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Aeromonas spp. diversity in U.S. mid-Atlantic surface and reclaimed water, seasonal dynamics, virulence gene patterns and attachment to lettuce

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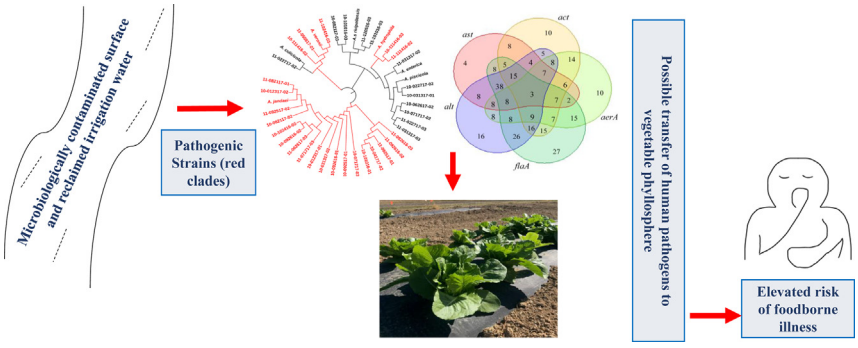
HIGHLIGHTS

- Highest *Aeromonas* diversity in rivers and winter season, lowest in ponds and summer
- Human pathogens *A. veronii, A. jandaei, A. hydrophila* detected in all water types
- Enterotoxin genes *ast* and *alt* were associated with isolates collected in summer.
- The gene *flaA* was associated with fresh rivers and abiotic surface adhesion.
- *A. veronii* associated loosely and strongly with lettuce, persisting for 120 h.

GRAPHICAL ABSTRACT



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ABSTRACT

Aeromonas, a ubiquitous taxon in water environments, is emerging as a foodborne pathogen of concern that remains understudied and under-reported. We evaluated the distribution of 331 *Aeromonas* spp. isolates collected from irrigation water over one year and characterised their virulence profile, attachment and ability to persist on lettuce. Water sources included non-tidal and tidal river, farm pond and reclaimed water. Twenty *Aeromonas* species were identified; *A. veronii*, *A. hydrophila* and *A. jandaei* predominated in all water types and seasons, comprising ~63% of isolates. Species distribution was most affected by water type. The highest and lowest diversity were detected in river and pond water, respectively. *A. hydrophila* and *A. veronii* ranked highest in frequency in fresh river and reclaimed water, while *A. jandaei* ranked first in pond water. Only two isolates carried all five virulence genes tested, while 46% of *A. hydrophila* ($n = 50$), 54% of *A. veronii* ($n = 61$) and 50% of *A. jandaei* ($n = 32$) isolates harboured multiple enterotoxin genes. Detection of *alt* and *ast* genes was more likely in summer collections, while *ast* detection was less likely in tidal brackish river and pond water isolates. Season was a factor in attachment to polystyrene, being strongest in spring isolates. The gene *flaA* was associated with strong attachment and was more likely to be detected in non-tidal fresh river isolates. *A. hydrophila* and *A. jandaei* isolates persisted on lettuce leaves for 24 h, but populations dwindled over 120 h, while loosely and strongly attached cells of *A. veronii* isolates persisted for 120 h. This study provides comprehensive data on *Aeromonas* species distribution and environmental traits. The associations revealed among diversity, water type, season, virulence factors and phyllosphere attachment capacity can inform agricultural water standards in novel ways. Moreover, understanding *Aeromonas*-plant interactions is an important step in advancing food safety of fruit and vegetables.

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1. Introduction

Climate change and population growth, excessive groundwater discharge and escalating water demands from the agricultural and non-agricultural sectors, necessitate exploration of alternative irrigation water sources for sustainable food production. In the mid-Atlantic region of the United States, tertiary treated wastewater (reclaimed water), surface water from rivers, creeks, ponds and recycled vegetable processing wash water have potential as irrigation water sources for fruit and vegetable crops that demand strict agricultural standards to ensure microbiological safety of food crops. Unfortunately, these water types often harbour coliforms, faecal coliforms, enterococci and human pathogens that can limit their use in agriculture (Haymaker et al., 2019; Sharma et al., 2020; Solaiman et al., 2020). Microbiologically contaminated irrigation water is frequently reported as the cause of many foodborne and waterborne outbreaks (Alegbeleye et al., 2018; Carstens et al., 2019; Jongman and Korsten, 2018; McDaniel and Jadeja, 2019). Therefore, in order to mitigate potential public health risks that come from using alternative irrigation water in the mid-Atlantic region, extensive research on both known and understudied bacterial pathogens residing in these water sources is required.

Aeromonas, a gram negative opportunistic bacterial pathogen, is a natural inhabitant of water environments (Cui et al., 2017) and an emerging food safety concern that remains understudied (Fernández-Bravo and Figueras, 2020). While both psychrophilic and mesophilic strains of the species are considered pathogenic, mesophilic strains of *Aeromonas* spp. are of major concern due to their ability to cause a variety of intestinal, extra-intestinal and systemic infections in humans (Beaz-Hidalgo et al., 2013; Dallaire-Dufresne et al., 2014). In addition to water environments, pathogenic species of *Aeromonas* have been isolated from several foods, including fruit and vegetables (Chang et al., 2008; McMahon and Wilson, 2001; Nagar et al., 2011; Szabo et al., 2000; Xanthopoulos et al., 2010), and patients with diarrhoeal disease (Igbinosa et al., 2012; Vila et al., 2003). Chang et al. (2008) found that *A. caviae* followed by *A. hydrophila* were the most frequently isolated species from foods suspected to have been linked to food poisoning incidents.

It has been postulated that the food safety risk of *Aeromonas* should be given more attention, on par with other notorious pathogens such as *Salmonella* and *Campylobacter*, following the finding from foodborne illness outbreak analyses that infective doses as low as 10^3 CFUs could cause gastroenteritis in humans (Teunis and Figueras, 2016). The emerging public health concern and food safety risk of *Aeromonas* spp. are attributed to cytotoxin and enterotoxin production and the ability to persist during refrigeration and in higher pH and saline environments (Vivekanandhan et al., 2003). Enteric disease resulting from the transmission of *Aeromonas* spp. from aquatic environments to crops depends on several factors including contamination level of the water, transfer and attachment potential to and persistence ability on the food surface, and finally virulence (Steele and Odumeru, 2004). Flagella, surface poly- and lipopolysaccharides, glucans, cytotoxins, haemolysins/aerolysin, secretory metabolites and extracellular enzymes are important virulence factors of *Aeromonas* (Tomás, 2012). Flagellar genes play a crucial role in attachment to both biotic and abiotic surfaces, while enterotoxin genes are prominent determinants for infection success and are frequently associated with gastroenteritis (Santiago et al., 1999; Sen and Rodgers, 2004; Von Graevenitz, 2007).

Aeromonas infection remains an unreportable disease in the United States and only two environment-associated outbreaks are listed in the CDC National Outbreak Reporting System (NORS), occurring in 1989 and 2013 (CDC, NORS, 2020). Data on *Aeromonas* spp. in irrigation water used for fresh fruit and vegetable crops is not available. We hypothesized that *Aeromonas* in irrigation water includes several potentially pathogenic species and that isolates from irrigation water carry virulence determinants and have the ability to persist on a lettuce crop. This study therefore aimed to address the knowledge gap on *Aeromonas* risk from irrigation water use in fresh produce agriculture by investigating the abundance, diversity and distribution of *Aeromonas* isolated from various irrigation water sources, including surface and recycled water, in the mid-Atlantic region. Isolates were obtained from a two-year longitudinal study as part of a U.S. Department of Agriculture funded Centre of Excellence (COE) called CONSERVE: *A Center of Excellence at the Nexus of Sustainable Water Reuse, Food and Health.* CONSERVE has a long-term goal of facilitating the adoption of safe reuse of water in agriculture of fresh fruit and vegetables. As such, virulence gene patterns of the *Aeromonas* spp. isolates and their attachment and transfer potential from irrigation water to abiotic and lettuce leaf surfaces, were also investigated.

2. Materials and methods

2.1. Sampling sites and sample collection

A total of 133 samples from 11 locations (current and prospective irrigation water sources) in the mid-Atlantic region were collected between September 2016 and October 2017. Sites included 5 non-tidal freshwater rivers (NF; includes sites MA03, MA04, MA05, MA07 and MA09), one tidal brackish river (TB; includes the site MA08), 3 highly treated reclaimed wastewater effluent points (RW; includes sites MA01, MA02 and MA06) and two on-farm ponds (PW; includes sites MA10 and MA11). Sampling frequency for each site is described in Table S1. Water was collected as described in Solaiman et al. (2020) and processed within 6–8 h of collection. Since the sites included farms, confidentiality of site location is maintained. The study was reviewed by the University of Maryland College Park Institutional Review Board (IRB) (project number 964795-1) and was approved as 'exempt' due to minimal risk to farm owners.

2.2. Isolation and identification confirmation for Aeromonas spp.

Aeromonas spp. were isolated by standard membrane filtration and incubation on Ampicillin Dextrin Agar (Criterion, Hardy Diagnostics, Santa Maria, CA, USA) supplemented with ampicillin, sodium salt (10 μg/ml) (Fisher Scientific, Hampton, NH, USA) and vancomycin (10 μg/ml) (Amresco, Randor, PA, USA) (ADA-V) using EPA method 1605 (USEPA, 2001) as described in Solaiman et al. (2020). Up to three yellow colonies from ADA-V plates were picked for further identification by PCR confirmation.

