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Slime producing, heavy metals and antibiotics resistance in *Aeromonas hydrophila* isolated in Tunisia

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Aeromonas hydrophila **strains isolated from different naturally polluted environments (ten from wastewater, six from bay used for aquaculture, eight from sea coast water and six from fish) were subjected to 13 antibiotics, and to four heavy metals (Copper, Cobalt, Zinc and Mercury) by using agar diffusion and agar dilution methods, respectively. In addition, effect of heavy metals on slime production was also investigated. Results of the antibiotic resistance agreed with those of heavy metals resistance, however, treated wastewater and bay strains were much tolerant than seawater and fish bacteria. The range of metal concentrations that was tolerated in the liquid media yielded information on the tolerance levels of *A. hydrophila* to different tested concentrations of metals. Copper and zinc were the best tolerated metals. Mercury was the most toxic component for all bacteria. Almost all *A. hydrophila* produced slime and a small number of strains have changed their morphotype under the heavy metals concentration. Our results have shown that Tunisian aquatic biotopes have a significant proportion of antibiotic and heavy metal resistant to *A. hydrophila.***

Key words: Antibiotic resistance, *Aeromonas hydrophila*, heavy metals and slime producing.

INTRODUCTION

The anthropogenic contamination of the environment with heavy metals is a serious problem. Aquaculture (Burridge et al., 2010) and agricultural practices (Han et al., 2002; Nicholson et al., 2003) contribute to this world wide pollution due to diverse applications of metals in feed additives, organic and inorganic fertilizers, pesticides, and anti-fouling products. Fish farmers frequently use pharmaceuticals (such as antibiotics) and metal containing products to prevent fouling, to feed and to treat fish in order to limit the spread of infections (Burridge et al., 2010).

Therefore, bacterial communities of aquacultures are strongly exposed to the combination of heavy metals and antibiotics. The exposure to both antimicrobial substances may increase the likelihood of selection and co-selection of antibiotic resistance. Moreover, the high nutritional value and the relatively low cost of wastewater, excreta, and sewage sludge convert such heavy metal containing waste to valuable fish feed, especially in developing countries (WHO, 2006).

In Tunisia, the persistence and proliferation of antibiotics and heavy metals resistance in bacterial pathogens, belonging to the *Aeromonas hydrophila*, in aquatic environments represents a considerable public health concern. Subsequent measures to control the emergence and propagation of antibiotic resistance have encountered limited success, and it persists in spite of the restricted use of several key antibiotics, which indicates that there are components governing the evolution, dissemination and perpetuation of these resistance systems that are yet to be understood.

Resistance to antibiotics can be conferred by chromo-

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somal or mobile genetic elements (for example, plasmids) and achieved using four main strategies: (i) reduction of membrane permeability to antibiotics; (ii) drug inactivation; (iii) rapid efflux of the antibiotic; and (iv) mutation of cellular target (s) (Krulwich et al., 2005). In addition, antibiotic sequestration has also been suggested as a potential resistance strategy (Pankaj et al., 2009). Overall, the structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance (Baker-Austin et al., 2006).

Although, bacterial exposure to metals predates human history, anthropogenic-derived sources of metals represent a major source of contamination in the environment. Importantly, a substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance (Alonso et al., 2001; Summers, 2002). This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics (Stepanauskas et al., 2005). Unlike antibiotics, metals are not subject to degradation and can subsequently represent a long-term selection pressure (Stepanauskas et al., 2005). Thus, there are concerns regarding the potential of metal contamination to maintain a pool of antibiotic-resistance genes in both natural and clinical settings. In addition to metals, other toxicants are implicated in the co-selection of antibiotic resistance, including quaternary ammonium compounds and antifouling agents and detergents (Sidhu et al., 2001; Chapman, 2003).

Several explanations have been proposed for the enhanced resistance of biofilm-associated cells to both metals and antibiotics (Baker-Austin et al., 2006). Both metal and antibiotic sequestration in the biofilm matrix and the presence of a small population of 'persister' cells might be contributing factors in the time-dependent tolerance of both planktonic cells and biofilms to high concentrations of antimicrobial agents (Harrison et al., 2005).

In Tunisia, on the east coast of the country bordering the Mediterranean Sea is a key location for the study of antibiotic resistant bacteria and heavy metals contamination in the aquatic environment. The bay is of great economic importance for fishing and aquaculture of numerous species of crustaceans and fish (Snoussi et al., 2006). In addition, domestic wastes, including industrial wastes are discharged into the bay and the sea.

To our knowledge, our present study is the first to determine the prevalence and resistance to antimicrobial agents and heavy metals of *A. hydrophila* isolated from wastewater, bay, seawater and fish. However, in this work, we focus on the current body of knowledge regar-ding (i) to characterize the *A. hydrophila* strains recovered from Tunisian aquatic biotopes; (ii) to determine the level of antibiotic resistance rates against widely used antibiotics in Tunisia; (iii) to determine the heavy metals resistance of the bacteria; (iv) to investigate the relationship between the antibiotics and heavy metals resistance and (v) to determine the heavy metals effect on *A. hydrophila* slime producing.

MATERIALS AND METHODS

Aeromonas hydrophila strains

This study includes 31 *A. hydrophila* strains: ten strains were isolated from treated wastewater (ONAS), six strains were isolated from the bay of Khenis (Aquaculture center, Monastir), eight strains were isolated from seacoast of Monastir, six strains isolated from ornamental fish and a reference strain *A. hydrophila* ATCC 7966^T [American Type Collection Culture (Manassas, Va.)] (Saidi et al., 2011).

All these strains were identified and characterized by Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and achieved by the conventional methods described by Balows et al. (1991). Gram staining method, cell morphology, the oxidase, catalase, motility (Mannitol-Motility agar, Pronadisa, Madrid, Spain), susceptibility to the vibriostatic compound O/129 (10 and 150 µg/disc) and ampicillin antibiotic (10 µg), growth at 30 and 37°C and growth on Rimler Shotts Agar (mRS) were the first tests employed to identify the organisms belonging to *Aeromonas* genus. Commercial miniaturized strips 20 NE Api (Non *Enterobacteriacae*, bioMerieux, France) were also used.

