies with black centers due to its production of hydrogen sulfide (Hashim, 2018). Isolates with this characteristic colony morphology were subcultured into Lysine Iron Agar for differentiation.

*Differentiation of Citrobacter from Salmonella in lysine iron agar*

After screening for *Citrobacter* on *Salmonella-Shigella* agar, selected isolates with pale colonies and black centers were sub-cultured onto Lysine iron agar for differential isolation. Isolates from SSA were inoculated by stabbing into the tubes containing Lysine iron agar and incubated in ambient air at 35 to 37°C for 18 to 24 hours. *Salmonella* can decarboxylate lysine which results in a purple slant, a purple butt, and blackening on the agar. On the other hand, *Citrobacter* are incapable of decarboxylating lysine and will produce a purple slant, a yellow butt, and blackening on LIA. This difference in color of butts in the tube will serve as the basis for distinguishing *Citrobacter* from *Salmonella*. Isolates that showed yellow butt on LIA and black-centered colonies on SSA will be presumptively identified as *Citrobacter* (Aryal, 2022).

*Definitive identification of isolated Citrobacter spp.*

After presumptive identification of *Citrobacter* on SSA, isolates that were noted to have shown black centers were transported to a laboratory that facilitates the VITEK MS system and was tested for definitive identification and characterization of *Citrobacter* spp. VITEK is an advanced colorimetry identification technology that provides a high level of discrimination between different species of isolates that allows precise identification (Biomerieux, n.d.). This system is known for its accuracy and effectiveness and is utilized for the identification of specific serotypes within the isolated strains of *Citrobacter*. The VITEK 2 cards can provide rapid and accurate antimicrobial susceptibility testing results and resistance detection for organisms such as gram-positive cocci, gram-negative bacilli, and yeasts. The isolated *Citrobacter* strains that were obtained from the *Salmonella-Shigella* agar plates were analyzed using VITEK and were characterized based on their biochemical, serotypical, and antimicrobial characteristics (Kim *et al.*, 2022).

*Statistical analysis*

Statistical Analysis was performed at a 0.05 level of significance to assess the presence of *Citrobacter* in different sources. The Proportion Test was used to determine if the observed presence of *Citrobacter* in each of the different sources is significant or only due to random chance. The Chi-square Goodness of Fit Test was used to determine if there was a significant difference in the presence of *Citrobacter* spp. among the different sources. Finally, the Chi-square Test for Independence was used was used to determine if there was a significant relationship between the presence of *Citrobacter* in duck eggs from different poultry farms and the presence of *Citrobacter* in the selected tributaries.

**Results**

The investigation into the presence of *Citrobacter* spp. in duck eggs and water samples from selected poultry farms and tributaries around Laguna de Bay revealed varying contamination levels within the two sources. Based on the data provided by the VITEK system (Fig. 1), *Citrobacter* spp. was found in different proportions within the sampled sources. Among the three specific tributaries surrounding the poultry farms, Pila River was the only tributary confirmed to have presented *Citrobacter* spp. in all its four samples. While both Pagsanjan and Sta. Cruz rivers followed with two positive samples out of four and three, respectively. Two samples from Pagsanjan revealed non enteropathogenic *Escherichia coli*, and one sample from Sta. Cruz was confirmed of having *Proteus* spp. As for the farms, both Farms B and Farm C had only one confirmed positive sample presenting *Citrobacter* spp. isolates.

For the analysis of duck eggs, Fig. 2 revealed the prevalence of *Citrobacter* in the three poultry farms. Among the farms, Farm A was the only one that showed no presence of *Citrobacter* or any pathological bacteria, with 12 of its samples tested negative. Meanwhile, Farm B and Farm C revealed similar prevalence of *Citrobacter*, with each farm having 8.33% of the samples testing positive for the bacteria. In total, the researchers recovered *Citrobacter* isolates from 5.56% of the duck egg samples.

Comparatively, the water samples showed a higher prevalence of *Citrobacter* spp. compared to the duck eggs. Fig. 3 shows the observed prevalence of *Citrobacter* in each tributary. Among the selected tributaries, Pila demonstrated the highest number of positive isolates with 33.33%, while Pagsanjan had the least positive isolates with 16.67%. Meanwhile, Sta. Cruz demonstrated a prevalence of 25% of the samples testing positive for the organism.

