Occurrence of antimicrobial resistant *Escherichia coli* in waterways of southeast Queensland, Australia

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Abstract

Antimicrobial resistance is a global health issue. The discharge, maintenance and transfer of antimicrobial resistance to the aquatic environment and the risk this presents is relatively unknown. This work describes the presence and distribution of antimicrobial resistant *Escherichia coli* in surface waters of seven rivers in south east Queensland, Australia. Resistance to four antimicrobials (ampicillin, tetracycline, sulfamethoxazole and ciprofloxacin) was determined using a method combining chromogenic microbial detection with breakpoint antimicrobial resistance analysis. *E. coli* concentration and antimicrobial resistance was significantly higher ($p < 0.05$) at sites receiving WWTP discharge compared to sites with no direct WWTP discharge. There was a positive correlation ($\rho < 0.001$, $r = 0.95$) between *E. coli* concentration and volume of WWTP discharge into these rivers. This would suggest that WWTPs are the primary source of antimicrobial resistant *E. coli* in the study region. There was no correlation ($\rho > 0.05$) between WWTP discharge volume into rivers and incidence of antimicrobial resistance for ampicillin, sulfamethoxazole and tetracycline. However, there was a positive correlation ($\rho < 0.01$, $r = 0.82$) between incidence of ciprofloxacin resistance and WWTP discharge volume into rivers. This would suggest that *E. coli* resistance to ciprofloxacin found in the study area is driven primarily by WWTP discharge, with resistance to other investigated antimicrobials influenced by additional sources and/or drivers.

Keywords: antimicrobial, antibiotic, resistance, wastewater, aquatic, environment, Australia

1. Introduction

The World Health Organisation has described antimicrobial resistance as one of the key global health issues and a profound threat to human health (1). Through overuse and poor management of antimicrobials, we now find ourselves in the precarious situation where careful management of antimicrobials is essential to combat the widespread emergence of resistant bacteria. Global responses to this have seen the development of taskforces such as the WHO and Australian Strategic and Technical Advisory Groups on Antimicrobial Resistance and the production of Strategic Action Plans in an attempt to address the issue (1, 2).

While much of this attention has been directed towards management of antimicrobial use and monitoring the prevalence of bacterial resistance within the community, antimicrobials and ARB in the aquatic environment have received comparatively little attention. This is surprising given the reliance on our water resources and the potential for the spread and maintenance of bacterial resistance to antimicrobials in this environment. Antimicrobials have recently been identified quite ubiquitously in the aquatic environment at sub-inhibitory concentrations (3-7). However, knowledge of sub-inhibitory effects of antimicrobials on environmental bacteria is scarce and contradictory (8-11).

Antimicrobial resistant bacteria can be considered a contaminant of emerging concern through their regular discharge to the aquatic environment from wastewater treatment plants (WWTPs) (12-18), aquaculture (19-24) and runoff from agricultural regions (25-31). Studies investigating the prevalence of antimicrobial resistance in the aquatic environment are limited, though they are starting to appear with increasing frequency (32-40).

Traditionally, the faecal coliform *Escherichia coli* has been the foremost indicator of faecal contamination in water quality monitoring. *Escherichia coli* is a causative agent of a number of infections in both humans (41, 42) and animals (43, 44), mostly caused by a number of pathogenic strains, such as *E. coli* O157:H7 (45). Additionally, *E. coli* has been shown as a significant reservoir of genes coding for antimicrobial drug resistance and therefore represents a useful indicator for antimicrobial resistance in bacterial communities (46). Therefore studies of antimicrobial resistance patterns in *E. coli* have implications on many levels since they can be used as an indicator for overall antimicrobial resistance and findings have direct impacts on risks to public health.

The aim of this study was to investigate the levels of antimicrobial resistance in *E. coli* isolated from surface waters in South-East Queensland. This information will contribute to our understanding of antimicrobial resistance in the aquatic environment and the potential environmental and public health risk associated with exposure to aquatic bacteria.

2. Materials and Methods

2.1. Preparation of media

Media was prepared as described in Watkinson *et. al. 2007* (47). Four antimicrobials were chosen for susceptibility testing: ampicillin; tetracycline; sulfamethoxazole; and ciprofloxacin due to their clinical relevance and their frequent identification in the aquatic environment (3, 6, 48).

