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Research Article

RESEARCH ARTICLE

Antibiotic resistance profiles of *Pseudomonas aeruginosa* isolated from various Greek aquatic environments

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One sentence summary: Resistant *P. aeruginosa* isolates circulate in water bodies, but the driving force for this process is not fully understood.
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ABSTRACT

A large number of antibiotic-resistant *P. aeruginosa* isolates are continuously discharged into natural water basins mainly through sewage. However, the environmental reservoirs of antibiotic resistance factors are poorly understood. In this study, the antibiotic resistance patterns of 245 isolates from various aquatic sites in Greece were analysed. Twenty-three isolates with resistance patterns cefotaxime–aztreonam–ceftazidime, cefotaxime–aztreonam–meropenem, cefotaxime–ceftazidime–meropenem, cefotaxime–ceftazidime–aztreonam–meropenem and cefotaxime–ceftazidime–cefepime–aztreonam–meropenem were screened phenotypically for the presence of extended spectrum β-lactamases (ESBLs), while 77 isolates with various resistant phenotypes were screened for the presence of class 1 and class 2 integrase genes. The aztreonam-resistant isolates and ESBL producers were the main resistant phenotypes in all habitats tested. In 13/77 isolates class 1 integron was detected, while all tested isolates were negative for the presence of the class 2 integrase gene. CTX-M group 9 β-lactamase was present in a small number of isolates (three isolates) highlighting the emergence of ESBL genes in aquatic environments. As a conclusion, it seems that Greek water bodies could serve as a potential reservoir of resistant *P. aeruginosa* isolates posing threats to human and animal health.

Keywords: *P. aeruginosa*; water samples; integrons; ATM resistance; ESBL; CTX-M group 9

INTRODUCTION

The human opportunistic pathogen *Pseudomonas aeruginosa* is a ubiquitous environmental bacterium that causes numerous opportunistic human infections. The emerging presence of multi-drug-resistant isolates resistant to almost all antimicrobials used for hospital patients has attracted the attention of many researchers in recent decades (Gomez, Vega-Baudrit and Nunez-Corrales 2012). The bacterium has intrinsic antimicrobial

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Table 1. Distribution of the water samples into the various geographic areas of Greece.

	Geographical sampling area							
Type of sample	Attica	Northern Greece	Dodecanese	Central Greece	Cycladic islands	Peloponnese	Ionian islands	Total no. (all types of sample)
Bottled water	3	2		1		3	1	10
Mains water	2	3	1	5		25	9	45
Swimming pools	20	4		1		20		45
Drilling water	2			3		2		7
Stream water		14		1		5		20
Thermal water		5		7		1		13
Water tanks			3	2	3	2		10
Total no. (all areas of Greece)	27	28	4	20	3	58	10	**150**

resistance due to low outer membrane permeability, chromosomally encoded AmpC and an extensive efflux pump system, and holds a prominent place in the development of acquired resistance mechanisms (Bonomo and Szabo 2006). The genome's large size and versatility are two important features in the acquisition of new resistance mechanisms. The bacterium's ability to obtain new resistance genes is enhanced by the dispersion in an aquatic environment that constitutes a potential reservoir for bacteria carrying other resistance traits (Mesaros *et al.* 2007). A large number of *P. aeruginosa* resistant isolates are continuously discharged into natural water basins through sewage (Daverio, Ghiani and Bernasconi 2004; Kummerer 2004). In the affected bodies of water, resistance is acquired through contact with sewage-derived *P. aeruginosa* resistant isolates, which retain their resistance either under the prolonged impact of antibiotics, regardless of their concentrations in the water body, or even in the absence of antibiotics (Kummerer 2004; Igbinosa, Odjadjare and Igbinosa 2012a). Horizontal gene transfer (HGT) plays a key role in the dissemination of resistance traits between the isolates of *P. aeruginosa* and other Gram-negative bacteria. It has been shown that various environmental factors enhances the HGT process (Shakibaie, Jalilzadeh and Yamakanamardi 2009; Tacao *et al.* 2014).

A search of the literature was carried out to investigate current knowledge on the occurrence of both intrinsic and acquired resistance mechanisms in environmental *P. aeruginosa* isolates as compared with clinical ones, which have been more thoroughly studied.

