Spread of antibiotic resistance in aquatic environments: *E.coli* as a case study

University of the Aegean
Department of Marine Sciences

M.A. Efstratiou[1], M. Bountouni[1] and E. Kefalas[2]

[1] Department of Marine Sciences, University of the Aegean, Mytilene 81100, Greece
[2] Laboratory of Water Quality, Regional Authority of North Aegean, Mytilene 81100, Greece

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Introduction :

Antibiotic resistant microorganisms have become, the last few decades, a challenge to infection control. Antibiotics appear with increased frequency to be less effective against microorganisms and, depending on the pathogen, the incurable infections are threatening to become an everyday phenomenon resulting in increased fatality. The current resistance to antimicrobial drugs has been estimated to account for 700 000 deaths a year. In the environment antibiotic resistant bacteria accumulate, arising either by accidental mutation, by mutation in humans or animals under antibiotic treatment selection or are naturally occurring environmental bacteria who, under selective pressures (an evolutionary response resulting from natural selection) have developed resistance for their own protection against competitors [1]. Resistance genes from environmental bacteria can be transferred to clinically important pathogens via horizontal gene transfer, pointing to the fact that environment is an important component of spread of antibiotic resistance to humans.

The amount of antibiotics consumed by humans is phenomenal. In livestock, too, excessive amounts are used for growth promotion and as feed additives, as well as in veterinary medicine. Antibiotic resistant bacteria from both sources are excreted through waste and are dispersed into aquatic environments either directly or by effluents of Waste Water Treatment Plants (WWTP) discharged in surface waters. In many countries deposition of manure or sludge on land as a fertilizer is a widespread practice. So is the use of WWTP effluents for irrigation and urban applications. Underground aquifers can be affected indirectly. Drinking water is a particularly important milieu for dissemination of such bacteria, which could infect the general population producing serious Pubic Health consequences. Several investigations have been conducted on the occurrence of antibiotic resistant and multi resistant microorganisms in drinking water. [3,4,5,6,7]

The widespread enrichment of environmental waters with antibiotic resistant bacteria and genes make it plausible that such bacteria find their way into drinking water sources, survive treatment processes and create public health issues. Should humans contact – or consume – antibiotic resistant microorganisms and submit to infection, the possibility of successful antibiotic treatment is severely reduced.

Materials & Methods :

1.Site description. Fresh water samples were collected from four Greek islands (Fig. 1). The drinking water was untreated and came from small rural communities; the ground water was collected from boreholes and wells from the same islands. It is used mostly for irrigation.

2.Sample collection. Sampling was conducted throughout 2017, according to ISO 19458:2006. The samples were collected in 500mL dark glass, sterile bottles and carried to the Laboratory on ice. Upon arrival their temperature was recorded, and was between 2.0- 8.0°C. They were processed within a maximum of 24 hours from collection.

3.*Escherichia coli* isolation. 235 strains of *E.coli* were isolated by membrane filtration (ISO 9308-1:2014). 158 strains came from the drinking, untreated water, 77 strains were from ground water sources. Membranes were placed on Chromocult Coliform Agar (Merck cat.no. 1.10426.0500). β-D-galactosidase positive colonies (dark-blue to violet color) were transferred and incubated on Tryptic Soy Agar (Merck cat.no. 1.05458.0500). Oxidase negative colonies were transferred in Tryptone Water (Merck cat.no.VM 610494.346) to perform Indole test (ISO 8199:2005). Colonies positive for indole and for β-D-galactosidase tests were considered as *Escherichia coli*.

4.Antibiotic resistance. The resistance of the 235 *E.coli* strains was tested against five antibiotics widely used in medicinal and veterinary practice: three quinolones (Norfloxacin -NXN, Ciprofloxacin -CIP, Levofloxacin –LVX) one cephalosporin (Cefaclor -CEC) and one penicillin (Amoxicillin -AMX) Becton Dickinson. *Escherichia coli* strain CRM09001L was used for quality control of the screening protocol. The bacteria were tested by the Kirby–Bauer disk diffusion susceptibility test. Interpretation of results was conducted based on the EUCAST Testing Guidelines - European Committee on Antimicrobial Susceptibility [2], and strains were classified as 'resistant', 'intermediate' or 'sensitive' (Table 1). Strains exhibiting "intermediate" resistance patterns were classified as "resistant" for the purposes of this study.