Each colony was cultured on Tryptic Soy Agar (TSA) (Oxoid, Lenexa, KS, USA) supplemented with ampicillin and vancomycin (TSA-VA). DNA was extracted from single *Aeromonas* colonies by rapid heat lysis (Micallef et al., 2012). Aliquots of 2.5 μl DNA suspensions were used as templates for genus-specific PCR amplification of the *gyrB* gene encoding the DNA gyrase β-subunit. DNA was mixed in a 22.5 μl PCR reactions containing 1× PCR buffer (New England Biolabs (NEB), Ipswich, MA, USA), 1.5 mM $MgCl_2$ (NEB), 0.2 mM dNTPs (VWR, Radnor, PA, USA), 0.3 μM each of forward and reverse primers (*gyrB* and 16S rRNA as internal control) (Integrated DNATechnologies (IDT), Coralville, Iowa, USA) (Table S2) and 0.24 μl of 5 Units of *Taq* DNA Polymerase (NEB). Amplification reactions were carried out with an initial denaturation step of 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 20 s and extension at 72 °C for 45 s, with a final extension of 7 min. PCR products were then electrophoresed in 1% agarose (Lonza, Rockland, ME, USA). *Aeromonas hydrophila* (Chester) Stanier ATCC 7966, *Aeromonas veronii* Hickman-Brenner et al. ATCC 35624 and *Aeromonas caviae* (ex Eddy) Popoff ATCC 15468 were used as reference strains throughout this study.

In total, 331 *Aeromonas* isolates, recovered using the selective culture technique, were confirmed through PCR screening and kept for further statistical analysis after confirmation of unique phylogeny (Section 2.3) and/or virulence gene profile (Section 2.4) for that sampling data and site.

2.3. Aeromonas species assignment based on sequencing RNA polymerase σ^{70} factor, rpoD

Two isolates from each sampling site and sampling date were randomly selected for phylogenetic analysis. This yielded 227 *Aeromonas* isolates for which an ~800 bp fragment of the *rpoD* gene was amplified by PCR and sequenced according to Martinez-Murcia et al. (2011) with some modification. PCR amplification was conducted in 50 μl reactions consisting of 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 20 mM $(NH_4)_2SO_4$ (Becton, Dickinson and Company (BD), Sparks, MD, USA), 0.2 μM each of forward and reverse primers and 0.24 μl of 5 Units of Taq DNA Polymerase (NEB). Amplification conditions are given in Table S2. Amplicons were confirmed through gel electrophoresis on 1% agarose (Lonza). Forward end *rpoD* (*rpoD* 4F or *rpoD* 70F) sequencing (700 to 800 bp reads) was conducted at Eurofins Genomics (Louisville, KY, USA) using industry standard Sanger sequencing. Sequence chromatograms were checked both manually and using BioEdit v7.0.5 (Ibis Therapeutics, Carlsbad, CA, USA) to remove any ambiguity and to identify insertions, deletions, sequencing errors or the presence of stop codon.

2.4. Detection of virulence-related genes in Aeromonas spp.

Virulence genes encoding enterotoxins, aerolysin and flagella (listed in Table S2) were evaluated in 331 *Aeromonas* spp. isolates. DNA was extracted using the QIAGEN DNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer protocol. Multiplex PCR were optimized in two protocols. Protocol I was designed for three sets of target genes, *act-alt*-16S rRNA, whereas protocol II targeted the *aerA-flaA-alt*-16S rRNA genes. PCR amplification was performed as described above but with 0.25 mM dNTPs (VWR) and 0.35 μM each of primer (IDT). Amplification conditions are described in Table S2. For each PCR reaction, 2.5 μl template DNA (~20 ng/μl) was used. PCR products were electrophoresed on 1% agarose (Lonza) gels to determine presence or absence of target genes.

2.5. In vitro bacterial attachment to a polystyrene surface

A loopful of fresh culture of *Aeromonas* isolates ($N = 331$) on TSA-VA were resuspended into Tryptic Soy Broth (BD) supplemented with ampicillin and vancomycin (TSB-VA). Sterile 96-well polystyrene plates were filled with 200 μl of culture suspension and incubated at 37 °C for 72 h, in replicates of 3 per isolate. Uninoculated TSB-VA served as a negative control. After incubation, planktonic cells were removed by inverting the plate and washing three times with sterile water. The remaining attached cells were fixed by adding 200 μl methanol and incubated at room temperature for 20 min, after which methanol was removed and the plate air-dried. The remaining attached cells were stained with 2% (w/v) crystal violet (TCI, Toshima, Kita-ku, Tokyo, Japan) for 5 min, washed with sterile water and air-dried. The cells were then suspended in 150 ml of 33% (v/v) glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) to bring the crystal violet adhering to attached cells back into solution. The optical density (OD) of each well was measured at 570 nm in a microplate reader (SYNERGY HTX Multi-Mode, BioTek Instruments Inc., Winooski, VT, USA) and interpreted as previously described (Stepanovic et al., 2000).

2.6. Aeromonas attachment to the lettuce leaf surface

Lettuce cultivation: Romaine lettuce cultivar 'Sparx' seeds were planted in the greenhouse. After germination, seedlings were transplanted and kept at a temperature of 20 °C during day for 13 h and 18 °C at night for 11 h. Seven-week-old plants were used for inoculation.

Isolate selection: Seven isolates were selected from a total of 13 strong biofilm formers on polystyrene with potential for enteric pathogen status (based on classification) that also harboured more than one enterotoxin gene. *Aeromonas hydrophila* (Chester) Stanier ATCC 7966 and *Aeromonas veronii* Hickman-Brenner et al. ATCC 35624 were used as reference strains.

Inoculum preparation and inoculation: 0.5 MacFarland standard bacterial suspensions were prepared for the seven selected isolates and the reference strains from overnight *Aeromonas* cultures (10^8 cells/ml). A 100 μl aliquot of this suspension was then inoculated onto the adaxial surface of the third and fourth leaves of 7-week-old lettuce plants in triplicate. Plants inoculated with water only were considered as negative control. Plants were processed 0, 24 and 120 h post inoculation (hpi; 3 plants each). Using sterile scissors, inoculated leaves were cut and placed in WhirlPak bags (Nasco, Baltimore, MD, USA) for bacterial retrieval and quantification. For retrieval of loosely attached bacterial cells, 30 ml 0.1% peptone water (BD Difco, Franklin Lakes, NJ, USA) were added to the bag and placed in the shaker for 5 min. Then, 100 μl of the leaf surface washes were plated on TSA supplemented with ampicillin and vancomycin (TSA-AV) as previously described and incubated at 37 °C for 24 h. In order to recover strongly associated cells, the suspension was discarded, and leaves were washed with sterile water twice to remove all loosely attached cells, then smashed using a pestle and diluted into 30 ml 0.1% peptone water. The bags were placed on the shaker for 5 min for homogenization and plated on TSA-AV and incubated for 24 h at 37 °C.

2.7. Sequencing data analysis

Reference strains were selected from NCBI BLASTn comparisons to classify isolates ($n = 227$) for building phylogenetic trees grouped by water type from which they originated and season of sample collection. Alignments were carried out in MEGA v. 7.0 (Pennsylvania State University, PA, USA) using built-in ClustalW (Kumar et al., 2016) with 1000 iterations in a neighbor joining clustering method. Every single sequence was aligned with a reference organism (type strain or lab strain from database), then all sequences were aligned together. All ambiguous characters or sequences (whole sequence) were excluded from the final alignment file. Amplicon sequences have been deposited at NCBI GenBank under accession number MW122064 - MW122082 and MW122083 - MW122290.

Isolates were placed into different categories based on water type and season. Kimura's 2-parameter model along with 1000 bootstrap replications were used to determine the genetic distance between isolates (Kimura, 1980) and evolutionary tree construction was performed by the Maximum Composite Likelihood model with MEGA v. 7.0 (Kumar et al., 2016). Trees were graphically displayed and annotated by iTOL v 4.2.3 (Letunic and Bork, 2016). Species diversity was calculated using the Margalef diversity index (Margalef, 1958):

$$D = \frac{S-1}{\ln N}$$

where S is the number of species detected in each water type, and N is the total number of isolates in that water sample.

2.8. Data management and statistical analysis

In total, 331 *Aeromonas* isolates were found to have unique phylogenetic classification and virulence gene profiles for site and sampling date and were included in data analysis. To check seasonal and water type effects on bacterial attachment to a polystyrene surface, data for $N = 331$ were pooled by water type (5 NF, 1 TB, 2 PW, 3 RW sites) and season. Season was defined as spring from March 01 to May 30, summer from June 01 to August 31, fall from September 01 to November 30, and winter from December 01 to February 28. The Pearson's Chi-square test was conducted to assess the effects of season and water type on the presence of virulence genes and on attachment to a polystyrene surface using R

packages "nnet" ver. 7.3.14 and "multcomp" ver. 1.4.15. Bacterial attachment and persistence capability on a lettuce surface was assessed using mixed effect model analysis using R package "lme4" ver. 1.1.26. The repeated measurements effects were controlled using the random effect of replication and fixed effects of attachment strength and inoculation time. Tukey's Honestly Significant Differences test with $\alpha = 0.05$ was used to assess significant differences between bacterial loads at three time points. Data visualization and graphical representation was done using the R packages "ggplot2" ver. 4.0.3 and "pheatmap" ver. 1.0.12. Multiple correspondence analysis (MCA) was conducted using virulence gene presence/absence data and water type, season, attachment and classification data. MCA is a method for visualizing associations among categorical variables. The adjusted inertia explaining the variability in each dimension on the MCA plots were calculated according to Greenacre (1984). MCA were performed in JMP Pro 14.1.0.

