Prevalence of *Enterobacteriaceae* and contaminants survey in sediments of the Atoyac River

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Abstract

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We identified the bacterial community in sediments from the Atoyac River within Puebla, Mexico by a 16S rRNA gene clone library. The *Enterobacteriaceae*, specifically *Shigella*, *Escherichia fergusonii* and *E. coli* species dominate the bacterial community which is consistent with the values obtained for total coliforms. Genes encoding Shiga toxins ($stx1$ and $stx2$) were not found. Higher alkanes (C11-C16), esters, organic acids, phenols and phthalates, classified as potential endocrine disruptors were recovered from the sediments, while the presence of coprostanol indicated fecal pollution. Element concentrations were also determined in sediments and they decreased in the following sequence: Ca > Fe > K > Ti > Sr > Ba > Mn > Zr > Zn > Cu > Rb, while As, Mo and Pb were below the detection limit. All element values but Cu are below those permitted by the United States Environmental Protection Agency (USEPA). This study highlights the dominance of *Enterobacteriaceae* and the low bacterial diversity with the ability to degrade hydrocarbons or persistent organic pollutants (POPs) in sediments of the Atoyac River in an area prior to the water storage in the Avila Camacho (Valsequillo) dam whose waters are used for irrigation in lands above for several crops.

Keywords: Microbial diversity, heavy metals, ecological monitoring, Atoyac, *Shigella*.

Resumen

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Se identificó la comunidad bacteriana en sedimentos del río Atoyac por medio de biblioteca genómica de clones. Encontramos una baja diversidad bacteriana, ya que la familia Enterobacteriaceae, *específicamente especies de* Shigella, Escherichia fergusonii *y E. coli dominan la comunidad bacteriana, lo cual es consistente con los valores obtenidos de coliformes totales por número más probable (NMP). No se encontraron los genes que codifican para toxinas Shiga (stx1 y stx2). Alcanos (C11-C16), ésteres, ácidos orgánicos, fenoles y ftalatos clasificados como potenciales disruptores endócrinos también fueron detectados en los sedimentos y la presencia de coprostanol indica contaminación fecal. Las concentraciones de elementos decrecieron de la siguiente manera: Ca > Fe > K > Ti > Sr > Ba > Mn > Zr > Zn > Cu > Rb, mientras que As, Mo y Pb estuvieron por debajo del límite de detección. Todos los elementos, excepto el Cu, no sobrepasan los límites de la Agencia de Protección Ambiental de los Estados Unidos (USEPA, 1977). Este estudio demuestra la dominancia de* Enterobacteriaceae *y la baja diversidad bacteriana con la habilidad de degradar contaminantes orgánicos en los sedimentos del río en un área previa a la presa Ávila Camacho, cuyas aguas son usadas para irrigar cultivos en tierras arriba.*