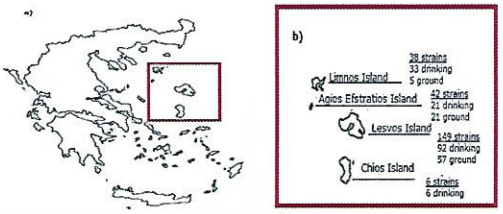

Figure 1. Sampling areas: (a) map of Greece; (b) the four Greek islands and number of isolated *E.coli* strains from each.

Results :

Of the 235 *E.coli* strains we examined more than half (54.9%) were resistant to at least one of the antibiotics tested. Of these 26.3% showed multiple resistance (to two or more antibiotics). 13.6% were resistant to two antibiotics, 9.8% were triply resistant, 0.8% were quardriply resistant and 2.1% were resistant to the five antibiotics tested (Table 2).

Strains from drinking water sources were overall more sensitive, although by little, exhibiting lower multiple resistance patterns (24.7% as opposed to 29.9% of the ground water strains). More specifically, 80 of the 158 *E.coli* strains from drinking untreated water (50.6%) were resistant to at least 1 antibiotic. Half of these (41 - 25.9%) were resistant to only one antibiotic, five (3.1%) were resistant to all 5 antibiotics tested. Ground water *E.coli* had higher percentages of resistance: of the 77 strains examined 49 (63.7%) showed resistance to one or more antibiotics. Although the percentage of resistant strains was higher, more than half of them (26 - 33.8%) were resistant to only one antibiotic, only two (2.6%) were resistant to 4 antibiotics, none resisted all 5 tested (Fig. 2). Amoxicillin was the antibiotic with the highest resistance response (38.3%) followed by Levofloxacin (28.5%), Cefalosporin, (18.3%), Ciprofloxacin (8.5%) and Norfloxacin (5.5%). In untreated drinking and in ground water the patterns of resistance were the same: in Amoxicillin the highest resistance, in Norfloxacin the lowest. There was no antibiotic, of those tested, to which 100% of the isolates were susceptible (Table 3).

Figure 2. Frequency of resistance of *E.coli* strains isolated from drinking and ground water to 5 antibiotics.

Table 1. Concentration (μg) of antibiotics and diameter (mm)of inhibition zones.

Antibiotic	Inhibition zone mm			
	concentration	resistant	intermediate	sensitive
Amoxicillin AMX	25μg	≤ 1,3	1,4 – 1,6	≥ 1,7
Cefaclor CEC	30 μg	≤ 1,4	1,5 – 1,7	≥ 1,8
Norfloxacin NXN	10 μg	≤ 2,0	2,1 – 2,4	2,5 – 3,1
Ciprofloxacin CIP	5 μg	≤ 2,4	2,5 – 2,7	2,8 – 3,5
Levofloxacin LVX	5 μg	≤ 2,5	2,6 – 2,8	2,9 – 3,6

Table 2. *Escherichia* coli strains resistant to one or more antibiotics.

Resistance to:	Drinking water	Ground water	Total
	Strains (%)**	Strains (%)**	Strains (%)**
1 antibiotic	41 (25.9)	26 (33.8)	67 (28.5)
2 antibiotics	23 (14.6)	9 (11.7)	32 (13.6)
3 antibiotics	11 (7.0)	12 (15.6)	23 (9.8)
4 antibiotics	-	2 (2.6)	2 (0.8)
5 antibiotics	5 (3.1)	-	5 (2.1)
Total strains	80 (50.6)	49 (63.7)	129 (54.9)

** Percentage of the total (235) number of strains examinated

Table 3. Resistance of *E.coli* strains to 5 antibiotics

	AMX number **.%	CEC number **.%	NXN number **.%	CIP number **.%	LVX number **.%
Drinking	54 – 34.2 %	29 – 18.4 %	9 – 5.7 %	11 – 7.0 %	42 – 26.6 %
Ground	36 – 46.8 %	14 – 18.2 %	4 – 5.2 %	9 – 11.7 %	25 – 32.5 %
Total	90 – 38.3 %	43 – 18.3 %	13 – 5.5 %	20 – 8.5 %	67 – 28.5 %

** Number of resistant strains , AMX - amoxicillin , CEC - cefaclor , NXN - norfloxacin , CIP - ciprofloxacin , LVX levofloxacin

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Discussion :

The results of our investigation showed that resistance to antibiotics is widespread amongst *E.coli* that proliferate untreated water used for drinking purposes and irrigation in small rural communities in four Greek islands. To the best of our knowledge in every published research project investigating *E.coli* or faecal coliforms in drinking water there were strains exhibiting resistance to antibiotics. In our research we found 50.6% to be resistant to at least one antibiotic. Our observations in multiresistance patterns are not similar to those of other research teams. We had multiresistance in 24.7% of our strains from drinking water sources. The findings of other authors ranged from 100% in India [3] to 3.7% in Canada [4]. The higher percentages in resistant strains were reported from countries where the use of antibiotics in humans and animals is less strictly controlled.

