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Program: OPTIMIZATION AND TESTING OF A NEW MOLECULAR APPROACH (GeneCARD-FISH) FOR THE *IN SITU* QUANTITATIVE DETECTION OF BACTERIAL CELLS CARRYING ANTIBIOTIC RESISTANCE GENES

1. INTRODUCTION

The spread of antibiotic resistance genes in the environment is raising a great concern due to the increased frequency of clinically relevant microorganisms unaffected by antibiotic treatment. However, the real abundance of bacteria carrying ARGs in the environment is not yet defined. The currently applied monitoring approaches, based on Q-PCR or cultivation, can only provide an estimate of the abundance of genes (ARGs) or of the cultivable fraction of resistant bacteria. A new approach, the GeneCARD-FISH, recently developed at IRSA-CNR for the *in situ* detection of target genes in bacterial cells, could instead provide a real estimate of the ARGs carrying bacteria level in environmental samples. The main objective of the proposed STM research activity is the evaluation and optimization of a GeneCARD-FISH for ARGs, using on purpose produced positive controls of pathogenic or non-pathogenic bacteria resistant to antibiotics (e.g. sulfonamides and macrolides).

In detail, the main objectives, related to STM activity, were to:

1. Knowledge acquisition on the methodologies for the cultivation of pathogen bacteria, isolation of antibiotic resistant bacteria from environmental samples and characterization of antimicrobial resistance of environmental isolates;
2. Production of bacterial positive controls carrying ARGs which will be used for the evaluation and optimization of GeneCARD-FISH for detection and quantification of bacterial cells carrying antibiotic resistance genes;
3. Characterization of antimicrobial resistance in environmental bacterial isolates by performing antibiotic susceptibility testing (AST)

2. ACTIVITIES AND RESULTS

2.1 Production of bacterial positive controls harbouring ARGs

The main objective of this activity was to produce pathogenic and non-pathogenic antibiotic resistant bacterial strains, in order to evaluate the GeneCARD-FISH assay on a wide group of antibiotic resistant bacteria differing in taxonomic affiliation and possibly in the number ARGs GC/cells. In particular, the-performed activity is reported below:

- cultivation of reference bacterial strains harbouring ARGs available in the Culture Collection at the Beuth University of Applied Science (Berlin, Germany);

- isolation of antibiotic resistance bacteria from environmental water samples:
  - bacterial species suggested as possible indicators of antibiotic resistance in the environment, e.g. *Aeromonas hydrophyla*, *Enterococcus* spp. (Berendonk et al., 2015);
  - or unknown resistant bacteria present in the environment (cultivation by Heterotrophic Plate Count with added antibiotics);

2.1.1 Materials and methods

Reference bacterial strains. Pathogenic and non-pathogenic bacterial strains belonged to *Enterococcus*, *Enterobacter* and *E.coli* species were selected from the Culture Collection available at the Beuth University of Applied Science (Berlin, Germany). These strains were cultivated and processed in a BSL-2 Laboratory (Biosafety Level 2), that is suitable for work with microbiological agents associated with human disease, namely pathogenic or infectious microorganisms posing a moderate hazard (e.g. hepatitis A, B, and C, *Salmonella* spp., MRSA, etc).

As culture media, TSA broth (Himedia Laboratories, Germany), BHI broth (Difco Laboratories, Germany) and LB broth (Himedia Laboratories, Germany) were used for *Enterococcus*, *Enterobacter* and *E.coli* species, respectively. All the media were prepared according to the manufacturers' instructions.

Sample collection. Surface water samples were collected from two different sites in Berlin, the Spree river and Landwehrkanal (Figure 1). Water samples were collected in sterile 50 ml plastic bottles, transported to the laboratory at 4°C, and stored until analysis. Inoculations into selective media were conducted within 24-48 h after collection of water samples.