The production of lipase (Tween 80), haemolysin (Sheep blood agar, Pronadisa, Madrid, Spain) and DNA hydrolysis (DNAse Agar, Sharlau Microbiology, Barcelona, Spain) were tested as described previously by Snoussi et al. (2006). The enzymes amylase and lecithinase were detected on media prepared with phosphate buffer saline (PBS) supplemented, respectively with 0.5% starch and 5% egg yolk emulsion. The caseinase activity was tested according the protocol described by Zanetti et al. (2000). *A. hydrophila* strains were cultured on Nutrient Agar containing 5% skim milk. After incubation for up to 72 h at 37°C, the formation of a clear zone caused by casein degradation was considered as a positive test.

Susceptibility testing

Antibiotic susceptibility was performed according to the national Committee for Clinical Laboratiry Standards (CLSI, 2007) method on Mueller–Hinton Agar (Difco) by the disk-diffusion method (Bauer et al., 1966). Resistance to the following antibiotics (BBL, Md, USA) of *A. hydrophila* strains (10^6 CFU/ml) was tested with disks containing nalidixic acid (NA, 30 µg), tetracycline (TE, 30 µg), gentamicin (GM, 10 µg), imipenem (IPM, 10 µg), neomycin (N, 30 µg), ticarciline (TIC, 75 µg), colistine (CL50, 50 µg), cefoxitine (FOX, 30 µg), cefalotine (CF, 30 µg) and flumequine (UB, 30 µg), oxolinic acid (OA, 10 µg), oxytetracycline (OTC, 30 UI), sulfamide/ trimethoprime (SULF/TMP, 200 µg/5 µg). The strain *A. hydrophila* ATCC 7966^T was used as control.

Multiple antibiotic resistances among *Aeromonas hydrophila*

The multiple antibiotic resistance (MAR) index when applied to a single isolate is defined as ***a/b***, where '***a***' represents the number of antibiotics to which the isolate was resistant and '***b***' represents the number of antibiotics to which the isolate was exposed.

For example if the isolate was exposed to twelve antibiotics and was tolerant to six antibiotics, the index for the isolate would be 6/12 or 0.50 (Liberto et al., 2007). MAR index value higher than 0.2 is considered to have originated from high-risk sources of contamination like human, commercial poultry farms, swine and dairy cattle where antibiotics are very often used. MAR index value of less than or equal to 0.2 is considered the origination of strain from animals in which antibiotics are seldom or never used.

Survival of *Aeromonas hydrophila* under heavy metals concentration

The heavy metals (E-Merck) were used to understand its impact on the growth and survival of *A. hydrophila.* The salts used for the study were Copper sulphate ($CuSO_4.5H_2O$), Cobaltous chloride ($CoCl_2.6H_2O$), Mercuric chloride ($HgCl_2$) and Zinc chloride ($ZnCl_2$).

The tendencies of growth were tested on Trypticase Soy Agar (TSA) medium mixed with different concentrations of heavy metals traces for all *A. hydrophila* strains and the plates were incubated for 24 h at 37°C. The average number on bacteria for every concentration of metal was calculated.

The survival test was also examined by filtering metals using Whatmann filter paper (0.2 µm), and stored at 4°C. From the stock solution, various concentrations like 100, 200, 300, 400, and 500 ppm (Copper, Cobalt and Zinc) and 1, 2, 3, 4 and 5 ppm (Mercury) of metal solutions were prepared and used for the study. Growing *A. hydrophila* strains in sterile nutrient broth at 37°C for 16 h was realized. After, the broth was centrifuged at 12000 rpm for 30 min. The cells were washed with sterile saline solution and transferred into 100 ml phosphate buffer solution and the initial optic density (OD) was taken (Thangavel, 2004). The flasks were kept at 37°C for 24 h and the OD was measured (copper, λ = 480 nm; zinc, λ = 213 nm; cobalt, λ = 425 nm and mercury, λ = 254 nm).

Minimal inhibitory concentration (MIC) of heavy metals

The MIC for each bacterial isolate for heavy metal was determined using Mueller-Hinton agar (Difco) containing heavy metals (Cu^{2+}, Zin^{2+}, and Co^{2+}) at concentrations ranging from 100 to 500 ppm and (Hg^{2+}) at concentrations ranging from 1 to 5 ppm. The isolates were considered tolerant if the MIC values exceeded that of the *Escherichia coli* K-12 strain which was used as the control (Akinbowale et al., 2007).

Slime production on Congo red agar (CRA)

The classic method most often used to phenotypically detect slime production in these bacteria is the Congo red agar (CRA) plate test as described by Freeman et al. (1989). The CRA plate test is performed on a solid medium, the Congo red agar. The direct analysis of the colonies formed on the solid medium allows the recognition of slime-producing strains (characterised by black colonies on the red agar) and of non-slime-producing strains (pink/red coloured colonies).

Congo red agar plate test was prepared by adding 0.8 g/L Congo red (Bio Basic INC) and 36 g of Saccharose (Merck), both of which had been previously autoclaved separately, to 1 L of Brain Heart Infusion Agar (Scharlau Microbiology, Pronadisa, Madrid, Spain). Plates were incubated for 24 h at 37°C and subsequently overnight at room temperature (Freeman et al., 1989). Slime-producing *A. hydrophila* grew as black colonies, while non slime-producing strains grew as red colonies. The original test was optimized by using a colorimetric scale with six tonalities: very black, black and approximately black were considered as positive results, while burgundy, red and very red were considered as negative results (Subashkumar et al., 2006). *Staphylococcus aureus* ATCC 25923 was used as positive control for slime production and *Staphylococcus epidermidis* CIP 106510 was used as negative control (Chaieb et al., 2007).