3. Results

3.1. Phylogenetic classification of Aeromonas isolates

A total of 331 isolates were confirmed to be *Aeromonas* spp.; 172 recovered from non-tidal fresh river water, 27 from tidal brackish river water, 69 from pond water and 63 from reclaimed water. A subset of 227 *Aeromonas* isolates, representative of water samples taken from all sources from all sampling dates, were classified phylogenetically and analyzed as a factor of water type and seasonality. Sequence alignments were generated from *rpoD* sequence fragments of 702 bp. In total, 20 different *Aeromonas* species were identified in all water samples: *A. veronii*, *A. culicicola*, *A. hydrophila*, *A. jandaei*, *A. bestiarum*, *A. dhakensis*, *A. popoffii*, *A. piscicola*, *A. enterica*, *A. allosaccharophila*, *A. salmonicida*, *A. encheleia*, *A. eucrenophila*, *A. aquatica*, *A. tecta*, *A. punctata*, *A. caviae*, *A. media*, *A. rivipollensis*, *A. bivalvium* and (Fig. 1). Although we kept these classifications separate since they aligned with different type strains, *A. punctata* and *A. caviae* are considered objective synonyms and *A culicicola* is considered a later subjective synonym of *A. veronii* (Janda and Abbott, 2010). Phylogenetic analyses showed that the most frequently encountered classification (making up ~63% of isolates) clustered with three major human pathogenic species *A. veronii*, *A. hydrophila* and *A. jandaei*. The next two most prevalent classifications, *A. bestiarum* and *A. piscicola* both known to cause disease in fish, together represented 11% of isolates. Two other species known to cause human disease, *A. caviae* and *A. dhakensis* were also detected but at lower frequencies (~2%) (Fig. 1).

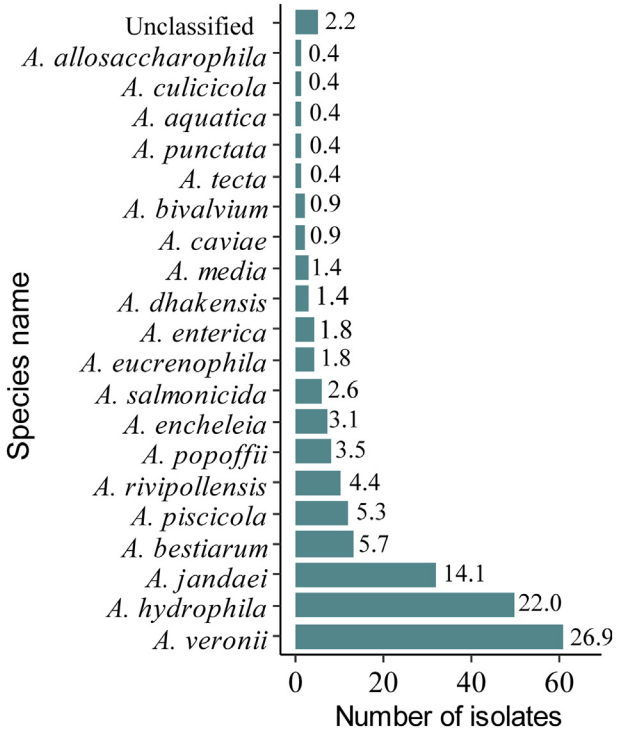

Fig. 1. Frequency distribution of *Aeromonas* species ($n = 227$) isolated from water collected at 11 sites in the mid-Atlantic region of the U.S., including tidal and freshwater rivers, ponds and reclaimed water. The *x*-axis denotes the total number of isolates and percentages are given alongside the bars for each species. Note: *A. punctata* and *A. caviae* are considered objective synonyms; *A culicicola* is considered a later subjective synonym of *A. veronii* (Janda and Abbott, 2010).

3.2. Diversity of Aeromonas species in different water types

Phylogenetic trees were generated with sequences grouped by water type. The phylogenetic tree constructed from non-tidal freshwater river/creek isolates collected from 5 sites was built using 277 similar and 425 variable nucleotide positions (Fig. 2A). Pairwise distances between nucleotide sequences of NF isolates ranged from 0.002 to 0.577. A total of 119 sequences clustered with 14 representative species, where Margalef diversity index was calculated as 2.72 (Table 1). Out of 119 isolates, 38 (31.9%) clustered with the reference strain *Aeromonas hydrophila* ATCC 7966 and 30 (25.2%) clustered with the reference strain *Aeromonas veronii* ATCC 35624. Three isolates remained unclassified (Fig. 2A; Table 1).

The phylogenetic tree generated using sequences from tidal brackish river water isolates was built with 493 similar and 209 variable nucleotide positions. Pairwise distances between nucleotide sequences ranged from 0.003 to 0.172. Only one tidal brackish water source was sampled, yielding 20 isolates used in this analysis. The 20 sequences clustered with 10 *Aeromonas* species with a Margalef diversity index of 3.00, the highest measure obtained for any water type (Fig. 2B; Table 1). One isolate form TB remained unidentified.

Phylogenetic analysis for pond water isolates was carried out with 35 sequences having 443 similar and 259 variable nucleotide positions. Pairwise distances between nucleotide sequences of PW isolates ranged from 0.001 to 0.234. These sequences clustered with 7 different species giving a Margalef diversity index value of 1.69, the lowest measure obtained for any water type. Eighteen out of 35 isolates clustered with *A. jandaei* (51.4%) and the rest clustered with six other species (Fig. 2C; Table 1).

The phylogenetic tree generated with isolates recovered from reclaimed water sites was constructed with 408 similar and 294 variable nucleotide positions on the *rpoD* gene. Pairwise distances between nucleotide sequences ranged from 0.01 to 0.23. Nine representative species were identified and the Margalef diversity index value was calculated at 2.02. Altogether 25 out of 53 total sequences (47.2%) clustered with *A. veronii*, 7 with *A. hydrophila* and 7 with *A. jandaei*. One isolate remained unclassified (Fig. 2D; Table 1).

Excluding the isolates that were not classified, *Aeromonas* species distribution appeared to be significantly affected by water type (Pearson's χ^2 ($n = 222$, df $= 57$) $= 183.410$, $p < 0.001$). *A. hydrophila* and *A. veronii* ranked highest in frequency in non-tidal fresh river and reclaimed water (Table 1). *A. veronii* and *A. hydrophila* were found in all water sources and prevalent at all sites. *A. jandaei* and the fish pathogen *A. piscicola* ranked first and second, respectively, in pond water but were also detected in all other water sources (Fig. 3). *A. rivipollensis* was also prevalent at both pond water sites. Three species, including *A. dhakensis* were found only in non-tidal fresh river water. *A. caviae* was detected only in reclaimed water. Three other possible human pathogens were detected sporadically; *A. popoffii* was only detected in NF and TB, *A. enterica* in PW and TB, and *A. tecta* in RW. Fish pathogens *A. eucrenophila* and *A. allosaccharophila* were prevalent in NF, *A. bestiarum* in both NF and RW, *A. salmonicida* in NF, RW and TB, and *A. encheleia* in NF and TB (Fig. 3).

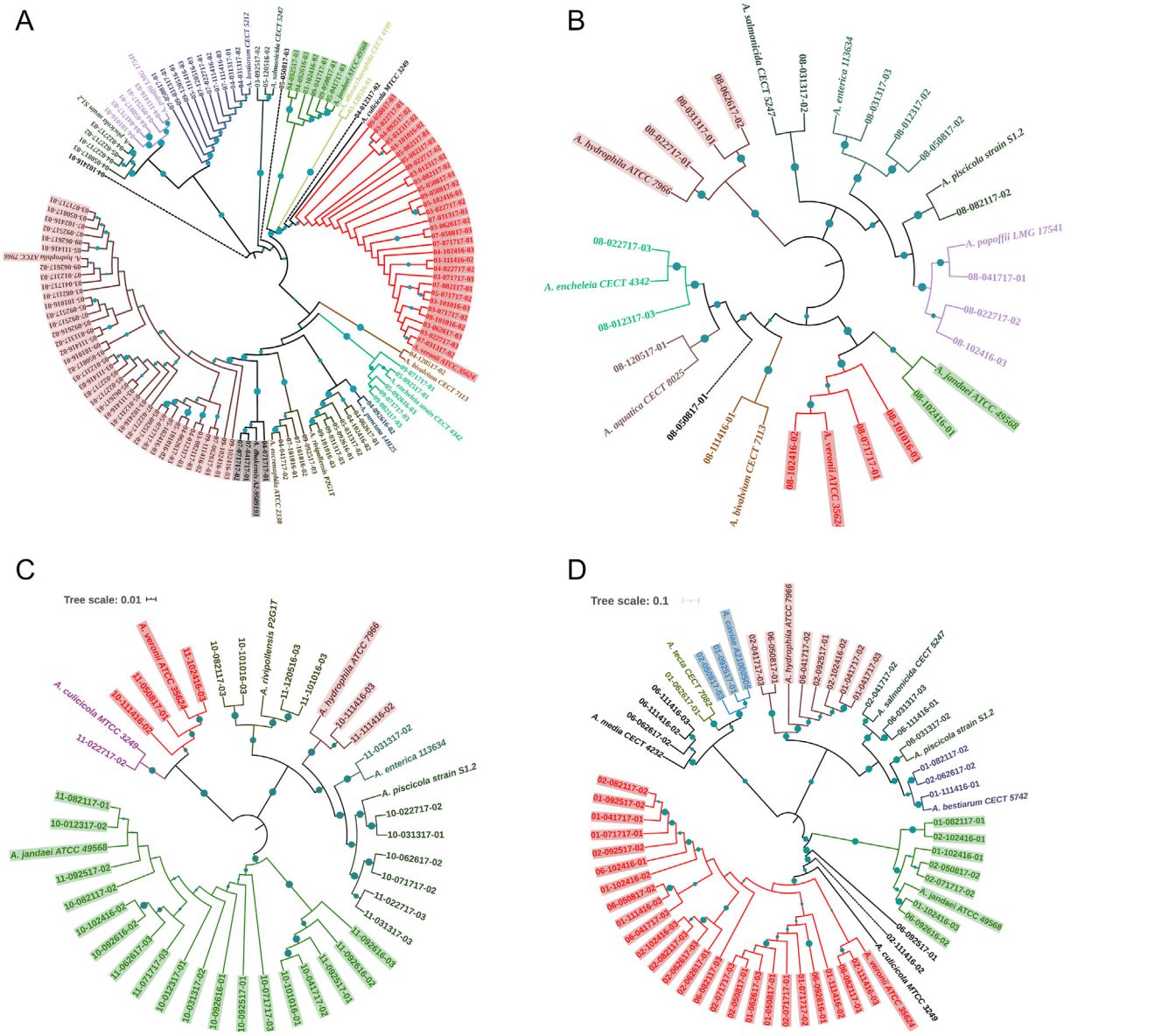

Fig. 2. Phylogenetic relationship among representative isolates (n = 227) of *Aeromonas* spp. collected from different water sources with potential for use as irrigation water. The trees were constructed from isolates collected from (A) non-tidal fresh rivers (NF; $n = 119$ from 5 sites), (B) tidal brackish rivers (TB; $n = 20$ from 1 site), (C) pond water (PW; $n = 35$ from 2 sites) and (D) reclaimed water (RW; $n = 53$ from 3 sites). Analyses were done using a maximum composite likelihood model. Isolate codes (denoted as site-date-isolate number) were aligned to reference strains from NCBI BLASTn. Each coloured node in the circular tree represents specific species. Blue dots in different sizes represent bootstrap values: small, medium and large dots indicate >50% to <70%, ≥70% to <90% and ≥90% values, respectively. Probable pathogenic species (from literature reports) were marked with a background colour on each leaf. Unidentified isolates (not phylogenetically classified) were denoted using a dashed line on that specific node. Note: *A. punctata* and *A. caviae*, and *A culicicola* and *A. veronii* are considered synonyms (Janda and Abbott, 2010).