2.2. Study Area

Water samples were collected on three occasions from 57 sites (n = 171 samples) across nine rivers in South-East Queensland, Australia (Figure 1). These rivers comprise

of freshwater, estuarine and marine sections and discharge either into a large semi-enclosed bay (Moreton Bay) or directly into the Pacific Ocean. Study sites were located within the estuarine-marine zones of these rivers and were classified as point source (PS, 18 sites) or non-point source (NPS, 33 sites) sites based on their proximity (< 50 m) to discharges with PS sites directly receiving discharge from a WWTP and NPS sites having no direct input. Each site was sampled on three occasions over a 6-month period. To assist in interpreting results, major practices within each river are summarised in Table 1.

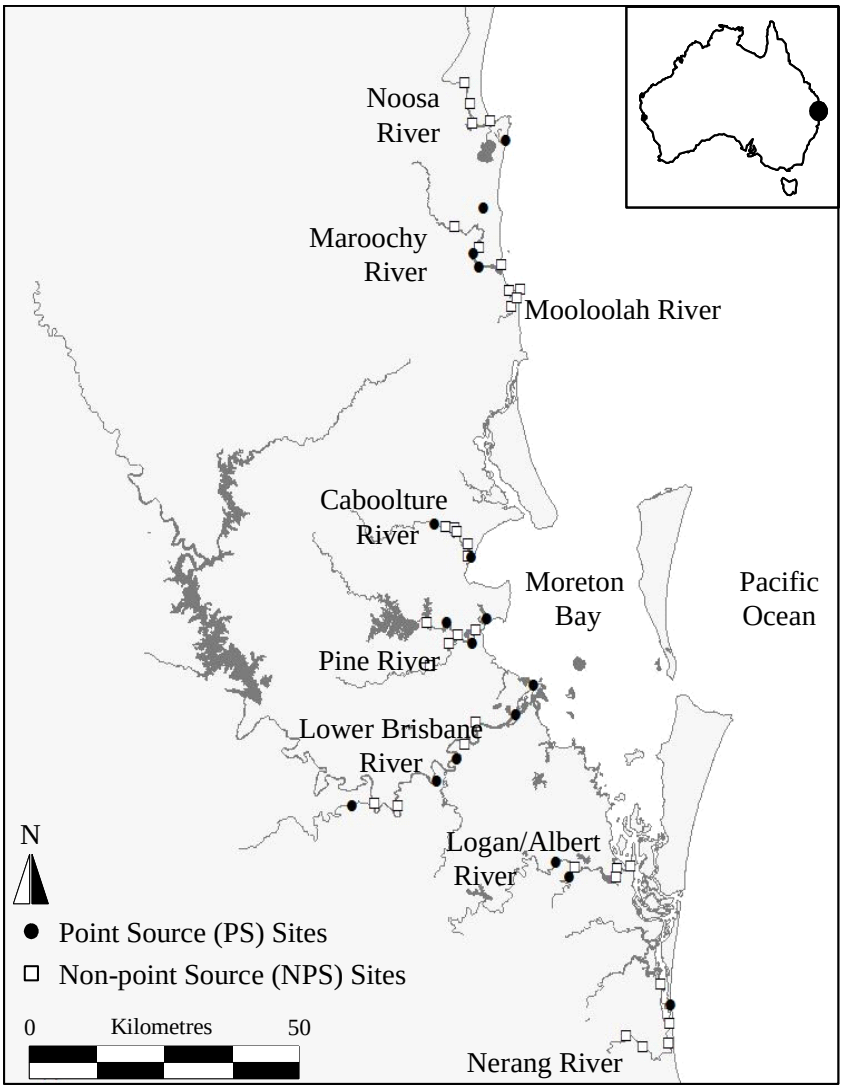

Figure 1: Location of eight investigated river systems in South- East Queensland. Point source and non-point source sites are indicated.