Aeromonas hydrophila morphology visualization by atomic force microscopy

To visualize the bacteria after heavy metal exposure on glass slides and to have an idea on the morphological changes in the cells during heavy metal stress, *A. hydrophila* ATCC 7966^T cells was used as a negative control. For the experiments, the cells enriched on PBS with different concentrations of mercury (1, 2, 3, 4 and 5 ppm) were collected, washed three times by PBS, centrifuged and the pellet was resuspended in PBS, fixed on a sterilized round microscope cover slide and the piece was examined by Atomic force Microscope (AFM, Nanoscope IIIA, Digital Instrument; Veeco) according to the method previously described (Braga and Ricci, 1998).

Statistical analysis

All results are shown as the average of at least three independent experiments; variation is expressed as standard deviation. The Pearson correlation coefficient was calculated to determine the possible relation between the resistance to heavy metals and the resistance to antibiotics. All statistics were performed using SPSS for Windows version 17.0.

RESULTS

Antibacterial resistance

The identified strains were multi-resistant to various antibiotics used including those exploited in the treatment of *Aeromonas* disease of the fish (flumequine, oxolinic acid, sulfamide + trimetoprime and oxytetracycline). Indeed, all bacteria were sensitive to gentamicin.

The results showed that bay, treated waste water *A. hydrophila* isolates were more resistant to almost tested antibiotics than sea water and fish *A. hydrophila* (nalidixic acid (70 and 60%), ticarcyline (60 and 50%), cefoxitine and cefalotine (100 and 90%)), respectively. While the isolates of seawater were most sensitive to the majority of antibiotics, all strains were sensitive for neomycin, tetracycline, fumequine, oxilinic acid, oxytetracycline and sulfamide-trimethoprime.

On the other hand, *A. hydrophila* strains isolated from moribund fish were fairly tolerant to certain antibiotics (colistine (50%), nalidixic acid and cefalotine (66.66%)) and completely sensitive to the oxilinic acid and sulfamide + trimethoprime (Figure 1). The study of the MAR index of these 31 isolates showed that these bacteria presented a high risk, indeed, the recorded values were higher than 0.2, what corresponded to 74.19% of the studied stocks (Table 1).

Aeromonas hydrophila resistance to heavy metals effects

In the present study, resistance to copper (Cu^{2+}), cobalt (Co^{2+}), zinc (Zn^{2+}) and mercury (Hg^{2+}) were studied for all the isolates. In the four sample types (treated waste water, bay, sea water and fish), resistance to heavy metals was described in the Table 2. Actually, all *A. hydrophila* are tolerant to various heavy metals tested and they presented a tolerance reaching 300 ppm (copper, zinc and cobalt) and 3 ppm (mercury). For a concentration reaching 400 ppm, all the strains were tolerant to copper,

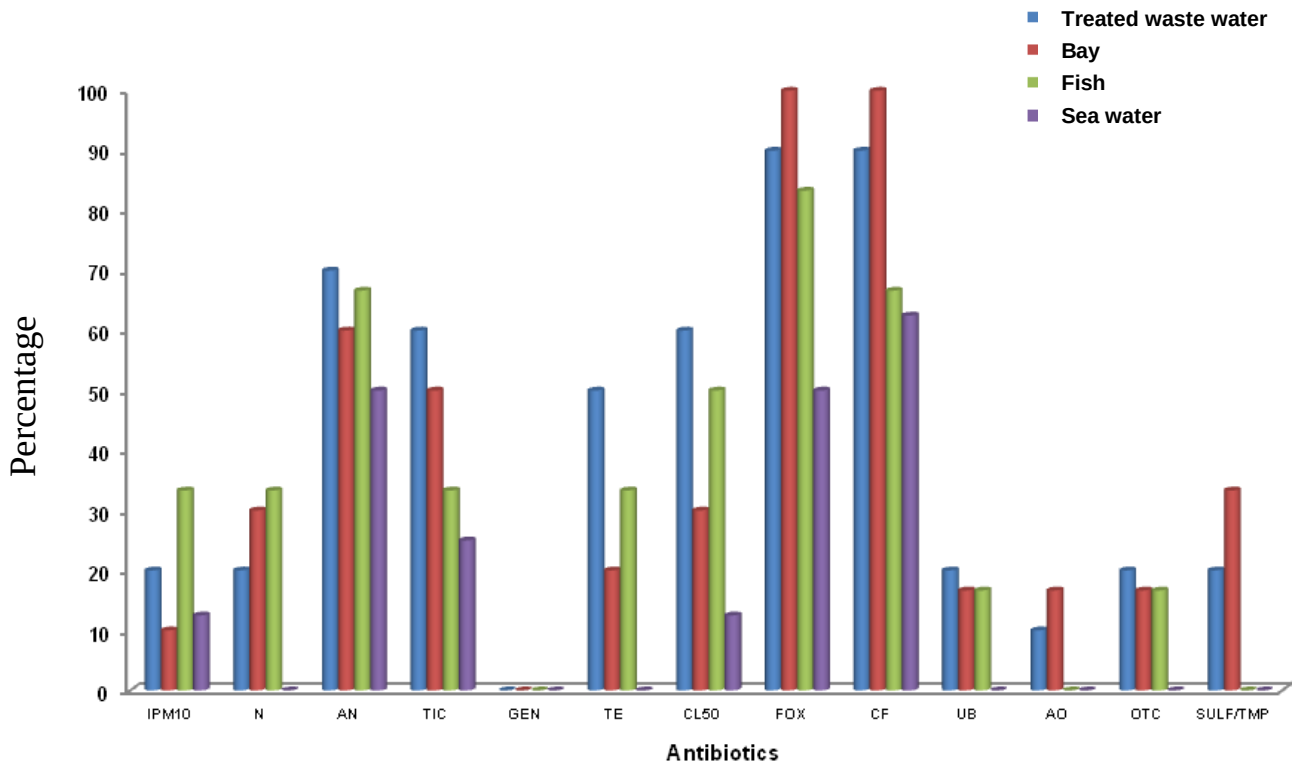


Figure 1. Percentage of resistance to 13 antibiotics of the 31 *A. hydrophila* strains isolated from treated wastewater, sea water, bay and fish. Antibiotics tested are as follow: **IMP**: Imipenem (10 µg), **N**: Neomycin (30 µg), **NA**: Nalidixic acid (30 µg), **TIC**: Ticarcilline (75 µg), **GEN**: Gentamicin (10 UI), **TE**: Tetracycline (30 µg), **CL50**: Colistine (50 µg), **FOX**: Cefoxitine (30 µg), **CF**: Cefalotine (30 µg), **UB**: Flumequine (30 µg), **OA**: Oxolinic acid (10 µg), **OTC**: Oxytetracycline (30 UI), **SULF/TMP**: Sulfamide/Trimethoprime (200 µg/5 µg).

zinc and cobalt, but, only 10% of the isolates from treated wastewater and bay were tolerant to mercury (4 ppm).

