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Abstract

Wastewater is increasingly being recognized as a key water resource, and reclaimed water (or treated wastewater) is used for irrigating vegetables destined for human consumption. The aim of the present study was to determine the diversity and prevalence of Aeromonas both in reclaimed water used for irrigation and in the three types of vegetables irrigated with that water. Seven of the 11 (63.6%) samples of reclaimed water and all samples of vegetables were positive for the presence of Aeromonas. A total of 216 Aeromonas isolates were genotyped and corresponded to 132 different strains that after identification by sequencing the rpoD gene belonged to 10 different species. The prevalence of the species varied depending on the type of sample. In the secondary treated reclaimed water A. caviae and A. media dominated (91.4%) while A. salmonicida, A. media, A. allosaccharophila and A. popoffii represented 74.0% of the strains in the irrigation water. In vegetables, A. caviae (75.0%) was the most common species, among which a strain isolated from lettuce had the same genotype (ERIC pattern) as a strain recovered from the irrigation water. Furthermore, the same genotype of the species A. sanarellii was recovered from parsley and tomatoes demonstrating that the irrigation water was the source of contamination and confirming the risk for public health.

Keywords	Reclaimed water, ready to eat , vegetables, irrigation water, Aeromonas
Manuscript category	Research from the Field
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Figure 1. ERIC-PCR.tiff [Figure]

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Reus, 23st October 2016

Dear Dr. José L. Domingo
Editor in Chief

Enclosed is the manuscript entitled: **Chlorinated and ultraviolet radiation -treated reclaimed irrigation water is the source of *Aeromonas* found in vegetables used for human consumption,** which is an unpublished paper. We would ask you to consider it for publication in the Environmental Research as a Original Research paper. All of the authors (Fadua Latif-Eugenín, Roxana Beaz-Hidalgo, Carolina Silvera-Simón and María J. Figueras) have read and approved the paper and it has not been published previously nor is it being considered by any other peer-reviewed journal.

Yours sincerely,

Prof. Maria José Figueras

Chlorinated and ultraviolet radiation -treated reclaimed irrigation water is the source of *Aeromonas* found in vegetables used for human consumption.

Running title: *Aeromonas* from vegetables and reclaimed water.

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Abstract
Wastewater is increasingly being recognized as a key water resource, and reclaimed water (or treated wastewater) is used for irrigating vegetables destined for human consumption. The aim of the present study was to determine the diversity and prevalence of *Aeromonas* both in reclaimed water used for irrigation and in the three types of vegetables irrigated with that water. Seven of the 11 (63.6%) samples of reclaimed water and all samples of vegetables were positive for the presence of *Aeromonas*. A total of 216 *Aeromonas* isolates were genotyped and corresponded to 132 different strains that after identification by sequencing the *rpoD* gene belonged to 10 different species. The prevalence of the species varied depending on the type of sample. In the secondary treated reclaimed water *A. caviae* and *A. media* dominated (91.4%) while *A. salmonicida*, *A. media*, *A. allosaccharophila* and *A. popoffii* represented 74.0% of the strains in the irrigation water. In vegetables, *A. caviae* (75.0%) was the most common species, among which a strain isolated from lettuce had the same genotype (ERIC pattern) as a strain recovered from the irrigation water. Furthermore, the same genotype of the species *A. sanarellii* was recovered from parsley and tomatoes demonstrating that the irrigation water was the source of contamination and confirming the risk for public health.

Key words: Reclaimed water, ready to eat, vegetables, irrigation water, *Aeromonas*

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1. Introduction

Wastewater is increasingly being recognized as a key resource, especially in areas with water scarcity where treated wastewater, i.e. reclaimed water, is used for irrigating fruits and vegetables that are destined for human consumption (Pianetti et al., 2004; Carvalho et al., 2012; Carey et al., 2016). The safety of reclaimed water and food products (shellfish, lettuces, meat etc.) is evaluated by the results obtained for fecal indicators (coliforms, *Escherichia coli,* etc.) during the stipulated controls fixed by legislation to determine their sanitary quality and potential risk (Figueras and Borrego, 2010; Fernandez-Cassi et al., 2016). However, related illness outbreaks still occur worldwide mainly due to the failure of the fecal indicator organisms to correlate with pathogens (Figueras and Borrego, 2010). Among the food and water borne pathogens considered to be emerging, some of them, such as *Aeromonas,* are of special interest because their significance to public health is still not clearly understood (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015).