Table 1: Characteristics of investigated rivers

	Catchment[a]				Dry Weather Effluent Discharge	
River	Area (km^2)	Population	Total Length of Streams (kms)	Major Land Uses	(ML d^{-1})	Discharge Point
Noosa	831	36,000	600	U, NB, CA, G	0	Pacific Ocean
Maroochy	636	136,000	438	U, NB, G, IA	37	Pacific Ocean
Mooloolah	223	83,000	322	U, NB, G, RR, MF	0	Pacific Ocean
Caboolture	468	115,000	795	U, RR, G, IA	18	Moreton Bay
Pine	816	177,000	553	U, RR, G, NB	35	Moreton Bay
Lower Brisbane	1,171	888,000	845	U, RR, NB, G	282	Moreton Bay
Logan/ Albert	4,133	407,000	2,828	RR, U, G, IA	50	Moreton Bay
Nerang	498	455,000	928	U, RR, IA, NB	0[b]	Pacific Ocean

NB=Native Bush; CA=Conservation Area; G=Grazing; U=Urban; IA=Intensive Agriculture; RR= Rural Residential; MF=Managed Forests

[a] SEQRWQMS (2001)

[b] effluent discharge at river mouth on ebb tide

2.3. Susceptibility Testing

One litre of surface water was collected at each site at approximately 0.5 m depth in autoclaved glass amber jar and transported back to the laboratory on ice. A dilution series of 100, 10, 1, 0.1 and 0.01 ml was filtered through 0.22 µm membrane filters (Millepore, Bedford, MA, USA), the latter three volumes combined with 10 ml peptone. All filters were rinsed with peptone. A dilution series was prepared for the control and each of the antimicrobial treatments for each site and plates were incubated at 35°C for 24 hrs. After incubation, blue colonies were counted under ambient light and confirmed under long-wave UV light (366 nm). For each antimicrobial, percent resistance was calculated by directly comparing counts on the antimicrobial plate with corresponding counts on the control plate:

$$\% \text{ Resistance} = \frac{[E.coli] \text{ antibiotic plate}}{[E.coli] \text{ control plate}} \times 100 \qquad (1)$$

Escherichia coli ATCC 25922 was chosen as a control strain carrying no resistance to selected antimicrobials. Ambient *E. coli* concentrations were calculated from control plate results for each site. A one-way ANOVA was used to determine significant differences ($P < 0.05$) for *E. coli* concentrations and each antimicrobial resistance between sources. A one-way ANOVA followed by a post hoc Tukey's HSD means test was used to determine significant differences ($P < 0.01$) between *E. coli* concentrations and each antimicrobial between rivers. Spearman's rank correlations ($\rho < 0.05$) were performed with total riverine effluent discharge and river catchment population investigated for correlation with median riverine *E. coli* concentration, and antimicrobial resistance.

3. Results

Recorded concentrations of *E. coli* varied greatly both within and between investigated rivers (Figure 2a). Highest *E. coli* concentrations were recorded in the lower Brisbane River (3330 cfu 100 ml^{-1}) which also had the highest median *E. coli* concentration (195 cfu 100 ml^{-1}) of all investigated rivers. The Maroochy and Pine

Rivers shared the second highest *E. coli* concentrations (95 cfu 100 ml^{-1}) followed by the Caboolture (55 cfu 100 ml^{-1}) > Logan (48 cfu 100 ml^{-1}) > Tweed (41 cfu 100 ml^{-1}) > Mooloolah (16 cfu 100 ml^{-1}) > Nerang (15 cfu 100 ml^{-1}) > Noosa (3 cfu 100 ml^{-1}). A median concentration of 55 cfu 100 ml^{-1} was recorded for the entire study.