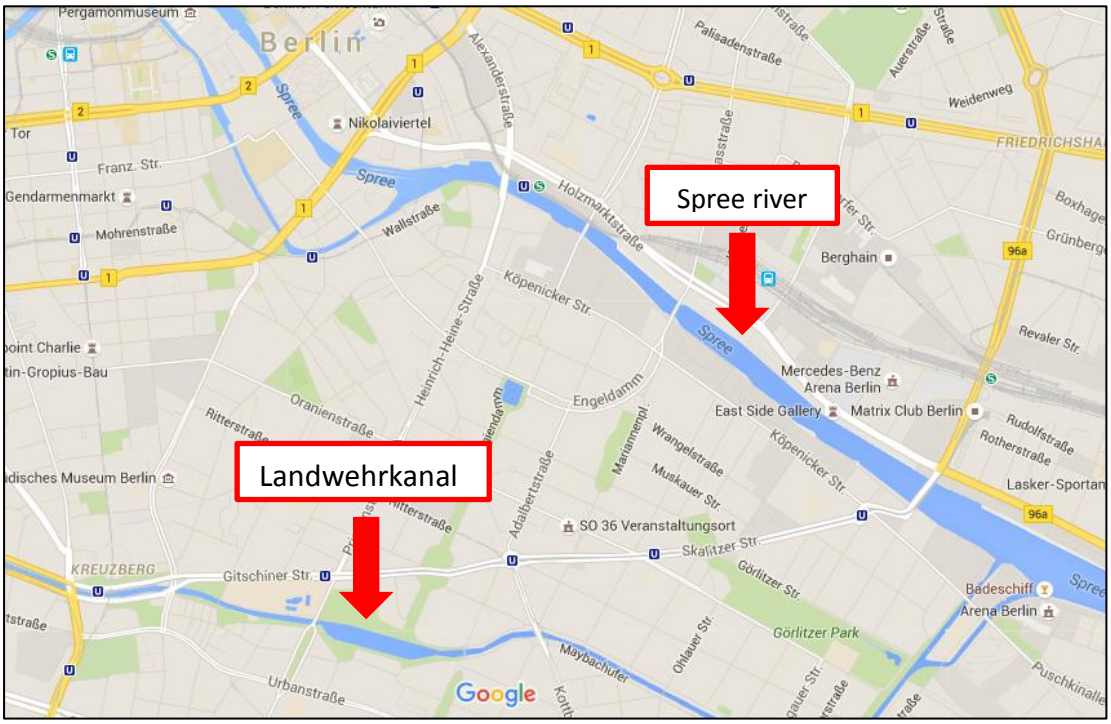

Figure 1. Map of the Spree River and Landwehrkanal (Berlin, Germany), identifying the locations from which samples were collected.

Isolation and morphological characterization of the antibiotic resistant bacteria. For all the samples, undiluted and diluted volumes were analysed in duplicate. Briefly, 500 µl undiluted, 100 µl undiluted and 100 µl of the 2 fold serial dilutions (1:10 and 1:100) were spread onto the plates with the appropriate selective media. SA Agar (Himedia Lab.) (incubated at 30°C for 24-48 hours) with ampicillin supplement (10 mg/L), and Chromocult Enterococcus Agar (Merck, Italy), (incubated at 37°C for 24-48 hours) were used for selective isolation of *Aeromonas hydrophyla* and *Enterococcus* spp., respectively. Typical cultural responses on the respective medium were starch hydrolysis clearing zone for *Aeromonas* and red colonies for *Enterococcus*.

The conventional approach of heterotrophic plate count (HPC) method was used to evaluate the concentration of antibiotic resistant bacteria in the samples. The HPC method was performed by plating samples on R2A agar medium (Difco Laboratories), amended with two different antibiotics: (1) erythromycin, at two different concentrations 2 µg/ml and 10 µg/ml and (2) sulfamethoxazole, at 5 µg/ml and 25 µg/ml. For each antibiotic, the lowest and the highest concentrations were chosen as the minimal inhibitory concentrations (MICs) of the susceptible bacterial population (MIC_{susc}) and that of the resistant bacteria (MIC_{res}), respectively. Plates were incubated for 2 days at 37°C and then for a period of 5 days at 27 °C (Munir et al., 2011). Total heterotrophic cultivable bacterial population was determined by plating samples on media without antibiotics. The colony forming units (CFU) on each plate were counted after incubation and CFU/ml was calculated to get Heterotrophic Plate Count (HPC). After enumeration, single colonies were picked and purified via one passage on R2A plates. After incubation, the colonies showed different characteristics. For observing cultural characteristics, discrete colonies on the agar surfaces were selected to evaluate their shape, size, consistency and pigmentation.