The genus *Aeromonas* consists of Gram negative, oxidase positive bacilli that are considered autochthonous of aquatic environments and are commonly isolated from clinical and environmental samples (Janda and Abbott, 2010; Beaz-Hidalgo and Figueras, 2013; Figueras and Beaz-Hidalgo, 2014, 2015). Several studies have shown that *Aeromonas* species are foodborne and waterborne pathogens of increasing importance (Altwegg et al., 1991; Demarta et al., 2000; Figueras and Borrego, 2010; Pablos et al., 2011; Khajanchi et al., 2010). *Aeromonas* can be readily isolated from treated sewage, reclaimed water, sea water, fresh water and from drinking water distribution systems, where they appear to survive well, to proliferate at low temperatures and to be linked to pipe biofilms where populations may survive at high chlorine levels (Emekdas et al., 2006; Figueras and Borrego, 2010; Jjemba et al., 2010; Khajanchi et al., 2010; Martone-Rocha et al., 2010; Figueira et al., 2011; Igbinosa and Okoh, 2013; Robertson et al., 2014; Al-Jassim et al., 2015). Several species of *Aeromonas* are recognized to be opportunistic pathogens to humans and can affect both immunocompromised and immunocompetent individuals being the most frequent clinical presentations gastroenteritis, wound infections and bacteremia (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015). Despite the role of *Aeromonas* in diarrheal cases have been questioned in some studies, a recent publication has provided evidences that support the true enteropathogenicity of these bacteria (Teunis and Figueras, 2016). It was proved recently that *Aeromonas* was the cause of two waterborne infections because strains showing the same genotypes were isolated from drinking water and from the feces of patients with diarrhea (Khajanchi et al., 2010; Pablos et al., 2011). Some studies have found *Aeromonas,* in reclaimed water used for agricultural irrigation, which is a risk for consumers when considering that this water can be the source of entry of the bacteria into the food chain (Pianetti et al., 2004; Al-Jassim et al., 2015; Fernandez-Cassi et al., 2016). So far, however, no studies have evaluated simultaneously the presence of *Aeromonas* in the water used for irrigation and in the irrigated vegetables. The aim of this study, therefore, was to determine, using molecular methods (genotyping and sequencing the *rpoD* gene), the prevalence, diversity and epidemiological relationship of the *Aeromonas* isolates recovered from the ready to eat vegetables and the reclaimed water used for their irrigation.

2. Material and methods

2.1. Water and vegetables sampling

Eleven reclaimed water samples were collected from a wastewater treatment plant, located in Catalonia North-East of Spain. Three water samples were collected after the secondary treatment, three after tertiary treatment that involved chlorination and ultraviolet radiation and five corresponded to irrigation water. The latter came from a hose pipe that extracted water from a well, where the tertiary treated water was accumulated before being use for irrigation. In addition three irrigated vegetables samples i.e. lettuces, tomatoes and parsley were analyzed. Drip irrigation was applied to the tomatoes and spray irrigation to the lettuces and parsley. The frequency of the irrigation depend on weather conditions but it was normally every two days.

2.2. Detection of faecal indicator bacteria

The determination of *E. coli* (EC) and intestinal enterococci (IE) was carried out using the 96-well microplate (BioRad, France) most probable number (MPN) methods ISO 9308-2 and ISO 7899-1 respectively. The detection of *E. coli* in the 96-well microplate is based on the expression β-D-glucoronidase enzyme, while the β-glucosidase is the target for intestinal enterococci.