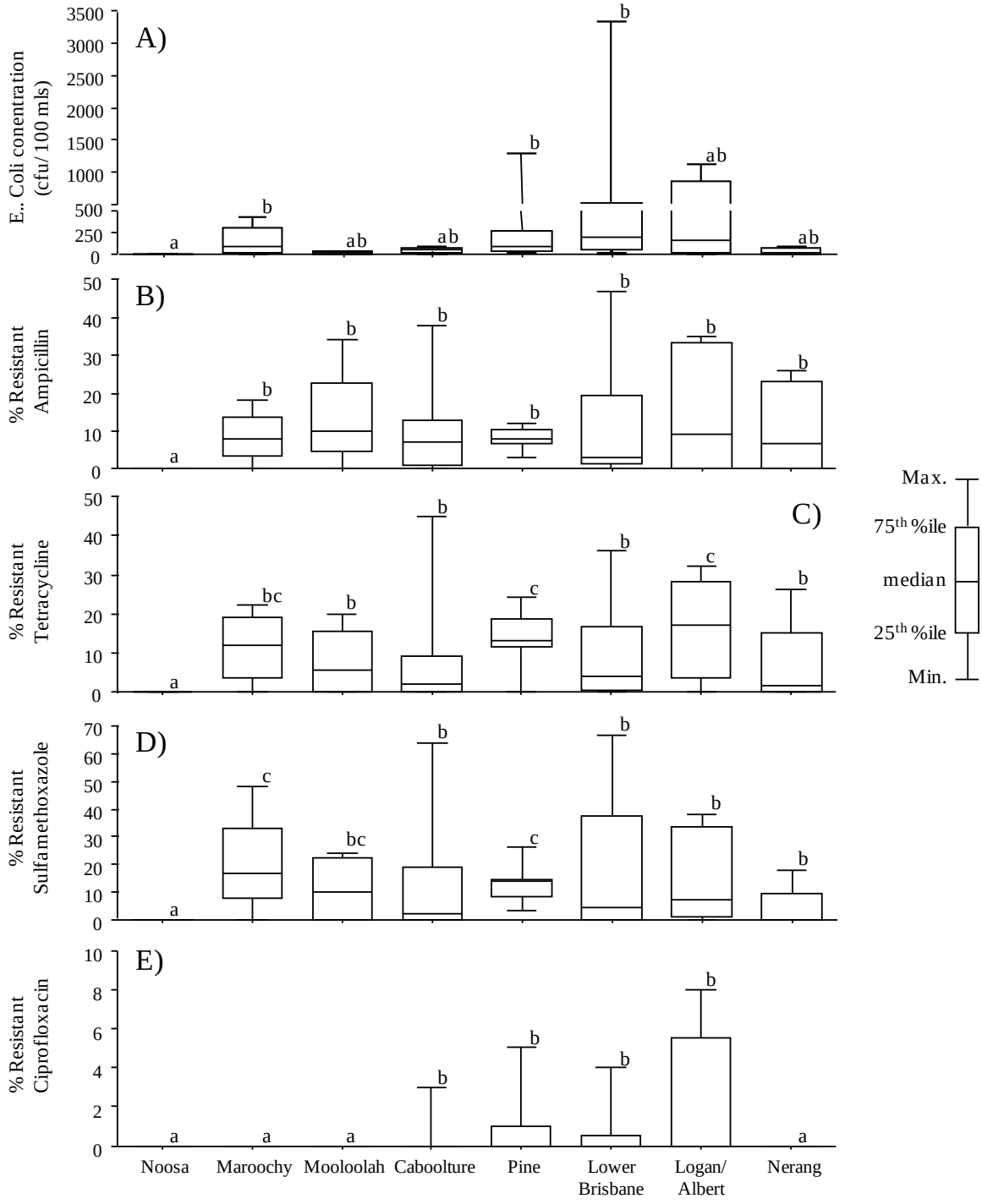

Figure 2: Comparison of South- East Queensland Rivers for A) average *E. coli* concentrations; B) % Ampicillin resistance; C) % Tetracycline resistance; D) % Sulfamethoxazole resistance; and E) % Ciprofloxacin resistance. Small different letters indicate significant differences ($p<0.05$) between rivers for each treatment.

Ampicillin resistance in *E. coli* was widespread but typically low with median values for all investigated rivers below 15% (Figure 2b). The highest median value was observed in the Logan/Albert River (14%) followed by the Mooloolah > lower Brisbane > Nerang > Caboolture > Pine and Maroochy > Noosa Rivers. The highest site ampicillin resistance was recorded in the lower Brisbane River (48%) with similar elevated values recorded across other sites in the Mooloolah, Caboolture, Logan/Albert and Nerang Rivers.

Overall resistance to tetracycline was approximately half that seen with ampicillin with a median value of 7% (Figure 2c). The highest median value was recorded in the Logan/Albert (16%) followed by the Pine > Maroochy > lower Brisbane > Mooloolah > Caboolture > Nerang > Noosa Rivers. Maximum tetracycline resistance was recorded at a site in the Caboolture River (45%).

