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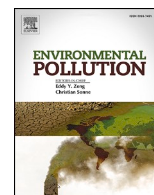

Indigenous food sources as vectors of *Escherichia coli* and antibiotic resistance☆

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ABSTRACT

The contamination of surface waters by fecal bacteria, measured by the number of *Escherichia coli*, is a significant public health issue. When these bacteria are also resistant to antimicrobials, infections are more complicated to treat. While water is regularly tested at recreational sites, wild-harvested foods, known as mahinga kai by the indigenous Māori people of Aotearoa New Zealand, are commonly overlooked as a source of exposure to potential pathogens and antimicrobial resistance (AMR). We investigate two likely sources of risk from harvesting aquatic wild foods. The first is water contact, and the second is contact with/ingestion of the harvest. We used *E. coli* as a proxy for microbial water quality at harvesting sites. Two popular mahinga kai species were also harvested and assessed. We found antibiotic-resistant bacteria on watercress (*Nasturtium officinale*) and cockles (*Austrovenus stutchburyi*). One-third of *E. coli* isolates were conjugative donors of at least one resistance phenotype. Tank experiments were used to track the internalization of *E. coli* by Greenshell/lip mussels (*Perna canaliculus*). Greenshell mussels kept at environmentally relevant concentrations of *E. coli* were colonized to levels considered unsafe for human consumption in 24 h. Finally, we measured horizontal gene transfer between bacteria within the shellfish, what we termed 'intra-shellular' conjugation. The transmission frequency of plasmid RP4 was significantly higher in mussels than in water alone. Our results indicate that shellfish could promote the dissemination of antibiotic resistance. They highlight the need to limit or reduce human pathogenic bacteria where food is gathered.

1. Introduction

Bacteria from human or animal feces are commonly observed in freshwater and coastal environments in Aotearoa New Zealand (Cornelisen et al., 2011; Larned et al., 2016; Land, Air, Water Aotearoa (LAWA), 2022b). Contaminating fecal coliforms are increasingly found to be antibiotic resistant (Burgess et al., 2021; Cooke, 1976a, 1976b; Larned et al., 2016; Paull, 2021; van Hamelsveld et al., 2019; van Hamelsveld et al., 2022; Winkworth-Lawrence & Lange, 2016; Winkworth, 2013).

Antimicrobial resistance/resistant (AMR) bacteria are recognized as a safety threat to water users worldwide (Nappier et al., 2020; O'Flaherty et al., 2019). Exposure pathways can include wild foods harvested from fresh and salt water (King et al., 2013; Schar et al., 2021). Māori refer to these foods as mahinga kai: foods and resources and the places where these are grown or extracted (Tau, 1990). Popular mahinga kai species include shellfish and freshwater plants such as watercress (Guy et al., 2021; Kainamu-Murchie et al., 2018; King & Lake, 2013), which are eaten raw or cooked, on their own or as ingredients in traditional dishes (Dixon et al., 2007; Tau, 1990).

Levels of fecal bacteria in mahinga kai can be high enough to make them unsafe for human consumption (Bell, 2019; Cornelisen et al., 2011; Donnison et al., 2009; Edmonds & Hawke, 2004). Contamination of food by fecal indicator bacteria such as *E. coli* is used as a proxy for potential exposure to pathogenic bacteria (Ministry for the Environment, 2017). If these pathogens are also resistant to antibiotics, the risk of adverse outcomes from eating contaminated foods is higher because the effectiveness of clinically relevant antibiotics may be reduced (Cassini et al.,

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2019; Goldstein et al., 2019).

The New Zealand government issues guidance on the use of aquatic animals harvested for food (Ministry for Primary Industries, 2022). However, at present, the guidance is based on a risk assessment that does not consider AMR; consequently, the levels of AMR in harvested foods are not determined on a regular basis. Indeed, the last report on mahinga kai was published almost 50 years ago (Cooke, 1976a, 1976b). There is reason to think that mahinga kai may be impacted by AMR. For example, overseas studies have established a link between contaminated watercress and infection by AMR *E. coli* (Jenkins et al., 2015; Ukah et al., 2018).

Bacteria populations can develop resistance after exposure to antibiotics from their environment, e.g., in medical settings or from livestock (Collection, 2015; Levin & Andreasen, 1999). Resistance determinants can be transferred between bacteria if they are located on conjugative plasmids (Amábile-Cuevas & Heinemann, 2004). These plasmids contain genes relevant for their own transfer between bacteria but can also feature a collection of other genes such as virulence determinants, pathogenicity islands or antibiotic resistance determinants (Villa et al., 2010).

We have assessed *E. coli* and AMR *E. coli* in watercress, cockles, and the sites where they are traditionally harvested in Waitaha/North Canterbury, New Zealand (Fig. 1). However, our findings are relevant well beyond Aotearoa New Zealand.

Due to environmental conditions or human activity, mahinga kai sites can experience transient flushes of fecal bacteria (Bell, 2019; Cornelisen et al., 2011). These spikes can easily go undetected because routine monitoring of water quality is not continuous. We therefore performed laboratory experiments using Greenshell mussels (*Perna canaliculus*) and seawater inoculated with *E. coli* (Dansted, 2021) to track the uptake of *E. coli* by mussels.

With a focus on how the animal might contribute to more efficient AMR gene transfer, we measured conjugation frequency inside mussels. The aim was to determine whether plasmid transmission frequencies were higher in mussels or their feces than in the water. This is the first study to assess whether shellfish could promote the spread of AMR through conjugative transmission of AMR genes, but the hypothesis has been posited before (Grevskott et al., 2017). Filter feeders such as shellfish internally concentrate bacteria at least ten-fold (Jozić et al., 2012), and the presence of AMR, conjugative bacteria inside shellfish has been confirmed (Grevskott et al., 2017). Bacteria population density was previously shown to be an important factor in determining conjugation frequency (Goodman et al., 1993). Inside shellfish, different species of bacteria may be in close proximity to one another at concentrations that do not occur in the water column and contact-dependent gene transfer by conjugation therefore might increase.

Besides gene transfer, conjugative plasmids can alter a variety of host phenotypes, including the tendency to clump or cling to surroundings (Amábile-Cuevas & Chicurel, 1992). We therefore tested whether bacteria infected with conjugative plasmids were more likely to be taken up by the mussels.