Further processing and analysis. All the positive controls to antibiotic resistance were cultivated in liquid media (TSA, BHI or LB broth depending on the strain) and subjected to other treatments, as described below:

- for GeneCARD-FISH analysis: 10 ml of overnight culture were collected with a sterile pipette, immediately fixed with formaldehyde (5% vol/vol final concentration) and kept for 3 h at 4°C. Then, one volume of ice-cold absolute ethanol was added and the fixed culture was stored at -20°C, until further processing;

- for DNA extraction: 2 ml of overnight culture were collected with a sterile syringe and centrifuged at 10,000 rpm for 2 min. The pellet was stored at -20°C, until DNA extraction;

- for qPCR analysis: 2 ml of overnight culture were collected with a sterile syringe and centrifuged 2 min at 10,000 rpm. The pellet was washed twice in PBS 1X and resuspended in one volume of PBS 1X and ice-cold absolute ethanol. The fixed culture was directly stored at -20°C.

2.1.2 Results

2.1.2.1 Antimicrobial resistance reference bacterial strains

Four reference strains from the Culture Collection at the Beuth University of Berlin were selected and cultivated on the basis of their well-characterized antimicrobial resistance. In detail,

- *Enterococcus faecalis RE25* harbouring *tetM* and *ermB* genes
- *Enterococcus gallinarum SF9117* harbouring *ermB* gene
- *Enterobacter cloacae DSM 46348* harbouring *vanA* gene
- *Escherichia coli SF100* harbouring *ampC* gene

2.1.2.2 Occurrence of *Aeromonas hydrophyla* and *Enteroccoccus* spp. in surface water samples

Surface waters from the Spree river and Landwehrkanal were analysed for the presence of *Aeromonas hydrophyla* and *Enterococcus* spp. as possible indicators of antibiotic resistance in the environment (Berendonk et al., 2015).

Only five colonies, 2 from the Spree river and 3 from the Landwehrkanal, were identified as belonged to presumptive *Aeromonas hydrophyla*. Colonies purified by twice subculturing using the streaking plate method are shown in Figure 2. The selected presumptive *Aeromonas* isolates will be identified by molecular analysis.

No *Enterococcus* spp. colonies were instead observed in both the surface water samples for all the volumes analysed.

Figure 2. *Aeromonas hydrophyla* colonies on SA Agar after incubation at 30°C for 48 hours.

2.1.2.3 Enumeration and isolation of antibiotic resistant bacteria by heterotrophic plate count (HPC)

Enumeration of antibiotic resistant bacteria. Total and antibiotic resistant heterotrophic plate counts (HPC) were determined using a low nutrient medium (R2 agar) without added antibiotics and with adding of sulfamethoxazole (5 and 25 µg/ml) and erythromycin (2 and 10 µg/ml). Some photos of heterotrophic plate count results are shown in Figure 3.