Previous studies dealing with clinical *P. aeruginosa* isolates have stated that there is a reduced outer-membrane permeability, which has been associated with an increase in drug efflux, a mechanism that confers cross-resistance to many unrelated antibiotic classes leading to multi-drug-resistant isolates (MDR) (Livermore 2002; Ikonomidis *et al.* 2008; Lee and Ko 2012). Nevertheless, the combination of the two intrinsic mechanisms, loss of oprD and major efflux pumps, can lead to imipenem-resistant *P. aeruginosa* isolates derived from hydropathic facilities (Pereira *et al.* 2011), water samples collected in hospitals (Deplano *et al.* 2005; Quick *et al.* 2014) and surface water samples (in Spain; Sanchez *et al.* 2014).

Some studies demonstrated the presence of extended spectrum β-lactamases (ESBLs) and metallo β-lactamases (MBLs) in clinical isolates of *P. aeruginosa* (Fournier *et al.* 2010; Ranellou *et al.* 2012; Fazeli *et al.* 2014). Fewer studies presented similar isolates recovered from aquatic ecosystems. Some published works indicated the presence of ESBL and MBL genetic determinants in aquatic environments such as rivers (Lu *et al.* 2010; Fontes *et al.* 2011; Tacao, Correia and Henriques 2012; Zhang *et al.* 2013), seawater (Alouache *et al.* 2012), freshwater and waste-water (Slekovec *et al.* 2012; Igbinosa *et al.* 2012b), drinking water and distribution systems (Xi *et al.* 2009). Other studies merely outlined the resistance of *P. aeruginosa* isolates recovered from water samples to commonly used antibiotics (Hirulkar and Soni 2011; Janam, Gulati and Nath 2011; Panda, Patra and Kar 2012).

Many ESBLs and MBLs have been described in *P. aeruginosa*. The ESBLs and MBLs examined in this study were the most commonly circulating in the aquatic ecosystems, as per the literature (Xi *et al.* 2009; Tacao, Correia and Henriques 2012; Zhang *et al.* 2013). In Greece, at least, they seem to be the only ones present in *P. aeruginosa*, but are also found in other Enterobacteriaceae (Vourli *et al.* 2004; Giakkoupi *et al.* 2008; Liakopoulos *et al.* 2013; Pournaras *et al.* 2013).

Integrons have been associated with the dissemination of β-lactamases encoded by class 1 and 2 integrons, such as OXA-type β-lactamases, IMP, VEB-1, VIM-2 and many more, both in clinical and environmental *P. aeruginosa* isolates (Martinez *et al.* 2012; Liakopoulos *et al.* 2013; Zanetti *et al.* 2013).

In a previous study, phenotypical and molecular typing of *P. aeruginosa* isolated from Greek aquatic and waste-water samples revealed a non-clonal population in these environments for the first time (Pappa *et al.* 2013). The aim of this study was to determine the antibiotic resistance profile of *P. aeruginosa* isolates deriving from various aquatic sites in Greece in an attempt to elucidate the main mechanisms of resistance (intrinsic or acquired) and to screen these habitats for the presence of class 1 and class 2 integrons. To our knowledge the prevalence and diversity of such phenotypes in aquatic *P. aeruginosa* isolates is still not clear.

MATERIALS AND METHODS

Sampling

Over the three-year period 2011–2014, 150 water samples of various types were collected from diverse areas in Greece (Table 1). We obtained the samples through the official monitoring sampling schedule of the Water Analysis Department, Central Public Health Laboratory (CPHL), Hellenic Center for Disease Control and Prevention (HCDCP). Water samples from all over the country are regularly delivered to the CPHL within the national water surveillance programme. Accordingly, the geographical distribution of the samples is more or less random, following the sole criterion that they are located within Greek territory.