The higher tolerance of *A. hydrophila* to 500 and 5 ppm concentrations of various metals traces had proven to be significant for the isolates of bay (100, 83.33, 66.66 and 10%) and of treated wastewater (60, 70, 50 and 10%), respectively for copper, zinc, cobalt and mercury. Whereas the small percentages of resistance were detected in the isolates of sea water (25, 37.5, 0 and 0%) and of the fish (25, 10, 0 and 0%), respectively for same metals quoted previously.

The Table 3 described the viability of *A. hydrophila* continuation of the different concentration effect from selected heavy metals. Indeed, for a concentration of 500 ppm copper, the number of the viable bacteria arrived at 5.01×10^4 CFU/ml (treated wastewater), 7.22×10^4 CFU/ml (bay), 5.16×10^2 CFU/ml (sea water) and 5×10^2 CFU/ml (fish), which was equivalent to 0.492, 0.43, 0.482 and 0.49 of OD, respectively. However, the less significant results were recorded for zinc and cobalt. On the other hand, at 5 ppm of mercury concentration, only the isolates of treated wastewater and bay presented viability up to 1.24 and 1.5 CFU/ml, corresponding to 0.043 and 0.023 OD.

Atomic force Micrography of the bacteria morphology (Figures 2a-b) showed that *A. hydrophila* after the mercury exposure (5 ppm), have changed form and become coccoid. These morphological modifications allow the adaptation to mercury stress.

Slime production under heavy metals

The objective to determine the effect of the tested metal on the slime production in isolated *A. hydrophila*, we found that after exposure to mercury, 3/10 of the treated wastewater isolates, 2/8 of sea water strains and only one strain from fish changed their phenotypical profile and became non slime producer and thereafter produced new morphotype (brown, pink and orange colonies). On the other hand, *A. hydrophila* of bay and those isolated from moribund fish did not modify their morphotype (Figure 3).

The Table 4 showed the resistance of all isolates to the effects of copper, zinc and cobalt and no morphotypic modification was registered in bay case. Similar results were found in the case of the treated wastewater isolates except for case of cobalt; indeed, only one strain changed profile and became non slime producer.

Whereas, *A. hydrophila* isolated from fish and sea water presented the most significant modifications, indeed, for copper, zinc and cobalt the percentages of morphotype

Table 1. MAR Index and Model of resistance of *the A. hydrophila*.

Strain	MAR index	Model of antibiotics resistance
Treated wastewater		
WT1	0.33	AN-TIC-FOX- SULF/TMP
WT2	0.41	IMP-AN-TIC-FOX -SULF/TMP
WT3	0.16	AN-FOX
WT4	0.16	TE -UB
WT5	0.33	TE-CL50-FOX- AO
WT6	0.33	AN-TIC-CL50-FOX
WT7	0.5	IMP-AN-TIC-CL50-FOX -OTC
WT8	0.5	AN-TIC-TE-CL50-FOX -OTC
WT9	0.58	N-AN-TIC-TE-CL50-FOX-UB
WT10	0.33	N-TIC-CL50-FOX
Bay		
R1	0.33	IMP-TIC-TE-FOX
R2	0.25	AN-TIC-FOX
R3	0.41	AN-CL50-FOX -AO-SULF/TMP
R4	0.33	AN-CL50-FOX -UB
R5	0.25	N-AN-FOX
R6	0.41	TIC-CL50-FOX- OTC-SULF/TMP
Fish		
E2	0.25	AN-FOX -UB
E3	0.5	AN-FOX -UB-AO-OTC-SULF/TMP
E4	0.33	N-TIC-FOX-OTC
E5	0.5	IMP-AN-TIC-TE-CL50-FOX
E6	0.33	IMP-N-AN-CL50
E7	0.66	IMP-N-AN-TIC-GEN-TE-CL50-FOX
Sea water		
S1	0.08	CL50
S2	0.5	AN-FOX -UB-AO-OTC-SULF/TMP
S3	0.66	IMP-N-AN-TIC-GEN-TE-CL50-FOX-CF
S4	0.08	FOX
S5	-	-
S6	0.08	AN
S7	0.16	AN-TIC
S8	0.16	AN-FOX

IMP: Imipenem. **N**: Neomycin. **AN**: Nalidixic acid. **TIC**: Ticarcilline. **GEN**: Gentamicin. **TE**: Tetracycline. **CL50**: Colistine. **FOX**: Cefoxitine. **UB**: Flumequine. **AO**: Oxolinic acid. **OTC**: Oxytetracycline. **SULF/TMP**: Sulfamide/Trimethoprime.

modification of the sea water isolates were 1/8, 2/8 and 2/8, respectively. On the other hand, these values were 0, 1/6 and 2/6 for *A. hydrophila* isolated from fish.

DISCUSSION

The *Aeromonas hydrophila* resistance to antibiotics

The study of antibiotic resistance in water organisms is important, as it might indicate the extent of alteration of water ecosystems by human action. Actually, water bacteria could be indigenous to aquatic environments, or exogenous, transiently and occasionally present in the water as a result of shedding from animal, vegetal, or soil surfaces.