Our work provides an up-to-date assessment of the infection risks associated with AMR bacteria in mahinga kai. To achieve this, we addressed the following questions:

1. Are AMR bacteria found in/on mahinga kai species and water from the sites of harvest?

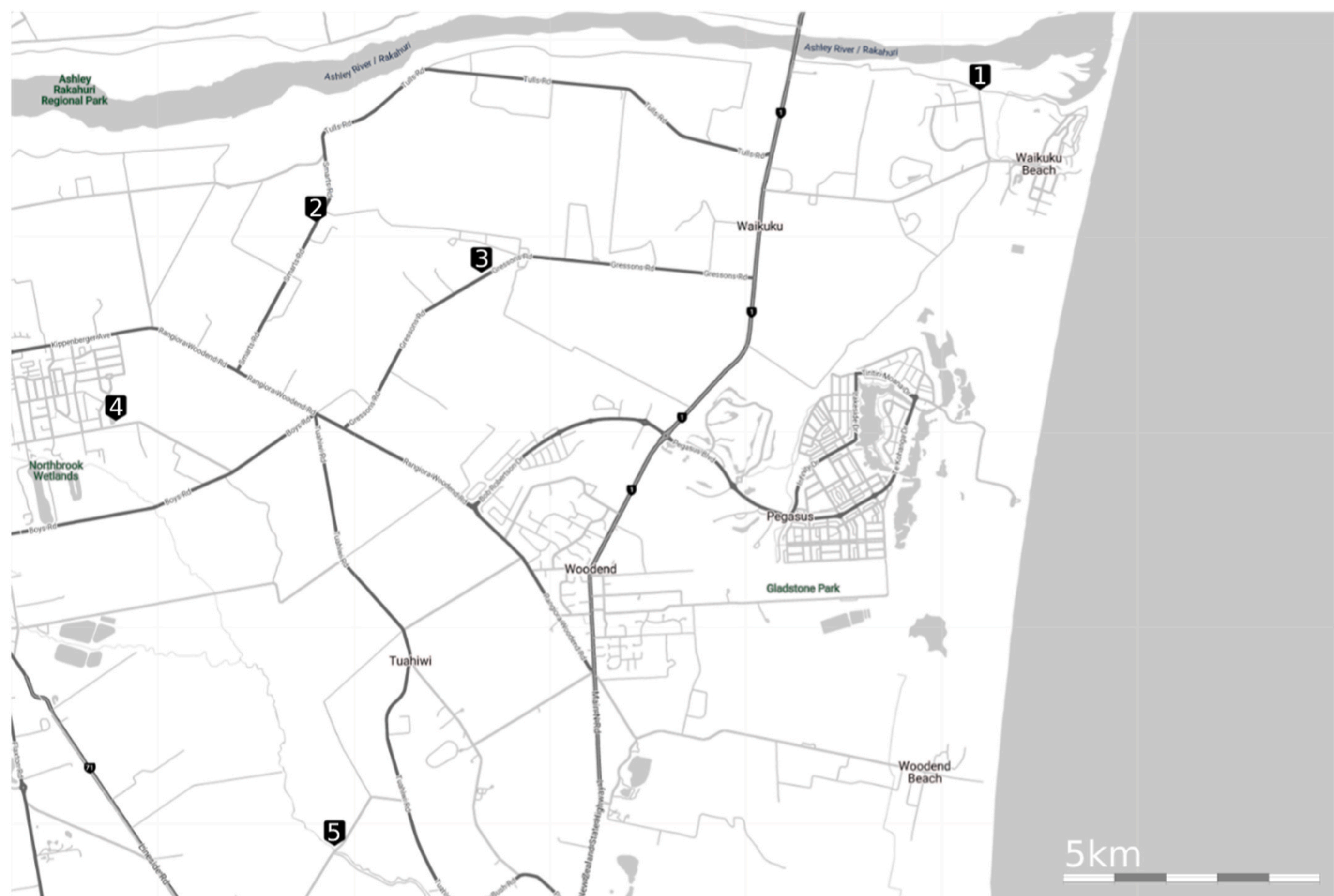

Fig. 1. Map of sampling locations in Waitaha/North Canterbury. 1: Te Akaaka (Ashley River Estuary); 2: Smarts Road; 3: Gressons Road; 4: Koura Reserve, 5: Ruataniwha at Bramleys Road Bridge.

2. Do Greenshell mussels hold or concentrate *E. coli*?
3. Do *E. coli* inside mussels conjugate and through this transmit antibiotic-resistance genes?

2. Methods

2.1. Environmental water sampling

Our study sites were chosen following discussion with Te Ngāi Tūāhuriri, the indigenous people of North Canterbury. The sites are where mahinga kai are commonly extracted. Tuaki/little-neck cockles (*Austrovenus stutchburyi*) were collected from Te Akaaka, the estuary at Waikuku Beach (Fig. 1). This site is a popular spot for cockle picking, fishing, and swimming. Wātākirihi/watercress (*Nasturtium officinale*) was collected near the Rangiora township. Watercress is frequently harvested at all four sites, especially the Koura Reserve and Ruataniwha/Cam River (Fig. 1). Consistent with mahinga kai being both the food and the place of harvest (Tau, 1990), water samples were also collected.

Quadruplicate 1 L water samples were taken alongside samples of watercress or cockles as they were collected. Water was collected by grab sampling in the flowing portion of the stream or river using sterile 1 L plastic bottles. Samples were stored on ice until processing. The water was filtered to isolate *E. coli* as previously described (van Hamelsveld et al., 2019) and as described in (United States Environemntal Protection Agency, 2002). In short, 100 mL was filtered and membrane filters were placed on Tryptone-Bile-X-Glucuronide (TBX) agar plates without antibiotic supplementation or supplemented with one of ciprofloxacin (1 μg mL^{-1}), chloramphenicol (6 μg mL^{-1}), or ampicillin (20 μg mL^{-1}). The plates were incubated for 12–16 h at 44 °C. If the bacteria were too numerous to be counted, the population density was conservatively recorded as 10^3 colony-forming units (CFU) 100 mL^{-1}.

A selection of presumptive *E. coli* (blue) colonies from all sampling conditions were isolated and stored at −80 °C for future analysis.

2.2. Watercress sampling procedure

E. coli in watercress was measured from watercress gathered seasonally from four sites between November 2019 and January 2021 (Fig. 1). Watercress was sampled in triplicate according to a Māori protocol (Donnison et al., 2009). Healthy plants without flowers were selected. Stems were cut to avoid disturbing the roots. Plants were gathered into 50 g bunches and rinsed in the stream flow. Bunches were kept in plastic bags on ice until processing.

In the laboratory, watercress leaves and stems were weighed into 50 g batches and blended with 150 mL sterile phosphate-buffered saline (PBS) for 2 min using a sterile kitchen blender. 1 mL aliquots of each sample were then spread onto five separate TBX agar plates. When the predicted number of *E. coli* was greater than 10^3 CFU 50 g^{-1}, this was treated as 10^3 CFU 50 g^{-1}, to align with water sample data.