***Palabras clave**: diversidad microbiana, metales pesados, monitoreo ecológico, Atoyac,* Shigella.

27

Introduction

The Atoyac River originates in the Calpulalpan sierra in the state of Tlaxcala and along its course, through the state, it is fed by the Zahuapan River forming the Alto Atoyac sub-basin with an area of 2013 km^2 (Carrera-Villacrés, 2007). The Atoyac River later finds its way into the state of Puebla and runs through its capital city before ending up on the Valsequillo dam. However, these rivers receive urban, industrial and agricultural effluents from the towns and industrial activities along with their course. The industrial activities are quite varied and they include the food, textile, chemical, petrochemical, pharmaceutical, metallurgic, electric, and automobile industries, among others (Sandoval-Villasana, Pulido-Flores, Monks, Gordillo-Martínez, & Villegas-Villareal, 2009). A previous study has assessed the quality of waters, sediments, and the genotoxic potential in the hydrological Atoyac-Zahuapan river system and the Avila Camacho dam (Mangas *et al.*, 2005). The genotoxic potential of sediments and superficial water of this system has been confirmed with the plant *Vicia faba* (Villalobos-Pietrini, Flores, & Gómez, 1994; Juárez-Santacruz *et al.*, 2012). Chromosomal aberrations and centromeric alterations were found in root tip cells of this plant after being exposed to the Atoyac River waters (ob. cit.) while the micronuclei frequency was increased after exposure to the river sediments in a recent study (Juárez-Santacruz *et al.*, 2012). Moreover, metals such as lead and arsenic have been detected in the Alto Atoyac sub-basin and their concentrations were above the Mexican and international permissible limits (García-Nieto *et al.*, 2011). Other studies have also revealed the number and location of effluents in the Atoyac River (Saldaña & Gómez, 2006; Sandoval-Villasana *et al.*, 2009) and have established them as the main source of contamination. Furthermore, concentrations of Mn and Fe in waters of the Valsequillo dam were also above the permissible limits (Díaz, Bonilla, Tornero, Cabrera, & Corona, 2005). These waters are used for irrigation of nearby crops such as maize and alfalfa and their soils also have Pb concentrations above the norm (Larenas-Bazán, 2010). The most recent study on these river sediments and waters reported severe pollution that includes hydrocarbons, metals, POPs, endocrine disruptors and potentially carcinogenic compounds (Greenpeace, 2014). Therefore, this study aims to elucidate the microbial diversity in a river area where it has received all the major domestic and industrial effluents prior to the water storage in the Valsequillo dam. The identification of the microbial diversity will provide a clearer idea of their catabolic potential in those sediments.

Materials and methods

A list of the different methods used in this study is included in table 1.

Table 1. List of methods used in this study.

Method	Objective	References
Clone libraries	Identification of the bacterial population	(Aburto *et al.*, 2009)
Shiga toxins by PCR	Detection of pathogens	(Fagan, Hornitzky, Bettelheim, & Djordjevic, 1999)
Coliforms enumeration	Fecal and total coliforms as indicators of water quality	(NMX-AA-42-1987)
Biochemical Oxygen Demand (BOD)	Water quality	(NOM-003-Semarnat-1997)
X-Ray fluorescence Analyzer (FPXRF)	Identification of heavy metals	(USEPA, 2007a)
Analytical chemistry (GC-MS)	Toxic compounds elucidation	(USEPA, 2007b), (USEPA, 2007c)

Site description and sampling

Sediment and water samples were collected from three different positions (A1-A3) located just prior the Valsequillo dam in central México. The river water at these positions has undergone wastewater treatment processes after receiving industrial and domestic effluents along the river course. The global positioning coordinates for the sampled sites and the relative position of the samples are indicated in table 2 and figure 1, respectively. The samples were collected during the dry season on 21 January 2013. Water samples were collected in sterile 1-litre glass bottles. Sediments from the bottom of the river were collected in 50 ml sterile centrifuge tubes. Then, they were immediately preserved in dry ice and protected from light.

Determination of elements in sediments

The sediments were dried to room temperature to prevent any volatilization. Each sample was homogenized and then a representative portion was milled with a mortar prior to passing it through a 60-mesh sieve before a further homogenization. The total element concentrations (As, Pb, Ca, Fe, K, Ti, Sr, Ba, Mn, Zr, Zn, Cu, Rb, Mo) were determined in 15 g of dried sediments following the Unites States Environmental Protection Agency Method 6200 (USEPA, 2007a), using a field portable X-Ray fluorescence Analyzer (FPXRF), NITON XL3t Thermo Scientific. Two reference materials were used to establish the method accuracy: the Standard Reference Material "NIST 2710" Montana Soil and the Reference Material unstratified glacial deposit Till-4. The accuracy percentage of all elements was in the $80-120\%$ range. Moreover, duplicate analyses were performed to establish the method precision and the relative difference percentage (% RDP) was always below 10%. To discard contamination interferences, blanks were analyzed between each interest sample. The analysis of clean quartz dioxide matrix (blank) was always below the detection limit for all elements.

Table 2. Coordinates of the sampled sites collected by GPS.