According to our results, bay and treated waste water *A. hydrophila* isolates were more tolerant to almost tested antibiotics than sea water and fish *A. hydrophila*. Martinez (2003) has found similar results and he has shown that more than 90% of bacterial strains originated from sea-water are tolerant to more than one antibiotic, and 20% are tolerant at least to five. The resistance of the strains to

Table 2. Tolerance of *A. hydrophila* isolated from treated wastewater. fish. bay and sea water to heavy metals.

Metal/environment	N	Heavy metal concentrations (ppm)				
		1*/100	2*/200	3*/300	4*/400	5*/500
Copper						
Treated wastewater	10	100	100	100	100	60
Sea water	08	100	100	100	100	25
Bay	06	100	100	100	100	100
Fish	06	100	100	100	100	25
Zinc						
Treated wastewater	10	100	100	100	100	70
Sea water	08	100	100	100	100	37.5
Bay	06	100	100	100	100	83.33
Fish	06	100	100	100	100	10
Cobalt						
Treated wastewater	10	100	100	100	100	50
Sea water	08	100	100	100	100	0
Bay	06	100	100	100	100	66.66
Fish	06	100	100	100	100	0
Mercury*						
Treated wastewater	10	100	100	100	10	10
Sea water	08	100	100	100	0	0
Bay	06	100	100	100	10	10
Fish	06	100	100	100	0	0

Reference of test: Minimal Inhibiting Concentration of the standard strain *Escherichia coli* K12.

antibiotics could be explained by the possibility of the heavy use of these compounds in aquaculture (bay), several of which are non biodegradable increases antibiotic selective pressure in water, facilitates the transfer of antibiotic resistance determinants between aquatic bacteria, including fish and human pathogens, and allows the presence of residual antibiotics in commercialized fish and shellfish products (Rhodes et al., 2000; Cabello, 2006). Antibiotic residues entering this aquatic environment from different sources may increase the distribution of potential drug-resistant pathogen bacteria (Matyara et al., 2008). However, some studies indicate that increasing heavy metal concentrations lead to a decrease of antibiotic resistance (Stepanauskas et al., 2005; McArthur and Tuckfield, 2008; Hölzel et al., 2012). The secontradicting results were investigated by Hölzel et al. (2012). In consequence of the addition of mercury chloride ($HgCl_2$) to the antimicrobial test procedure, the MIC for a wide range of antibiotics decreased. This observation could be due to an interaction of Hg with enzymes or nucleic acids which cause antibiotic resistance. $HgCl_2$ could also have acotoxic effect with antibiotics that interfere with ribosomes because the generation of the Hg-degraded enzyme would be inhibited. Furthermore, Hölzel et al. (2012) mentioned also a possible metal induced shift within the bacterial community to ward Hg tolerant bacteria where by the benefit of antibiotic resistance in the presence of antibiotics would be out competed. The increased antibiotic susceptibility in consequence of Hg exposure could also play a role in the observations of other field studies (Seiler and Berendonk, 2012).

Multiple antibiotic resistance (MAR) index of *Aeromonas hydrophila*

Like Gram negative bacilli, the emergence of resistance among Aeromonads will be accelerated by the extensive clinical use of antibiotics (Ko and Chung, 1995; Chaudhury et al., 1996). Such high level of multiple drug resistance may arise from selective pressure due to the indiscriminate use of antibiotics. The variation in the drug resistance may be related to the source of *A. hydrophila* isolated and the frequency prescribed for treating *Aeromonas* infections in geographical areas (Radu et al., 1997).

These reports revealed that geographical, socio economical parameters and local selective pressures could influence antibiotic resistance among *Aeromonas* spp. Growing incidence of MAR among *A. hydrophila* strains isolated from various sources has been reported from many parts of the world (Radu et al., 2003). In our study,

Table 3. The viability of *A. hydrophila* isolated from the treated wastewater; sick and healthy fish; bay and sea water of ornamental fish after the heavy metals effect.