2.3. Detection and isolation of Aeromonas

All samples were submitted to an enrichment step with Alkaline Peptone Water supplemented with Ampicillin (APW-A) at a concentration of 10 mg/l. For this, 10 ml of water samples were mixed with 90 ml of APW-A (1:10 vol/vol). For the vegetables, 10 g were homogenized in 90 ml of APW-A (1:10 wt/vol). The enrichment culture was incubated at 30ºC for 24 hours, 10-fold serial dilutions were performed and then 100 µl were plated on 3 different culture media: Dextrin Ampicillin Agar (ADA= M-*Aeromonas* Agar, Biolife, Italy, Havelaar et al., 1987), Starch Ampicillin Agar (SAA, Palumbo et al., 1985) and Bile Irgasan Brilliant Green- modified (BIBG-m, Neyts et al., 2000) and incubated at 30ºC for 24 hours. Typical *Aeromonas* colonies were transferred to Trypticase Soy Agar (TSA, BD, France) to obtain a pure culture from which to perform the DNA extraction for the molecular genotyping and molecular identification. The density of *Aeromonas* was determined by direct count using ADA culture media being this also the culture media used for the verification of the positive tubes obtained by the MPN method.

2.4. Genotyping and molecular identification

DNA was extracted using InstaGene Matrix (BioRad, France) according to manufacturer's instructions. All isolates were identified to genus level using the PCR detection of Glicerophospholipid Cholesterol Acyltransferase (GCAT) characteristic of the genus *Aeromonas* (Soler et al., 2002). All isolated were genotyped with the Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) technique using the primers and conditions described by Versalovic et al. (1991) and used for *Aeromonas* in several other studies (Soler et al., 2003a; Beaz-Hidalgo et al., 2012). Molecular identification to species level was performed by sequencing the *rpoD* gene using the primers and conditions described by Soler et al. (2004). Sequence corrections and analysis were performed with the DNAstar Seqman program (Lasergene, USA). The

sequence alignments and the Neighbor Joining phylogenetic tree were performed using the MEGA program version 5.0 (Tamura et al., 2011).

2.5. Statistical analysis

The values obtained by the two analytical methods were transformed into $\log_{10}$ by Excel (Microsoft Office 2007). This same program was used to calculate the geometric mean. A two-way ANOVA, followed by the Bonferroni post-hoc test was used for comparing the bacteria indicators and *Aeromonas and* their concentration in the different types of samples. All the analyses were performed using SigmaPlot 11.0 (SSI, California, USA) and significance was fixed at $P<0.05$.

3. Results

3.1. Detection fecal indicator bacteria and Aeromonas

Reclaimed waters were positive for *E. coli* (EC) and intestinal enterococci (IE) in 45.5% of the samples (**Table 1**). The geometric mean of EC and IE in the secondary treated wastewater were reduced by the tertiary treatment in 3.35 and 3.43 logs (99.9% and 100% reduction) respectively (**Table 1**). Statistically significant differences were only observed for the densities of IE that were considerably reduced in the tertiary treated water and in the irrigation water ($P < 0.05$).

A total of 63.6% (7/11) water samples and all vegetables (3/3) were positive for the presence of *Aeromonas* (**Table 1**). The four negative water samples were the three that were submitted to a tertiary treatment with chlorination and ultraviolet radiation and one irrigation water sample. The density of *Aeromonas* was 1 to 3 logs higher with the MPN enumeration method that with the direct plate count. *Aeromonas* were not detected after the tertiary treatment and this represented a reduction of 4.50 to 5.66 logs (**Table 1**). However, four of the five irrigation water samples were positive for *Aeromonas* with levels that reached up to 2.22×10^3 MPN/100ml **(Table 1).** The *Aeromonas* density was significantly higher ($P < 0.05$) in the secondary than in the tertiary treated water and the latter was higher ($P < 0.05$) than the density found in the irrigation water.