Colony isolation

The samples were analysed for the detection of *Pseudomonas aeruginosa* using a standard method based on the membrane filtration technique (ISO 16266; International Organization for Standardization 2006). According to this method, colony identification (phenotypic identification) is presumptively based on the production of pyocyanin. Pyocyanin-producing colonies are considered as confirmed *P. aeruginosa*; other fluorescing or reddish brown colonies require confirmation (ISO 16266; International Organization for Standardization 2006). Some strains with atypical biochemical features were subjected to molecular identification targeting the lipoprotein gene *oprL* (De Vos *et al.* 1997). Three reference strains were used as control strains: (i) *P. aeruginosa* ATCC 27853, (ii) a clinical control provided by HPA/NEQAS (the HPA External Quality Control Scheme), and (iii) *P. aeruginosa* PAO1 (Collection of Institute Pasteur CIP104116, www.crbip.pasteur.fr).

Antibiotic susceptibility testing

Susceptibility tests were performed by the Kirby–Bauer method (an agar dilution method according to Clinical and Laboratory Standards Institute Guidelines 2011/M100S21; http://clsi.org; Clinical and Laboratory Standards Institute 2011). All isolates were tested for susceptibility to 14 commonly used antibiotics belonging to four different classes: non-carbapenem β-lactams: ceftazidime (CAZ; 30 μg), cefotaxime (CTX; 30 μg), cefepime (FEP; 30 μg), piperacillin (PIP; 75 μg), ticarcillin (TIC; 75 μg), piperacillin/tazobactam (TZP; 100 μg/10 μg), ticarcillin/clavulanate (TCC; 75 μg/10 μg) and aztreonam (ATM; 30 μg); carbapenems: imipenem (IPM; 10 μg) and meropenem (MEM; 10 μg); aminoglycosides: amikacin (AN; 30 μg), tobramycin (TOB; 30 μg) and gentamicin (GM; 30 μg); and fluoroquinolones: ciprofloxacin (CIP; 5 μg). The interpretation of the resistant phenotypes was performed according to published literature (Livermore, Winstanley and Shannon 2001).

Detection of ESBLs and MBLs

ESBL isolates were phenotypically detected by a modified double disk synergy (DDS) test with the addition of boronic acid to the antibiotic disks as described in Ranellou *et al.* (2012). MBL detection was performed according to Giakkoupi *et al.* (2008).

Isolation of genomic DNA

Pseudomonas aeruginosa genomic DNA was extracted using the Purelink Genomic DNA mini kit (Invitrogen) following the manufacturer's instructions after 48 h growth in nutrient broth and nutrient agar.

PCR amplification of ESBL and MBL genes

Isolates phenotypically positive for ESBL production were subjected to PCR for the detection of 10 different ESBL and six MBL genes (PER-1, OXA-2, VEB-1A, GES-1A, TEM-A, SHV-A, CTX-M groups 1, 2, 8/25 and 9; and VIM-2, IMP, SIM-1, GIM-1, SPM-1 and NDM). PCR conditions and the specific primers (Supplementary Table S1) for the ESBL genes $bla_{\text{PER-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{VEB-1A}}$, $bla_{\text{GES-1A}}$, $bla_{\text{TEM-A}}$, $bla_{\text{SHV-A}}$ and bla_{CTXM} (groups 1, 2, 8/25 and 9) were chosen from the published literature (Weldhagen, Poirel and Nordmann 2003; Woodford, Fagan and Ellington 2006; Libisch *et al.* 2008); the detection of MBL genes was performed using specific primers (Supplementary Table S1) for $bla_{\text{VIM-2}}$, bla_{IMP}, $bla_{\text{SIM-1}}$, $bla_{\text{GIM-1}}$, $bla_{\text{SPM-1}}$ and bla_{NDM}; PCR conditions have also been previously published (Castanheira *et al.* 2004; Lee *et al.* 2005; EuScape, 2013).

Detection of *intI1* and *intI2* genes

Seventy-seven isolates, of various sample-types and geographical sites, and with various resistant phenotypes, were screened for the presence of class 1 and class 2 integrase genes (*intI1* and *intI2*, respectively). The isolates were selected in order to reveal any relationship between the presence of integrons to both resistant phenotypes and to the type of sample and geographical sampling site. Specific primers for *intI1* and *intI2* integrase genes were used for the detection of class 1 and class 2 integrons (intI1F and intI1R, intI2F and intI2R, Supplementary Table S1). The variable region of class 1 integron positive isolates was further amplified using a pair of oligonucleotide primers with homology to the conserved ends 5′-CS and 3′-CS of class 1 integron in order to determine the size of the integrons (5′-CS and 3′-CS, Supplementary Table S1). Finally, a PCR amplification using specific primers (ant(3″)Ia and ant(3″)Ib, Supplementary Table S1) was performed for the identification of the *aadA1* aminoglycoside-resistance gene. For all the above PCR experiments the amplification conditions as described in Levesque *et al.* (1995) were used adjusting the annealing temperature for each pair of primers (Supplementary Table S1).

