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Characterization and antibiotic susceptibility of *Salmonella* strains isolated from wastewater treated by infiltration percolation process

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Abstract

Municipal wastewater contains a high variety of pathogenic microorganisms than may present potential risks for public health and the environment. We were interested in our study to isolate and study *Salmonella* in effluents throughout the wastewater treatment plant process steps. The results showed the presence of *Salmonella* strains all along of the treatment steps and especially at its exit. A total of 19 *Salmonella* serotypes were identified in three wastewater types: raw water, decanted water and purified water. Despite of treatment, results revealed the presence of *Salmonella* strains in the purified water at the station exit. A high diversity of identified serotypes was noted. Antibiotic susceptibility showed that all *Salmonella* serotypes were susceptible to all antibiotics tested, except for one isolate of *S.* Chester and too isolates of *S.* Infantis strains which were multi-resistant and resistant only to nalidixic acid, respectively.

Keywords: Antibiotic susceptibility; Infiltration percolation; *Salmonella*; Wastewater treatment.

Introduction

Agadir city is one of many major coastal cities in the world that discharge their wastewaters into the marine environment. Wastewater may consist of a combination of domestic, industrial and agricultural effluent.

The wastewater treatment plants (WWTPs) are mostly designed to effectively remove fecal bacteria, some chemical pollutants but are rarely been provided specifically to eliminate pathogenic microorganisms (Koivunen *et al.*, 2003). Furthermore, most of these treatment systems remain defective and continue to reject varying concentrations of enteric bacteria in the environment (Koivunen *et al.*, 2003; Huang *et al.*, 2012; Bouki *et al.*, 2013; Naidoo & Olaniran, 2014).

Generally, an optimal wastewater treatment process can attain 90 to 99% of reduction of microbial load (Kayser *et al.*, 1987; Kümmerer, 2009; Mimouni *et al.*, 2009). However, in some cases the reduction level decreases are mostly due to a non-respect of the operating mode or to bad or inadequate plant maintenance.

Pathogenic bacteria become normally more harmful to environment and humans when they acquire resistance to antibiotics. Moreover, many studies (Hartig *et al.*, 1999; Huang *et al.*, 2012; Michael *et al.*, 2013) confirmed the detection of antibiotic concentrations in wastewater that treatment systems generally fail to eliminate. Therefore, wastewater could be an ideal environment at a time of contact between bacteria and antibiotics traces, exchange of genetic material between bacteria and acquisition of antibiotic resistance (Espigares *et al.*, 2006; Huang *et al.*, 2012; Michael et al., 2013; Vaz-Moreira *et al.*, 2014).

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Antibiotic resistant bacteria (ARB) have been detected widely in wastewater samples (Karthikeyan & Meyer, 2006; Czekalski *et al.*, 2012; Michael *et al.*, 2013). Available data show significantly higher proportion of antibiotic resistant bacteria contained in raw and treated wastewater comparative to surface water. According to these studies, conditions in wastewater treatment plants (WWTPs) are favorable for the proliferation of ARB (Bouki *et al.*, 2013; Rizzo *et al.*, 2013).

The *Salmonella* genus consists of two species, *Salmonella enterica* and *Salmonella bongori*, six subspecies and over 2500 serotypes that all supposed to be able to cause human diseases, such as typhoid fever, paratyphoid fever and other non-typhoidal salmonellosis (Porwollik *et al.*, 2004; Hsu *et al.*, 2011). *Salmonella* is one of pathogen bacteria that could be conveyed and may end up in environmental waters through sewage pollution. Therefore, the determination of the presence of *Salmonella*, its levels and its antibiotic resistance at the same time in wastewaters and environmental waters are crucial and required to assess the related health risk.

Previous studies have been conducted to study the presence of *Salmonella* in wastewater (Bandart *et al.*, 2000; Koivunen *et al.*, 2003; Howard *et al.*, 2004; Espigares *et al.*, 2006; Levantesi *et al.*, 2010; Ben Salem *et al.*, 2011) but their diversity and antibiotic resistance profiles remains a poorly researched subject.

In Morocco, *Salmonella* in wastewater is also a subject little discussed and bibliography concerning is very scarce. In this context, our study is the first one led in the Morocco southern region focused on the identification, serotyping and antibiotic susceptibility of *Salmonella* strains isolated from Agadir wastewater treatment plant.

The aim of this study is firstly to provide qualitative information on the capacity of this treatment process to eliminate *Salmonella* considered among the most pathogenic bacteria easily transmissible in water. Secondly, to determine the serotypes of our *Salmonella* isolates and then to establish their antibiotic susceptibility using a wide range of the most commonly used antibiotics.