3.2. Genotyping and molecular identification of Aeromonas

From the seven reclaimed water samples where *Aeromonas* was detected, 216 isolates were recovered which corresponded to 124 different genotypes (after the analysis by ERIC-PCR) representing a 57.4% genetic diversity (**Table 2**). No statistically significant differences were observed between the number of different genotypes found in the secondary treated water and the irrigation water. The 39 isolates recovered from vegetables belonged to eight different strains showing a genetic diversity of 20.5%. The 132 genotypes (strains) recovered from reclaimed water and vegetables samples belonged to 10 *Aeromonas* species, nine of which were present in irrigation water and five in the secondary treated water (**Table 3**). However, the eight strains detected in vegetables belonged all to *A. caviae* (75.0%), except two strains that belonged to *A. hydrophila* and *A. sanarellii* (**Table 3**). The distribution and prevalence of the species varied depending on the type of samples analyzed. For instance 91.4% of the total

strains recovered from secondary treated reclaimed water corresponded to *A. caviae* (71.4%) and *A. media* (20.0%) with no detection of *A. salmonicida* and *A. popoffii*. However, *A. salmonicida* (22.2%), *A. media* (18.5%), *A. allosaccharophila* (18.5%) and *A. popoffii* (14.8%) were the most prevalent species in irrigation water. One *A. caviae* isolate (AAR-15B) recovered from irrigation water showed the same ERIC pattern than an isolate (Sle-15D) recovered from lettuce and two *A. sanarellii* strains identified in parsley (Bpe-16B) and tomato (ATot-15A) showed the same ERIC pattern (**Table 3** and **Fig. 1**).

4. Discussion

4.1. Detection of fecal indicator bacteria and Aeromonas

Both the fecal indicators (EC and EI) and the *Aeromonas* were present in high concentrations in all the secondary treated wastewater samples in agreement with results of other studies (Pianetti et al., 2004; Jjemba et al., 2010; Martone-Rocha et al., 2010; Al-Jassim et al., 2015). However, after the tertiary treatment with chlorine and ultraviolet radiation, these microorganisms were not detected, with exception of one sample where EC was found in a relatively low concentration (147 MPN/100ml). These results show that the use of chlorine and ultraviolet radiation were highly effective for the reduction of the bacterial load. The 100% reduction of *Aeromonas* and IE corresponded to a decrease of 5.66 and 3.43 logs respectively, while the 99.9% reduction for EC represented 3.35 logs. However, 80% of the irrigation water samples showed to be positive for *Aeromonas*, and 20% for EC. The amount of *Aeromonas* in these samples was significantly higher ($P < 0.05$) than those present in the tertiary treated water. This data indicated that these bacteria (*Aeromonas* and EC) were not totally eliminated by the tertiary treatment and that had the capacity to re-grow when the tertiary treated water was stored in a well (**Table 1**). However, IE were not detected in any sample after the tertiary treatment. Our results are in agreement with two previous studies that found *Aeromonas* in irrigation water (Pianetti et al., 2004; Al-Jassim et al., 2015). In one of the studies 84.6% (11/13) of the water samples used for irrigation of agricultural products were positive (Pianetti et al., 2004) while in the other all samples from a chlorinated effluent were positive (Al-Jassim et al., 2015). However, *Aeromonas* were not recovered after chlorination treatment in the four water samples that Figueira et al. (2011) analyzed.

In relation to the ability of the bacteria to regrow, Jjemba et al. (2010) found that despite fecal indicator bacteria and *Aeromonas* were effectively removed from wastewater using different disinfection mechanisms (chlorine, ozone and ultraviolet radiation) the bacteria were again detected in the distribution system as a result of their ability to regrow. Al-Jassim et al. (2015) also found regrowth of *Aeromonas* and other microbes and to minimize the risk they discourage the use of treated wastewater for irrigation. Also using metagenomics (454 pyrosequencing) targeting the V4 or the V6 region of the 16S rRNA gene several authors showed that wastewater samples contained *Aeromonas* as one of the dominating bacteria (McLellan et al., 2010; Ye and Zhang et al., 2011; Al-Jassim et al., 2015). It has been suggested that the regrowth of *Aeromonas* in the irrigation water could be due to: i) the decrease of the chlorine concentration that occurs during storage of the water and in the irrigation distribution systems, ii) the capacity of *Aeromonas* to produce biofilm and iii) the amount of organic matter present

in the reclaimed water (Jjemba et al., 2010 and Figueras and Beaz-Hidalgo, 2014). These are the same reasons that explain the presence of *Aeromonas* in drinking water distribution systems (Figueras and Borrego, 2010).