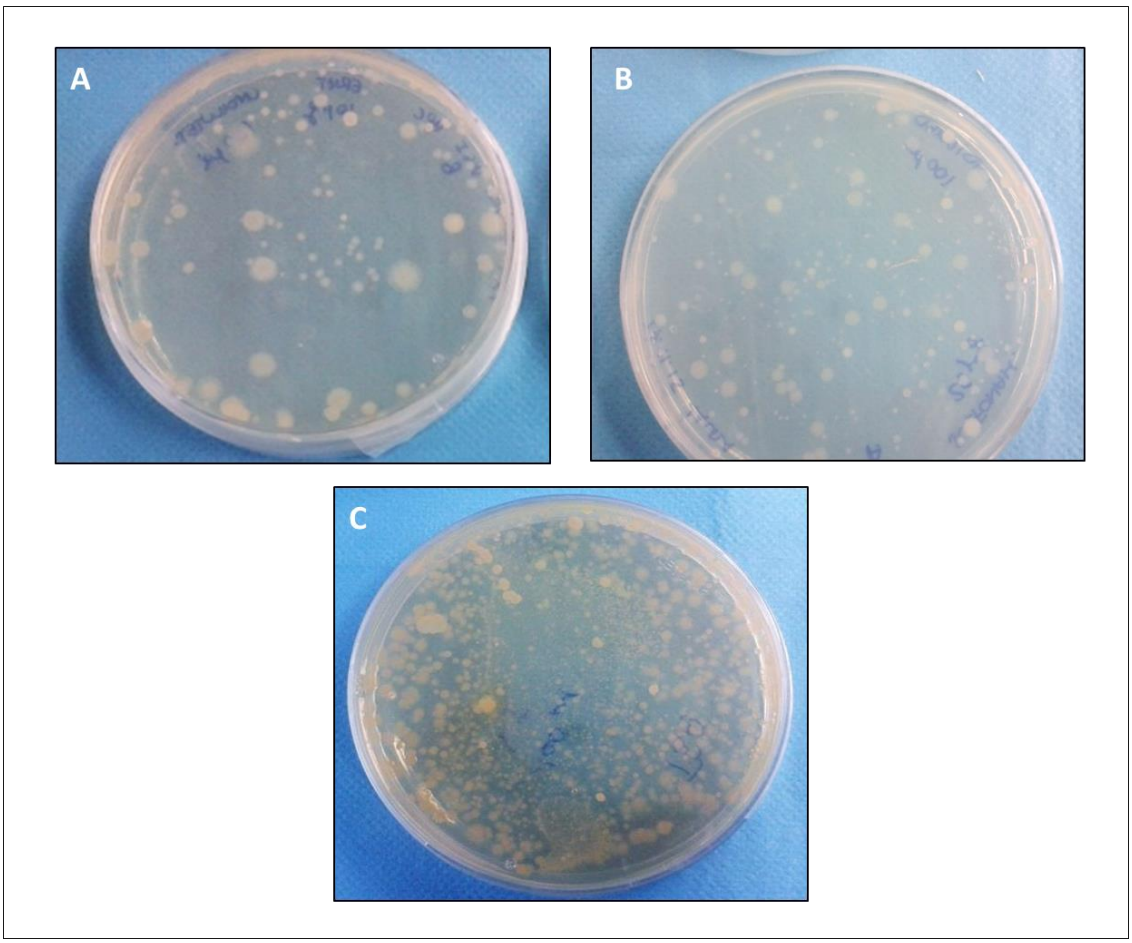

Figure 3. A) Heterotrophic plate count (HPC) bacteria on R2A Agar amended with erythromycin (10 µg/ml); B) heterotrophic plate count (HPC) bacteria on R2A Agar amended with sulfamethoxazole (25 µg/ml); heterotrophic plate count (HPC) bacteria on R2A Agar without adding of antibiotics.

The average counts of HPC bacteria and antibiotic resistant bacteria with their respective proportions of the total bacteria are reported in Figure 4.