Metal element	Concentration (ppm)	CFU/ml ±SD				OD* (A)			
		Treated waste water	Sea water	Bay	Fish	Treated wastewater	Sea water	Bay	Fish
Copper	100	$35.00 \pm 1.37 \times 10^4$	$29.76 \pm 0.36 \times 10^2$	$65.64 \pm 2.75 \times 10^4$	$26 \pm 1.15 \times 10^2$	0.211	0.231	0.153	0.18
	200	$25.38 \pm 0.69 \times 10^4$	$22.3 \pm 1.00 \times 10^2$	$52.39 \pm 0.91 \times 10^4$	$17 \pm 1.15 \times 10^2$	0.261	0.295	0.21	0.311
	300	$19.45 \pm 0.43 \times 10^4$	$15.65 \pm 0.70 \times 10^2$	$35.22 \pm 0.60 \times 10^4$	$11.5 \pm 1.73 \times 10^2$	0.334	0.315	0.277	0.323
	400	$12.36 \pm 0.91 \times 10^4$	$10.41 \pm 0.34 \times 10^2$	$22.27 \pm 0.94 \times 10^4$	$6.5 \pm 0.57 \times 10^2$	0.414	0.398	0.36	0.4
	500	$5.01 \pm 0.50 \times 10^4$	$5.16 \pm 0.50 \times 10^2$	$7.22 \pm 0.36 \times 10^4$	$5 \pm 1.15 \times 10^2$	0.492	0.482	0.43	0.49
Cobalt	100	$26.68 \pm 0.97 \times 10^4$	$24.86 \pm 0.51 \times 10^2$	$56.72 \pm 0.25 \times 10^4$	$20.5 \pm 0.57 \times 10^2$	0.221	0.201	0.113	0.212
	200	$16.48 \pm 0.43 \times 10^4$	$20.8 \pm 0.64 \times 10^2$	$42.3 \pm 0.62 \times 10^4$	$14.5 \pm 1.73 \times 10^2$	0.225	0.261	0.221	0.267
	300	$15.58 \pm 0.24 \times 10^4$	$14.31 \pm 0.88 \times 10^2$	$27.4 \pm 0.64 \times 10^4$	$12.5 \pm 1.73 \times 10^2$	0.305	0.324	0.287	0.331
	400	$10.83 \pm 0.68 \times 10^4$	$9.41 \pm 0.46 \times 10^2$	$19.65 \pm 0.32 \times 10^4$	$5 \pm 1.15 \times 10^2$	0.298	0.274	0.3	0.288
	500	$4.63 \pm 0.42 \times 10^4$	0 ± 0.0	$4.76 \pm 0.90 \times 10^4$	0 ± 0.0	0.322	0.394	0.2	0.411
Zinc	100	$30.44 \pm 0.63 \times 10^4$	$19.65 \pm 0.34 \times 10^2$	$36.17 \pm 0.80 \times 10^4$	$15 \pm 1.15 \times 10^2$	0.103	0.2	0.1	0.231
	200	$21.16 \pm 0.98 \times 10^4$	$15.33 \pm 0.68 \times 10^2$	$21.21 \pm 0.92 \times 10^4$	$10.5 \pm 0.57 \times 10^2$	0.141	0.291	0.125	0.322
	300	$16.49 \pm 0.53 \times 10^4$	$13.45 \pm 0.88 \times 10^2$	$17.92 \pm 0.13 \times 10^4$	$8.5 \pm 0.57 \times 10^2$	0.177	0.304	0.215	0.331
	400	$11.06 \pm 0.10 \times 10^4$	$7.63 \pm 0.39 \times 10^2$	$10.81 \pm 0.28 \times 10^4$	$3.5 \pm 0.57 \times 10^2$	0.26	0.414	0.208	0.425
	500	$3.41 \pm 0.46 \times 10^4$	$1.9 \pm 0.15 \times 10^2$	$4.42 \pm 0.21 \times 10^4$	$1 \pm 0.0 \times 10^2$	0.3	0.462	0.3	0.485
Mercury	1	$9.65 \pm 0.48 \times 10^2$	$6.65 \pm 0.48 \times 10^2$	$15.44 \pm 0.67 \times 10^2$	$2.5 \pm 0.57 \times 10^2$	0.143	0.199	0.11	0.211
	2	$7.92 \pm 0.97 \times 10^2$	6.5 ± 0.44	$13.06 \pm 0.48 \times 10^2$	2 ± 0.0	0.11	0.23	0.1	0.235
	3	$1.66 \pm 0.51 \times 10^2$	2 ± 0.0	$9.96 \pm 0.76 \times 10^2$	2 ± 0.0	0.107	0.322	0.055	0.342
	4	$3.5 \pm 0.57 \times 10^2$	0 ± 0.0	$3.62 \pm 0.48 \times 10^2$	0 ± 0.0	0.073	0.431	0.043	0.445
	5	1.24 ± 0.21	0 ± 0.0	1.5 ± 0.57	0 ± 0.0	0.043	0.452	0.023	0.463

*: λ = 480 nm (copper). λ = 213 nm (zinc). λ = 425 nm (cobalt) and λ = 254 nm (mercury).

the MAR index of the 31 isolates ranged between 0.08 and 0.66. Hence, almost all isolates were from the high risk source contamination like faecal-oral contamination. Due to indiscriminate use of antibiotics, the microorganisms might have developed resistance towards several antibiotics. Under these circumstances, it will be worth while to find out prevalence of antibiotic resistance of *A. hydrophila* strains that may be considered as an emerging pathogen and to identify the high-risk source. Indeed, multidrug resistant pathogens are the serious problem nowadays faced by the clinicians.

Such a multiple antibiotic resistant strains enter the community, and hybridize with non-MAR strains resulting in the transfer of resistant plasmids and become a serious problem in controlling these strains.

Co-resistance of antibiotic and metal-resistance traits

There is growing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance. Documented associations between the types and levels of metal contamination and specific patterns of antibiotic resistance suggest that several mechanisms