4.2. Genotyping and molecular identification of Aeromonas

A total of 10 *Aeromonas* species were recovered from reclaimed, irrigation water and vegetables. However, nine of this species came from the irrigation water, which seems to indicate that cleaner water favors a higher diversity of species, than the more contaminated secondary treated wastewater where only five species were detected. In this sense 91.4% of the total strains recovered from the secondary treated water corresponded to two species *A. caviae* (71.4%) and *A. media* (20.0%). The latter were followed by far by *A. hydrophila* (5.7%), *A. allosaccharophila* and *A. veronii* (1.4% each). The dominance of this two species is in agreement with results reported by Figueira et al. (2011), that molecularly identified 19 *Aeromonas* isolates from treated wastewater and found that the 68.4% of them belonged to *A. media* (7/19, 36.8%) and *A. caviae* (6/19, 31.6%) followed by *A. dhakensis* (3/19, 15.8%) and *A. sanarellii* (3/19, 15.8%).

In the present study the prevailing species found in the irrigation water were *A. salmonicida* (22.2%), *A. media* (18.5%), *A. allosaccharophila* (18.5%) and *A. popoffii* (14.8%) followed by *A. caviae* (13.0%), three species with an equal prevalence of 3.7% each (*A. hydrophila*, *A. veronii*, and *A. bestiarum*) and finally by *A. eucrenophila* (1.9%). This high diversity of species is in agreement with the species found from freshwater samples by other authors using molecular method (Soler et al., 2002; Figueira et al., 2011). The abundance of species typically found in freshwater may indicate that not all the species detected in the irrigation water originated by regrowth in the storage tank or distribution system. Some could come from the recontamination with freshwater.

The diversity of *Aeromonas* species may be mask by using phenotypic identification methods like the API 20E system used by Pianetti et al. (2004), that identified only three species i.e. *A. caviae* (48.64%), *A. sobria* (35.13%) and *A. hydrophila* (16.22%) among 111 *Aeromonas* isolates recovered from 11 irrigation water samples. The phenotypic misidentification problems of *Aeromonas* species associated to API 20E and other methods have been demonstrated in previous studies (Soler et al., 2003b; Beaz-Hidalgo et al., 2010; Lamy et al., 2010).

In this study, the strains recovered from vegetables belonged predominantly to *A. caviae* (75.0%), except two strains that belonged to *A. hydrophila* and *A. sanarellii* (**Table 3**). These results are in agreement with the ones obtained by Nishikawa and Kishi (1988) that analyzed several types of vegetables (cabbage, carrot, cucumber, eggplant, lettuce, onion, tomato, potato and spinach) and identified *A. caviae* (63.0%) as the most prevalent species, followed by *A. hydrophila* (37.0%). Identical results were reported by Nagar et al. (2011), that identified *Aeromonas* in 2/80 (2.5%) sprouts samples analyzed by sequencing of 16S rRNA gene and found only three *Aeromonas* isolates, two (66.6%) of them were identified as *A. caviae* and the remaining isolate (33.3%) as *A. hydrophila*. Other authors have found other prevalent species associated with vegetables, like McMahon and Wilson (2001), who using biochemical identification found that 29/86 (33.7%) of the vegetables samples i.e. lettuce, potato,

carrot, onion, celery were positive for the presence of *Aeromonas*, being the most frequently isolated species *A. schubertii* (54.5%), *A. hydrophila* (15.2%), *A. trota* (15.2%), *A. caviae* (9.1%) and *A. popoffii* (6.0%). However, these authors did not investigate the irrigation water in parallel with the vegetables as we did in our study neither they performed a genotyping study to investigate if there existed an epidemiological relationship among the isolates.

