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Full Length Research Paper

Antimicrobial susceptibility of aeromonads and coliforms before and after municipal wastewater treatment by activated sludge under arid climate

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Two hundred and twenty four-strains of motile *Aeromonas* species and *E. coli* were isolated from three sampling points (influent, oxidation pond and effluent) in an activated sludge wastewater treatment plant in Nouakchott, Mauritania. Antimicrobial susceptibility tests were performed in accordance with the diffusion technique and fifteen of the most frequently used antibiotics in the antibiotic treatment at the national level were tested on these isolates. The statistical analysis showed insignificant differences (P>0.05) in the resistance patterns between influent and effluent isolates. All strains were found to be monoresistant, primarily to Vancomycin. Nearly 4.5 % of *A. hydrophila* and 3.1 % *A. caviae* were resistant to Cefoperazone, while greater than 97.1 % of *A. sobria* were found to be susceptible to this drug. The overall resistance rates to Amikacin and Chloramphenicol did not exceed 4.6 and 10.6 % respectively. The results indicate that despite the important removal rate given by the treatment process, antimicrobial resistance incidence among pathogenic aeromonads has not been decreased and remains significant to potentially compromise the reclaim of the treated effluent in urban agricultural practices in the wastewater spreading area of Nouakchott where water reclaim permits to the urban agriculture to survive water scarcity.

Keywords: Activated sludges, *Aeromonas*, Antibiotics, Irrigation, Polyresistance, Removal, Sanitation, Urban agriculture, Wastewater, Water reclaim.

INTRODUCTION

Municipal wastewater purification has become more important during the last decades due to increased awareness of wastewater associated risks to both human health and the environment. Problems related to municipal wastewater have become increasingly critical due to the growing numbers of slums that result from accelerated urbanization as a consequence of the drought in rural areas.

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Mauritania is a sahelian country characterized by a hot and arid climate, with a permanent water shortage. Therefore, there is a great need for the reclaim of wastewater. In the capital, Nouakchott, more than 28% of the population suffers from lack of drinking water and urban agriculture begins to be highly dependent on drinking water availability.

The activated sludge wastewater purification process is one of the most efficient wastewater treatment technologies (Bond et al., 1998). Nevertheless, bacteria remain present in the effluent and represent a potential risk of infection for those who come in contact with it.

Furthermore, toxic effects may result from the remaining chemical pollutants including heavy metals.

An assessment of the wastewater activated sludges purification system has given satisfactory results regarding the removal of bacteria, however, considerable quantities of micro organisms were still present in the treated effluent (Gagneux et al., 1999; Martone-Rocha et al., 2010). Motile aeromonads often remain in the effluent. These species have long been reported to cause various outbreaks in humans and poikilothermic animals (Dumontet et al., 2000; Kannan et al., 2001, Mahmoud and Mohamed, 2011).

In the city of Nouakchott, Mauritania, motile aeromonads have been identified as the source of several categories of human infections associated with raw vegetables consumption. The frequent isolation of *Aeromonas* strains in stool samples from diarrheic patients in the area indicates that these infections raise serious health concerns, calling for an effective antibiotic therapy.

In addition, the widespread use of antibiotics in agriculture as growth promoters and therapeutic or prophylactic agents have been reported (Imziln et al., 1998). This practice could increase the risk of bacterial resistance proliferation among environmental flora, resulting in reduced effectiveness of antimicrobial chemotherapy in the event of an infection.

This study investigates the effect of the activated sludge treatment process on the antibiotic resistance of three motile *Aeromonas* species and faecal coliforms. Antibiotic resistance profiling has been restricted to the aeromonads in regard to their recorded high infection rate in Nouakchott. In addition, *E. coli* is known as a representative faecal and opportunistic bacteria frequently associated with urinary infections. The goal of this research is to assess the health risks associated with the use of treated effluent for irrigation of vegetables in Nouakchott's urban agricultural area and to determine whether or not this practice could be seen as acceptable in terms of human health.

The most frequently used standards allow restrictive irrigation of crops with purified wastewater when water quality requirements meet those of the World Health Organization (WHO) standard B category. The limit for the faecal coliforms bacteria corresponding to this standard is 10^5/100 ml, under conditions where adult farm workers are exposed to spray irrigation (Blumenthal et al., 2000).

MATERIALS AND METHODS

Study area

Nouakchott (18°7 N, 15°05 W), the main urban center and captial of Mauritania, is located in the south-west of the country, along the Atlantic coast.