In ground water 63.6% of our strains showed resistance to one or more antibiotics. Of these 29.9% were multiresistant. This is not different from results elsewhere. In an early study in the US [5] it was reported that 16% of faecal coliforms isolated from well water was multiresistant. In a study published 26 years later [6] 87% of coliform bacteria isolated from ground water in the US were multiresistant. Of these 14% were *E.coli*. In Egypt [7] 100% of the *E.coli* from groundwater exhibited multiple resistance. All investigators report single resistance to be more often met than multiple resistance. It is worth noting that the more antibiotics the strains are tested against, the more multiresistant findings.

Because of the very small number of relevant studies internationally, and to the fact that in each study a different set of antibiotics is examined, it is not possible to perform a comparison as to resistance patterns of waterborne *E.coli* strains. Amoxicillin is an antibiotic against which most bacteria from our study had resistance genes (34.2%), 95.0% resistance was documented in India [3]. Ciprofloxacin and Levofloxacin, second and third generation quinolones, are antibiotics heavily prescribed, and this reflects in the fact that we observed high percentage of resistant *E.coli* strains (11.7% and 32.5%) to these two antibiotics in ground water.

The existence of *E.coli* in drinking water is in itself disturbing. In countries with high standards of living it is attributed mostly to the fact that many families live in farms and remote locations, where there is no mains connection and water used for drinking comes from private, untreated sources. The existence of so many antibiotic resistant strains of this bacterium is alarming, given the fact that not only *E.coli* itself could under certain circumstances cause illness, but even more because of the ability of the resistance genes to transfer amongst microorganisms, implanting the resistance to pathogens. Our work demonstrates that untreated drinking and ground water can contribute to the spreading of antibiotic resistance to the general population, posing a serious challenge to infection control.

References :

1. Seiler, C.; Berendonk, T. U. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front Microbiol* **2012**, *3*, 399. Available online: https://doi.org/10.3389/fmicb.2012.00399 (accessed on 09/01/2018).
2. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 8.0, 2018. http://www.eucast.org/clinical_breakpoints/.
3. Pantak, S.P.; Gopal, K. Prevalence of bacterial contamination with antibiotic resistant and enterotoxigenic fecal coliforms in treated drinking water. *J Toxicol Env Heal A* **2008**, *71*, 427-433. Available online: http://www.tandfonline.com/doi/abs/10.1080/15287390701838796 (accessed on 12/01/2018).
4. Coleman, B.L.; Louie. M.; Salvadori, M.I.; McEwen, S.A.; Neumann, N.; Sibley, K., Irwin, R.J.; Jamieson, F.B.; Daignault, D.; Majury, A.; Braithwaite, S.; Crago, B.; McGeer, A.J. Contamination of Canadian private drinking water sources with antimicrobial resistant Escherichia coli. *Water Res* **2013**, *47*, 3026-3036, 10.1016/j.watres.2013.03.008. Available online: https://www.ncbi.nlm.nih.gov/pubmed/23548566 (accessed on 19/02/2018).
5. Amundson, D.; Lindholm, C.; Goyal, S.M.; Robinson, R.A. Microbial pollution of well water in southeastern Minnesota. *J envir sci hlth A* **1988**, *23*, 453-468. Available online: http://www.tandfonline.com/doi/abs/10.1080/10934528809375425 (accessed on 19/02/2018).
6. McKeon, D.M.; Calabrese, J.P.; Bissonnette, G.K. Antibiotic resistant gram-negative bacteria in rural groundwater supplies. *Water Res* **1995**, *29*, 1902-1908. Available online: https://doi.org/10.1016/0043-1354(95)00013-B (accessed on 19/02/2018).
7. AbdelRahim, K.A.A.; Hassanein, A.M.; AbdelAzeiz, H.A.ElH. Prevalence, plasmids and antibiotic resistance correlation of enteric bacteria in different drinking water resources in Sohag, Egypt. *Jundishapur J Microbiol* **2015**, *8*, e18648. Available online: http://dx.doi.org/10.5812/jjm.18648 (accessed on 19/02/2018).