An important finding in this study was the recognition of one *A. caviae* isolate (AAR-15B) recovered from irrigation water that showed the same genotype as another isolate (Sle-15D) recovered from lettuce (**Table 3** and **Fig. 1**). This epidemiological relation demonstrated that water acted as the vehicle of dissemination of these bacteria. The epidemiological relationships among *Aeromonas* strains have so far only been reported in few studies. For instance among environmental strains the same persistent clone was found from distant sites (up to 4 km) in a drinking water supply system or from a fountain and the storage tank (Martínez-Murcia et al*.,* 2000; Figueras et al*.,* 2005). However, other authors found the epidemiological link between the isolates recovered from the environment and those found in diarrheal cases. For instance, Altwegg et al. (1991) demonstrated that the *A. hydrophila* strain recovered from human stool of a patient with diarrhea was identical to the one isolated from the left over shrimp cocktail that he consumed. Demarta et al. (2000) reported that four *A. caviae* strains isolated from feces of children with diarrhea presented the same ribotype that the isolates found in the household environment (tap water and wet surfaces, mainly from the kitchen and the bathroom). More recently, the epidemiological relationship was established between the *Aeromonas* found in the human stools and in the drinking water (Khajanchi et al., 2010; Pablos et al., 2011). In the study by Khajanchi et al. (2010), two *A. caviae*/*A.media* strains isolated from drinking water showed indistinguishable PFGE patterns from the ones isolated from the feces of two patients with diarrhea in the United States. The same genetic relationship was established by Pablos et al. (2011) in Spain that found one *A. caviae* clinical isolate and two water isolates with identical PFGE patterns. These discoveries reinforce the believe that the route of infection of *Aeromonas* would be the drinking water or the water contaminated food (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2014).

In the present study the same *A. sanarellii* strain, was recovered from parsley and tomato, being this the first time that this species, originally found in association with human wound infection (Alperi et al., 2010) is recovered from vegetables. So far this species have only been recovered from treated wastewater as mentioned above (Figueira et al., 2011) and in association with chironomid egg masses found in wastewater stabilization ponds (Beaz-Hidalgo et al., 2012; Laviad and Halpern, 2016). In 2013, *A. sanarellii* was again isolated from wound infections (Chen et al., 2013) and recently, the same authors identified for the first time this species from human feces (Chen et al., 2015), which indicated that this species can also act as enteropathogen. Also in the latter study among the 13 cases of *Aeromonas* diarrhea one case was associated with the consumption of lettuce (Chen et al., 2015). This is in agreement with our findings that showed the same genotype of *A. caviae* in the irrigation water and in the lettuce, because this species is the most prevalent in diarrhea cases (Figueras and Beaz-Hidalgo, 2015). Furthermore it has been described that the *Aeromonas* already present in ready to eat vegetables possess the capacity to multiply during storage at refrigerating temperatures (5ºC) and this represents an extra risk for consumers (Wadhwa et al.,

2012; Figueras and Beaz-Hidalgo, 2014). In this sense in two foodborne outbreaks of *Aeromonas* diarrhea the attributed source of infection was a contaminated salad (Krovacek et al., 1995; Zhang et al., 2012), while in others it was fermented fish (Granum et al., 1998) and oysters (Abeyta et al., 1986). All these findings suggest that reclaimed waters can act as a contamination source for pathogens like *Aeromonas* that may enter the food production chain from where they can contaminate food products used for human consumption.

5. Conclusions

The high number of investigated isolates from the irrigation water and the vegetables have probably favored the recognition of the epidemiologically related isolates found in our study. Despite the use of reclaimed water is a good way to compensate scarcity of water, it is necessary to considered the potential risk for human health derived from the presence of opportunistic pathogens like *Aeromonas*. The results showed that the tertiary treatment by chlorination and ultraviolet radiation was effective in reducing the *Aeromonas* loads below their detection limit. However, these microorganism were detected again in the irrigation water either as result of a contamination from surface water or from soil and/or by their regrow capacity during the storage of the treated water. In the present study it was clearly demonstrated that the irrigation water acted as a vehicle of transmission of *Aeromona*s to the vegetables used for human consumption. Considering the failure of *E. coli* to predict the presence of potential pathogenic microorganisms such as *Aeromonas*, the latter bacteria should be considered as new potential indicators in order to increase the protection of public health. Moreover, due to the high capacity of contamination of this ready to eat vegetables by soil or water, additional interventions before consumption to minimize the risk of the presence of pathogens seems necessary.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the design and development of the study and approve it for publication. In addition authors declare no conflict of interest.