The studied waste water treatment plant is found in the suburb of Sebkha near the most important urban agricultural area in Mauritania. It became operational in 1996 and receives an average influent of 18000 m^3/day. The facility consists of a typical activated sludge plant with primary and secondary treatment followed by retention in an oxidation channel, which is continuously oxygen-enriched by two pumps. The average inflow rate is about 37 litres / sec and the outflow rate is 32 litres / sec. The plant was designed for 432,000 equivalent inhabitants and is currently managed by the national office for sanitation (ONAS).

This treatment process generates significant quantities of sludge which are dried then used as fertiliser. The microbiological characterization of the dried sludge is tested, in order to assess its suitability for utilization as fertilizer outside the studied area.

Sampling

Water samples were collected in pre sterilized glass bottles and stored in cold boxes at 4°C until processing. Three sampling points were considered: E at the plant influent, C in the oxidation pond and S corresponding to the purified effluent wastewater at the end of the clarification phase. Three subsamples were taken from each sampling point. Four seasonal samples were collected between January and December 2010 and between twenty and thirty strains were isolated and screened from each sample. Samples were generally taken at about midday and analysed within two hours.

Bacteriological methods

Water samples were shaken gently for about five minutes, then ten-fold diluted in sterile saline solution 0.85 % (w/v of NaCl in demineralized water). Aliquots of 0.1 ml from suitable dilutions were spread onto PADE agar of Imziln et al. (1997) and Mac Conkey agar. Plates were incubated respectively at 37°C and 44.5°C for PADE and Mac Conkey. Typical *Aeromonas* and coliforms colonies were sub cultured on trypto-casein-soja agar (TSA, Diagnostic Pasteur 64554) plates. Isolates were considered as presumptive *Aeromonas* strains when they were: Gram-negative rods, oxydase positive, motile, fermentative of glucose (O/F test Hugh-Leifson medium, Merck 10282), arginine dihydrolase positive (ADH, Möller), resistant to the vibriostatic agent 2,4 diamino-6,7-diisopropylpteridine phosphate (O129, 150 µg. Diagnostic Pasteur 53872). Other biochemical profiles were considered as non *Aeromonas* and rejected from the antimicrobial susceptibility testing.

Isolates were identified to the species level according to the biochemical profiles described by Popoff (1984). These biochemical tests were: fermentation of salicine,

Table 1. Specific composition and origins of the *Aeromonas* and *E. coli* isolates

Species	Influent	Effluent	Total
Aeromonas caviae	23	41	64
Aeromonas sobria	11	33	44
Aeromonas hydrophila	17	18	35
Atypical isolates	06	02	08
Escheichia coli	35	38	73
Total	***92***	***132***	***224***

production of acetoïne (Voges-Proskauer reaction), esculin hydrolysis, production of gas from glucose, decarboxylation of lysine (Möller), fermentation of L-arabinose and production of H2S from L-cysteine. API 20 E strips were utilized in parallel to identify either one of the three motile species of *Aeromonas* or an atypical isolate.

Presumptive coliforms strains were screened through the IMViC tests (Indole, Methyl red, Voges-Proskauer and Simmon's Citrates).

Antibiotic susceptibility testing

Antimicrobial susceptibility of *Aeromonas* strains was determined using the standard diffusion method of Kirby Bauer (Bauer et al., 1966). Isolates were checked for purity, sub cultured in brain heart broth tubes (Oxoid), grown on TSA plates, and then inoculated by spreading on Mueller-Hinton agar (Mueller Hinton 2, bioMérieux 51861). Antibiotic concentrations (µg/ml) were : Ticracillin 75 µg; Sulphamides 200 µg; Imipenem 10 µg; Colistin 50 µg; Vankomycin 30 µg; Piperacillin 100 µg; Cefoperazone 30 µg; Cefsulodin 30 µg ; Fosfomycin 50 µg; Amikacin 30 µg; Oxacillin 1 µg; Tobramycin 10 µg; Ampicillin 10 µg; Erythromycin 15 µg and Chloramphenicol 30 µg. All chemicals were from bioMérieux (Marcy, France) and were of an analytical grade.

Chemical methods

The biochemical oxygen demand (BOD) and the total suspended solids (TSS) were measured according to the procedures of the American Public Health Association (APHA, 1998).