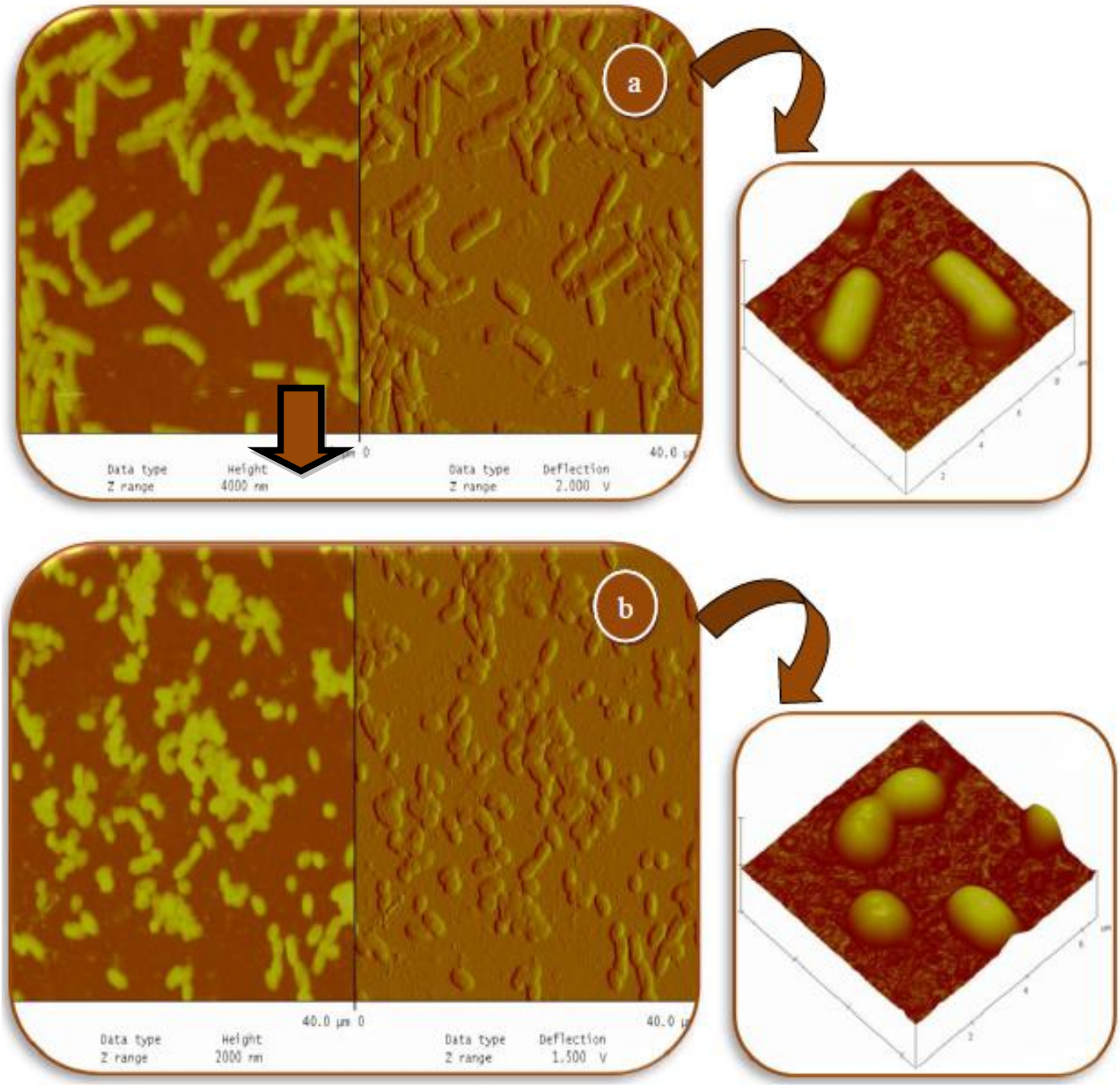
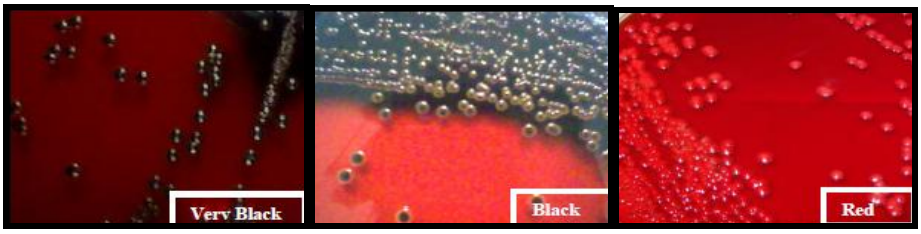


Figure 2. Morphological modification of *A. hydrophila* examined by Atomic force Microscope (AFM): (a) bacillus form to (b) coccoid form after mercury exposure.

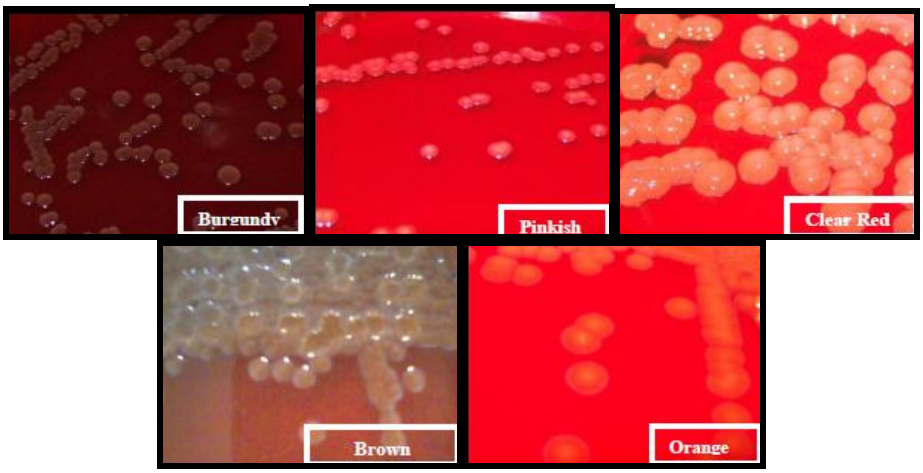
underlie this co-selection process. These co-selection mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals) (Clutterbuck et al., 2007).

Our results revealed that the wastewater and bay isolates were more tolerant to heavy metals (copper, zinc, cobalt and mercury) than sea water and fish strains; similar results of resistances have been shown for antibiotics.

The association of antibiotic-resistance and resistance to heavy metals is very frequent in the same organism (also in the same plasmid, transposon, or integron) so that industrial pollution probably selects for antibiotic-resistance and vice versa (Baker-Austin et al., 2006). The studies of Seiler and Berendonk (2012) have investigated the co-selection in the environment and they showed the presence of correlation between increased heavy metal



(a) Before heavy metal exposure



(b) After heavy metal exposure

Figure 3. Morphotypes of *A. hydrophila* based on the colorimetric scale obtained on Congo red agar before heavy metals exposure **(a):** Very Black colonies (A. *hydrophila* ATCC7966); Black colonies; Red colonies and after heavy metals exposure **(b):** Brown colonies; Burgundy colonies; Orange colonies and Pinkish colonies. *Staphylococcus epidermidis* producing and non-producing slime were used as negative and positive controls.

concentrations with increased phenotypic or genotypic antibiotic resistance. In addition, others researches proved that metal contamination represent a long-standing, widespread, and recalcitrant selection pressure for multi-resistant organisms (Pathak and Gopal, 2005). For the non aquatic organisms, obviously the density of antibiotic - resistance organisms and antibiotic-resistance genes in fresh water varies with the proximity to areas with increased antibiotic consumption, metal pollution, and between seasons, being more frequently found in rainy seasons (Peak et al., 2007).

Evidence for co-selection of antibiotic and metal resistance in the environment originates from diverse habitats contaminated with a variety of metals, which indicates that co-selection is not limited to a subset of metals, environments or microbial taxonomic groups. The strength of evidence presented by these studies ranges considerably between anecdotal reports of co-resistances to experimental studies that unambiguously implicate metals in antibiotic resistance co-selection (Baker-Austin et al., 2006).