2.3. Cockle sampling procedure

Cockles were sampled according to a Māori protocol (*Te Rūnanga* o Ngāi *Tahu, 2015*). At low tide, cockles were located in the sand by touch. Triplicate samples of approximately 20 cockles were taken at the sampling site. Sand was removed by rinsing in the nearest area of flowing water before the cockles were stored in sterile plastic bottles. Only large cockles at least 5 cm across were taken. Water samples were collected from free-flowing water nearest the cockle bed, and from the main river flow about 1.5 km upstream of the cockle beds. Samples were kept on ice until processing.

E. coli were enumerated from cockle samples as described by (Bell, 2019). In short, cockles were cleaned using paper towels misted with 70% ethanol, then individuals were shucked until 100 g of meat and water from inside the shell (shellfish liquor) was collected. Samples were blended for 2 min with 100 mL sterile PBS. To enumerate *E. coli*, five 1 mL aliquots of each sample were spread onto TBX agar plates.

2.4. Strains, plasmids, media, and chemicals

Strains of bacteria and plasmids are listed in Supplementary Table 1. *E. coli* was routinely cultured on TBX agar (Himedia) or Luria–Bertani (LB) broth (Invitrogen). Antibiotics were purchased from Becton Dickinson, Sigma, Oxoid or Duchefa. Stock solutions were prepared according to the manufacturers' instructions.

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using the supplemented agar plate procedure described in van Hamelsveld et al. (2019). *E. coli* isolates from water, watercress and cockles were tested against a panel of antibiotics at concentrations corresponding to the Clinical Laboratory Standards Institute (CLSI) susceptible, intermediate, and resistant concentrations for each antibiotic (CLSI, 2022). The antibiotics used were chloramphenicol, tetracycline, ciprofloxacin, trimethoprim, gentamicin, kanamycin, ampicillin, cefotaxime, and ceftazidime. Strain BW25113 (Supplementary Table 1) was used as a control.

2.6. Conjugation assays

A random selection of *E. coli* displaying resistance to one or more antibiotics at concentrations equal to or exceeding the CLSI breakpoint concentration (CLSI, 2022) were tested for the ability to transmit their resistance phenotype(s) by conjugation as described previously (Heinemann & Ankenbauer, 1993; van Hamelsveld et al., 2019; van Hamelsveld et al., 2022). The rifampicin resistant strain JB570 was used as the recipient (Supplementary Table 1). The donor selective marker was one of these antibiotic(s) to which it was resistant: chloramphenicol (6 μg mL^{-1}), ampicillin (20 μg m^{-1}), tetracycline (8 μg mL^{-1}), trimethoprim (8 μg mL^{-1}), kanamycin (32 μg mL^{-1}), cefotaxime (8 μg mL^{-1}) or ceftazidime (8 μg mL^{-1}).

2.7. Procuring and acclimating live animals

All mussels were grown in the Marlborough Sounds, Marlborough, New Zealand by either Hairy Mussel Co. or Mills Bay Mussels. Mussels were purchased from a supermarket. Individuals of a similar size (± 1 cm) were selected. Mussels were allowed to recover from shipping in an aquarium system at approximately 12 °C for at least one week. The water in this system was collected from a local bay (−43.610610, 172.703457) and regularly checked for the carriage of *E. coli*, which were never detected in 1 L water samples. Prior to the start of the experiment, mussels were transferred to 40 L tanks in a temperature-controlled environment set at 15 °C and acclimated in 1 μm filtered seawater for at least two days with regular water changes. Mussels were fed twice per 24 h with 5 mL Reef Phytoplankton™ (Seachem).

2.8. Measuring removal of bacteria from water by mussels

20 L of 1 μm filtered seawater was added to four tanks. 10 mussels were added to two of the tanks. Each tank had an air bubbler and a clear plastic cover. The room that housed the tanks was kept at a constant 15 °C with a 12-h light-dark cycle.

The bacteria used were CMB44_Rif^R and/or L3Cip3_MAR2018, both isolated from the environment (Supplementary Table 1). When measuring the uptake of *E. coli* itself, only strain CMB44_Rif^R was used. When measuring the uptake of a susceptible *E. coli* in comparison to a multidrug resistant (MDR) *E. coli*, both strains were used simultaneously. *E. coli* were grown to an OD_{600} of 0.05 (approximately 10^7 CFU mL^{-1}). The cultures were then diluted 100-fold in PBS to a concentration

of approximately 10^5 CFU mL^{-1}.

At time zero, 2 mL of diluted *E. coli* culture was added to each tank to achieve a starting concentration of approximately 2 × 10^2 CFU 100 mL^{-1}. Immediately after the addition of *E. coli*, water samples were taken from each tank to confirm initial *E. coli* concentrations. Thereafter, water samples were taken at two, four, six, eight and 24 h, under the assumption that *E. coli* were either inside the mussels or in the surrounding water. At the eight- and 24-h time points, three mussels were also removed from tanks containing mussels. During the experiment, mussels were fed with 5 mL Reef Phytoplankton™ 1 h before and 8 h after bacteria were added.

E. coli were enumerated from water samples by membrane filtration. 100 mL and 10 mL aliquots of water samples were filtered. Filters were placed onto TBX agar plates supplemented with Rif (100 μg mL^{-1}). This was to further decrease the probability that *E. coli* from external sources would be counted on plates. When two strains were used, the plates were supplemented with either Rif (100 μg mL^{-1}) to select for CMB44_Rif^R, or Tet (16 μg mL^{-1}) to select for L3Cip3_MAR2018.

E. coli were enumerated from mussels by the following method. The outsides of the shells were cleaned using paper towels misted with 70% ethanol. Mussels were shucked and the flesh added to a sterile glass beaker until 100 g of flesh and liquor was obtained (approximately three mussels). The samples were blended for 2 min with 100 mL sterile PBS. Five 1 mL aliquots of homogenate from each replicate were aseptically spread on TBX agar plates and dried in a laminar flow cabinet. Plates were incubated at 44 °C for 12–16 h. The tank exposure trials were run independently three times, each with two replicates of each treatment (tank with mussels or no mussels).

2.9. Conjugation in mussels

A pair of isogenic strains (Supplementary Table 1) that differed only by their resistance to rifampicin or streptomycin were used as donor and recipient. Plasmid RP4 (Supplementary Table 1) was transferred into CMB44_Str^R by liquid culture mating as described previously (Heinemann & Ankenbauer, 1993).

The RP4 transmission frequency was measured in mussels, mussel feces and the surrounding water at 15 °C. Live animals were acclimated as described above. Five individuals were placed in separate glass containers each with 1 L of 1 μm filtered seawater (Supplementary Figure 6). Each container received an air bubbler for oxygenation and was tilted to allow mussels to elevate themselves above the water. Five control containers without mussels were included, with all other parameters kept the same.