Data analysis

Bacterial abundances were expressed in a 10-basis logarithmic scale. The comparison of the antibiotic resistance index (ARI) was performed by the Wilcoxon Signed rank non parametric statistical test. The antibiotic resistance index (ARI) was calculated according to Hinton and Linton (1983) using the following formula: ARI = x/ny, where x represents the number of resistant determinants in a population y, and n represents the number of antibiotics tested. StatView software was utilized for the statistical comparison for the all data.

RESULTS

This study involved 224 bacterial strains, including 151 *Aeromonas* and 73 *E. coli*. The *Aeromonas* population consisted of 35 *A. hydrophila*, 64 *A. caviae*, 44 *A. sobria* and 8 atypical *Aeromonas* isolates. 57 strains representing 37.8 % of the entire aeromonads population originated from raw wastewater, while 94 strains representing 62.2 % were obtained from the plant effluent.

All of the 73 coliforms strains were formally identified as *E. coli*, of which, 35 (47.9 %) were isolated from raw wastewater and 38 (52.1 %) were isolated from the effluent.

The specific composition and origin of the 224 tested bacterial isolates is given in table 1. This research has shown that *A. caviae* strains dominated in both raw sewage (sampling point E) and in activated sludge plant effluent (sampling point S).

Furthermore, a bacteriological and physico-chemical 12-months survey undertaken at the plant, which recorded average removal rates, showed only an average of 0.39 logarithmic unit (U. Log) removal (33.3 %) for *Aeromonas* species and 0.68 logarithmic unit (61.2 %) for the *E. coli* population during the whole 15-month survey period.

Moreever, in a way to assess the bacterial abundance and the antimicrobial susceptibility before the purified wastewater is released outside the process, the monitoring involved an additional intermediate sampling point (C) at the oxidation pond of the plant.

Overall results of the monitoring showed that counts of motile aeromonads were found to be 3,69 U.Log in the raw wastewater, 3,36 U.Log in the oxidation pond and 3,23 U.Log in the effluent. Therefore, data indicated that aeromonads bacterial population is removed primarily in the oxidation pond (0,32 U.Log) corresponding to 71,11 % of the overall removal efficiency for this population.

The average quantities of the aerobic heterotrophic bacteria in the raw wastewater (E), the oxidation pond (C), and the exit of the treatment plant (S) were 7,3 U.Log, 6,88 U.Log and 6,15 U.Log respectively.

In regard to the faecal streptococci, the overall averages for the three considered sampling points were 3,52 U.Log, 3,21 U.Log and 3,05 U.Log respectively. Removal efficiency percentages corresponding to the aerobic heterotrophic bacteria and the faecal streptococci were 91,2 % and 41,8 % respectively.

Results regarding the biochemical oxygen demand (BOD), the averages obtained for the complete period of the survey, were 184 mg/l in raw wastewater, 91 mg/ml in the oxidation pond (C) and 35 mg/l in the effluent at the exit of the clarification phase (S).

Analysis regarding the three *Aeromonas* species polyresistance patterns in relation to the origin is shown in the Table 3. Data were calculated for the combinations of resistance from 2 to 7 antibiotics.

DISCUSSION

The results of the present study are consistent with other studies (Kannan et al., 2001) and in agreement with many reports which suggest that *A. caviae* is the dominating species in the human faeces and consequently, in raw municipal wastewater. It has been found elsewhere that *A. caviae* abundances correlate with those of faecal coliforms (Araujo et al., 1991). In addition, many authors have reported that the stabilization ponds select *A. sobria* strains, leading to its domination in the effluent (Monfort and Baleux, 1990; Stecchini and Domenis, 1994).

Despite the relevance of these overall removal rates, this could not be considered as satisfactory, given the risk associated with the consumption of the crops produced in the spreading area and the occurrence of a significant antibiotic resistance.

Nevertheless, the faecal coliforms loads in the effluent (<1000 CFU /100 ml) do not meet the requirements of WHO wastewater category A, henceforth prohibiting irrigation of the various crops for human consumption.

These records correspond to the removal rates of 60,21% and 50,54 % for the oxidation pond and the clarification phase respectively, showing that the main removal is obtained during the oxidation phase in the process.

Furthermore, these results indicate that the removal of organic chemical loads proceeded primarily, as expected, from the oxidation pond process. The average of total suspended solid load for the complete survey period was 261 mg/l for the raw wastewater, 173 mg/l in the oxidation pond and 16 mg/l in the clarification phase. The removal rate of the total suspended solids appears to be occurring during the clarification phase (90,75%).