Furthermore, the statistical analysis indicates that there is insufficient evidence showing the significant difference between the number of positive and negative results from the three tributaries of Laguna de Bay, as the p-value is greater than 0.05.

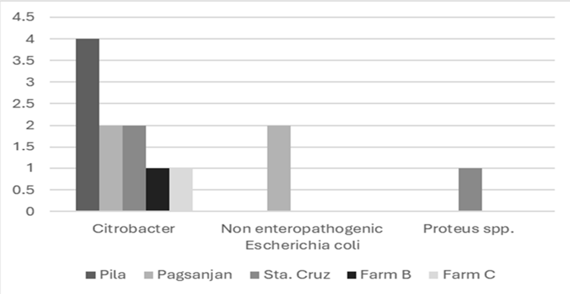


**Fig. 1.** Bacterial pathogens detected by VITEK

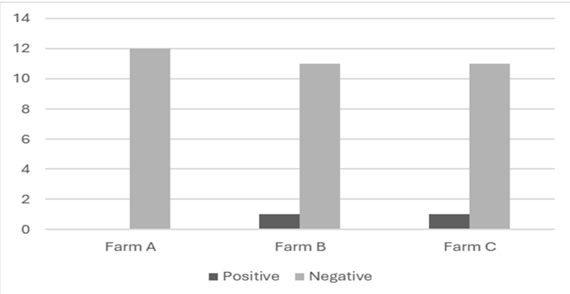


**Fig. 2.** *Citrobacter* spp. in farms

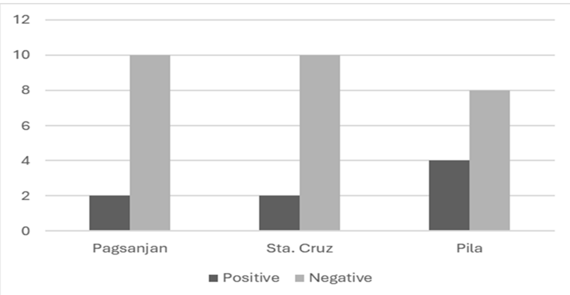


**Fig. 3.** *Citrobacter* spp. in tributaries

The Sensitivity Test was done to determine the susceptible pattern of the obtained *Citrobacter* isolates. Table 1 shows the antibiotic susceptibility of the isolated *Citrobacter* to the three selected antibiotics: Azithromycin, Ampicillin, and Tetracycline. Among the three, *Citrobacter* showed the most resistance to Azithromycin with 90.91% of the isolates exhibiting high resistance. The isolates also exhibited moderate resistance to Ampicillin, with 54.55% showing complete resistance and 18.18% showing intermediate resistance. On the other hand, *Citrobacter* was observed to be most susceptible to Tetracycline, with only 36.36% of the isolates being resistant.

**Table 1.** Antibiotic susceptibility of *Citrobacter* spp. from selected farms and tributaries

| Antibiotics | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| Ampicillin | 3 (27.27%) | 2 (18.18%) | 6 (54.55%) |
| Azithromycin | 1 (9.09%) | 0 (0%) | 10 (90.91%) |
| Tetracycline | 7 (63.64%) | 0 (0%) | 4 (36.36%) |

## Discussion

The emergence of antibiotic-resistant bacteria, particularly in agricultural industry, poses global health concerns. Antibiotics are extensively used in livestock production that led to development of resistance among various pathogens, including *Citrobacter* spp. (Mann *et al.*, 2021). *Citrobacter*, a gram-negative bacterium found in the intestinal tracts of hosts and environmental sources has been identified as opportunistic pathogenicity and antibiotic resistance (Hasan, 2019). The interaction between ducks and their environment increases the likelihood of cross-contamination between the two, indicating a serious public health threat (Jabeen *et al.*, 2023).

The study revealed a low isolation rate of *Citrobacter* spp. in duck eggs sourced from the three selected poultry farms in Victoria, Laguna. Among the three farms, only two reported a presence of *Citrobacter*, with Farm B and Farm C each having one sample (2.5%) testing positive for the bacteria while Farm A reported no presence of bacteria. Statistical analysis indicated that the prevalence of *Citrobacter* spp. in duck eggs from these farms is not significant. Factors such as farm management practices, hygiene protocols, and environmental conditions may influence the prevalence of *Citrobacter*. The low prevalence of *Citrobacter* may be attributed to the farms' strict adherence to health standards. However, the low presence observed does not eliminate the risks of *Citrobacter* infection, highlighting the importance of continued monitoring (Li *et al.*, 2015). This uniformity in the prevalence indicates the adequate waste management and sanitation practices.