3.3. Seasonal variability in Aeromonas species detected

The impact of seasonal variability on *Aeromonas* spp. distribution revealed by phylogenetic analysis (Fig. 4) approached significance (Pearson's χ^2 (n = 222, df = 60) = 74.121, $p = 0.063$). The 86 isolates collected in the fall clustered with 13 different species giving a Margalef Diversity Index of 2.69 (Table 1). Sequences from 36 isolates recovered in the winter clustered with 14 different classifications. Species diversity was found to be higher in winter than other seasons, with a Margalef Diversity Index of 3.62. In spring, 52 isolates clustered with 12 different classifications giving a Margalef Diversity Index value of 2.77. Sequences from the 53 isolates retrieved in summer clustered with 10 different species yielding a Margalef Diversity Index of 2.27. The majority of *A. veronii* isolates ($n = 61$) were retrieved in summer (36.1%) and fall (32.8%) (Fig. 4). *A. hydrophila,* and *A. jandaei* were mostly isolated in the fall (44.0% and 53.1%, respectively). *A. caviae* was only isolated in fall (50.0%) and spring (50.0%) while *A. dhakensis* was most prevalent in summer (67%). For all these species, occurrence was lowest in winter. All other species were distributed uniformly across seasons (Fig. 4).

3.4. Virulence gene patterns in different Aeromonas species

All isolates ($N = 331$, including ones that were not phylogenetically classified) were screened for five virulence genes, the enterotoxin genes *ast*, *alt*, *act*, the haemolysin gene *aerA* and the flagellar gene *flaA*. Of these, 319 (~96%) isolates harboured at least one of the virulence genes, 68 harboured only 1 gene, 12 harboured none, and 2 harboured all 5 genes (Fig. S1). The *flaA* gene was the most prevalent gene, with 200/331 (~60%) isolates carrying it. The next most prevalent gene was *alt*, detected in 181/331 (~55%) isolates. The genes *act, ast* and *aerA* were detected in 136 (~41%), 126 (~38%) and 116 (~35%) isolates, respectively (Table 2).

Table 1
Aeromonas species distribution and diversity measures by water type and season (n = 227).

	N°. of Sites	N°. of Isolates	Species Richness	Margalef Diversity Index	Most Frequent Classification (%)
Water type					
Non-tidal fresh	5	119	14	2.7	*A. hydrophila* (32) *A. veronii* (25)
Tidal brackish	1	20	10	3.0	*A. enterica* (15) *A. hydrophila* (15) *A. popoffii* (15) *A. veronii* (15)
Pond	2	35	7	1.7	*A. jandaei* (51) *A. piscicola* (17)
Reclaimed	3	53	9	2.0	*A. veronii* (47) *A. hydrophila* (13) *A. jandaei* (13)
Season					
Fall	11	86	13	2.7	*A. hydrophila* (26) *A. veronii* (23) *A. jandaei* (20)
Winter	08	36	15	3.6	*A. hydrophila* (22) *A. veronii* (19)
Spring	11	52	12	2.8	*A. veronii* (23) *A. hydrophila* (19)
Summer	11	53	10	2.3	*A. hydrophila* (42) *A. veronii* (19)

Focusing on the phylogenetically classified 227 representative isolates showed that those carrying all three enterotoxin, aerolysin and flagellar genes were classified as *A. popoffii* ($n = 1$) and *A. eucrenophila* (n = 1). Among the 50 isolates classified as *A. hydrophila*, the genes *alt* (60%) and *flaA* (60%) were the most frequently detected (Table 2). The most common gene pattern was *ast* + *alt* + *flaA* (11 isolates), while18 isolates carried two enterotoxin genes (1 *ast* + *act*, 13 *ast* + *alt*, 4 *act* + *alt*) and 5 carried all three enterotoxin genes along with *aerA* or the *flaA* gene. Among 61 probable *A. veronii* isolates, the most frequently detected genes were *flaA* (69%), *alt* (57%) and *ast* (51%). Several *A. veronii* isolates harboured gene combinations. The gene pattern *ast* + *alt* + *flaA* (8 isolates) was also common, while 27 isolates carried two enterotoxin genes (7 *ast* + *act*, 16 *ast* + *alt*, 4 *alt* + *act*) and 6 carried all three enterotoxin genes along with *aerA* or *flaA* genes. Among 32 probable *A. jandaei* isolates, *alt* (69%) and *flaA* (63%) were also the most prevalent genes. Common gene combinations included 13 isolates carrying two enterotoxin genes (2 *ast* + *act*, 5 *ast* + *alt*, 5 *alt* + *act*) and 3 harbouring all three enterotoxin genes along with or without *flaA*. A high prevalence of *flaA* carriage was detected in *A. piscicola* (83%) and *A. bestiarum* (62%), followed by *aerA* and *alt* (in *A. piscicola* only). Only one probable *A. dhakensis* isolate collected in the summer harboured a combination of *ast* + *alt*. Another 2 isolates carried *ast* and *act* separately. Likewise, only one probable *A. caviae* isolate carried *ast* + *act*, the second isolate harboured only the *act* gene.

An MCA plot of 199 classified *Aeromonas* isolates, including all classifications with $n \geq 6$, was plotted to assess the association between virulence gene detection in isolates and assigned species (Fig. 5A). The plot corroborated the associations between gene patterns and species assignments. The genes contributing most to inertia were presence or absence of *alt*, and presence of *aerA* and *ast* for dimension 1, and presence or absence of *act* and absence of *flaA* for dimension 2. The MCA plot supported the association between *A. hydrophila* ($n = 50$) and the presence of *flaA* and *alt* genes, both detected in 60% of isolates, as well as *ast* and *act* (Fig. 6A). The position of *A. veronii* ($n = 61$) on the plot reflected the high prevalence of the genes *flaA, alt, ast* and *act* in that species. *A. jandaei* ($n = 32$) was positioned closest to $flaA^+$ and alt^+ on the plot, driven by approximately two-thirds of isolates found to harbour those genes, and influenced by the absence of *aerA*. The subsequent, most prevalent classifications, *A. bestiarum* ($n = 13$) and *A. piscicola* ($n = 12$) were divergent from the other species (Fig. 5A), attributed to the higher number of gene patterns identified in relation to spp. richness. *A. caviae* associated with reclaimed water. Gene patterns:spp.

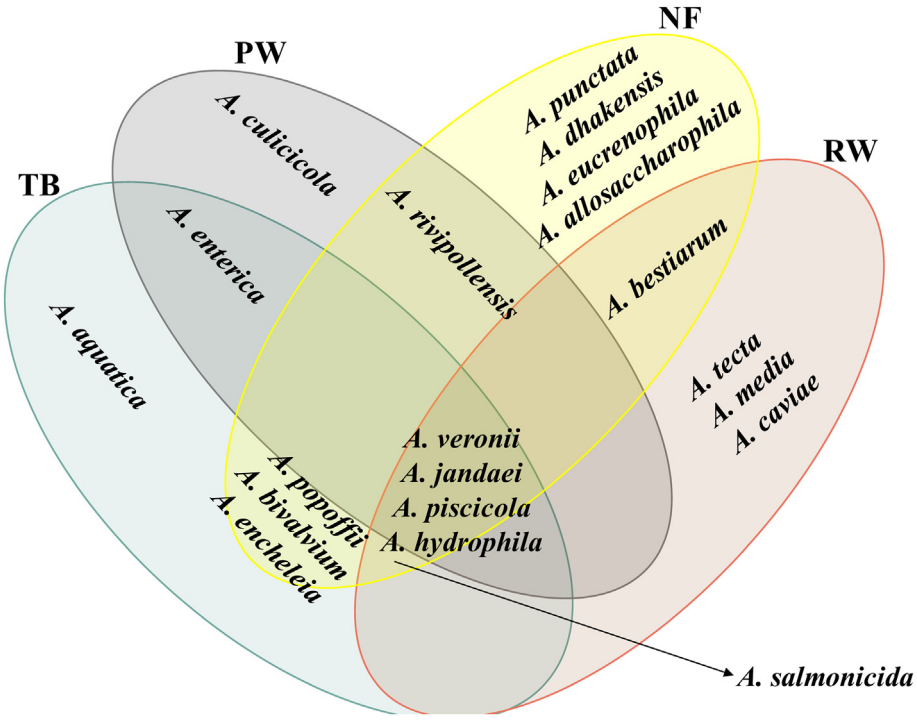

Fig. 3. Venn diagram showing *Aeromonas* species detection by water type, non-tidal fresh rivers (NF; yellow balloon), tidal brackish rivers (TB, green balloon), pond water (PW, grey balloon) and tertiary treated reclaimed water (RW, red balloon) for 227 isolates. Four species, *A. veronii, A. jandaei, A. piscicola* and *A. hydrophila*, were detected in all water types. Note: *A. punctata* and *A. caviae,* and *A culicicola* and *A. veronii* are considered synonyms (Janda and Abbott, 2010).