Moreover, a moderate removal of suspended solids (33,7%) occurs during the primary treatment of the raw wastewater owing to the additional dissolution of the suspended particles during intensive mechanical aeration in the oxidation pond. Results corresponding to the antimicrobial susceptibility of the isolates to the fifteen tested antibiotics are shown in Table 2.

The results related to the antimicrobial susceptibility to the fifteen tested antibiotics are given in the Table 2. The antibiotic resistance levels are expressed in percentages of the whole population and as antibiotic resistance index numbers. Due to the fact that the isolation medium already contained Ampicillin, absolute resistance to this drug was expected to be confirmed and therefore it was not practical to consider these resistance levels, although (Figueira et al. (2011) have reported high to absolute rates of Ampicillin resistance among motile *Aeromonas* species). Genghesh et al. (2001) who studied the antibiotic susceptibility of aeromonads in untreated wells water found absolute resistance rates for Ampicillin. The high rate of Ampicillin resistance among *Aeromonas* strains has been confirmed by other reports (Rippey and Cabelli, 1979; Ansary et al., 1992) and has thus led to the frequent utilization of Ampicillin as a selective agent in the most common *Aeromonas* culture media (Rogol et al., 1979; Palumbo et al., 1985; Havelaar et al., 1987; Imziln et al., 1997; Vila et al., 2003). Furthermore, Lauria (1996) who compared resistance to Ampicillin among two *Aeromonas* populations isolated with and without Ampicillin as a selective agent, found that the resistance to Ampicillin was at least 62.5 % among the total tested population.

In addition, our research indicated that 148 of the 151 (98.01) % of *Aeromonas* isolates exhibited resistance to Vancomycin, which confirmed the results of Iversen et al. (2002) and Vandan et al. (2011) who reported only 3 % of *A. hydrophila* strains sensitive to this drug.

The recorded resistance rates to Vancomycin could confirm the reliability of using this drug as a selective agent in several media like Vancomycin Ampicillin Blood Agar of Koehler and Ashdown (1993). Resistance rate to Cefsulodin was 4.6 % (7 strains). This resistance level is supporting the results of Alonso et al. (1996), who suggested that Cefsulodin may be a useful selective agent for *Aeromonas spp*. growth when faecal coliform isolation is targeted and high levels of background flora are expected.

However, incidence of resistance to Imipenem, Sulphamides, Cefoperazone and Fosfomycin were respectively 7.9, 0.7, 4 and 2.6 % while appearing to be intermediate for the different *Aeromonas* species.

The resistance recorded for the total *E. coli* population showed an absolute susceptibility to Colistin and Piperacillin. The highest resistance rate corresponds to Cefsulodin (91.8%) with 67 resistant isolates.

The resistance levels discovered for Ampicillin and Chloramphenicol were respectively 42 and 2,7%. These

Table 2. Percentages of monoresistance and antibiotic resistance index among *Aeromonas spp.* and *E. coli* isolates

Antibiotic	*Aeromonas caviae*		*Aeromonas hydrophila*		*Aeromonas sobria*		*Atypical isolates*		*Escheichia coli*	
	Num	%	Num	%	Num	%	Num	%	Num	%
Amikacin	03	4.7	01	2.3	02	5.7	01	12.5	02	2.7
Chloramphenicol	06	9.4	04	9.1	02	5.7	04	50	02	2.7
Ticracillin	12	18.8	08	18.2	06	17.1	04	50	06	8.2
Cefsulodin	02	3.1	02	4.5	02	5.7	01	12.5	67	91.8
Erythromycin	09	14.1	04	9.1	03	8.6	05	62.5	04	5.5
Fosfomycin	01	1.6	01	2.3	02	5.7	00	00	02	2.7
Cefoperazone	02	3.1	02	4.5	01	2.9	02	25	09	12.3
Sulphamides	00	00	01	2.3	00	00	00	00	01	1.4
Vancomycin	64	100	44	100	33	94.3	07	87.5	02	2.7
Colistin	01	01.6	00	00	00	00	00	00	00	00
Piperacillin	14	21.9	07	15.9	09	25.7	08	100	00	00
Imipenem	06	09.4	02	4.5	04	11.4	06	00	08	11
Oxacillin	17	26.6	05	11.4	14	40	03	37.5	09	12.3
Tobramycin	02	03.1	01	2.3	03	8.6	02	25	06	8.2
Ampicillin	64	100	44	100	35	100	08	100	42	57.5
ARI*	**0.2115**		**0.1909**		**0.2210**		**0,2104**		**0.3750**	

* ARI : antibiotic resistance index

results agree with those of Hassani et al. (1999), who have reported 44 and 9 % respectively for Ampicillin and Chloramphenicol resistance among a wastewater originated faecal coliform population.