However, caution is still advised due to the potential risks associated with *Citrobacter* contamination, as it can cause gastrointestinal infections if it is ingested (Anderson *et al.*, 2018).

Similarly, the study detected the presence of *Citrobacter* in all three tributaries of Laguna de Bay. The isolation rates varied between the tributaries, with Pila having the greatest number of samples positive for *Citrobacter* (33.33%), followed by Sta. Cruz (25%) and Pagsanjan (16.67%). Statistical analysis confirmed that the presence of *Citrobacter* in all the tributaries is significant. The contamination is attributed to various sources such as runoff from farms, sewage discharge from canals, and even industrial wastes from nearby factories. A study by Khatri and Tyagi (2014) reported that sewage contributes the most to coliform bacteria contamination of aquatic ecosystems. The high prevalence of *Citrobacter* in the tributaries underscores the need to improve wastewater management practices, and stricter regulations on industrial discharge and land use.

The study reported an overall isolation rate of 25% for the water samples from all three tributaries, with 25% showing growth for *Citrobacter*. This rate is significantly higher than a similar study in Helsinki, Finland which only recorded an isolation rate of 8.77% (Heljanko *et al.*, 2023). These results suggest a higher prevalence of *Citrobacter* contamination in Laguna de Bay's tributaries, indicating a potentially elevated risk to both environmental and public health. Furthermore, the Chi-square Goodness of Fit test revealed that there is no significant difference in the isolation of *Citrobacter*, implying consistent contamination levels. All three tributaries pose equal risks of *Citrobacter* infection to those who come in contact with them, particularly in activities like recreational water use, fishing, or agricultural practices near these tributaries. Heavy rains, common in the flood-prone province of Laguna, can exacerbate contamination risk that increases risk of health concerns for nearby communities (Lim, 2015). In such instances, the potential for *Citrobacter* contamination is heightened, posing additional health concerns for communities residing in flood-prone areas. These findings can be used to pinpoint potential areas for intervention to effectively reduce *Citrobacter* contamination in both duck eggs and nearby tributaries. Similarly, the lack of association between *Citrobacter* presence in tributaries and poultry farms suggests that addressing poultry farm waste management alone may not be sufficient to mitigate bacterial contamination in surrounding water bodies. Instead, a more comprehensive and widespread approach must be applied to reduce the risk of bacterial infection from both agricultural and recreational water sources.

The Chi-Square test for Independence was done to determine if the presence of *Citrobacter* in duck eggs from poultry farms was related to the presence of the bacteria in the nearby tributaries, or vice versa. The Chi-square test reported a p-value of 0.434, which is not less than 0.05. This means that there is no sufficient evidence to conclude that there is a significant relationship between the presence of *Citrobacter* in duck eggs from poultry farms and the presence of the bacteria in the nearby tributaries. In other words, the data does not provide strong support for the hypothesis that the presence of *Citrobacter* in duck eggs is associated with its presence in the tributaries. This means that the presence of *Citrobacter* in ducks is not due to the exposure to contaminated tributary waters and other sources of infection, such as the soil or feces of other animals, maybe the cause. Similarly, this also means that the *Citrobacter* contamination observed in the surrounding tributaries is not due to contamination by waste from the poultry farms. Instead, other sources such as agricultural runoff, or human sewage could be contributing to the presence of *Citrobacter* in the tributaries. These findings can be used to pinpoint potential areas for intervention to effectively reduce *Citrobacter* contamination in both duck eggs and nearby tributaries. Similarly, the lack of association between *Citrobacter* presence in tributaries and poultry farms suggests that addressing poultry farm waste management alone may not be sufficient to mitigate bacterial contamination in surrounding water bodies. Instead, a more comprehensive and widespread approach must be applied to reduce the risk of bacterial infection from both agricultural and recreational water sources.