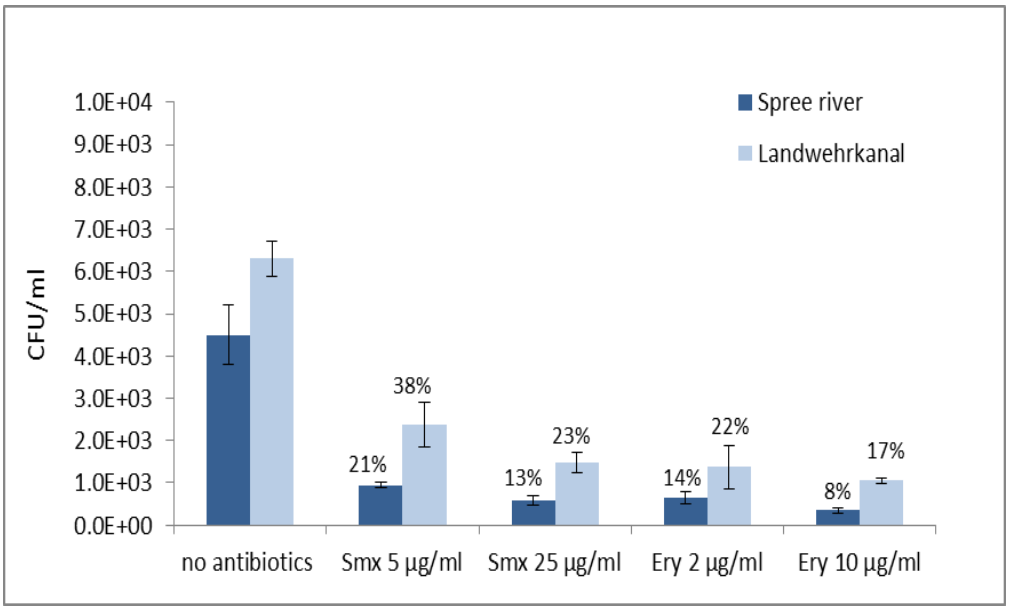

Figure 4. Concentration (CFU/ml) of sulfamethoxazole resistant bacteria, erythromycin resistant bacteria and total heterotrophic plate count in water samples collected from the Spree river and the Landwehrkanal.

Total bacterial count (HPC) ranged from $4.5x10^3 \pm 7.1x10^2$ CFU/ml to $6.3x10^3 \pm 4.2x10^2$ CFU/ml in Spree river and Landwehrkanal, respectively. These values are comparable to the range of HPC populations reported in surface and drinking waters, i.e. <0.02 to 10^4 CFU/ml or higher (Allen et al., 2004). Antibiotic resistant bacteria, with higher levels of resistance to sulfamethoxazole, were always observed in both the water samples. Specifically, Landwehrkanal was more contaminated by antibiotic resistant bacteria compared to Spree river. In detail, 38% ($2.4x10^3 \pm 5.2x10^2$ CFU/ml) of the total bacteria were resistant to sulfamethoxazole (5 µg/ml) and 22% ($1.4x10^3 \pm 5.2x10^2$ CFU/ml) to erythromycin (2 µg/ml). In presence of 5-fold higher antibiotic concentrations, the percentage of resistant bacteria was reduced at 23% ($1.5x10^3 \pm 2.3x10^2$ CFU/ml) and 17% ($1.1x10^3 \pm 7.1x10^1$ CFU/ml), respectively for sulfamethoxazole and erythromycin. In the Spree river, 21% ($9.5x10^2 \pm 7.1x10^1$ CFU/ml) and 14% ($6.4x10^2 \pm 1.4x10^2$ CFU/ml) of the HPC were resistant to sulfamethoxazole and erythromycin (low doses), respectively. At high doses of sulfamethoxazole and erythromycin, this percentage was declined at 13% ($5.8x10^2 \pm 1.1x10^2$ CFU/ml) and 8% ($3.6x10^2 \pm 6.4x10^2$ CFU/ml), respectively.

Characterization of antimicrobial-resistant isolates. After enumeration of antibiotic-resistant bacteria, 10 isolates resistant to sulfamethoxazole (5 bacterial isolates at 5 µg/ml and 5 at 25 µg/ml) and 10 to erythromycin (5 bacterial isolates at 2 µg/ml and 5 at 10 µg/ml) were selected as representatives of the different colony morphology types, and collected from the two surface water samples. All the isolates were subjected to morphological characterization on the basis of colony

form, margin, surface property and pigmentation. The colony characteristics of bacterial isolates are listed in Table 1.

Table 1. Colony characteristics of the isolates from the surface water samples in Berlin.