Code	GPS positioning	Elevation (m)
A1	N19°1´31.55´´, W98°13´27.78´´	2092
A2	N19°1´18.48´´, W98°13´43.21´´	2092
A3	N19°0´56.00´´, W98°14´47.00´´	2093
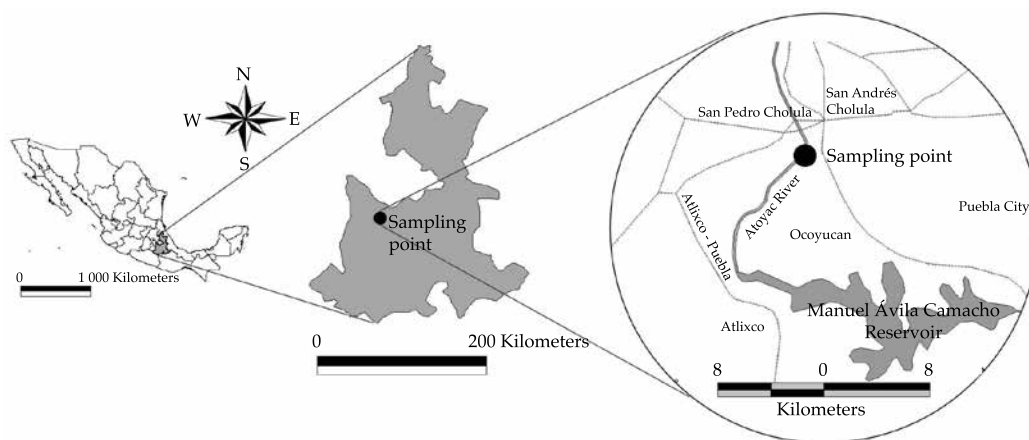
Figure 1. Location of sampled site. GPS coordinates of the sampling sites are shown in table 2.

29

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30

Determination of organic compounds

The organic compounds were extracted from dried sediments by sonication Method 3550C, (USEPA, 2007b) using 3 g of soil and 95 ml of a mixture 1:1 n-hexane-ketone (Mallinckrodt, HPLC grade). The extracts were concentrated by roto-evaporation and changed to n-hexane as a solvent. The recovery percentage of this extraction method was validated using a soil sample with known concentrations of 17 reference compounds. The percentage of recovery of all compounds was higher than 90%. Regular controls with this soil were performed to ensure the quality of the data. The extracts were analyzed to identify organic compounds by gas chromatography Method 8270D (USEPA, 2007c) coupled with a mass spectrum detector (Agilent 6890N, MSD 5975B, USA) using a 5 MS column (Agilent, USA). The temperatures of the detector and injector were 250°C and 220°C, respectively. The initial and final temperatures of the oven were 70°C and 250°C at a rate of 7°C/min. Helium was used as the carrier gas and the mass scan range was from 50 to 450 z/m at 70 eV. Identification was made using the NIST05 Mass Spectral library.

Determination of total and fecal coliforms

The Most Probable Number (MPN) of total coliforms, fecal coliforms, and *Escherichia coli* was obtained for the three sampling points (A1-A3) and followed the Mexican Norm NMX-AA-42-1987, which agrees with the ISO norm: ISO/DP 9308/2.

Biochemical Oxygen Demand (BOD)

The biochemical oxygen demand for five days for the three sampling points (A1-A3) was obtained with a Hach BOD Track II Respirometric apparatus according to manufacturers´ instructions.

Determination of organic carbon

The organic carbon content was evaluated in water samples for the sampling point A1 using a TOC-LSCH analyzer (Shimadzu, Japan) which principle of detection method is the combustion catalytic oxidation at 680 ºC. Total and inorganic carbon (TC and IC) were measured automatically in triplicates by the equipment and organic carbon was calculated by the difference between those values. The detection limit of TC and IC was 4 µg/L with a maximum coefficient of variation of 1.5% between replicates.