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Table 1. Detection of *Aeromonas* sp. and fecal indicators in samples from reclaimed water and irrigated vegetables.

Type of sample analyzed	n	Number (%) of positive samples for *Aeromonas*			Number (%) of positive samples for fecal indicators		
		Positive samples	Geometric mean[a] (minimum-maximum)		Positive samples	Geometric mean (MPN/100ml)[a] (minimum-maximum)	
			Direct plate[b]	MPN/100ml		EC	IE
Reclaimed water	**11**						
Secondary treated	3	3 (100.0)	$3.19x10^4$ ($1.5x10^4$-$6.8x10^4$)	$4.60x10^{5c}$ ($4.60x10^5$-$4.60x10^5$)	3 (100.0)	$1.69x10^4$ ($1.30x10^3$-$5.60x10^5$)	$2.68x10^{3e}$ ($7.30x10^2$-$6.40x10^4$)
Tertiary treated	3	0 (0.0)	ND	ND	1 (33.3)	$7.61x10^0$ (ND-$4.40x10^2$)	ND
Log reduction			***4.5***	***5.66***		***3.35***	***3.43***
Irrigation water	5	4 (80.0)	$3.74x10^1$ (ND-$1.5x10^3$)	$2.22x10^{3d}$ (ND-$1.60x10^4$)	1 (20.0)	$1.72x10^0$ (ND-$1.50x10^1$)	ND
Total		**7 (63.6)**	**$1.06x10^4$**	**$1.54x10^5$**	**5 (45.5)**	**$5.64x10^3$**	**$8.94x10^2$**
Vegetables	**3**	3 (100.0)[f]	NP	NP	1 (33.3)	$9.8x10^0$ (ND-$9.40x10^2$)	ND

[a]For the geometric mean calculation all values corresponding to ND were replaced for 1 before the log transformation; [b]The plate count results were expressed in cfu/100ml; [c]The amount of *Aeromonas* was higher ($P < 0.05$) in the secondary treated water and [d]in the irrigation water, as occurred also for the EI[e]; [f]The 3 samples corresponded to a lettuce, tomato and parsley that were positive after an enrichment step and were recovered from the following culture media: ADA, SAA and BIBG-m simultaneously in the case of the lettuce and tomato samples and only from BIBG-m in the case of the parsley sample; EC: *Escherichia coli*; IE: intestinal enterococci; ND: not detected; NP: not performed.

Table 2. Number of *Aeromonas* isolates and genotypes recovered from reclaimed water and irrigated vegetables.

Type samples		Total of isolates GCAT-PCR (+)	Total number of genotypes	% Genetic diversity
Reclaimed water				
Secondary treated		108	70	64.8
Irrigation water		108	54	50.0
	Total	**216**	**124**	**57.4**
Vegetables				
Lettuce		13	6[a]	46.2
Tomato		13	1[b]	7.7
Parsley		13	1[c]	7.7
	Total	**39**	**8**	**20.5**