Both strains were grown to approximately 10^9 CFU mL^{-1} in LB broth. A 2 mL aliquot of donor and recipient cells was added to each of the ten containers to achieve a starting titre of approximately 2 × 10^6 CFU mL^{-1} for each strain. The titres of the starting cultures were determined by plate count assay. Immediately after donors and recipients were added, 10 mL aliquots (time zero) were removed from each container and placed on ice. The titre of donors, recipients and transconjugants was immediately determined by plating 10-fold dilution series in agar plates containing the appropriate antibiotics. Thereafter, 10 mL of water was sampled from each beaker at two and 4 h post addition of bacteria.

At 2 h and 4 h, mussel fecal samples were also collected from all beakers with mussels using sterile forceps. 1 mL sterile PBS was added to each fecal sample, which was then homogenised by vortexing and a sterile pestle. The titres of donors, recipients and transconjugants in fecal samples were determined as described above. At 4 h, each of the five mussels was removed from the beakers and wiped with a paper towel misted with 70% ethanol. Mussels were stored separately in labelled plastic bags on ice until processing as described above. Blended mussel homogenate was diluted to determine the plate count titres of donors, recipients and transconjugants.

For all conditions, transmission frequency of RP4 was calculated by dividing the number of transconjugant bacteria in the sample by the number of limiting parent, which in our case was always the donor. The experiment was performed once, with five replicates of each treatment (mussel or no mussel).

2.10. Statistical methods

R Studio was used for all statistical analyses and producing figures (R Software Core Development Team, 2020). The relationship between *E. coli* counts in water and in cockle or watercress samples was modelled using a generalised linear model with a Poisson error distribution. We tested whether the amount of *E. coli* measured in cockles or in the shellfish harvesting waters was significantly greater than New Zealand Food Safety guideline limits for *E. coli* (Dansted, 2021) using a one tailed *t*-test. The choice to use a one tailed test was made *a priori*, and checked for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). To test whether the concentration of *E. coli* in the water where cockles were harvested exceeded the food safety guideline limit for *E. coli* in shellfish harvesting waters we used a one sample *t*-test (Dansted, 2021). To test whether the concentration of *E. coli* in the watercress or cockles was significantly greater than the water from which the food was harvested, we used an independent samples *t*-test (R Software Core Development Team, 2020).

The difference between *E. coli* counts in mussels and in water was confirmed to be statistically significant using a Welch Two Sample *t*-test (Welch, 1947). The concentration of *E. coli* in mussels was confirmed to be significantly greater than the food safety limits using a one-tailed *t*-test. To measure the relationship between time and treatment (mussel versus no mussel) on *E. coli* concentrations in the water, we used a linear mixed effects model (Bates et al., 2015). To control for replication, we used blocks fitted as a random effect (Bates et al., 2015). Treatment and time were fitted as fixed effects. *E. coli* counts were $\log_{10}$-transformed prior to fitting the model as this improved the appearance of residual plots.

We used a second linear mixed effects model to test the relationship between time and treatment (mussel versus no mussel) on AMR versus susceptible *E. coli* concentrations in the water (Bates et al., 2015). 'Block' was fitted as a random effect, while time, treatment (mussel or no mussel) and strain (CMB44_Rif^R or L3Cip3_MAR2018) were fixed effects. *E. coli* counts were $\log_{10}$-transformed prior to fitting the model as this improved the residual plots. P-values for mixed effects models were calculated with a Chi Square error distribution (R Software Core Development Team, 2020).

3. Results

3.1. E. coli and AMR in watercress and surrounding water

In all but three samples, the concentration of *E. coli* in and on watercress was higher than in corresponding water samples. *E. coli* were detected in the water and on watercress in every sample (Fig. 2). The *E. coli* concentration on watercress across all four sites was between 2 × 10^1 CFU 50 g^{-1} and 1 × 10^3 CFU 50 g^{-1}.

The average number of *E. coli* on watercress was significantly higher than in water at all individual sites ($P = 0.03$). Furthermore, when data from all four sites were pooled, there was a significant relationship between the number of *E. coli* in water and on watercress ($P < 2e\text{-}16$).

The concentration of *E. coli* resistant to ampicillin, chloramphenicol, or ciprofloxacin was also measured from water samples at each sampling event (Supplementary Figure 1). *E. coli* resistant to ampicillin was detected in all but two samples. *E. coli* resistant to chloramphenicol was detected occasionally at the Ruataniwha/Cam River and Gressons Road sites. *E. coli* resistant to ciprofloxacin was detected three times and all at the Ruataniwha/Cam River site (Supplementary Figure 1). The average proportion of *E. coli* with resistance to ampicillin in water samples across all four sites was 0.22. The average proportion of *E. coli* with chloramphenicol or ciprofloxacin resistance was less than 0.01.