The study also evaluated the antibiotic susceptibility patterns of the isolated *Citrobacter* bacteria. The results indicated that the isolated *Citrobacter* spp. were highly resistant to Azithromycin and Ampicillin classifying them as Multidrug-Resistant (MDR) Pathogens. Isolates showed most resistance to Azithromycin (90.91%). Notable resistance to Ampicillin were also observed, with 54.55% displaying high resistance and 18.18% displaying intermediate resistance. However, the isolates showed lower resistance to Tetracycline, with only 36.36% exhibiting resistance. The resistance pattern observed aligns with previous research, indicating the emerging trend of antibiotic resistance among *Citrobacter* bacteria. Liu *et al.* (2021) demonstrated *Citrobacter* is highly resistant to Azithromycin, penicillin and cephalosporins. Additionally, previous studies reported resistance to ampicillin, tazobactam, gentamicin, and cefazolin (Tariq *et al.*, 2023). The increasing resistance of *Citrobacter* observed is due to the widespread misuse of antibiotics in various community sectors, including agriculture and healthcare. Antibiotics are extensively used for prophylactic and growth-promoting purposes in livestock, leading to the development of antibiotic resistance, Moreover, inappropriate antibiotic prescribing practices and inadequate infection control measures exacerbate the problem. Antibiotics consumed by humans are excreted into sewage systems and treatment plants that contribute to the emergence of antibiotic-resistant bacteria posing a serious threat to human health (Afroz *et al.*, 2023; Samrot *et al.*, 2023).

*Citrobacter* infections can lead to various health implications particularly in high-risk populations such as infants and immunocompromised adults (Jabeen *et al.*, 2023; Emery *et al.*, 2020). The emergence of multi-drug resistant strains of *Citrobacter* poses a challenge in clinical management, especially in healthcare settings where broad-spectrum antibiotics are commonly used. The high resistance, particularly against Azithromycin, is concerning as it is often a first-line treatment for bacterial infections. Moreover, the intermediate resistance observed against Ampicillin further complicates treatment decisions, emphasizing the importance of conducting susceptibility testing to guide appropriate antibiotic therapy.

Effective strategies for mitigating contamination and minimizing the risk of infection from multidrug-resistant *Citrobacter* spp. in both duck eggs and tributaries should be applied consistently across all affected areas. These strategies may include improved hygiene practices during egg collection, storage, and transportation, regular monitoring of ducks for signs of infection and frequent monitoring of water quality to enable early detection and intervention. Public awareness campaigns can educate residents about the risks associated with contact with contaminated water and promote proper hygiene practices. Implementing regulations to control agricultural runoff and industrial discharge into the tributaries can help reduce contaminants. Moreover, identifying the antibiotics to which the strain is susceptible can aid in treatment choices. Combination antibiotic therapy may also be used against resistant strains and may aid in preventing the development of further resistance to antibiotics.

## Conclusion

The study marks the discovery of antibiotic resistant *Citrobacter* species in both tributaries of Laguna de Bay and poultry farm in Victoria, Laguna, Philippines. This discovery emphasizes the importance of addressing antibiotic resistance, as the recovered bacteria have varying susceptibility patterns to antibiotics such as Ampicillin, Azithromycin, and Tetracycline. The variation in susceptibility patterns across various environments underlines the complicated processes of antibiotic resistance spread, emphasizing the critical need for extensive observation and approaches to intervention. This discovery holds significant consequences for public health and food safety, as antibiotic resistant bacteria in tributaries and duck eggs pose a serious risk not just to the environment, but most especially to human health.

**Recommendation(s)**

In the current paper at hand, the sampling size of the study leans more on the smaller population that yielded no significance regarding the presence of *Citrobacter* spp. on most samples. In contrast, samples that yielded a positive significance may still indicate discrepancy as the p-value used was near the result value yielded, which indicates that isolating *Citrobacter*-positive samples may only be by fluke. Hence, future studies may opt to increase the sample size so that the value from the samples tested will more likely yield a greater deviation from the p-value to cancel out the possibility of fluke significance within samples.

Additionally, future researchers can opt to investigate further the dynamics of *Citrobacter* contamination in different ecological niches, notably, water sources. As identifying the sources and pathways of bacterial contamination and assessing the impact of environmental factors including urbanization, agricultural runoff and waste management methods are critical. This improved understanding would help shape strategies for reducing pollution risks and protecting the environment and public health. Given the possible consequences of elevated contamination levels, more research is needed to inform successful management and conservation efforts for aquatic ecosystems.

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