	Form	**Margin**	**Surface**	**Pigmentation**
Sulfamethoxazole resistance				
Isolate 1 [A]	Circular	Entire	Smooth	White
Isolate 2 [A]	Irregular	Undulate	Concentric	Transparent
Isolate 3 [A]	Circular	Entire	Smooth	Yellow
Isolate 4 [A]	Punctiform	Entire	Smooth	Transparent
Isolate 5 [A]	Irregular	Undulate	Radiate	Transparent
Isolate 6 [B]	Irregular	Filamentous	Radiate	Transparent
Isolate 7 [B]	Circular	Entire	Smooth	White
Isolate 8 [B]	Punctiform	Entire	Smooth	Transparent
Isolate 9 [B]	Irregular	Undulate	Smooth	White
Isolate 10 [B]	Irregular	Undulate	Radiate	Transparent
Erythromycin resistance				
Isolate 1 [C]	Irregular	Undulate	Smooth	Light yellow
Isolate 2 [C]	Irregular	Lobate	Smooth	White
Isolate 3 [C]	Punctiform	Entire	Smooth	Transparent
Isolate 4 [C]	Circular	Entire	Smooth	White
Isolate 5 [C]	Circular	Entire	Smooth	Transparent
Isolate 6 [D]	Irregular	Undulate	Smooth	Light yellow
Isolate 7 [D]	Irregular	Lobate	Smooth	Light yellow
Isolate 8 [D]	Circular	Entire	Smooth	White
Isolate 9 [D]	Punctiform	Entire	Smooth	White
Isolate 10 [D]	Circular	Entire	Smooth	White

[A]: sulfamethoxazole, 5 µg/ml; [B]: sulfamethoxazole, 25 µg/ml; [C]: erythromycin, 2 µg/ml; [D]: erythromycin, 10 µg/ml.

2.2 Antibiotic resistance profiling of different bacterial isolates from surface water samples

The present activity aimed also to evaluate the susceptibility of different (pathogenic and non-pathogenic) environmental bacterial isolates to the main antibiotics currently used in clinical settings. Resistance of the bacterial isolates to specific antibiotics was determined by the disc diffusion method, performed according to CLSI guidelines (CLSI, 2012).

2.2.1 Method

Antibiotic susceptibility test (AST). The antimicrobial susceptibility test was performed using the Kirby-Bauer disk diffusion method, according to Clinical and Laboratory standards Institute guidelines (CLSI, 2012) with the following antibiotic discs (Oxoid, Wesel, Germany): ampicillin (25 µg), erythromycin (10 µg), sulfamethoxazole (SMX; 25 µg), chloramphenicol (30 µg), kanamycin (30 µg), and tetracycline (30 µg). *E. coli* ATCC 25922 was used as a control. Single colonies of 5 presumptive *Aeromonas hydrophyla* isolates and of 5 heterotrophic bacterial isolates were diluted in tryptic soy broth (TSA) to an OD_{630} of 0.16 and streaked with a cotton swab on TSA agar plates. Antibiotic discs were applied on the plates using sterile forceps and the plates were incubated at 30°C for 24 h. After incubation, the antibiotic inhibition zone diameters (IZD) were measured. Results obtained were used to classify isolates as being resistant, intermediate resistant, or susceptible to a particular antibiotic using standard reference value according to CLSI (2012, 2014). Some picture of the disk diffusion test are shown in the Figure 5 below.