DNA extraction and PCR

Microbial community DNA was extracted directly from the slurries (A1) collected on January 21, 2013, using the UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach CA) and PCR amplification of 16S rDNA genes was performed with the following primer pair: 63F (CAG GCC TAA CAC ATG CAA GTC) and 1389R (ACG GGC GGT GTG TAC AAG) (Marchesi *et al.*, 1998) for bacteria as described previously (Aburto *et al.*, 2009). The cycling conditions were as follows: 1 cycle at 94 °C for 2 min, 30 cycles of 1 min at 94 °C, 1 min at 55°C and 2 min at 72 °C and a final elongation at 72 °C for 10 min.

PCR amplification of genes encoding Shiga toxins 1 and 2 was performed on the A1 slurries with the primer pair *stx*1 and *stx*2 as described previously (Fagan *et al.*, 1999). The cycling conditions were as follows: an initial 95°C denaturation step for 3 min followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s. The final cycle was followed by a 72°C incubation for 5 min.

Cloning and sequencing

PCR products were cloned with a CloneJet cloning kit (Thermo) as described in the manufacturer´s instructions using One Shot TOP10 chemically competent *E. coli* cells. Recombinant colonies were recovered from LB

agar plates containing ampicillin (50 μg ml⁻¹). The screening of inserts from transformants was performed by direct PCR amplification from white colonies using the 16S primers 63F (CAG GCC TAA CAC ATG CAA GTC) and 1389R (ACG GGC GGT GTG TAC AAG). Heating at 94°C for 10 min preceded standard cycling conditions (as above). Amplified inserts were grouped on the basis of restriction fragment length polymorphism (RFLP) patterns using a combination of the two restriction endonucleases *Cfo*I and *Alu*I (Sigma) at 37°C for 3 hrs. Representative clones of each RFLP group were sequenced. The sequencing analysis was performed at the UNAM biotechnology institute (Mexico). The sequences length was 600 to 700 nucleotides in average.

Phylogenetic analyses

The sequences obtained were compared with the European Bioinformatics Institute and Genebank databases by online FastA searches (Pearson & Lipman, 1988) and to the Check Chimera program (Cole *et al.*, 2003). The profile alignment technique of ClustalW (Thompson, Higgins, & Gibson, 1994) was used to obtain preliminary alignments that were manually refined in GeneDoc Multiple Sequence Alignment Editor ver. 2.6.002 (Nicholas & Nicholas, 1997) taking into account of secondary structure. Phylogenetic analyses were performed with the Phylip software package (Felsenstein, 2001), using the Jukes-Cantor distance and the neighbour-joining methods. The significance of the branching order was determined by bootstrap analysis with 1000 re-sampled data sets.

Results and discussion

Water quality

Total coliforms, fecal coliforms, and biochemical oxygen demand (BOD) values are shown in table 3. These values indicate that the waters are heavily contaminated according to the Mexican maximum limits (NOM-003-Semarnat-1997).

Metals, semimetals and other elements in sediments

Element concentrations for As, Ba, Ca, Cu, Fe, K, Mn, Mo, Pb, Rb, Sr, Ti, Zn, and Zr were obtained for sampling point A1 (table 4). The concentrations of As, Mo and Pb were below the detection level. The mean of values for Fe, Mn, Pb, and Zn detected in this study are below the permissible limits of the USEPA (1977) and the Ontario Ministry of the Environment and Energy (OME, 1976) except for Cu, which is slightly above the USEPA limit. They are also below the values determined for lead and arsenic in river sediments collected near the river origin in the state of Tlaxcala (García-Nieto *et al.*, 2011) but above for Fe and Mn in agricultural soils located further south Puebla city that have been irrigated with the river waters for more than 30 years (Méndez-García, Rodríguez-Domínguez, & Palacios-Mayorga, 2000). This suggests that heavy metal concentrations in sediments vary

Table 3. MPN of total and fecal coliforms and BOD for sampling points A1-A3. A: Atoyac River.

Code	Fecal coliforms (thermal tolerance) (MPN/100 ml)	Total coliforms (MPN/100 ml)	BOD (mg/l)
A1	9	240	123
A2	14	210	125
A3	15	240	120
MON	240	___	20

MON: Maximum limits of Mexican Official Norm (NOM-003-Semarnat-1997).