Materials and methods

Plant description

The Agadir wastewater treatment plant is built on coastal dunes of Mzar in Ait Melloul within the Souss-Massa National Park at about 8.5 km south of the city of Agadir. This resort provides treatment of wastewater in the whole city of Agadir.

The WWTP process is based on the principle of infiltration percolation. Indeed, it consists of 13 settling ponds at which the wastewater undergoes a primary treatment settling aimed at eliminating of suspended matter and fats. The treated water is channeled to 24 sand filters where the water having undergone the primary treatment percolates in an unsaturated granular medium (secondary treatment) (Figure 1). The purifying biomass present on the sand filter allows to oxidize organic matter, nitrify ammonia nitrogen but also to reduce pathogens.

Isolation and identification of *Salmonella*

The three types of water: raw (RW), decanted (DW) and purified (PW) have first been pre-enriched in buffered peptone water (BPW) (Biokar). Two aliquots of 0.1 ml and 1 ml of each sample were then enriched respectively in 10 ml of Rappaport Vassiliadis broth (RV) (Biolife) and Muller-Kauffman with Tetrathionate-Novobiocine (MKTTn) (Biolife).

Both enriched suspensions were then plated on Xylose Lysine Desoxycholate environment (XLD) (Biolife) and on the Rambach agar

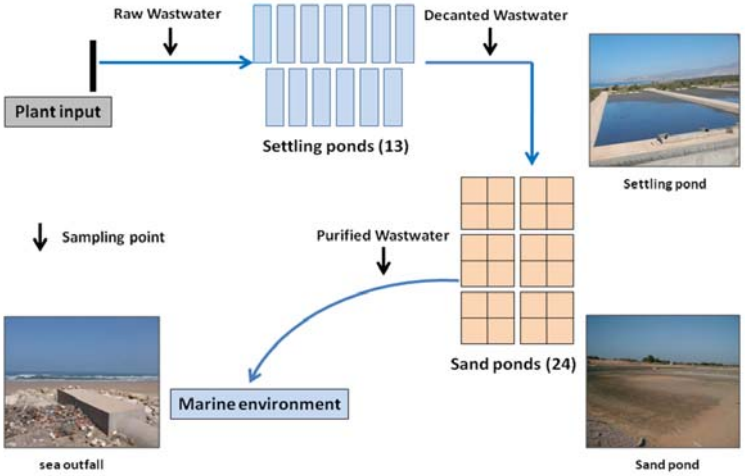

Figure 1. Schema of Agadir wastewater treatment plant.

(CHROMagar, Paris, France). Mauve colonies on Rambach and grey with black center on XLD medium were removed for identification and further analysis. Suspect colonies were all identified using the API20E system (bioMérieux).

Salmonella serotyping

Salmonella serotyping was performed using the direct agglutination technique blade, involving strains with different antisera (Bio-Rad, Marnes-la-Coquette, France). Serotyping was performed according to the antigenic formulae of Kauffmann-White-Le Minor scheme (Grimont & Weill, 2007).

Antibiotic susceptibility testing

The sensitivity to 32 antibiotics were tested by the agar diffusion method following the recommendations of the Antibiogram Committee of the French Society for Microbiology (CA-SFM 2014): Flood of Mueller-Hinton agar medium with a bacterial suspension equal to McFarland 0.5 standard diluted to 1 / 100th and filing of antibiotic discs (BioRad Marnes-La-Coquette, France) as follows: amoxicillin (AMX), amoxicillin-clavulanic acid (AMC), ticarcillin (TIC), ticarcillin-clavulanic acid (TCC), piperacillin (PIP), piperacillin-tazobactam (TZP), cephalothin (CEP), cefamandole (MAN), cefoperazone (CFP), aztreonam (ATM), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), cefoxitin (FOX), moxalactam (MOX), imipenem (IPM), streptomycin (STR), spectinomycin (SPT), kanamycin (KAN), tobramycin (TOB), netilmicin (NET), gentamicin (GEN), amikacin (AMK), isepamicin (ISE), nalidixic acid (NAL), ofloxacin (OFX), ciprofloxacin (CIP), sulfonamides (SSS), trimethoprim (TMP), chloramphenicol (CHL), cotrimoxazole (SXT) and tetracycline (TE). Reading boxes was carried out by the micro SirScan+ machine after 24 hours incubation at 37°C.