In regard to the antibiotic resistance variability between the three *Aeromonas* species, the results did not reveal significant differences. The comparison of the susceptibility rates of the three *Aeromonas* species and *E. coli* to the fifteen antibiotics is shown in the table 2. Slight differences can be observed. The obtained ARI values for *A. caviae*, *A. hydrophila*, *A. sobria,* the atypical *Aeromonas* isolates and *E. coli* were 0.2115, 0.1909, 0.2210, 0,2104 and 0.3750 respectively.

Nevertheless, *A. hydrophila* strains appear to be the most sensitive with an ARI of 0.1909. In many reports, it has been suggested that the species *A. sobria* is the most susceptible due to its high sensitivity to Cephalotin (Janda and Motyl, 1985). According to these results, Cephalotin resistance has been proposed as a potential marker for the *A. sobria* species.

Meanwhile, the Wilcoxon's signed rank non parametric test does not reveal significant statistically differences ($P > 0.05$) between the values of ARI obtained for the three species.

Comparison of the resistance rates obtained from the influent and effluent originated strains revealed negligible statistically significant differences between the two sampled populations. The overall ARI values calculated for the fifteen antibiotics tested on the influent and the effluent originating populations were 0.2082 and 0.2071 respectively. Although the results showed the existence of a slight variability for some antibiotics, they did not show any significant differences in the antimicrobial susceptibility behaviour at the inflow and the outflow of the wastewater purification plant.

The analysis related to the three *Aeromonas* species polyresistance patterns is shown in the table 3. In regard to the influent population, 100 % of the isolates were found to be resistant to at least two antibiotics. The combination was Vancomycin and Ampicillin. The percentages of aeromonads possessing only this double resistance were 100 % at both the influent and the effluent, while 36 strains (63,2 %) at the influent and 54 strains (57,4 %) at the effluent were found to possess a maximum triple resistance pattern. This antibiotic resistance profile consisted of Ampicillin, Vancomycin and one of the following drugs: Oxacillin, Erythromycin, Piperacillin, Cefoperazone or Ticracillin.

Quadruple resistance among aeromonads existed in only 21 isolates (36.8 %) from the influent and 20 (21.3%) from the effluent. Simultaneous resistance toward six antibiotics was recorded in only 2 strains from the influent and 5 isolates from the effluent. The combination involved Ampicillin, Vancomycin, Piperacillin, Oxacillin, Ticracillin and Erythromycin. No isolates were found to develop multiple resistances to seven antibiotics.

The Wilcoxon's signed ranks did not reveal significant differences between the percentages of polyresistance at the influent and effluent of the plant.

Our results showed that the antibiotic resistance rates obtained for the process surviving bacteria remain at

Table 3. Percentages of polyresistance among motile *Aeromonas* in relation to the origin

	Influent		Effluent	
	Number	%	Number	%
Number of strains resistant to 2 ATB	57	100	94	100
Number of strains resistant to 3 ATB	36	63.2	54	57.4
Number of strains resistant to 4 ATB	21	36.8	20	21.3
Number of strains resistant to 5 ATB	13	22.8	18	19.1
Number of strains resistant to 6 ATB	02	03.5	05	05.3
Number of strains resistant to 7 ATB	01	01.8	00	00

levels of those existing in raw wastewater. In other cases, the resistance levels have increased between up-stream and down-stream sampling points in urban effluent (Goni-Uriza et al., 2000). Nevertheless, the incidence of resistance may increase considerably among the effluent population since it has been suggested that this resistance is essentially transferable plasmid mediated (Adams et al., 1998).

The present study provided valuable information regarding the impact of the municipal wastewater purification (using the activated sludge process under sahelian climate conditions) on the incidence of antimicrobial susceptibility among aeromonads and *E.coli*. The results showed that the antibiotic resistance levels remain significant in the effluent of the plant and could lead to a probable proliferation throughout the bacterial population, seriously compromising the use of reclaimed effluent for irrigational purposes.

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