31

Tecnología y Ciencias del Agua, vol. VIII, núm. 3, mayo-junio de 2017, pp. 27-37

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along the river course and at the dam. Another study has also determined low concentrations of metals in the river waters (Cedeño, Téllez, Pacheco, Rosano, & Ascencio, 2008) and the water hyacinth (*Eichhornia crassipes*) has been suggested as a phytoaccumulation species within the Valsequillo dam (Cedeño *et al.*, 2008). However, it has also been established that the concentrations of metals have accumulated in soils throughout the time because of the irrigation activities (Méndez-García *et al.*, 2000) requiring constant monitoring. Moreover, further studies have established that these waters should not be used for the irrigation of crops (Díaz *et al.*, 2005; Bonilla, Vázquez, Silva, & Cabrera, 2013) and this is supported by a previous study where long-term irrigation of soils with wastewater in Germany caused significant heavy metal contamination and a potential impact on the underlying aquifers (Lottermoser, 2012).

Organic compounds detection

A total of 20 organic compounds were detected in the river sediments of sampling point A1 and they are higher alkanes (C11-C16), esters, organic acids, phenols, phthalates, among others (table 5). The detection quality of most of the compounds ranged from 80 to 98% (table 5). These compounds have been reported as common contaminants of river waters (Pennell, Abriola, & Weber, 1993; Gracia, Cortés, Sarasa, Ormad, & Ovelleiro, 2000). Ten of the twenty organic compounds detected in the sediment

sample are classified by the USEPA as chemicals for potential endocrine disruptor screening and testing and they include n-hexadecanoic acid, octadecanoic acid, phenol, 2,4-bis(1,1-dimethylethyl)-, dodecane, cholestan-3-ol, (3.beta, 5.beta.)-, hexadecane, tridecane, undecane, tetradecane and Cyclopenta[g]-2-benzopyran, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl- (USEPA, 2012). Compounds such as cholestan-3-ol, (3.beta, 5.beta.), known also as coprostanol, have been used as indicators in faecal pollution source tracking (FST) to identify the origin(s) of pollution in water, in areas where diffuse faecal contamination are often marked by the co-existence of human and animal sources (Roslev & Bukh, 2011; Derrien *et al.*, 2012). The presence of coprostanol was correlated with the microbial populations found in situ, as discussed later. Moreover, a recent study detected other persistent organic pollutants (POPs) in the river sediments that included hexachlorocyclohexane isomers, hexachlorobenzene, many polychlorinated biphenyl congeners and plaguicides such as DDT, DDE, mirex, and aldrin, (Juárez-Santacruz *et al.*, 2012). Therefore, the presence of all these organic pollutants could pose a health risk to the population because the crops in the sub-basin and those located lands above the dam are irrigated with these waters. Crops that are irrigated with polluted waters may contain pesticide residues; chlorpyrifos, cypermethrin, and omethoate have been usually detected in green vegetables such as spinach (Kobayashi *et al.*, 2011).

Table 4. The content of metals, semimetals and other elements in the Atoyac River sediments and comparison with the USEPA limits.

Code	Elements (mg/kg)													
	As	**Ba**	**Ca**	**Cu**	**Fe**	**K**	**Mn**	**Mo**	**Pb**	**Rb**	**Sr**	**Ti**	**Zn**	**Zr**
Av A1	<DL	417	157 019	54.8	21 554	5 881.7	407.2	<DL	<DL	24.4	667.8	3 026.3	89.6	169.2
EPA	8	---	---	50	25000	---	500	---	60	---	---	---	200	---
DL	14.5	27.1	70	42.7	113	200	107.3	10	20	10	10	209.5	61	8.1

Av: Average value; A: Atoyac River; EPA: USEPA Guideline (1977); DL: Detection limit.
As, Mo and Pb were below the detection limit.

Table 5. Organic compounds identified by GC-MS from sediments of the Atoyac River sampling position A1.