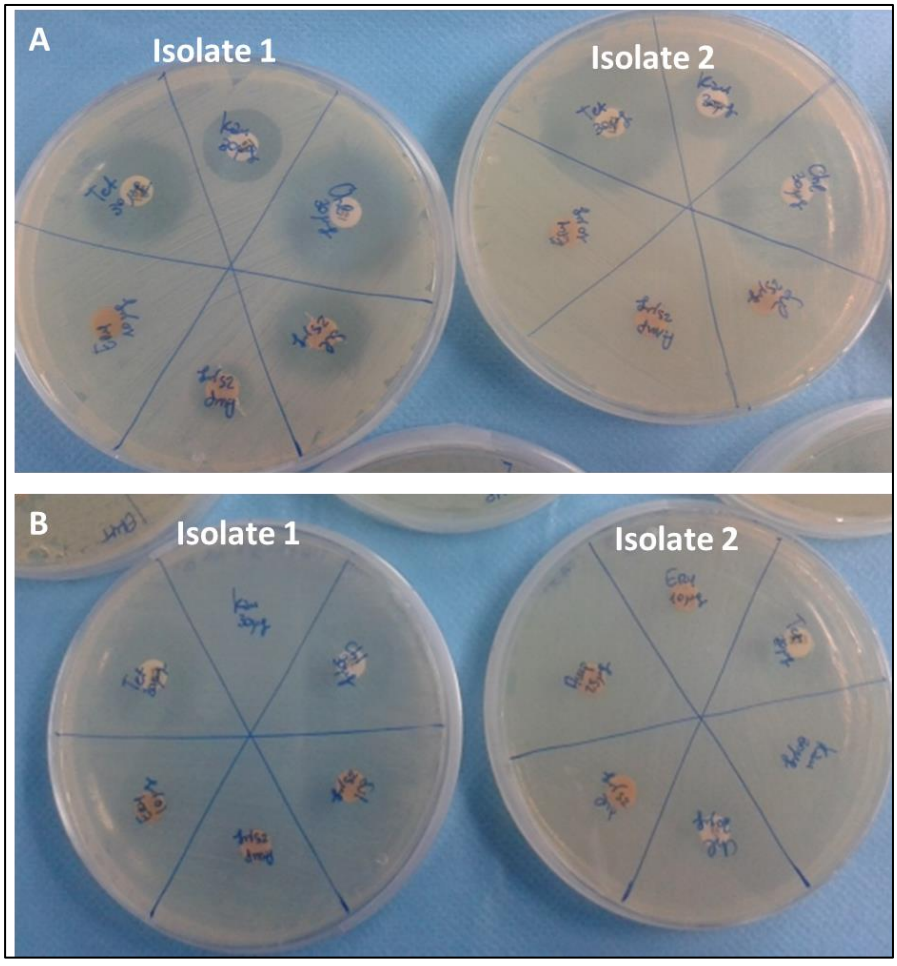

Figure 5. Disk diffusion test. A) *Aeromonas hydrophyla* isolates 1 and 2; B) total heterotrophic isolates 1 and 2.

2.2.2 Results

Presumptive *Aeromonas hydrophyla* and total heterotrophic isolates were tested for susceptibility to 6 antibiotics. The antimicrobial susceptibility profiles of the isolated strains are reported in Table 2.

Table 2. Antimicrobial susceptibility profiling of the bacterial isolates from surface water samples, performed according to disk diffusion method (CLSI, 2012).

	Ery 10	**Amp 25**	**Smx 25**	**Kan 30**	**Chl 30**	**Tet 30**
Heterotrophic bacteria (*n*= 5)						
Isolate 1[A]	R	R	R	-	R	S
Isolate 2[A]	R	R	I	-	S	S
Isolate 3[B]	R	R	R	-	R	S
Isolate 4[B]	R	R	R	-	S	S
Isolate 5[B]	R	R	R	-	R	S
***Aeromonas hydrophyla* (*n*= 5)**						
Isolate 1[A]	R	R	I	I	S	S
Isolate 2[A]	R	R	R	I	S	S
Isolate 3[B]	R	R	S	I	I	S
Isolate 4[B]	R	R	R	S	R	S
Isolate 5[B]	R	R	R	S	R	S

[A]: Spree river; [B]: Landwehrkanal

Resistance to 4 and intermediate resistance to 3 antibiotics were observed, out of 6 antibiotics tested. Overall, results showed that over 70% of the isolates in this study are resistant to more than 3 antibiotics (multiple drug resistances), which could lead to occurrence of newly emerging resistant bacteria potentially transmitted to humans. The prevalence of intrinsic multi-resistance to common antimicrobial agents has been documented (Wright, 2007; D'Costa et al., 2011; Cox and Wright, 2013).

Interestingly, heterotrophic bacteria were all resistant to erythromycin and ampicillin. Resistance to sulfamethoxazole and chloramphenicol was also common with 40% and 30% of isolates showing resistance, respectively. No resistance to tetracycline was shown. A similar antibiotic resistance profiling was observed for *Aeromonas hydrophyla*, excluding ampicillin, for which a natural resistance was described (Saavedra et al., 2004). All the isolates were resistant to erythromycin. Sulfamethoxazole resistance was the next most common, with 30% of resistant isolates, then chloramphenicol (20% being resistant and 10% intermediate), and finally kanamycin (30%

intermediate). Similarly to heterotrophic bacteria, none of *Aeromonas* isolates were susceptible to tetracycline.