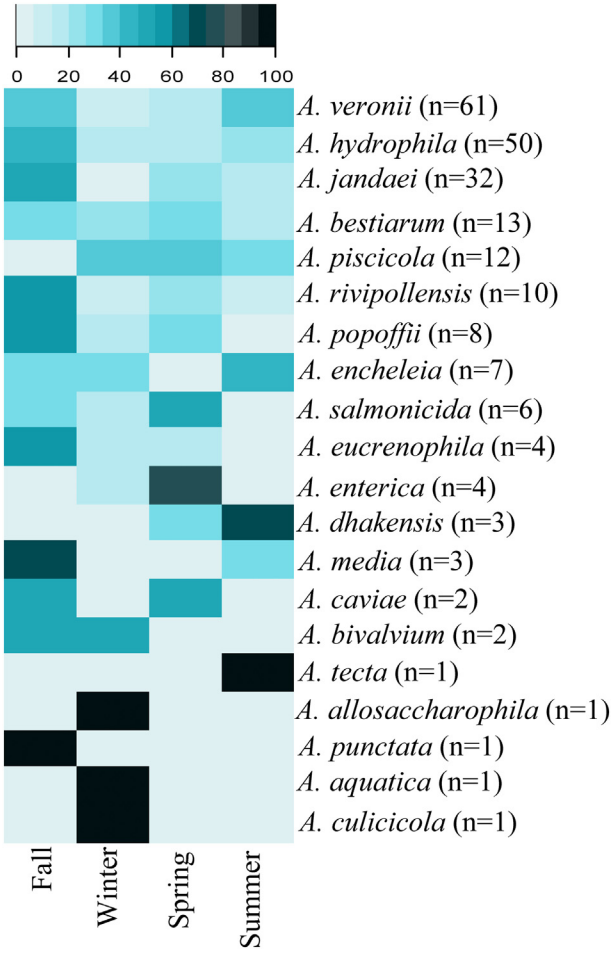

Fig. 4. Heat map showing the seasonal variation in distribution of *Aeromonas* species among 227 isolates. The colour key shows percent of each species in a specific water type. Colour gradation from blue to black denotes frequency increase. Note: *A. punctata* and *A. caviae*, and *A culicicola* and *A. veronii* are considered synonyms (Janda and Abbott, 2010).

richness ratios were 9:13 (0.7) and 8:12 (0.7) for *A. bestiarum* and *A. piscicola*, respectively, compared to e.g. *A. veronii* (26:61; 0.4) and *A. hydrophila* (22:50; 0.4).

3.5. Aeromonas spp. attachment to an abiotic surface assessed by water type and season

Biofilm production for each isolate was interpreted according to the scheme in Table S3. A large number of isolates in our study were not able to or only weakly able to attach to an abiotic (polystyrene) surface. Out of 331 isolates tested, 211 (63.7%) formed weak biofilms on polystyrene while 70 (21.1%) isolates were strong biofilm producers. An additional 50 (15.1%) were classified as moderately capable of attaching to the polystyrene surface.

Isolates collected from different water types exhibited variability in their attachment capability (Fig. 6A). Non-tidal fresh water isolates and reclaimed water isolates had the highest proportion of strong biofilm formers, at 41/172 (23.8%) and 16/63 (25.4%), respectively. The other non-tidal fresh water isolates were characterised as weak (105/172; 61.0%) and moderate (26/172; 15.1%) biofilm formers. Similarly, a larger proporiton of reclaimed water isolates were determined to be weakly (39/63; 61.9%) compared to moderately (8/63; 12.7%) able to attach to polystyrene. None of the 30 tidal brackish water isolates attached strongly to the polystyrene and these isolates were grouped as weak (22 isolates; 73.3%) and moderate (5 isolates; 16.7%) biofilm formers. From the 69 pond water isolates, 45 (65.2%) were categorized as weak, 11 (15.9%) as moderate and 13 (18.8%) as strong biofilm formers. The apparent effect of water type on bacterial attachment to a polysterene surface was not statistically significant (Pearson's χ^2 (df = 6, N = 331) = 9.08, p = 0.16) (Fig. 6A).

Our data indicated that season of isolate collection exerted a stronger influence on attachment capability than water type. A significant seasonal variation was detected (Pearson's χ^2 (df = 6, N = 331) = 15.71, $p < 0.05$); isolates collected in spring exhibited a stronger attachment capabilty to an abiotic surface than in other seasons (Fig. 7B). From the 79 isolates recovered in the spring (median water temperature 17.7 °C), 26 (32.9%) attached strongly to the abiotic surface, while 43 (54.4%) were categorized as weakly and 10 (12.6%) moderately attached. Out of 121 isolates collected in the fall (median water temperature 15.2 °C), 81 (66.9%) were categorized as weak, 19 (15.7%) as moderate and 21 (17.3%) as strong biofilm formers. From 80 isolates retreived in the summer (median water temperature 26.1 °C), 46 (57.5%) attached weakly, 16 (20.0%) moderately and 18 (22.5%) strongly to the polystyrene surface. From the 51 isolates collected in the winter (median water temperature 8.0 °C), 41 (80.4%) were grouped as weak, 5 as moderate (9.8%) and 5 (9.8%) as strong biofilm formers (Fig. 6B).

Table 2
Detection of virulence genes in phylogenetically classified isolates (excludes 5 isolates that remained unclassified; n = 222).

Classification	n	Genes									
		ast		*act*		*aerA*		*flaA*		*alt*	
		+	−	+	−	+	−	+	−	+	−
A. allosaccharophila	1		1		1	1		1			1
A. aquatica	1		1		1		1	1		1	
A. bestiarum	13	2	11	5	8	6	7	8	5	3	10
A. bivalvium	2		2	1	1	1	1	1	1		2
A. caviae	2	1	1	2		1	1	1	1		2
A. punctata	1	1			1		1	1		1	
A. dhakensis	3	2	1	1	2		3	2	1	1	2
A. encheleia	7	3	4	5	2	1	6	5	2	6	1
A. enterica	4		4	1	3	2	2	1	3		4
A. eucrenophila	4	3	1	2	2	1	3	4		2	2
A. hydrophila	50	21	29	20	30	14	36	30	20	30	20
A. jandaei	32	12	20	13	19	11	21	20	12	22	10
A. media	3	1	2	1	2	1	2	1	2	2	1
A. piscicola	12	4	8	4	8	6	6	10	2	6	6
A. popoffii	8	2	6	3	5	6	2	6	2	5	3
A. rivipollensis	10	5	5	3	7	2	8	6	4	6	4
A. salmonicida	6	6		6		4	2	4	2	3	3
A. tecta	1	1		1		1			1		1
A. veronii	61	31	30	27	34	26	35	42	19	36	25
A. culicicola	1		1	1		1		1		1	
n		95		96		84		145		125	

3.6. Association between virulence genes, season, water type and attachment capability

Out of 70 isolates from different water types collected in different seasons that were capable of strong attachment to an abiotic surface, 44 were assigned a phylogenetic classification and comprised of *A. veronii* (12), *A. hydrophila* (8), *A. jandaei* (7), *A. bestiarum* (3), *A. piscicola* (3), *A. encheleia* (2), *A. salmonicida* (2), *A. caviae* (2) and 1 isolate each of *A. enterica*, *A. eucrenophila*, *A. media*, *A. popoffii*, and *A. rivipollensis*. The detection of the genes *ast* (Pearson's χ^2 (df = 3, N = 331) = 8.21, $p < 0.05$) and *flaA* (Pearson's χ^2 (df = 3, N = 331) = 11.72, $p < 0.01$) differed by water type, with *ast* less likely to be detected in tidal brackish river and pond water and *flaA* more likely to be detected in non-tidal fresh river water. The detection of the genes *ast* (Pearson's χ^2 (df = 3, N = 331) = 105.97, $p < 0.001$) and *alt* (Pearson's χ^2 (df = 3, N = 331) = 25.09, $p < 0.001$) differed by season. The gene *ast* was more likely to be detected in the summer and less likely to be detected in the winter and