Results and Discussion

Identification and serotyping

Twenty *Salmonella* strains were isolated in three wastewater types analyzed between the period of February and September 2014. Nineteen of them were characterized and serotyped only one strain wasn't identified by serotyping as *Salmonella*. On the 19 identified strains, 10 different serotypes have been identified (Table 1). The major one identified was Muenster (n=5), followed by Infantis and Senftenberg (n=3 each) and Kentucky (n=2) (Table 1). The least frequent serotypes were Agona, Chester, Menston, Oranienburg, Reading and Schwarzengrund with one isolate each. Their repartition revealed the presence of different *Salmonella* serotypes in the same wastewater type and the same serotypes were observed in different wastewater types (case of *S.* Muenster, *S.* Infantis and *S.* Senftenberg) (Table 1).

The major serotype Muenster isolated from Mzar WWTP wastewaters has been detected at a time at the entry and at the treatment plant exit. In addition to Muenster, serotypes Infantis, Oranienburg and Kentucky were also present in purified water at the treatment plant exit. Their presence in treated wastewater indicated that they were able to escape from the whole treatment process steps.

Table 1. *Salmonella* strains isolated from raw, decanted and purified wastewater.

Serotypes	Number of isolations			
	RW	DW	PW	Total
Agona	-	1	-	1
Chester	-	1	-	1
Infantis	-	2	1	3
Menston	1	-	-	1
Muenster	2	-	3	5
Oranienburg	-	-	1	1
Kentucky	-	-	2	2
Reading	-	1	-	1
Schwarzengrund	1	-	-	1
Senftenberg	1	2	-	3
Total	5	7	7	19

RW: Raw Water, **DW**: Decanted Water, **PW**: Purified Water

Our finding revealed high serotype diversity of *Salmonella*. It was previously described in other studies showing that *Salmonella* serotypes Muenster, Infantis and Senftenberg were identified in wastewater in France (Baudart *et al.*, 2000). Muenster, Schwarzengrund, Senftenberg and Infantis *Salmonella* serotypes were also found in Finnish wastewater treatment plants (Koivunen *et al.*, 2003). Data from Spain showed that the most frequent serotypes isolated from wastewater were Hadar (38.1%), Enteritidis (23.8%), London (14.3%), Anatum (9.5%) followed by the least frequent serotypes Typhimurium, Gold coast, and Newport, with one isolate each (4.8%) (Espigares *et al.*, 2006). In Morocco, available data show that *Salmonella* serotypes isolated from wastewater were: Typhimurium (57%), Hadar (21%), Senftenberg (7%), Give (4%), Nexport (4%) and Anatum (4%) (Oubrim *et al.*, 2012).

As illustrated in Table 1, more *Salmonella* strains of the present study were isolated from decanted and purified wastewaters than from the raw wastewater. This could be explained by the fact that raw wastewater was characterized by a very high organic pollution that acted on certain physicochemical parameters such as increased turbidity and dissolved oxygen quantity reduction, so this made isolation of *Salmonella* in raw wastewater more difficult than in decanted and purified wastewater.

From an epidemiological point of view, the presence of *Salmonella* strains in purified water at the plant output is very important. This revealed a considerable risk of the transmission of *Salmonella*. Many previously results have also shown that municipal treated wastewater continued to carry *Salmonella* (Howard *et al.*, 2004; Espigares *et al.*, 2006; Becerra-Castro *et al.*, 2015).

Actually, Mzar WWTP wastewaters are released after treatment into the marine environment. Interestingly, the passage of *Salmonella* in sewage improves subsequent survival after discharge into the seawater, and their survivability is even more important than the length of stay of *Salmonella* in wastewater is longer (Dupray & Derrien, 1995).

Our findings show the presence of *Salmonella* in Mzar WWTP. So, the discharges of these wastewaters in marine environment would have a role in the contamination of this ecosystem. Senftenberg and Kentucky Serovars isolated from wastewater in our investigation have also been identified previously in mussels, seawater and sediments in the marine environment of Agadir city (Setti *et al.*, 2009).

From an efficiency point of view of the station's process system, the infiltration percolation wastewater treatment enables a very significant reduction of chemical

contamination concentration, fecal indicator bacteria, and pathogenic bacteria but it doesn't completely eliminate them (Mimouni *et al.*, 2009; Eddabra, 2011). Although the important purifying capacity of this treatment system, our results confirmed that infiltration percolation remained a defective treatment system for total *Salmonella* elimination. Therefore, the WWTP plant continued to reject *Salmonella* strains in the marine environment. This fact might contribute to the dissemination of a large diversity of *Salmonella* strains in aquatic environment.