Sequencing

Isolates positive for the CTX-M group 9 gene and class 1 integrase gene were sequenced by CeMIA SA (http://cemia.eu/sangersequencing.html) using the primers shown in Supplementary Table S1 (CTX-M group 9 forward and reverse; 5′-CS and 3′-CS). All chromatograms were imported and edited in Sequencer 5.3. Sequences obtained were subjected to BLAST for the identification of the appropriate product (CTX-M β-lactamase and integrase). The ones with the class 1 integrase gene were additionally searched against the INTEGRALL database in order to compare the obtained sequence with the ones submitted in the integron database (http://integrall.bio.ua.pt).

Statistical analysis

The statistical analysis was performed in order to elucidate any correlations between the high resistance of a specific antibiotic or combination of antibiotics (resistance pattern) and any of the study sampling sites of Greece. The following statistical packages were used: SPSS v17 (descriptive statistics calculation, χ^2 tests, presented graphs), SAS v9.3 (data manipulation), χ^2 test (categorical variables comparison), one-way ANOVA and Kruskal–Wallis test (correlation of antibiotic resistance to any of the study sampling areas).

RESULTS

Identification of *P. aeruginosa*

Three hundred presumptive *P. aeruginosa* colonies (pyocyanin-producing, other fluorescing or reddish brown) were initially collected by selecting a number of colonies depending on their density in each plate (the numbers of presumptive *P. aeruginosa* colonies are obtained by counting the number of characteristic colonies on the membrane filter according to ISO 16266; International Organization for Standardization 2006). In total

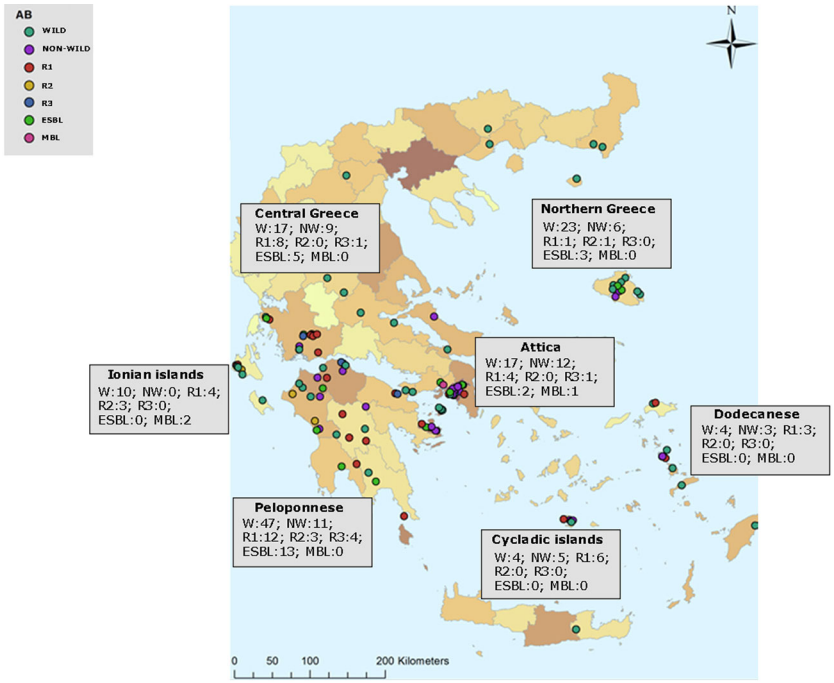

Figure 1. Geographic information system map presenting the distribution of the resistant phenotypes for 245 *P. aeruginosa* isolates in the various geographical areas of Greece (official monitoring sampling schedule of the CPHL, HCDCP). Abbreviations: ESBL: extended spectrum β-lactamases; MBL: metallo β-lactamases; NW, non-wild: susceptible to all antibiotics except ticarcillin, ticarcillin + clavunalic acid and cefotaxime; R1: AmpC, partially/fully derepressed with resistance to aztreonam; R2: increased efflux; R3: loss of OprD with resistance to imipenem; W, wild: susceptible to all antibiotics presenting no acquired resistance mechanism while the intrinsic mechanisms are not expressed.