Resistance to sulfamethoxazole in all investigated rivers was nearly half of what was observed with tetracycline (4%) (Figure 2d). Highest median sulfamethoxazole resistance was seen in the Maroochy River (19%) followed by the lower Brisbane > Logan/Albert > Pine > Caboolture > Mooloolah > Nerang > Noosa Rivers. Maximum site sulfamethoxazole resistance was seen in the lower Brisbane River (67%) with elevated resistance also recorded at sites within the Maroochy and Caboolture Rivers.

E. coli resistance to ciprofloxacin was very low with a median value of 0% overall with only the lower Brisbane (1%), Logan/Albert (2%) and Pine (1%) Rivers demonstrating medians greater than 0% (Figure 2e). Maximum site resistance to ciprofloxacin was observed in the Logan/Albert River (8%).

Point-source (PS) sites appeared to be the primary source of both *E. coli* and antimicrobial resistance with significantly greater ($p < 0.05$) colony counts and percent resistance than non-point source sites (NPS) (Figure 3).

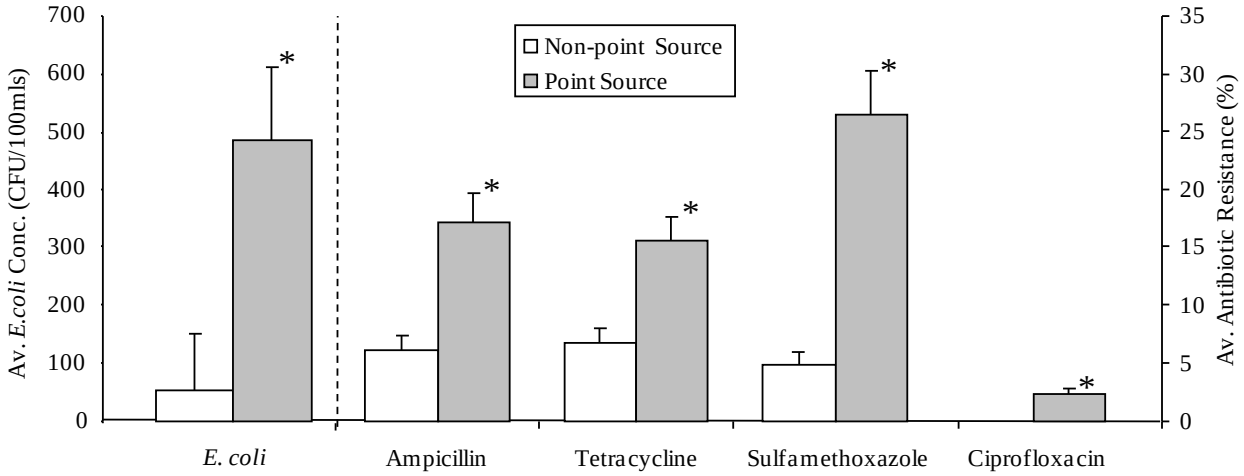

Figure 3: Comparison of *Escherichia coli* concentrations and associated antimicrobial resistance between investigated point and non-point source sites. *values indicate significant differences ($p < 0.05$) between sources.

Significant correlations ($\rho < 0.05$) were identified for *E. coli* concentration and both river WWTP discharge and river catchment population (Table 2). Significant correlations ($\rho < 0.05$) were identified between median riverine ciprofloxacin resistance and total river WWTP discharge. No other relationships were significant ($\rho > 0.05$).

Table 2: Spearman's rank correlations for selected river parameters and *E. coli* concentration and antimicrobial resistance.

	River WWTP Discharge Volume		River Population	
Median River Parameter	Spearman r	ρ value	Spearman r	ρ value
E.coli Concentration	0.95	0.001	0.76	0.03
Ampicillin Resistance	0.69	0.06	0.32	0.42
Tetracycline Resistance	0.61	0.11	0.26	0.53
Sulfamethoxazole Resistance	0.21	0.6	0.08	0.83
Ciprofloxacin Resistance	0.82	0.01	0.49	0.21