Antibiotic susceptibility

Among 19 *Salmonella* isolates, 16 strains (84%) were found to be susceptible to all antibiotics tested (Table 2). Two strains *S.* Infantis presented resistance exclusively to nalidixic acid and only one serotype *S.*Chester showed a multiple antibiotic resistance to nalidixic acid, ofloxacin, tetracycline, sulfonamides, trimethoprim and cotrimoxazole.

The distribution of resistant strains (Table 2) showed that serotypes which were resistant have been detected only in decanted wastewaters. Generally, bacteria in wastewater are in permanent contact with other element including antibiotics and other chemical products. Actually, studies showed that high quantities of antibiotics are released into municipal wastewater due to incomplete metabolism in humans or because of the mismanagement of unused antibiotics (Duong *et al.*, 2008; Bouki *et al.*, 2013). Furthermore, WWTPs are not designed to remove antibiotics and pharmaceuticals personal care products which then can find their ways in different compartments of the natural environment (Thomas & Foster, 2005). Previously data showed that antibiotic resistant bacteria (ARB) have been detected extensively in wastewater samples which can pass through WWTPs and arrive to the receiving environment (Kümmerer, 2009; Czekalaski *et al.*, 2012; Rizzo *et al.*, 2013). Compared to surface water the higher proportion of ARB has been reported in wastewater relative to surface water (Bouki *et al.*, 2013).

Table 2. Antibiotic susceptibility patterns of *Salmonella* strains isolated from raw, decanted and purified wastewater

Strains	Resistance
Raw Water	
S. Schwarzengrund	Susceptible
S. Muenster	Susceptible
S. Menston	Susceptible
S. Senftenberg	Susceptible
S. Muenster	Susceptible
Decanted Water	
S. Senftenberg	Susceptible
S. Agona	Susceptible
S. Infantis	NAL
S. Reading	Susceptible
S. Infantis	NAL
S. Senftenberg	Susceptible
S. Chester	NAL, OFX, TE, SSS, TMP, SXT
Purified Water	
S. Kentucky	Susceptible
S. Kentucky	Susceptible
S. Muenster	Susceptible
S. Muenster	Susceptible
S. Muenster	Susceptible
S. Oranienburg	Susceptible
S. Infantis	Susceptible

NAL: Nalidixic Acid, **OFX**: Ofloxacin, **TE**: Tetracycline, **SSS**: Sulfonamides, **TMP**: Trimethoprim, **SXT**: Cotrimoxazole.

Others studies suggest that the coexistence and competition among strains, species and even different genera in wastewater can allow interchanges of genetic material and favor the selection of resistant strains in treatment plant exit (Espigares *et al.*, 2006; Vaz-Moreira *et al.*, 2014).

The effect of the passage and stay of *Salmonella* in wastewater treatment plant on their antibiotic resistance is widely discussed but it remains very ambiguous. The high resistant *Salmonella* strains are almost isolated in purified wastewater at the treatment plant exit compared to raw wastewater (Schwartz *et al.*, 2003; Volkmann *et al.*, 2004; Vaz-Moreira *et al.*, 2014).

Salmonella Chester was the only serovar that showed antibiotic multiresistance. Chester is a rare *Salmonella* serotype, which has become recently emerging and often linked to several cases of salmonellosis (Taylor *et al.*, 2012). Moreover, multi-resistant bacteria has become a significant public health problem and infection that can caused would be much more difficult to treat because the panel of effective antibiotic will be reduced (Roberts *et al.*, 2009; Magiorakos *et al.*, 2012). The presence of resistant *Salmonella* strains in wastewater constitutes a source of potential risk to human and environment. However, the presence of resistant serotypes in decanted water and their absence after treatment at the station exit can be a positive sign to the capacity of this treatment system to reduce the risk of dissemination of resistant *Salmonella* strains in the environment.

Conclusion

Our results provide accurate and interesting information about the *Salmonella* serotype variety present in the wastewater effluent from Mzar WWTP and their resistance to antibiotics commonly used by health services. Our results confirm the inefficiency of infiltration percolation treatment system to the total elimination of *Salmonella* strains, which could be released into the environment. However, following to our findings this treatment system doesn't favorite the selection of resistant strains after treatment. *Salmonella* infections remain a major public health problem, and the risk on human and environment contamination is always important; hence, the need for epidemiological, microbiological and environmental surveillance especially with the permanent emergence of multi-resistant *Salmonella* strains.

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