245 colonies (9 from bottled water, 102 from mains water, 53 from swimming pools, 7 from drilling water, 25 from stream water, 21 from thermal water, 28 from water tanks; Supplementary Table S2) were confirmed as *P. aeruginosa* using first the ISO 16266 confirmation procedures and second molecular identification (De Vos *et al.* 1997).

Antimicrobial susceptibility profiles and detection of β-lactamase producers

Initially, the isolates were classified into three categories: 52% (127/245) of the isolates were characterized as wild (W: susceptible to all antibiotics presenting no acquired resistance mechanism while the intrinsic mechanisms are not expressed); 16.3% (40/245) of the isolates as non-wild (NW: susceptible to all antibiotics except ticarcillin (TIC), ticarcillin + clavunalic acid (TCC) and cefotaxime (CTX)); and 32% (78/245) of the isolates as resistant (R: resistance or intermediate to more than three antibiotics). The resistant isolates (32%) were categorized into three resistance profiles representing the main intrinsic resistance mechanisms of *P. aeruginosa*: R1 (AmpC, partially/fully derepressed with resistance to aztreonam; 52.5%, 41/78), R2 (increased efflux; 7.7%, 6/78) and R3 (loss of OprD with resistance to imipenem; 6.4%, 5/78). A substantial portion of the resistant isolates (29.5%, 23/78) with resistance patterns CTX–CAZ–ATM, CTX–ATM–MEM, CTX–CAZ–MEM, CTX–CAZ–ATM–MEM and CTX–CAZ–FEP–ATM–MEM (Supplementary Table S2) were screened phenotypically for the presence of ESBLs. All 23 isolates produced positive results during the DDS test (synergy between amoxicillin + clavunalic acid (AMC) and ceftazidime (CAZ) or cefotaxime (CTX)), which is indicative of the presence of ESBL. These isolates were characterized as ESBL producers and were isolated mainly from mains water (eight isolates) and swimming pools (five isolates), while the remainder derived from other sources. Three isolates (two from mains water and one from swimming pool samples) presented the characteristic synergy between meropenem (MEM)/imipenem (IPM) and the disk with EDTA, and were characterized as MBL producers (3.8%, 3/78). The resistance profiles of the 245 *P. aeruginosa* colonies are shown in Supplementary Table S2. The resistant phenotypes were distributed in all geographical areas (Fig. 1), and the Peloponnese presented the highest percentage of all the resistant profiles. ESBL isolates appeared in four geographically unrelated areas of Greece together with other resistant mechanisms (R2, R3; Fig. 1). The high resistance of ATM, MEM, IPM and CTX was tested separately in relation to geographical areas. Antibiotic resistance of MEM did not show any difference in relation to the geographical area ($P = 0.417$; >0.05), while ATM seems to have higher resistance in Northern Greece ($P = 0.087$; <0.10) and CTX presented higher resistance in Northern Greece and the Ionian islands ($P = 0.108$, marginal significance level $\alpha = 0.10$); finally, IPM seems to have higher resistance in the Dodecanese and Cycladic islands ($P = 0.004$; $\ll 0.05$). A statistically significant indifference interaction was observed with IPM combined with ATM–CTX vs. sampling location, indicating that IPM-resistant isolates (R3 phenotype) corresponded exclusively to the loss of OprD mechanism. ATM and CTX presented statistically significant antibiotic synergy with MEM ($P = 0.08$; <0.10) in relation to Northern Greece. Although resistance to MEM is not associated to any specific region of Greece, it seems that when it appears in this pattern (ATM–CTX–MEM) it contributes to the high resistance of the other two antibiotics in the area.

Table 2. Characteristics of the three CTX-M group 9 positive *P. aeruginosa* isolates. Abbreviations: ATM: aztreonam; CAZ: ceftazidime; CTX: cefotaxime; MEM: meropenem; ESBLs: extended spectrum β-lactamase.