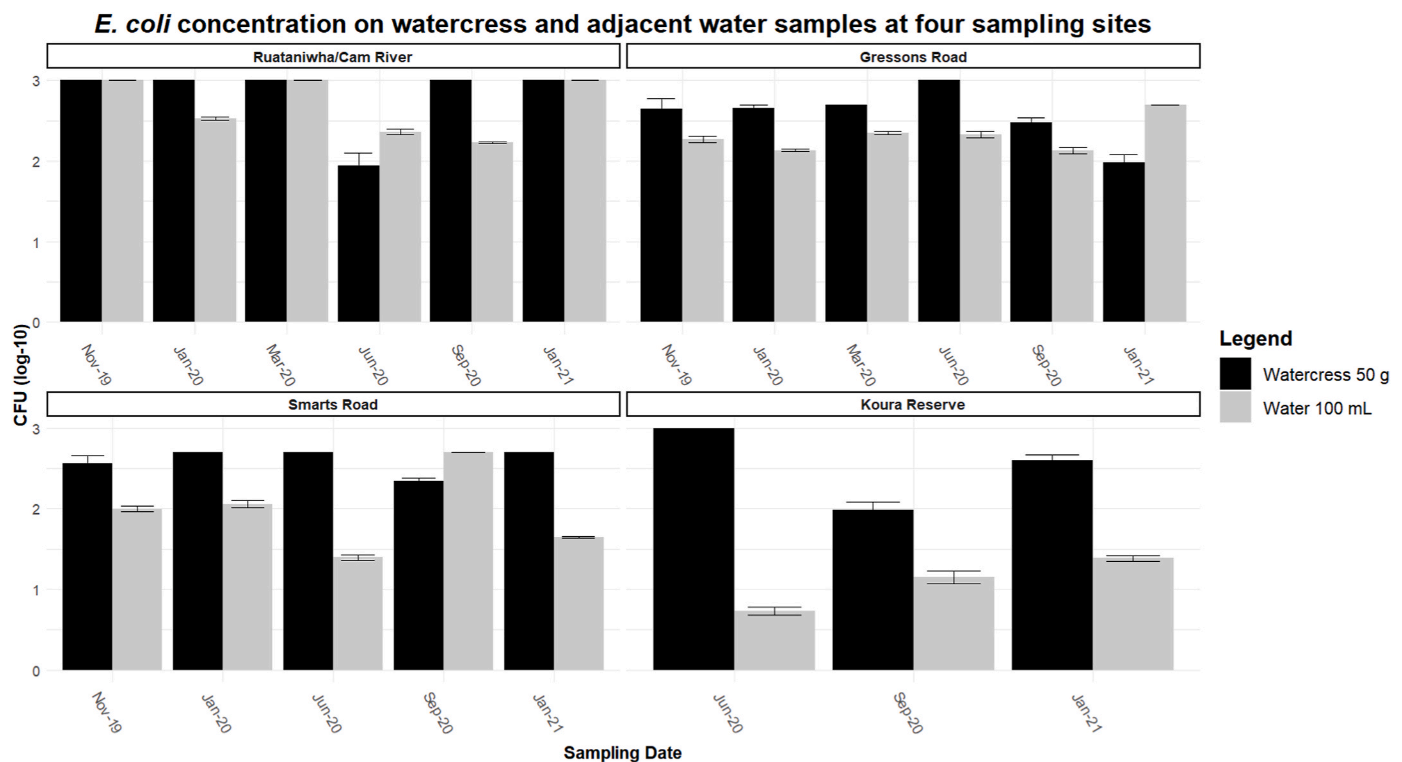

Fig. 2. *E. coli* isolated from water samples (CFU 100 mL^{-1}) and watercress (CFU 50 g^{-1}) at four locations. CFU: colony forming units. 'Sampling Date' is the month and year when the sample was collected. Colony counts are log_{10}-transformed.

3.2. E. coli and AMR in cockles and surrounding water

E. coli concentrations in cockles was consistently higher than in water. Cockles were sampled five times between September 2019 and January 2021 (Fig. 3). *E. coli* concentrations in cockle samples ranged from 4 x 10^2 to 7 × 10^4 CFU 100 g^{-1}. This was significantly higher than in congruent water samples (P = 0.002). The concentration of *E. coli* in cockle samples was up to 60 times higher than in water. However, *E. coli*

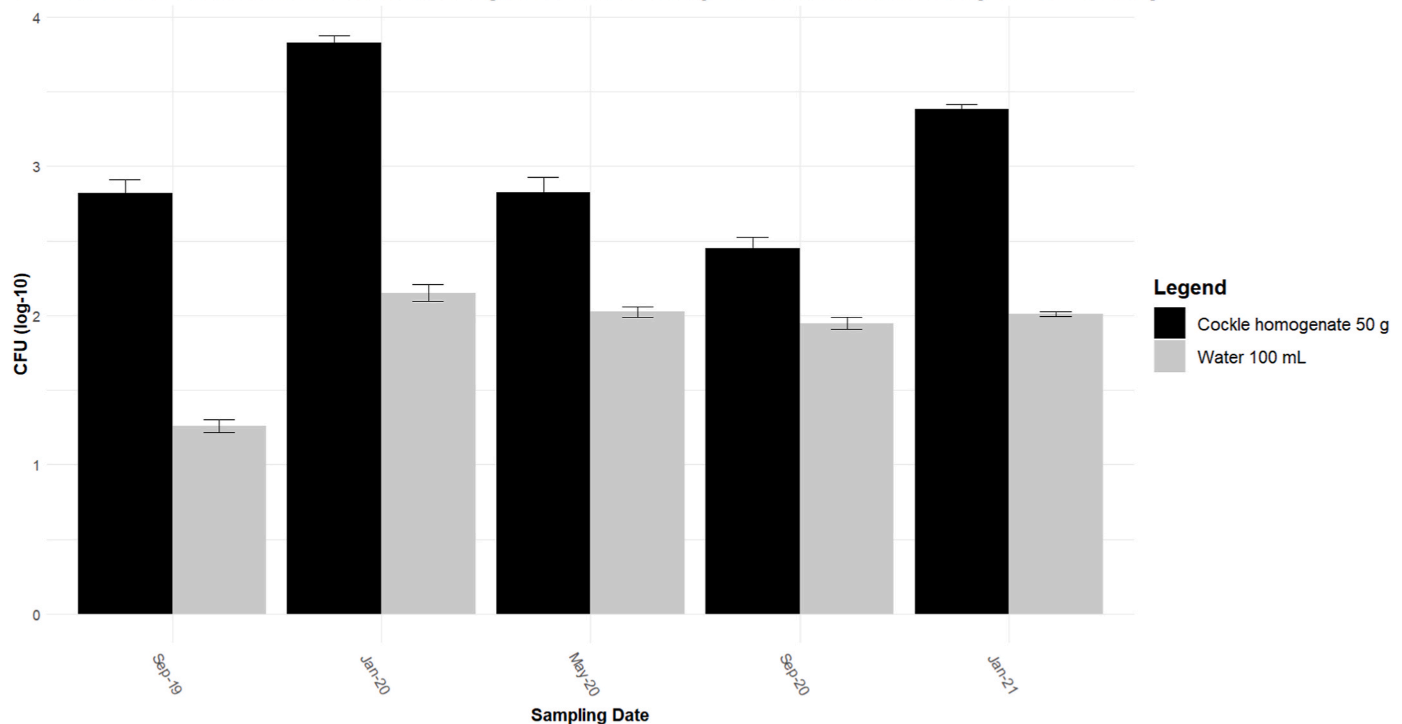

Fig. 3. *E. coli* isolated from water (CFU 100 mL^{-1}) and cockle (CFU 50 g^{-1}) samples at Te Akaaka/Ashley River Estuary. 'Sampling Date' is the month and year when the sample was collected. Colony counts are log_{10}-transformed. CFU: colony forming units.

concentration in water was not a significant predictor of *E. coli* concentration in cockle samples (P = 0.066).

E. coli resistant to ampicillin, ciprofloxacin and chloramphenicol were enumerated from water samples taken from a site adjacent to the cockle bed ('estuary site') and from an upstream site ('lower Rakahuri'). Ampicillin-resistant *E. coli* were detected in every sample at concentrations of 3–8 x 10^1 CFU 100 mL^{-1} (Supplementary Figure 2). Chloramphenicol resistance was detected in water samples once at each of the estuary site and the lower river site. Ciprofloxacin resistance was detected once in a water sample from the estuary. In all cases, the numbers of ciprofloxacin and chloramphenicol resistant *E. coli* were less than 5 CFU 100 mL^{-1} (Supplementary Figure 2).