Actually, the results of this work has proven that *A. hydrophila* isolated from fish were tolerant to high concentration of heavy metals (copper, zinc and cobalt (100 to 400 ppm) and mercury (3 ppm)). Although these studies do not directly address the hypothesis that metal exposure co-selects for antibiotic resistance, they highlight the fact that metal and antibiotic resistances are commonly found within the same bacteria. Indeed, potential public health concerns for the co-resistance of metal and antibiotic resistances were raised by Pathak and Gopal (2005), who observed that bacterial isolates obtained from fish tissue commonly consumed by humans exhibited resistance to multiple metals and antibiotics.

As evidenced in previous studies, subsequent exposure to elements of heavy metal leads to direct selection for metal-resistance while co-selection for antibiotic resistance. Maintenance of the co-selection antibiotic resistance was accomplished by co-resistance, cross resistance, and co-regulation of the resistant genes (Miranda and Castillo, 1998; Spain and Alm, 2003; Stepanauskas et al., 2005; Wright et al., 2006).

Nonetheless, bacterial resistance to heavy metal was

Table 4. The effect of heavy metals on the slime production of *A. hydrophila* isolated from treated wastewater, sea water, bay and fish.

Origin/metal	N	Slime production (%)	
		Before	After
Copper			
Treated wastewater	10	07/10	07/10
Bay	06	03/06	03/06
Sea water	08	04/06	03/06
Fish	06	06/06	06/06
Zinc			
Treated wastewater	10	07/10	07/10
Bay	06	03/06	03/06
Sea water	08	04/08	02/08
Fish	06	06/06	05/06
Cobalt			
Treated wastewater	10	07/10	06/10
Bay	06	03/06	03/06
Sea water	08	04/08	02/08
Fish	06	06/06	04/06
Mercury			
Treated wastewater	10	07/10	04/10
Bay	06	03/06	03/06
Sea water	08	04/08	02/08
Fish	06	06/06	05/06

emphasized in the present study because substantial number of reports have been alerting on maintenance and proliferation of antibiotic resistance. Resistance genes to both substances were presumably residing closely on the bacterial plasmid and transported together in the environment (Sabry et al., 1997; Spain and Alm, 2003; Wright et al., 2006).

Interaction between antibiotics, heavy metals resistance and slime production in *A. hydrophila*

Many bacteria in the environment exist in surface-attached communities; in fact, the initial bacterial monolayer adhering to polymeric surfaces is converted to a typical biofilm consisting of bacteria plus an extracellular substance (Heilmann et al., 1996). As compared with planktonic bacteria, biofilm bacteria are more tolerant to several antimicrobial agents or other environmental stresses. It has been postulated that large amounts of biofilm formed by these microorganisms play an important role in the degradation and transformation of pollutants in the increasingly polluted soil and water environment (Meng-Ying et al., 2009).

Moreover, biofilm bacteria are usually embedded in an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, and nucleic acids (Whitfield, 1988; Flemming and Wingender, 2001; Sutherland, 2001; Whitchurch et al., 2002). Furthermore, the production of this nature substance termed “slime” appears to play a relevant role (Cristhensen et al., 1982; An and Friedmann, 1998).

The result of this work has shown that the antibiotic and heavy metals resistant strains were biofilm positive and producing slime on CRA. Besides, *A. hydrophila* isolated from treated wastewater and bay has presented the important viability under heavy metals effect than fish and sea water strains. Therefore, these findings were confirmed by Liberto et al. (2007), these researchers have shown that adhesion, bacterial proliferation and slime production increase antibiotic resistance, since drugs may not be able to reach bacteria kept in rein in biofilm.

Further, this work proved the importance of the function played by slime to protect the water environment from selective events caused by the antibiotic and heavy metal release and reduced antibiotic susceptibility, which are acting more effectively on planktonic bacteria (Baquero et al., 2008) and proved that the degree of penetration is dependent on the biofilm and the antimicrobial agent. Clutterbuck et al. (2007) have demonstrated that EPS also can act as an ion exchange and is able to sequester hydrophobic and positively charged antibiotics such as aminoglycosides. On the other hand, Teitzel and Parsek (2003) have suggested that bacteria have developed a variety of resistance mechanisms to counteract heavy

metal stress. These mechanisms include the formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell (Outten et al., 2000).

A proposed mechanism that contributes to this increased resistance is binding and sequestration of antimicrobial agents by EPS components, such as negatively charged phosphate, sulphate, and carboxylic acid groups (Hunt, 1986). Another factor that may contribute to the resistance of biofilms is that many antimicrobial agents target metabolically active cells (Teitzel and Parsek, 2003). However, slime production and association in biofilm are two parameters of great complexity; they are highly correlated with the environment.

The present study focuses on a part of the northern Mediterranean region, wastewater, bay and seawater samples from a polluted aquatic environment. It was established that there is a possible association between heavy antimicrobial consumption within a population and the frequent recovery of antibiotic resistant bacteria. However, it is apparent that a range of other agents might represent important mechanisms that drive the selection of antibiotic-resistance determinants.

Current advances in microbial genomics, physiology and biochemistry could provide the basis for the precise determination of important processes involved in metal–antibiotic resistance interactions. Areas of particular interest include the multifunctional properties of co-resistance determinants and the relative contributions of these resistance systems to the fitness of bacteria in different environmental and clinical settings. It is necessary to evaluate potential mechanisms at several levels of biological organization to comprehensively assess the role of metal contaminants as a selective force in maintaining and propagating the pool of antibiotic-resistance determinants in the environment.

The geographic scope of this study should include other parts of the Tunisian coasts on the Mediterranean Sea. Furthermore, more studies should be developed - cheap and reliable: first, bacterial clones and resistance genes source tracking; second, detection of antibiotics in water environments; third, identification of the mechanisms involved in the association between antibiotic, metal resistances and slime producing, fourth, disinfection of water from antibiotic-metal-resistant populations and the resistance gene pool, and removal of antibiotics from wastewater.

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