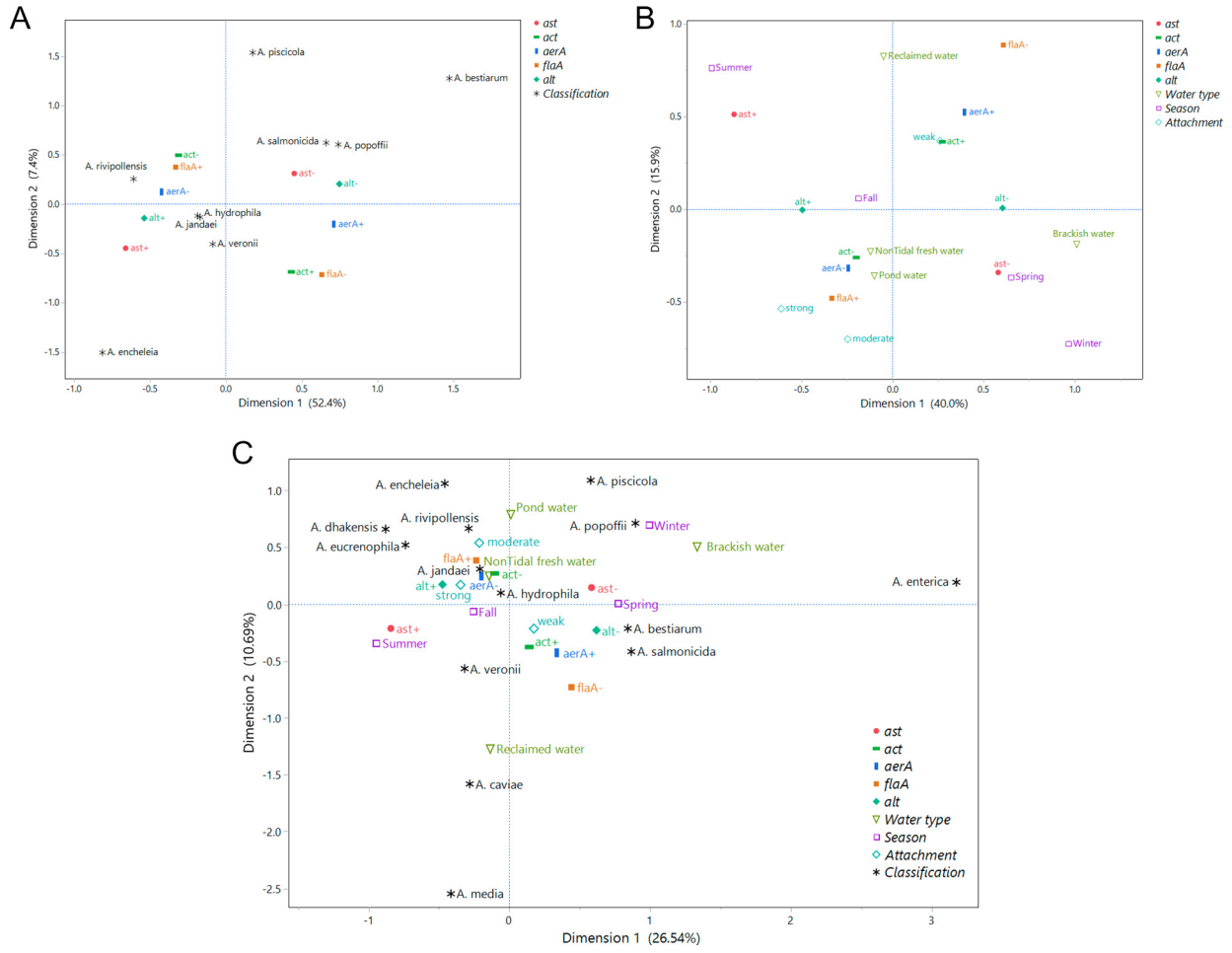

Fig. 5. Multiple Correspondence Analysis (MCA) plot showing the association between assigned species, harboured virulence genes and attachment to polystyrene surface. MCA plot (A) displays the association between virulence gene profiles and *Aeromonas* species where number of each species ≥6, ($n = 199$), (B) displays the association between virulence genes and attachment in various seasons and water types ($N = 331$) and (C) shows the association among assigned species with $n \geq 3$ ($n = 214$), harboured virulence genes and attachment to polystyrene in various seasons and water types ($n = 217$). *A. punctata* and *A. caviae* are pooled in (C); *A culicicola* and *A. veronii* are pooled in (A) and (C). The adjusted inertia explains the variability in each dimension on the MCA plots, calculated according to Greenacre (1984).

spring, while *alt* was more likely to be detected in the summer and less likely in the winter. All 5 strongly attaching isolates (*A. hydrophila* (2), *A. encheleia* (2) and *A. popoffii* (1)) that carried all enterotoxin genes (*ast, alt* and *act*) were collected from non-tidal fresh river water, in summer (4) and fall (1).

The MCA plot exploring the associations between virulence genes, attachment, season and water type for all isolates (N = 331), revealed a strong association bewteen summer and the gene *ast* (Fig. 5B). For the MCA plot in Fig. 5B, presence or absence of *ast* and the summer season were the strongest contributors to inertia for dimension 1, while the gene *flaA* contributed most inertia to dimension 2. The presence of *flaA* was strongly associated with strong and moderate attachment while the presence of genes *aerA* and *act* were associated with weak attachment capability (Fig. 5B and C). Out of 44 strongly and 40 moderately attaching isolates only 2 (5%) and 6 (15%) were *flaA* negative. On the other hand, the presence or absence of *flaA* was equivalent among weakly attaching isolates.

Strong attachment associated most closely with *A. jandaei* and *A, hydrophila* (20/32 and 30/50 were $flaA^{+}$, respectively). The detection of *alt* and non-tidal fresh river water and fall samplings also converged with strong attachment (Fig. 5C). This plot strongly supported the connection between summer and ast detection. Strongly attaching isolates classified as *A. veronii* and carrying enterotoxin genes were collected from RW (4) and NF (3), in fall (4) and summer (3); associations evident in the MCA plot in Fig. 5C. Brackish water isolates associated with winter collection, and the absence of the *ast* and *alt* genes, with *A. popoffi, A. bestiarum* and *A. salmonicida* relating to these factors (Fig. 5C). *A. encheleia*, *A. piscicola* and *A. rivipollensis* associated with pond water, with the latter also relating to moderate attachment and *flaA* (Fig. 5C). None of the isolates of the potentially enteropathogenic *A. caviae*, *A. dhakensis*, *A. enterica* and *A. popoffii* were categorized as strong biofilm producers and enterotoxin gene carriers simultaneously. Altogether, 13 isolates were found to harbour more than one enterotoxin gene and had the capability of strongly attaching to an abiotic surface. Seven of these isolates were selected to assess the attachment to lettuce leaves based on water type, virulence gene pattern and attachment to polysterene surface (Table 3).

3.7. Attachment and persistence of potential pathogenic species to biotic surface

To evaluate the possibility of transfer of presumptive pathogenic species of *Aeromonas* from irrigation water to vegetable crops, we investigated the attachment and persistence capability of select *Aeromonas* isolates to the lettuce leaf surface. Seven representative isolates classified as *A. hydrophila, A. veronii* and *A, jandaei*, determined to carry multiple virulence genes and having the ability to attach strongly to a polystyrene surface, were assayed in a lettuce model (isolates 1–7 in Table 3).

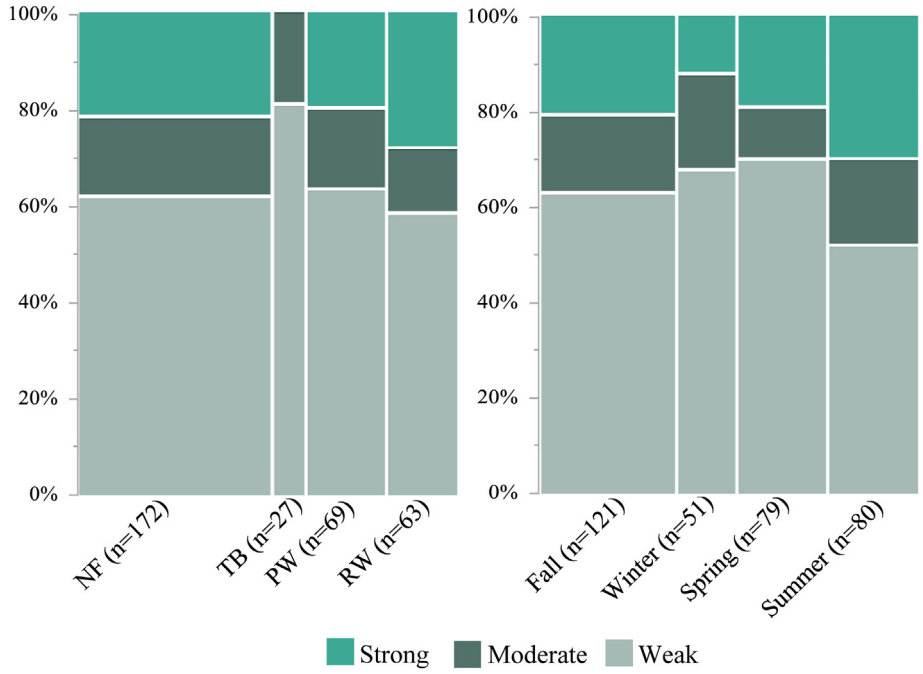

Fig. 6. Mosaic plots showing attachment capability of *Aeromonas* spp. (N = 331) onto polystyrene surface (biofilm formation) in (A) different water types and (B) different seasons. The *y* axis shows the proportion of isolates forming a biofilm. The length on the *x* axis of each rectangle is proportional to the percentage of isolates for each water type (non-tidal fresh river, NF = 52%, tidal brackish river, TB = 8%, pond water, PW = 21% and reclaimed water, RW = 19%) and seasons (fall = 36%, winter = 15%, spring = 23%, summer = 24%). n denotes the number of isolates.

Loosely and strongly attached cells were recovered from lettuce leaves for all test isolates and the two reference strains, *A. veronii* ATCC 35624 and *A. hydrophila* ATCC 7966. Out of 7 isolates, one (isolate 5, classified as *A. veronii* retrieved from RW in summer) showed no significant reduction of microbial load between time of inoculation and 120 h ($p > 0.05$), while two other *A. veronii* isolates (isolate 6 – collected from RW in fall and isolate 7 – collected from RW in summer) exhibited an initial reduction in loosely adhering cells ($p < 0.01$ and $p < 0.001$, respectively) after 24 h, followed by an increase after 5 days ($p < 0.01$ only for isolate 7). The rest of the isolates (isolates 1, 3 and 4, classified as *A. hydrophila* and isolate 2, classified as *A. jandaei*) showed a significant reduction in loosely attached bacterial cell counts over time (from 0 h to 5 days) ($p < 0.001$), similar to the reference strain *A. hydrophia* ATCC 7966 (Fig. 7).

Isolates 1 to 4 also exhibited a steady decline in bacterial cells strongly adhering to lettuce leaves, with a significant reduction in bacterial count ($p < 0.05$ for all) over time (from 0 h to 5 days). No significant change in strongly attached cells was detected for isolates 5 and 7, while isolate 6 exhibited an increase over time ($p < 0.05$) (Fig. 7).

We calculated the ratio of strongly to loosely attached cells (S:L) to assess attachment strength for each isolate (Table S4). Isolates 1–4 had a high level and isolates 5–7 a moderate level of strongly attached cells immediately after inoculation. This ratio remained proportional in isolates 1 and 2 after 24 h but decreased slightly in isolates 3 and 4, indicating a higher proportion of loosely to strongly attached cells. In all these isolates, strongly attached cells were an insignificant component of the population after 5 days. This was similar to the reference strain *A. hydrophila* ATCC 7966 (S:L from 0.66 to 0 by 120 h). Isolates 5, 6 and 7 exhibited an increase in the proportion of strongly attached cells after 24 h, as was detected for both reference strains. In isolates 6 and 7, the S:L ratio was proportional at 24 h. However, in contrast to *A. hydrophila* ATCC 7966, attachment strength in the *A. veronii* ATCC 35624 reference strain continued to augment over 120 h. This increase only occurred in one test isolate, isolate 5, which attained a proportional ratio of S:L cells by 120 h (Table S4). Attachment strength in isolate 6 remained high to also retain a proportional ratio. On the other hand, isolate 7 exhibited a decline in the S:L ratio but unlike isolates 1–4, maintained a strongly attached population of cells.