4. Discussion

It is well established in the literature that the presence of *Escherichia coli* in environmental surface waters is associated with degraded water quality (49-51). This study supports the literature with elevated *E. coli* found in the more degraded catchments. For example, the lower Brisbane and Logan/Albert Rivers frequently demonstrate poor water quality with elevated nutrients and suspended sediments, and low dissolved oxygen (52) and also recorded the highest median *E. coli* concentrations in this study. In contrast, the Noosa, Mooloolah and Nerang Rivers are frequently characterised by excellent water quality (52) and exhibited very low *E. coli* concentrations. While there are many reasons for these differences in water quality, such as varying flow and catchment land use, WWTP discharge has been implicated as a major contributor to these degraded rivers (53). This study has also indicated that WWTP discharge is highly correlated with *E. coli* concentrations.

Research has shown that antimicrobial resistance increases with the level of exposure of faecal bacteria from humans (54) which may further support the increase in resistance with proximity to WWTP discharge. Further work would suggest wastewater treatment plants offer a favourable environment for the transfer and exchange of genetic information and could enhance the development of antimicrobial resistance (12, 55-58). Additionally, Mao *et al 2015* (59) demonstrated that bacteria surviving the treatment process (including chlorination) have a greater abundance of antimicrobial resistance genes (ARG) and suggest this may be due to the selective pressure of significant concentrations of antimicrobials through the treatment stream and Novo *et al 2013* (60) showed that resistance was positively correlated with the occurrence of antimicrobial residues. There is some evidence to suggest that ARG carrying *E.coli* have a higher number of virulence genes and therefore may present a greater risk for re-association with the human microflora (61).

There was no correlation between volume of wastewater discharge and prevalence of antimicrobial resistance for all antimicrobials investigated, with the exception of ciprofloxacin. This would suggest that other factors are more of an influence on the rates of antimicrobial resistance seen within investigated *E. coli*.

This may include antimicrobial use and concentration within different source catchments or treatment and disinfection practices at WWTPs. Reinthaler *et al*. *2003* (12) have previously proposed that wastewater treatment will select for more antimicrobial resistant organisms and that effluent disinfection, while lowering the total bacterial count, has also been shown to increase the prevalence of antimicrobial-resistant bacteria (62, 63) and not be an effective control for antimicrobial resistance at typical doses (18, 64). Therefore, variations in treatment and disinfection are likely to lead to variations in antimicrobial resistance in discharged organisms.

Quinolone resistance amongst *E. coli* was also significantly lower ($p < 0.05$) compared to the resistance to other investigated antimicrobials. There are a number of possible explanations for this and the difference is probably driven by a combination of these. Firstly, quinolone antimicrobials were only introduced into clinical practice in the early 1970s, and as the first synthetic antimicrobials, the development of resistance to these drugs is mostly limited to spontaneous mutants that alter target protein or increase pump expression (65) and the spread of this resistance is relatively restricted within the bacterial community. In contrast resistance to the β-lactams, originally derived from a mould of the genus *Penicillium*, is moderated by highly specific catalytic enzymes that were probably present before human development of antimicrobials, either in other roles (for instance, housekeeping genes regulating sugar kinases or acetyl-transferases also confer aminoglycoside resistance) or as part of microbial competition for an ecological niche (66). Secondly, due to the comparatively low incidence of clinical resistance and the fact their synthetic nature makes production relatively expensive, quinolones are generally reserved for treatment of infections resistant to cheaper drugs (67). And lastly, due to their restricted use in agriculture as growth promoters, there is little opportunity for the development and spread of resistance from low-dose exposure to these antimicrobials as is typically seen with other agricultural antimicrobials. This is highlighted by the development of vancomycin resistance in *Enterococci* following the use of avoparcin as a growth promoter (68).

E. coli resistance to ciprofloxacin was the only investigated resistance correlated with WWTP discharge volume. Previous investigations have identified a strong correlation between the presence of quinolone antimicrobials, such as ciprofloxacin, norfloxacin and enrofloxacin, and their proximity to WWTP discharge (7). The use of quinolone antimicrobials have recently been banned in agriculture in Australia with their application confined solely for human and domestic animal treatment (69), indicating a potential driver for their confinement to WWTP discharge. While the level of resistance to ciprofloxacin appeared low it was comparable to clinical estimates in both Queensland (3.4%) and Australia (3.4%), which are very low themselves on a global scale (70).