3.3. AMR and conjugative phenotypes

AMR phenotypes of 132 randomly selected *E. coli* isolates from water, watercress, and cockles were determined (Supplementary Figures 3 and 4 and a list of *E. coli* isolates is in Supplementary Table 2). The most common resistance (≥ CLSI intermediate concentration) was to ampicillin, with a proportion of 0.22 across all isolates, followed by tetracycline, with a proportion of 0.22 across all isolates. Resistance to antibiotic concentrations exceeding the CLSI breakpoint was comparatively rare for kanamycin, cefotaxime, and ceftazidime, with proportions less than 0.04 in all cases. Most of the resistant strains were isolated from water samples. A small number of *E. coli* isolated from either watercress or cockles were resistant to one or more antibiotics (e.g., Supplementary Figure 3). Several strains, including some isolated from cockle samples, were resistant to four or more antibiotics.

Resistance was often associated with conjugative plasmids. Sixteen out of 41 strains (0.39) tested were able to transmit one or more resistance phenotypes to an antibiotic susceptible recipient by conjugation (Supplementary Tables 3 and 4). Tetracycline resistance was transmitted by 11 of 16 strains (0.69). The next most frequent were ampicillin, chloramphenicol, and trimethoprim, each transmitted by seven strains (0.43). Two isolates, including one isolated from a cockle, transmitted four different antibiotic resistances simultaneously. The transmission frequency was between 10^{-4} and 10^{-6} transconjugants per limiting parent (Supplementary Tables 6 and 7).

3.4. E. coli moves from water to mussels

The *E. coli* concentration in the water declined significantly faster in tanks with mussels than in those without (P < 0.0001) (Fig. 4). At 8 h, the concentration of *E. coli* in the water of tanks with mussels was on average five times lower than in tanks without mussels, but this difference was not significant (P = 0.08). At 24 h, the difference in *E. coli* concentration (CFU 100 mL^{-1}) in the water of tanks with mussels was on average 39 times lower than the control tanks (P = 0.049).

At time zero, we added *E. coli* to each tank, resulting in a starting concentration of approximately 2 × 10^2 CFU 100 mL^{-1}. After 24 h of exposure to this inoculum, the average concentration of *E. coli* in mussels had increased from not detectable in 100 g, to 3 × 10^3 CFU 100 g^{-1} (data not shown). This represented a concentrating factor of at least ten times, and was significantly higher than the food safety limit proposed by New Zealand guidelines (P = 0.02) (Dansted, 2021).

Neither the antibiotic resistance nor conjugative phenotypes contributed to uptake by the animals. Mussels were equally effective at removing from water a MDR strain carrying a conjugative plasmid. When tanks were inoculated with either CMB44_Rif^R or L3Cip3_-MAR2018 (Supp fig 5) there was no effect of strain (MDR vs susceptible) (P = 0.90).

3.5. Bacteria conjugated in mussels

Because mussels remove bacteria from water and concentrate them, they may be accelerants of horizontal gene transfer in general and bacteria conjugation in particular. We tested this possibility using mixtures of *E. coli* donors of plasmid RP4 (Supplementary Table 1;

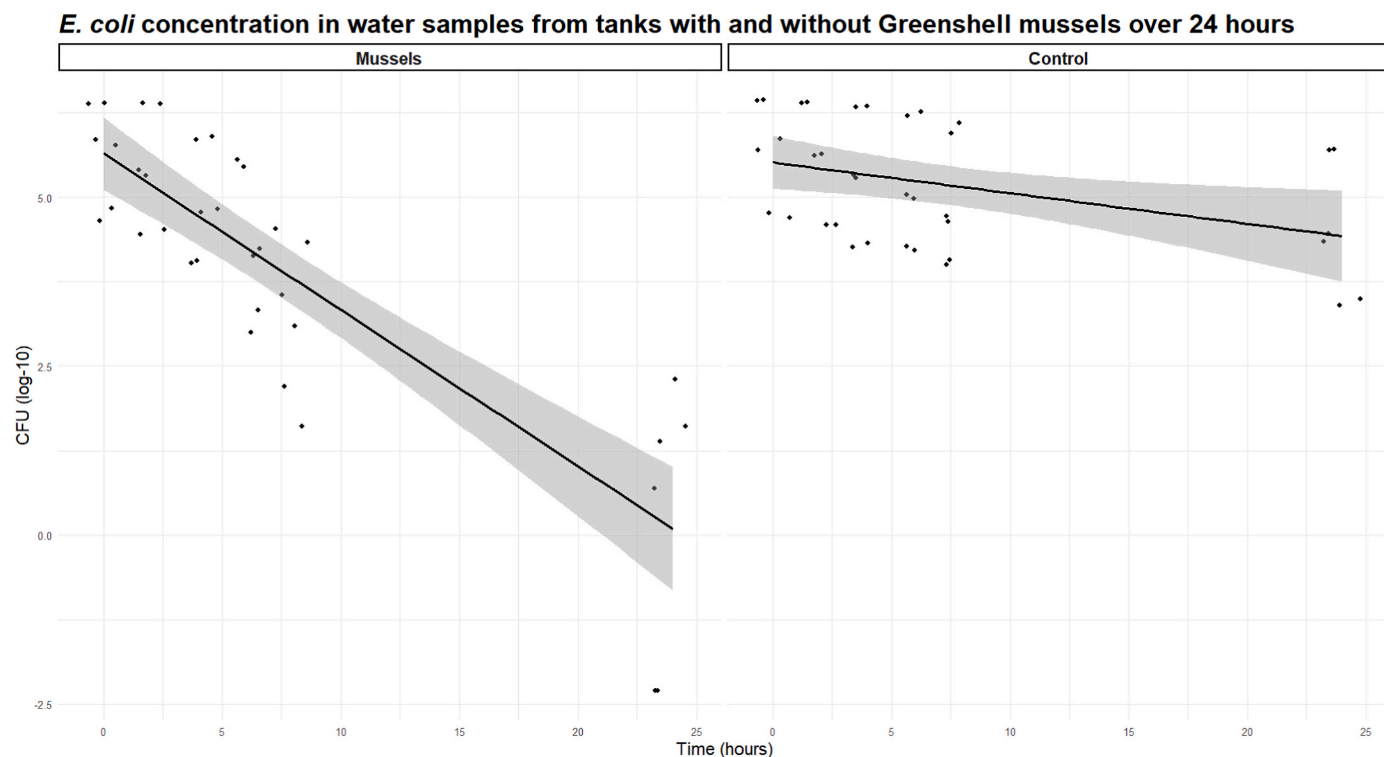

Fig. 4. Decline in CMB44_RIF44 (CFU 100 mL^{-1}) over 24 h in the water of tanks with mussels (left) versus tanks without mussels (right; control). *E. coli* concentration was log-10 transformed. Shaded areas represent standard error of the mean. Data were plotted with jitter to minimize over-plotting. CFU: colony forming units.

Pansegrau et al., 1994) and recipient strains under four simulated marine conditions (Fig. 5).