Taken together, this indicated that for *A. veronii* isolates 5, 6 and 7 and the reference *A. veronii* ATCC 35624 strain, strongly attached cells became a dominant component of the population of leaf-associated cells (Fig. 7). The assayed isolates classified as *A. hydrophila* and *A. jandaei* behaved similarly to the reference strain *A. hydrophila* ATCC 7966, all exhibiting declines in loosely and strongly attached cells by the end of the 5-day incubation.

4. Discussion

Foodborne disease outbreaks of fruit and vegetable crops are frequently associated with irrigation water (Gelting and Baloch, 2013; Gelting et al., 2015). Understanding the ecology and pathogenicity of under-studied pathogens prevalent in agricultural water will help to ensure that strategies developed to mitigate foodborne illness are universally effective. Using phylogenetic analysis, spatial and temporal distribution analysis, virulence gene profiling and attachment assays, this study characterised *Aeromonas* isolates recovered from representative irrigation water sources and evaluated potential for establishment onto a leafy green crop. Water type and season of collection affected the spatial and temporal distribution of *Aeromonas* species detected in water environments. The highest diversity was observed in fresh and tidal rivers and the lowest in ponds. Seasonally, the highest diversity was obtained in winter and the lowest in summer. *Aeromonas* species known to cause human disease were isolated from all water types with the highest incidence in non-tidal fresh river water and reclaimed water; both of which are identified as potential alternative irrigation water sources for fresh crops. Bacterial isolates collected from non-tidal fresh river, on-farm pond and reclaimed water were identified predominantly as *A. hydrophila, A. veronii* and *A. jandaei* and almost 75% of these isolates harboured multiple virulence genes. We also detected seasonal and water type effects on bacterial attachment to an abiotic surface. Importantly, *A. veronii* was able to attach and persist on Romaine lettuce leaves for up to 5 days. This study provides an ecological assessment of the under-studied bacterial taxon *Aeromonas* in surface

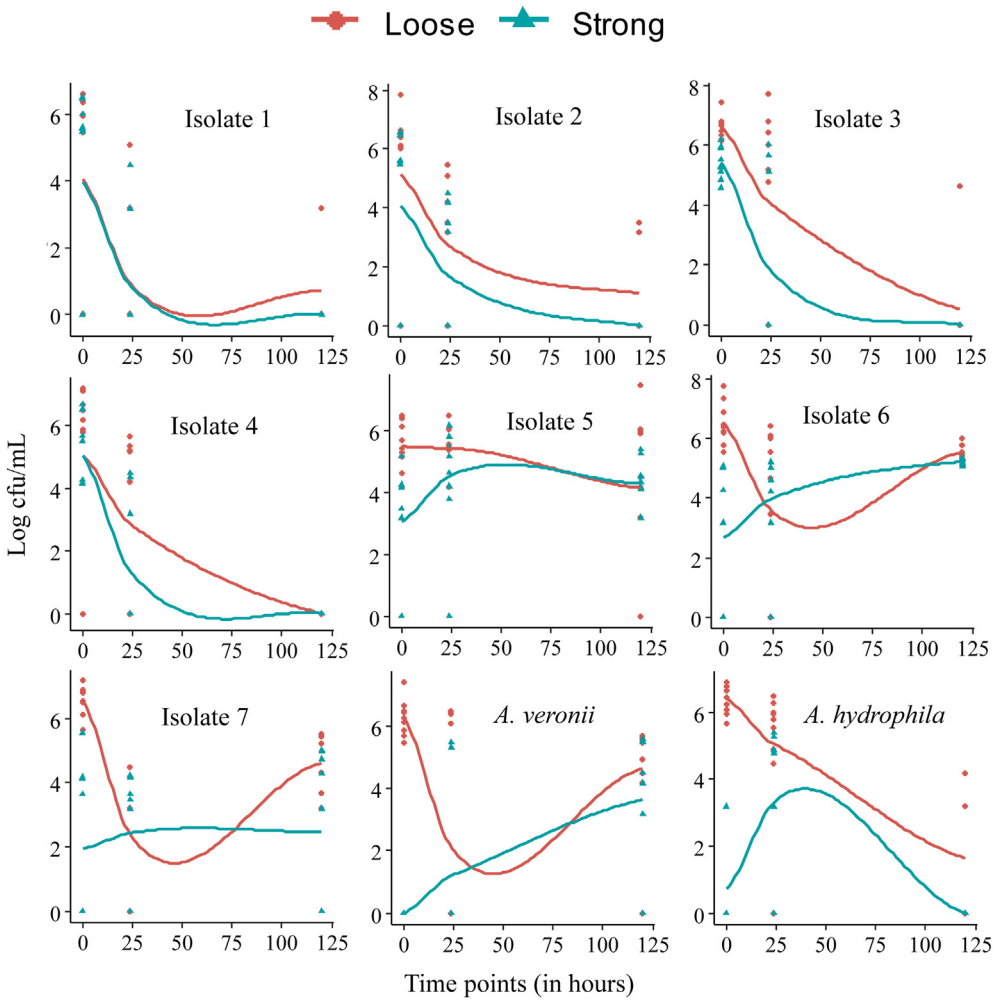

Fig. 7. Change in number of strongly to loosely (S:L) attached *Aeromonas* spp. on a biotic surface (adaxial side of Romaine lettuce leaves cv. 'Sparx'). Seven isolates used were classified as *A. hydrophila* (isolates 1, 3 and 4), *A. jandaei* (isolate 2) and *A. veronii* (isolates 5–7), recovered from different water types and harbouring different combinations of virulence genes as described in Table 3. *A. hydrophila* (Chester) Stanier ATCC 7966 and *A. veronii* Hickman-Brenner et al. ATCC 235624 were used as reference strains. Time points for cell counting were 1 h (0) post-inoculation, 24 h (24) post-inoculation and 120 h (120) post-inoculation of leaf surfaces.

and reclaimed waters and characterizes traits that may be relevant to food safety of fruit and vegetable crops that can contact this potential pathogen via irrigation water.

Aeromonas spp. is ubiquitous in water environments and is hence expected to be present in surface and recycled irrigation water sources. Monitoring the proportion of species within this large genus that are associated with human gastrointestinal disease in any given irrigation water source may provide a more appropriate indication of food safety risk. In this study, *A. hydrophila*, *A. veronii*, *A. jandaei* were the predominant species among 20 identified species in freshwater samples (freshwater

Table 3
List of the isolates exhibiting strong attachment to an abiotic surface. NF = non-tidal fresh river water, PW = pond water; RW = reclaimed water. Isolates 1–7 were selected for evaluating attachment to and persistence on a biotic surface (lettuce leaves).

Isolate Nº.	Classification	Season of Collection	Water Type	Virulence genes	Attachment to polystyrene	Attachment to lettuce
1	*A. hydrophila*	Summer	NF	*ast, alt, flaA*	Strong	Yes
2	*A. jandaei*	Summer	PW	*ast, alt, flaA*	Strong	Yes
3	*A. hydrophila*	Fall	NF	*ast, alt, flaA*	Strong	Yes
4	*A. hydrophila*	Summer	NF	*ast, alt, act, flaA*	Strong	Yes
5	*A. veronii*	Summer	RW	*alt, act, aerA, flaA*	Strong	Yes
6	*A. veronii*	Fall	RW	*alt, act, flaA*	Strong	Yes
7	*A. veronii*	Summer	RW	*ast, alt, aerA, flaA*	Strong	Yes
8	*A. hydrophila*	Summer	NF	*ast, alt, act, aerA*	Strong	No
9	*A. veronii*	Fall	NF	*ast, alt, flaA*	Strong	No
10	*A. veronii*	Fall	NF	*ast, alt, act, flaA*	Strong	No
11	*A. veronii*	Fall	RW	*ast, act, flaA*	Strong	No
12	*A. veronii*	Summer	NF	*ast, alt, flaA, aerA*	Strong	No
13	*A. veronii*	Summer	NF	*ast, alt, flaA*	Strong	No
Reference	*A. hydrophila*		ATCC 7966	*ast, alt, act, flaA*	Moderate	Yes
Reference	*A. veronii*		ATCC 35624	*ast, alt, act, aerA, flaA*	Strong	Yes

river and pond water). A study from China that included 40 isolates reported *A. veronii* as the predominant species, with *A. hydrophila* and *A. jandaei* comprising only 5% each in pond water (Hu et al., 2012). In Malaysia, a collection of 122 *Aeromonas* isolates recovered from a freshwater lake were dominated by *A. veronii* and *A. jandaei*, with a much lower proportion of *A. hydrophila* (Khor et al., 2015). In our study we also recovered known pathogenic species of *Aeromonas* (*A. hydrophila*, *A. caviae*, *A. veronii* and *A. jandaei*) from reclaimed water (from storage lagoons of tertiary treated wastewater treatment plants). *Aeromonas* spp. were not detected from three samples collected from tertiary treated wastewater in Spain, although all these species except for *A. jandaei* were isolated from secondary treated wastewater (Latif-Eugenín et al., 2017). Jjemba et al. (2010) reported a higher recovery rate of *Aeromonas* spp. compared to indicator microorganisms in storage reservoirs and distribution systems, but no detection in wastewater treatment plant effluents in Florida, California, Massachusetts and New York. Detection of *Aeromonas* in chlorinated effluent has been reported, however, attributed to its ability to multiply in low concentrations in the presence of residual chlorine (Al-Jassim et al., 2015). These and our reports attest to the adaptable capacity of *Aeromonas* and the importance of garnering a better understanding of its ecology and dynamics in the agricultural environment. It is unclear whether our current agricultural water standards and guidelines are effective in minimizing the risk of transfer of potentially pathogenic *Aeromonas* species from surface water to food crops. The use of reclaimed water requires strict compliance to recommendations for the adoption of this water type for fruit and vegetable crop irrigation (https://www.epa.gov/waterreuse/water-reuse-action-plan), and safety should be validated in relation to *Aeromonas* spp.