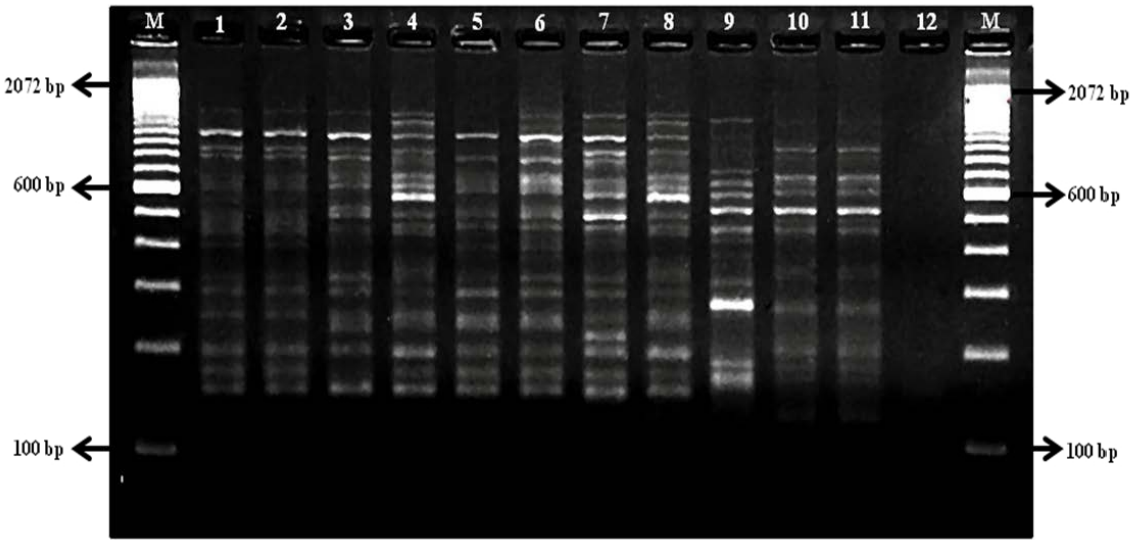
[a,b]The same genotypes were recovered with using the three culture media ADA, SAA and BIBG-m only after enrichment; [c]This genotype was only recovered from the BIBG-m culture medium after enrichment and was identical to the genotype found from the tomato sample.

Table 3. Prevalence and diversity of *Aeromonas* spp. among the 132 strains recovered from the reclaimed water and the irrigated vegetables.

Total Nº (%) of *Aeromonas* spp.			
	Secondary treated water	**Irrigation water**	**Vegetables**
63 (47.7) *A. caviae*	50 (71.4)	7 (13.0)[a]	6 (75.0)
24 (18.2) *A. media*	14 (20.0)	10 (18.5)	0 (0.0)
12 (9.1) *A. salmonicida*	0 (0.0)	12 (22.2)	0 (0.0)
11 (8.3) *A. allosaccharophila*	1 (1.4)	10 (18.5)	0 (0.0)
8 (6.1) *A. popoffii*	0 (0.0)	8 (14.8)	0 (0.0)
7 (5.3) *A. hydrophila*	4 (5.7)	2 (3.7)	1 (12.5)
3 (2.3) *A. veronii*	1 (1.4)	2 (3.7)	0 (0.0)
2 (1.5) *A. bestiarum*	0 (0.0)	2 (3.7)	0 (0.0)
1 (0.8) *A. sanarellii*	0 (0.0)	0 (0.0)	1 (12.5)[b]
1 (0.8) *A. eucrenophila*	0 (0.0)	1 (1.9)	0 (0.0)
Total	**70 (53.0)**	**54 (40.9)**	**8 (6.1)**

[a]One *A. caviae* isolate from irrigation water showed the same ERIC-genotype as one isolate recovered from lettuce. [b]The same ERIC-genotype was found for two isolates of *A. sanarellii* one was recovered from parsley and the other from tomato.

FIGURE 1. Epidemiological relationship, on the basis of the ERIC-PCR patterns, between *Aeromonas* strains isolated from irrigation water (lane 1) and the irrigated vegetable samples (lanes 2-8: lettuce; lanes 9,10: tomato; lane 11: parsley). Lanes: 1, AAR-15B= 2, Sle-15D; 3, Ale-15A; 4, Ale-15D; 5, Ale-16B¸6, Ale-16C; 7, Ble-15D; 8, Ble-16A; 9, ATot-15B; 10, ATot-15A= 11, Bpe-16B; 12, negative control; M, molecular weight ladder (100 to 2072 bp, Invitrogen). Left and right numbers indicate the molecular weight.



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