No transconjugants were detected in the water surrounding mussels. Transconjugants were detected at low frequencies in tanks without mussels. Transconjugants were found at higher concentrations in mussel feces than in water without mussels after 4 h ($P = 0.006$). Likewise, transconjugants were found in significantly higher concentrations in mussel homogenate than in water without mussels at 4 h ($P = 0.009$).

The much higher numbers of transconjugants found in extracts of internal tissues and feces than in water indicates that plasmid transmission occurred more frequently between bacteria that had been internalized by the animals.

4. Discussion

The practice of harvesting wild food is a valued cultural activity (Fromentin et al., 2022; Kainamu-Murchie et al., 2018) and important for food security and diet diversity (Bharucha & Pretty, 2010; Schunko & Brandner, 2022; Sulaiman et al., 2022). Importantly, it is an activity that binds different generations of the same family and community and ensures the retention of skills for identifying useful species and processing the harvest (Bharucha & Pretty, 2010; Kainamu-Murchie et al., 2018). Our work is important for this activity because it shows that mahinga kai harvesters and consumers may be at risk of exposure to fecal bacteria and bacteria with resistance to antibiotics.

Water quality influences the reliability and safety of aquatic wild foods. In Aotearoa New Zealand, guidance is provided based on the number of fecal coliforms in the water column (Dansted, 2021; Ministry for the Environment, 2017). This is a useful and effective measure, but it does not inform about other sources of harm including infection by bacteria resistant to antibiotics. The guidance therefore increasingly underestimates the risk to those in the water and eating food harvested from the water as the frequency of AMR in the environment increases.

4.1. Antibiotic-resistant bacteria were found in watercress and cockles

Antibiotic-resistant *E. coli* were found at sites where watercress and shellfish are regularly harvested, and in the foods themselves. Resistance to ampicillin and tetracycline was readily detectable. This is concerning because antibiotics from these classes are among the most-commonly prescribed in New Zealand (Williamson et al., 2016). Ours was the first study which assessed antibiotic resistance in watercress in New Zealand. The last available data for AMR in shellfish is from 1976. However, Cooke (1976) also detected resistance to ampicillin and chloramphenicol in marine shellfish, indicating that AMR has been a feature of these mahinga kai for many decades.

We isolated *E. coli* with a similar resistance profile to those described by Harris et al. (2017), in their study of cephalosporin resistant *E. coli* from bloodstream infections, which included *E. coli* isolated in New Zealand and Oceania. Recreational water contact was previously shown to be a risk factor for carriage of pathogenic *E. coli* in New Zealand (Jaros et al., 2013). Future studies should seek to establish whether a similar link exists between consumption of wild-harvested foods and carriage of AMR or of pathogenic bacteria.

4.2. *E. coli* are found in/on mahinga kai species and water in aotearoa New Zealand

The popular food plant watercress was shown to be contaminated with levels of *E. coli* considered to be unsatisfactory by existing guidelines (Food Standards Australia New Zealand FSANZ, 2022). *E. coli* concentrations on watercress were usually higher than in the surrounding water. The rare exceptions we saw might be explained by transient spikes in *E. coli* levels in waterways (Cornelisen et al., 2011).

E. coli may adhere to the watercress leaf and stem surfaces or even be internalized by the plant, leading to a concentrating effect (Xicohtencati-Cortes, 2009). Our findings are consistent with those from the North Island of New Zealand and from Mauritius (Donnison et al., 2009; Edmonds & Hawke, 2004; Googoolee et al., 2020). Of particular concern was the concentration of *E. coli* on watercress at the Koura Reserve, a purpose-built watercress harvesting bed (Fig. 1). Watercress harvested from this site would have failed to meet the requirements for a 'satisfactory' rating under the New Zealand guidelines for ready to eat salad vegetables on two out of three sampling events (Food Standards Australia New Zealand FSANZ, 2022). This was despite water in the Koura Reserve having the lowest average *E. coli* concentration.

The significant relationship between *E. coli* in water and watercress demonstrates the potential for government water quality monitoring

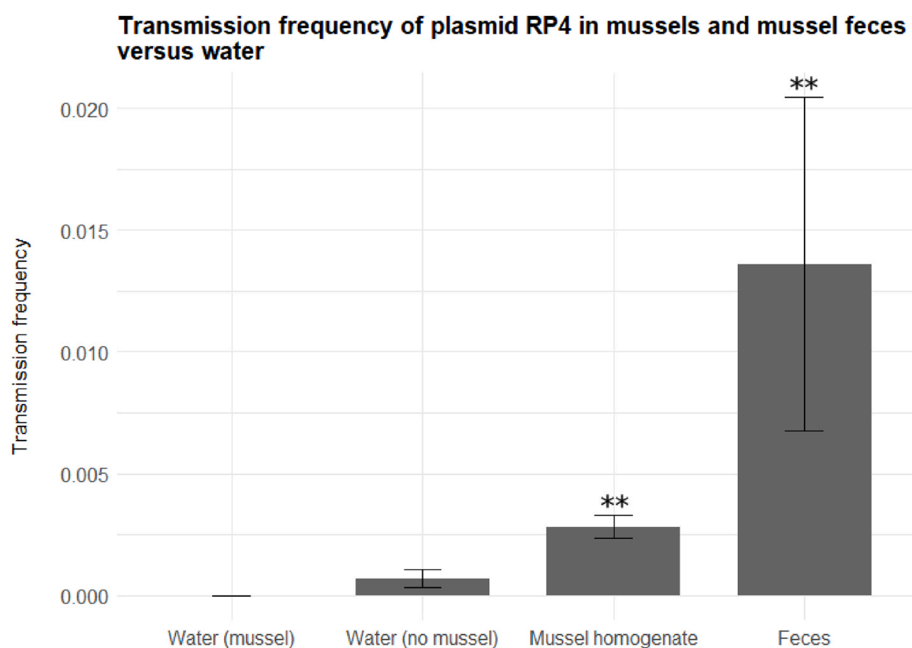

Fig. 5. RP4 transmission frequency per limiting parent in water (without mussels), water (with mussels), in mussels (extrapolated from transconjugants in homogenate) and in mussels (extrapolated from transconjugants in feces) at 15 °C after 4 h.

regimes to help predict which sites are safest for watercress harvesting. However, only two of the four sites studied here (Ruataniwha/Cam River and Gressons Road) are currently monitored (Land, Air, Water Aotearoa (LAWA), 2022a; Land, Air, Water Aotearoa (LAWA), 2022c).

Cockles from Te Akaaka were at times unsafe for human consumption. Under New Zealand guidelines, no more than 10% of samples should exceed 700 *E. coli* CFU 100 g^{-1} (Dansted, 2021). Three of our samples (60%) exceeded this limit. The water from which cockles were harvested would also fail to meet the requirements for a shellfish harvesting site (Dansted, 2021). The levels of *E. coli* in the cockles were up to 60 times higher than the water. This indicates that the animals may be useful indicators of the recent past fluxes of bacteria through the river. A limitation of our study was the small number of samples from this site.