Seasonal dynamics in species diversity were detected; although higher diversity was found in the winter, the communities had higher evenness and several species not associated with human disease were identified. By contrast, *A. hydrophila* and *A.veronii* predominated in summer and fall communities. This is concordant with other studies and might explain the frequent gastroenteritis cases/outbreaks in summer (Humphries and Linscott, 2015; USEPA, 2006). Interestingly, in this study, the summer and fall seasons were associated with the presence of two entertoxin genes *ast* and *act* while the winter and spring were associated with the absence of these genes. The presence of virulence genes does not equate to pathogenicity, however, and the ability of aeromonads to cause gastroenteritis in humans is multifactorial and depends on a wide range of virulence determinants (Fernández-Bravo and Figueras, 2020). Albert et al. (2000) reported that the presence of multiple enterotoxin gene patterns (*alt* + *ast* or *alt* + *ast* + *act*) might be responsible for both pediatric and adult diarrhoea. In our study almost half of *A. hydrophila, A. veronii, A. jandaei, A. dhakensis* and *A. caviae* carried different combinations of enterotoxin genes along with attachment (*flaA*) and aerolysin (*aerA*) genes. Other studies report virulence gene detection in *A. hydrophila* from surface water, sediment, phytoplankton and zooplankton in Bangladesh (Albert et al., 2000) and from drinking water samples in the United States (Sen and Rodgers, 2004). Considering the high prevalence of virulence genes in environmental isolates, it is plausible that clinical and environmental strains are cycling through the host and the environment.

Biofilm formation in pathogenic microorganisms facilitates the disease transmission from contaminated irrigation water via food crops (Choi et al., 2013). In the present study, strong biofilm formation on a polystyrene surface was detected in *A. caviae*, *A. jandaei*, *A. veronii* and *A. hydrophila* retrieved from three water types (NF, PW, RW). A previous analysis of 58 environmental isolates from Turkey reported that *A. hydrophila* and *A. veronii* but not *A. caviae* were capable of strong biofilm formation (Ormanci and Yucel, 2017). In both cases, *A. veronii* showed a higher potential to form biofilms on abiotic surfaces than *A. hydrophila*. In our study, isolates classified as non-human pathogens were more likely to show strong attachment capabilities; *A. salmonicida*, *A. encheleia* and *A. piscicola* compared to *A. veronii* and *A. hydrophila*. Moderate and strong attachment associated with the presence of the *flaA* gene which was also detected in high proportions in the former group of species. Spring samplings were also more likely to yield an isolate exhibiting strong attachment, with summer and fall following. It is possible that weather related factors such as higher temperature and rainfall, which are lower in the winter months in the mid-Atlantic, may select for more efficient biofilm formers. The occurrence of isolates with combinations of various enterotoxin genes and stronger attachment ability may be coinciding with the crop growing seasons of April–October.

There is evidence that *Aeromonas* can transfer from irrigation water to fresh fruit and vegetables and act as a vehicle of transmission of infections (Latif-Eugenín et al., 2017). Indirect evidence also comes from sequence identity between two strains collected from irrigation water and fresh produce from a farm in Catalonia North-East of Spain (Latif-Eugenín et al., 2017). Assessing the attachment and biofilm formation capability of *Aeromonas* isolates gives us some insight on the potential for persistence in food processing environments and possible risk of contamination of fresh crops, but the data may not be directly translatable as was previously shown for *A. hydrophila* on cabbage and lettuce (Elhariry, 2011). We turned our attention to the five *A. hydrophila,* seven *A. veronii,* and one *A. jandaei* isolates from our study that exhibited strong attachment to the abiotic surface, carried the *fla* gene for which we identified an association with stronger attachment, and simultaneously carried multiple enterotoxin genes. These representative isolates, selected to assess their attachment to Romaine lettuce, did reveal additional capabilities relevant to agriculture and highlighted the caveat that attachment to abiotic surfaces may not be directly equivalent to interactions with vegetable surfaces. Both loosely and strongly associated cells of these isolates were retrieved from lettuce leaf surfaces, indicating the ability for *Aeromonas* to quickly adapt to this niche. Although three *A. hydrophila* and one *A. jandaei* isolates displayed a gradual decrease of both loosely and strongly associated cells after 120 h of leaf inoculation, the three *A. veronii* isolates exhibited a strong attachment capability and persisted in the lettuce phyllosphere for the duration of the 5-day incubation. These behaviors followed the same patterns as the reference strains and indicated that measurements on attachment to an abiotic surface can miss important characteristics pertaining to attachment to biotic surfaces which differed by species.

The on-plant assay not only strongly suggests that certain *Aeromonas* species or strains can become established on lettuce but indicates a possible differential *Aeromonas* food safety risk for vegetable production based on species. Moreover, all the *A. veronii* test isolates were recovered from reclaimed water in summer and fall, which were also the seasons found to associate with the carriage of the enterotoxin genes *ast* and *alt*. Identifying bacterial traits and environmental determinants that increase the propensity for successful bacterial transfer and persistence and integrating this information with data that suggests potential virulence capability can help devise better management practices that minimize this risk. A high prevalence of *Aeromonas* spp. has been reported on packaged lettuce (55% positive, $n = 120$), although most of those isolates were identified as presumptive *A. hydrophila* (Szabo et al., 2000). Our findings suggest *Aeromonas* persistence could span the period between an irrigation event and sale to a consumer and we identify *A. veronii* as a species particularly well adapted to persist on lettuce. *A. veronii* biotype *sobria* was a predominant cause of traveller's diarrhoea caused by *Aeromonas* spp. (Vila et al., 2003). For irrigation water that is compliant to microbial standards based on *Escherichia coli* levels, there is no recommendation regarding the time interval between the last application of spray or overhead irrigation and harvest. Moreover, some *Aeromonas* species can pose a significant public health risk due to their ability to multiply under refrigerated conditions and tolerate sanitizers. Foodborne illness outbreaks linked to fresh produce contaminated with *A. hydrophila* have been previously reported (Krovacek et al., 1995; Zhang et al., 2012). Therefore, contamination should be minimized as much as possible at pre-harvest levels, necessitating more research that investigates risks associated with the establishment of *Aeromonas* in fresh vegetable crop systems.

Due to the complexity of this taxon, phenotypic identification methods are not sufficient to classify *Aeromonas* species. Multilocus phylogenetic analysis (MPLA) incorporating seven housekeeping gene fragments (*gyrB*, *rpoD*, *recA*, *dnaJ*, *gyrA*, *dnaX* and *atpD*) determined that *rpo*D was among the genes that exhibited the highest substitution rate between inter-species variation (Martinez-Murcia et al., 2011). This prompted us to use the *rpoD* gene to identify isolates collected from four different water types. Approximately 65% of the isolates were classified as *A. hydrophila, A. veronii, A. jandaei, A. caviae* and *A. dhakensis*. Studies have shown that four of these – *A. hydrophila, A. veronii, A. caviae* and *A. dhakensis* – were more frequently associated with various human infections, while *A. jandaei* less so (Fernández-Bravo and Figueras, 2020; Parker and Shaw, 2011). Five isolates remained unclassified and concatenating the gene *gyrB* could increase resolution to provide a classification. Despite the diversity we uncovered in this study, other rarer, diarrhoeagenic species such as *A. trota* were not recovered from any water sample. This is attributed to methodological limitations as no single method can recover all species (Latif-Eugenín et al., 2016) and *A. trota* is susceptible to ampicillin (Carnahan et al., 1991), an antibiotic that was used to enhance the selectivity of our agar medium to *Aeromonas* spp.

5. Conclusion

Our study provides an integrated assessment of the diversity, distribution and potential for pathogenesis of *Aeromonas* prevalent in irrigation water. We investigated a variety of typical or potential water sources over a period of one year, revealing spatial and temporal patterns in species richness, evenness, virulence gene carriage and attachment behaviors. Taken together, these data can be used to infer risks of transfer and establishment potential of possible pathogenic strains from irrigation water sources to fresh produce crops that are minimally processed and may be consumed raw. We found that river, pond and reclaimed water sources are all major reservoirs of species of *Aeromonas* known to cause gastrointestinal disease, raising the possibility that these species enter the food production chain and persist for extended periods when these water sources are used for irrigation. This genus, that poses a greater health problem in the elderly, children and immunocompromised populations, has so far been under-recognized as a food safety risk, yet its ubiquity and ability to withstand sanitizers, antimicrobials, high salt concentrations, low pH and temperature conditions, elevate its threat. Data provided by epidemiological studies showed that infectious doses of *Aeromonas* are relatively low for vulnerable populations (Fernández-Bravo and Figueras, 2020; Tomás, 2012). Moreover, *E. coli*, the microorganism used as a microbial standard to assess irrigation water quality, failed to indicate the presence of this bacterium (Solaiman et al., 2020), supporting the need to include this taxon in future irrigation water quality assessments and enteropathogen-plant interaction studies. Moreover, assays with plants, as reported in this study and others (Elhariry, 2011), provide data that have direct application for food safety. This study links *Aeromonas* spp. seasonal distribution with virulence gene carriage and attachment to lettuce, providing novel data for assessing risk posed by this understudied foodborne pathogen for fresh crop food safety.

CRediT authorship contribution statement

Sultana Solaiman: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft.

Shirley A. Micallef: Conceptualization, Methodology, Formal analysis, Visualization, Writing – parts of original draft, reviewing and editing, Resources, Supervision, Project administration, Funding acquisition.

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.

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Appendix A. Supplementary data

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