Quality (%)	Compound	CAS number
91	Undecane	001120-21-4
91	Dodecane	000112-40-3
91	Tridecane	000629-50-5
96	Tetradecane	000629-59-4
90	Hexadecane	000544-76-3
81	Undecane, 2,6-dimethyl-	017301-23-4
93	Dodecane, 2,6,11-trimethyl-	031295-56-4
89	2-Bromo dodecane	013187-99-0
95	Hexadecane, 2,6,10,14-tetramethyl-	000638-36-8
96	Phenol, 2,4-bis(1,1-dimethylethyl)-	000096-76-4
80	4-tert-Butylphenyl acetate	003056-64-2
90	Cyclopenta[g]-2-benzopyran, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-	001222-05-5
98	n-Hexadecanoic acid	000057-10-3
81	Octadecanoic acid	000057-11-4
87	Sulfurous acid, butyl heptadecyl ester	1000309-18-4
91	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	004376-20-9
92	1-Eicosene	003452-07-1
90	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	001786-12-5
87	[Bi-1,4-cyclohexadien-1-yl]-3,3′,6,6′-tetrone, 4,4′-dihydroxy-2,2′,5,5′-tetramethyl-	010493-51-3
91	Cholestan-3-ol, (3.beta, 5.beta.)-	000360-68-9

Bacterial diversity in situ

A total of 53 clones were retrieved from the clone library and they were related to members of the *Enterobacteriaceae* within the Gammaproteobacteria (figure 2). The bacterial diversity of the surveyed sediments is low; species related to *Shigella flexneri*, *Escherichia fergusonii* and *E. coli* dominated the clone library. The degradation of hydrocarbons has been reported recently by *Escherichia fergusonii* (Sriram *et al*., 2011; Pasumarthi, Chandrasekaran, & Mutnuri, 2013) suggesting the use of some of the detected compounds by the *Escherichia fergusonii*-related bacteria, however, these and the other members of the *Enterobacteriaceae* may also pose potential health risks to the population. *Shigella flexneri* (B serogroup) is a common cause of bacillary dysentery in humans. Infection by *Shigella* is usually via ingestion invading the colonic mucosa and producing an intense inflammatory reaction leading to tissue destruction (Kotloff

et al., 1999). Therefore, we tested for the presence of Shiga genes within our samples but they were not found. However, future studies would be necessary to look for further genes encoding other toxins or virulence and invasion plasmids (Sansonetti, Kopecko, & Formal, 1982; Ménard, Sansonetti, & Parsot, 1993; Fagan *et al*., 1999) in this strain in order to have a proper assessment of the *Shigella*-related clones pathogenicity.

The high number of clones related to *Enterobacteriaceae* is not uncommon since the river receives industrial, agricultural and domestic effluents; however, our samples were collected after the wastewater treatment process suggesting the presence of further domestic effluents or the lack of efficacy during the wastewater treatment process. This is also constant with the high levels of coliforms (240 MPN 100 ml^{-1}) found at the same sampling point and with previous studies (Silva, Muñoz, Isla de Bauer, & Infante, 2002; Cabrera, Bonilla, Tornero, & Castro, 2005; Sandoval-Villasana *et al*., 2009).