We found no significant relationship between the *E. coli* concentration in estuary water and in cockle samples, indicating that existing monitoring protocols do not predict shellfish safety at this site. Similarly, no relationship between cockles and water was found in a study performed near Dunedin, New Zealand (Bell, 2019). This may be because cockles are usually collected at low tide in the estuary that is influenced by both the conditions of the sea and the river and may therefore be variable on short time scales. Hence, the water and bacteria trapped inside the shells may reflect the previous high tide, rather than current conditions when sampling. While water from the Lower Rakahuri/Ashley River is regularly monitored for *E. coli*, the addition of cockle and other shellfish to existing sampling regimes should be considered to provide stronger safety predictions to consumers.

4.3. Greenshell mussels hold or concentrate E. coli

Mussels in tanks inoculated with *E. coli* became unsafe for human consumption at between eight and 24 h after exposure to *E. coli*. The speed at which the food safety limit was reached highlights the need for frequent water testing at sites where shellfish are harvested. Transient spikes in the levels of fecal bacteria should be rapidly communicated to the public to protect those who harvest this mahinga kai. The inoculum of *E. coli* used is within the order of magnitude frequently encountered in New Zealand coastal settings (Land, Air, Water Aotearoa (LAWA), 2021a, Land, Air, Water Aotearoa (LAWA), 2021b, Land, Air, Water Aotearoa (LAWA), 2021c, Land, Air, Water Aotearoa (LAWA), 2022a, Land, Air, Water Aotearoa (LAWA), 2022b; Land, Air, Water Aotearoa (LAWA), 2022c).

We had hypothesized that the increased cell to cell adhesion demonstrated by strains with conjugative plasmids might increase *E. coli* uptake and survival in shellfish through increased adherence to solid surfaces (Amábile-Cuevas & Chicurel, 1992). However, the conjugative, MDR *E. coli* strain we used did not accumulate more efficiently inside mussels than the strain without a plasmid. Both strains appeared to be taken up by mussels with equal efficiency.

4.4. Intra-shellular conjugation: E. coli inside mussels conjugate and through this exchange antibiotic resistance genes

More than one third of *E. coli* isolates from cockles could transmit conjugative plasmids that carried resistance genes. Through whole genome sequencing, we previously showed that genes diagnostic of incompatibility-group F plasmids were a feature of at least some of the strains isolated during that study (van Hamelsveld et al., 2022). F plasmids often confer several resistances, including to antibiotics for which we did not perform antimicrobial susceptibility testing, and genes associated with pathogenicity (Ragupathi, 2019). This was true of at least some of the *E. coli* we isolated (van Hamelsveld et al., 2022).

We therefore wondered whether the frequency of carriage of conjugative plasmids could be influenced by the animals. To test this, we used Greenshell mussels in tanks exposed to a mixture of *E. coli* plasmid donors and recipients.

The RP4 transmission frequency was significantly higher inside mussels than in water alone. We conclude this because the concentration of transconjugants was significantly lower in water than in samples taken from inside the mussel or recently excreted by the animal. The concentration of fecal bacteria in water may usually be too low for conjugation to be measured (Fernandez-Astorga et al., 1992; Goodman et al., 1993). However, as filter feeders, shellfish help bacteria overcome the dilution effect. Indeed, previous studies have shown that evidence of the transfer of AMR genes can be seen in *E. coli* isolated from marine mussels (Grevskott et al., 2020; Grevskott et al., 2017; Vignaroli et al., 2016). However, ours was the first study that assessed plasmid transmission by conjugation that had to have occurred intra-shellularly, or “between the shells” of the mussels. Not surprisingly, transconjugant concentrations were also higher in mussel feces, which are excreted as mucous-bound sacks. In this environment, bacteria may benefit not only from proximity, facilitating horizontal gene transfer, but also from increased availability of food, protection from UV radiation and predatory microorganisms. This might allow them to persist in the environment for longer.

It was recently shown that *E. coli* can receive plasmids with AMR genes from environmental microbes (Guzman-Otazo et al., 2022). Further studies, for example whether shellfish facilitate horizontal gene transfer between potential pathogens of humans, and environmental strains resident in seawater, would be valuable. The role that pseudo-feces play in potentially augmenting the survival of fecal bacteria is another area where research is warranted.

5. Conclusion

Here we have presented the first update of antibiotic resistance in mahinga kai/wild food in recent decades. The high levels of *E. coli* in mahinga kai indicate significant fecal contamination of waterways adjacent to the city of Otautahi Christchurch. While not all of our sampling sites are monitored by the authorities, they fit the trend of official data for *E. coli* in waterways (Land, Air, Water Aotearoa (LAWA), 2022b). This study also adds to the growing body of evidence that AMR is widespread in the New Zealand environment, and may have increased in frequency since the first studies of the 1970s (Cooke, 1976a, 1976b). We demonstrated that contamination of water with antibiotic-resistant bacteria is sufficient evidence to predict that the bacteria will also be found in the foods gathered from these environments, often in much higher concentrations due to accumulation effects.

This is the first study that tracked the internalization of *E. coli* by *P. canaliculus* in seawater tanks. We also considered the possibility that shellfish might increase horizontal gene transfer via what we have termed ‘intra-shellular’ conjugation. The finding that plasmid transmission frequencies were higher in Greenshell mussels and their feces than in water alone is novel.

Wild foods are regularly extracted from environments contaminated with *E. coli* and AMR *E. coli*. Better stewardship of water resources is therefore crucial to improve the safety of mahinga kai. However, actions of stewardship are undermined by several factors.

First, existing food safety guidelines underestimate food safety and cultural risk. For example, the guidelines assume that food which fails to meet microbial standards will in any case be cooked or depurated. This is not consistent with Māori expectations of water quality (Tau, 1990). Second, monitoring of sites relevant to the collection of mahinga kai is insufficient both in frequency and site density to allow for a more sophisticated consumer guidance. AMR is not one of the characteristics routinely determined from marine or freshwater samples. Hence the needs of the most vulnerable groups that rely upon mahinga kai are unmet. New Zealand’s food safety and water quality monitoring guidelines should more faithfully address the expectations and needs of Māori, taking the additional health risks posed by AMR into account.

Credit author statement

Sophie van Hamelsveld: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original draft, Writing – Review & editing, Visualization. **Brigitta Kurenbach**: Methodology Investigation, Review & editing**. Deborah J. Paull**: Methodology, Investigation. **William A. Godsoe**: Methodology, Formal analysis, Writing – Review & editing. **Gayle C. Ferguson**: Visualization, Writing – Review & editing. **Jack A. Heinemann**: Conceptualization, Methodology, Writing – Original draft, Writing – Review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122155.

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