It has been previously demonstrated that sites receiving wastewater discharge (PS) typically have a higher incidence of antimicrobial resistance amongst their bacterial populations than NPS sites (36, 39, 47, 71, 72) and this study is no exception. As the majority of bacteria in wastewater is of faecal origin, is it highly likely that the antimicrobial resistance seen in this study is acquired either within the original host or during wastewater treatment; not from within receiving environment. However the potential role of the environment in the transfer, development and storage of antimicrobial resistance should not be ignored. Additionally, the selective pressure

of antimicrobial residues present in these waters may help maintain or even further develop resistance (73). Regulation of these PS facilities does not currently consider antimicrobials or the level of resistance in discharged bacteria in effluent licensing. Recent advancements and flexibility in the licensing approach has allowed for significant innovation in the region to include voluntary market-based mechanisms for nutrient management to provide a better environmental and cost-effective outcome for eutrophication (74). It will be important, however, in the future to ensure this flexibility adequately considers the role these point sources may play in the antimicrobial dissemination of resistance, how to mitigate this process and its link to other aspects of the water cycle (e.g. Irrigation and drinking water).

Recent studies have identified an important environmental reservoir of antimicrobial-resistance determinants, termed the antimicrobial resistome, in soil micro-organisms (75). As the environment, particularly the soil microbial community, is the primary source of the antimicrobials we develop, it is not surprising that amongst these communities strategies have evolved to resist their actions. Even antimicrobial producers themselves harbor resistance elements for self-protection that are often clustered in operons (76). D'Costa and colleagues (76) recently screened 480 soil *Streptomyces* representatives against 21 antimicrobials (both natural and semisynthetic) and found that without exception, all strains demonstrated some level of multi-resistance. Dantas *et. al. 2008* (75) have even gone so far as to show that soil bacteria can utilize a number of antimicrobials as a carbon source. This may have implications for the use of effluent to irrigate pasture and crops.

While it is suggested that most ARG originated from environmental isolates, due to the presence of clinically relevant ARG on the chromosome of environmental bacteria (77), this is significantly different to the clinical role ARG play through rapid horizontal and mobile transfer on genetic elements, and then how these elements may be maintained, transferred or further developed in the environmental setting. Czekalski *et al 2015* (39) has demonstrated the potential for freshwater lakes to preserve ARG and that background levels of resistance may be influenced by low level anthropogenic activities. Further, studies have shown the similarity of genes encoding for CTX-M, an extended spectrum β-lactamase found in clinical isolates, with chromosomal β-lactamases from an environmental *Kluyvera* spp. (78, 79) and quinolone resistance (qnrA) on the chromosome of *Shewanella algae* (80). Given the relatedness of many of these environmental microbes to pathogenic individuals, the further study of this environmental resistome, and the mechanisms driving it, could hold the key to the development of new means to combat antimicrobial resistance.

5. Conclusions

The aquatic environment is becoming of increasing interest as a sink for antimicrobial resistant bacteria due to the regular release of our waste streams (sewage, aquaculture and agricultural) carrying antimicrobial residues and antimicrobial resistant bacteria. This study has identified antimicrobial resistant *E. coli* in rivers of South-East Queensland with resistance greatest at sites receiving discharge from WWTPs. The evidence presented in this study highlights the need for investigations into the mechanism of antimicrobial resistance of discharged *E. coli* and the potential for this resistance to be transferred and maintained to the native bacterial population.

A modern-day cholera pandemic in Ecuador affected an estimated 8000 people and was highlighted by the presence of a multi-resistance strain of *V. cholera*, which had been recently identified in multiple areas around the globe (81). While initial concerns around the use of antimicrobials in local shrimp farms and its potential for the development of this resistance were unfounded, the emergence of multiple antimicrobial resistance in a virulent opportunistic pathogen found in the environment highlights the potential risk of the presence of antimicrobial resistance in the aquatic environment. The impact of these observations on public health and the potential for the emergence and spread of antimicrobial resistance through the aquatic environment demands further investigation to maintain our vigilance against the spread of infectious disease. While the battles rage on the clinical front as the justified primary focus against antimicrobial resistance, we cannot lose sight of the importance of the strategic role of the environment if we are to win the war.

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