Figure 6 shows the frequency of resistance in bacterial isolates from the two surface waters to antimicrobial agents.

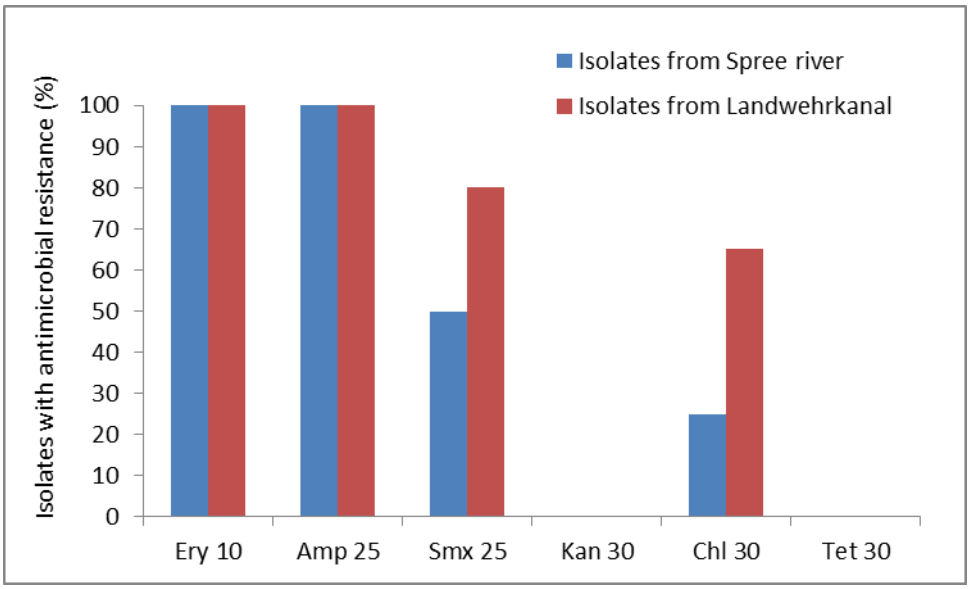

Figure 6. Percentages of antibiotic-resistant isolates from Spree river and Landwehrkanal. Ery, erythromycin (10 µg); Amp, ampicillin (25 µg); Smx, sulfamethoxazole (SMX; 25 µg); Chl, chloramphenicol (30 µg); Kan, kanamycin (30 µg); Tet, tetracycline (30 µg). Resistance to kanamycin was tested only for *Aeromonas* isolates.

In the present study, the percentage of antibiotic-resistant isolates from Spree river and Landwehrkanal ranged from 25% to 100%. All the isolates tested showed resistance to erythromycin and ampicillin. A higher frequency of resistance to sulfamethoxazole (80%) and chloramphenicol (65%) was also found in isolates from the Landwehrkanal compared to the Spree river. Wide distribution of antibiotic-resistant bacteria in surface waters has been reported in previous studies (Kummerer et al., 2009; Garcia-Armisen et al., 2011; Ozaktas et al., 2012).

2.3 NEXT ACTIVITIES

The GeneCARD-FISH assay will be developed and calibrated on positive controls harbouring ARGs and then applied on environmental samples. A real estimate of the ARGs carrying bacteria level may provide information that allows the understanding of ARG contamination level normally estimated as gene copy number by q-PCR methods. Briefly, the next activities are listed as follows:

1- Selection and characterization of the isolates useful as positive controls:

1a) analysis of the antimicrobial resistance by PCR;

1b) taxonomical and morphological characterization by Gram staining and sequencing;

2- Estimate of ARGs gene copy numbers per cell and per 16S rDNA gene copies by q-PCR;

3- Development and application of Gene-CARDFISH assay for in situ detection of bacteria carrying ARGs.

References

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