33

Tecnología y Ciencias del Agua, vol. VIII, núm. 3, mayo-junio de 2017, pp. 27-37

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Thus, there may be health risks since these waters are later used to irrigate several crops such as maize, sorghum, and alfalfa in lands above the dam (Larenas-Bazán, 2010). Therefore, it is of great importance to identify the effluents located after the wastewater treatment process and / or review the water quality coming out of those treatment facilities, another aid would be the use of microbial source tracking markers such as the stanols (Derrien *et al.*, 2012) in order to identify faecal contamination sources. It is equally important to look for catabolic and pathogenicity genes in the environmental samples in order to properly assess the microbial diversity degrading potential and its pathogenicity.
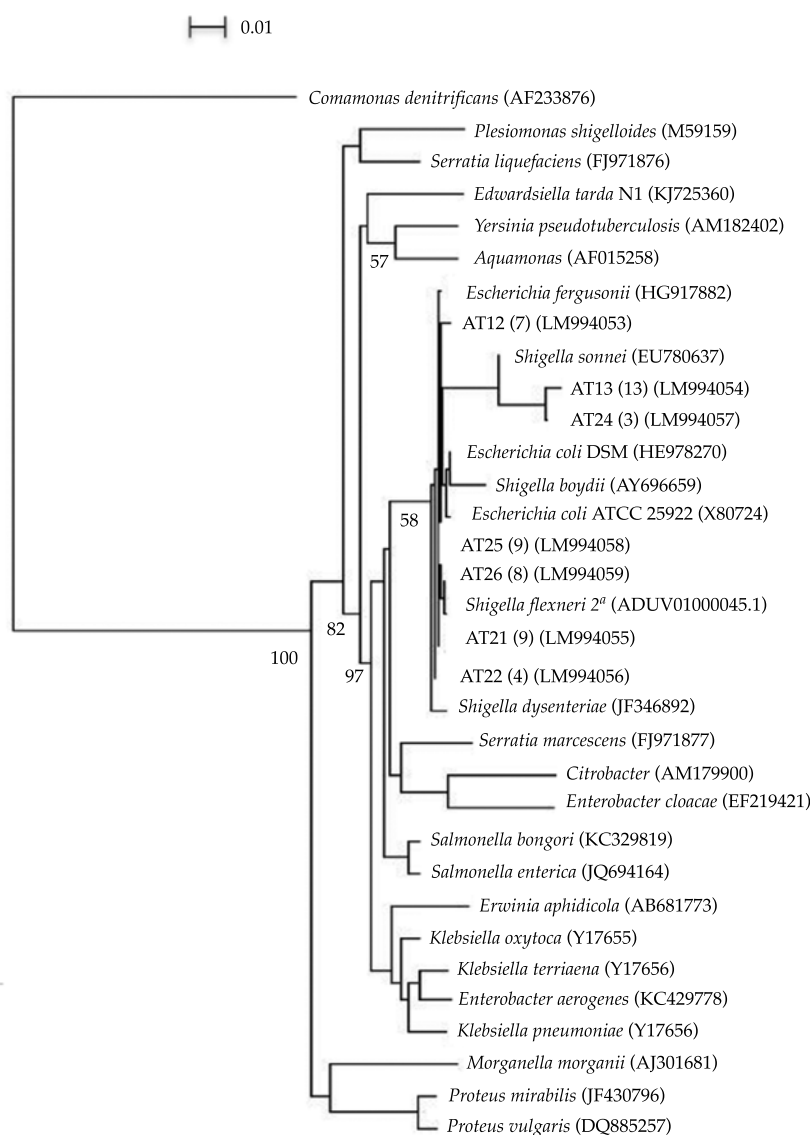
Figure 2. Phylogenetic tree of the *Enterobacteriales* order based on partial 16SrDNA sequences showing the relationships among bacterial clone sequences from this study and members of the *Enterobacteriaceae* family found in the database. Jukes and Cantor distance, neighbour-joining method, outgroup: *Comamonas denitrifications*. Clones from this study are in bold, A: Atoyac. A number in parenthesis indicates the number of clones obtained for each sequence. Accession numbers of the sequences retrieved from the databases are also indicated in parenthesis. Bootstrap values above 50% are shown at branch nodes (per 1000 trials). 16S rRNA sequence similarity over the region 800 to 1390 based on *E. coli* numbering.

Conclusion

This study provides further evidence of the environmental degradation of the Atoyac River. It is the first to describe the bacterial communities in its sediments and to elucidate the contaminants present just prior to the dam. Moreover, it shows a low bacterial diversity in situ, usually related to gut diseases and although it lacks Shiga toxins encoding genes, further studies are required to discard the presence of all other toxin-encoding genes since these waters are later used for crops irrigation.

Acknowledgements

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35

Tecnología y Ciencias del Agua, vol. VIII, núm. 3, mayo-junio de 2017, pp. 27-37

ISSN 0187-8336 •

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36

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37

Tecnología y Ciencias del Agua, vol. VIII, núm. 3, mayo-junio de 2017, pp. 27-37