Isolate code no.	Type of sample	Sampling site	Resistant pattern	Resistance phenotype	*intI1*	*intI2*
121	Thermal water	Central Greece	CTX–ATM–MEM	ESBL	0	0
129	Thermal water	Central Greece	CTX–ATM–CAZ–MEM	ESBL	0	0
131	Mains water	Attica	CTX–ATM–MEM	ESBL	0	0

Table 3. Characteristics of the 13 integron positive *P. aeruginosa* isolates. Isolates shown in bold present the *aadA1* aminoglycoside gene. Abbreviations: DW, drilling water; MW, mains water; SP, swimming pool; SW, stream water; TW, thermal water; NR, no resistance; NW, non-wild: susceptible to all antibiotics except ticarcillin, ticarcillin + clavunalic acid and cefotaxime; R1: AmpC, partially/fully derepressed with resistance to aztreonam; R2: increased efflux; R3: loss of OprD with resistance to imipenem; W, wild: susceptible to all antibiotics presenting no acquired resistance mechanism while the intrinsic mechanisms are not expressed; ESBL: extended spectrum β-lactamases; MBL: metallo β-lactamases.

Isolate Code No	Type of sample	Sampling site	Resistant pattern	Resistance phenotype	Variable region of integrons
119	SP	Attica	CTX–ATM–MEM	R1	500, 1400, 1500 bp
142a	SP	Attica	GM–AN	NW	1000 bp
142b	SP	Attica	GM–AN	NW	700, 900, 1000 bp
144	MW	Peloponese	MEM–AN	R2	1250, 1500 bp
146a	MW	Northern Greece	GM–AN–TOB	NW	**1250 bp**
151	DW	Central Greece	CTX–GM	NW	**1250 bp**
152	TW	Central Greece	CTX–GM	NW	**1250 bp**
171a	MW	Peloponese	NR	W	900 bp
176	MW	Peloponese	CTX–ATM–GM	R1	600, 800, 1250 bp
193a	SP	Attica	CTX–CAZ–ATM–MEM	R1	1000 bp
257	SP	Peloponese	CTX–ATM–GM	R1	**1250 bp**
269	MW	Central Greece	CTX–ATM–MEM	R1	500 bp
317a	SW	Northern Greece	NR	W	>1000 bp

PCR amplification of ESBL and MBL genes and sequence analysis

All 23 ESBL producers were screened for the presence of β-lactamase genes. Out of the 10 ESBL genes tested only the CTX-M group 9 was detected in three isolates (Table 2), where sequence analysis identified the CTX-M group 9 β-lactamase. None of the remaining ESBL genes was detected in any of the 23 isolates tested with the primer sets used in this study. The three phenotypically MBL positive isolates did not produce positive results for the six MBL genes tested.

Detection and sequence analysis of integrons

Of the strains tested, 13/77 (16.9%) were positive for the presence of the class 1 integrase gene. The sizes of the regions encompassed by the 5′-CS and 3′-CS conserved sequences ranged from 500 to 1500 bp (Table 3). For four isolates, a single product of 1250 bp was observed; sequencing of all four amplicons with 5′-CS and 3′-CS primers identified through the integron database search (accession number: DQ157701) the *aadA1* aminoglycoside gene, which confers resistance to aminoglycosides. Seventy-seven isolates with various antimicrobial resistance profiles were tested for the presence of the class 2 integrase gene; none of these isolates produced a positive electrophoretic signal.

DISCUSSION

To the best of our knowledge, this is the first time that an attempt has been made to determine *P. aeruginosa* resistance profiles circulating in various Greek aquatic ecosystems. Additional information is provided for the presence of class 1 and class 2 integrons in these environments and their likely participation in the occurrence of resistance to several classes of antibiotics. Theoretically all wild isolates should be susceptible to all antibiotics presenting no acquired resistance mechanism while the intrinsic mechanisms are not expressed (Livermore 2002). The presence of 50% resistant *P. aeruginosa* isolates in such diverse aquatic environments is considered high and worrying (Daverio, Ghiani and Bernasconi 2004; Kummerer 2004). In clinical isolates the intrinsic resistance mechanisms of *P. aeruginosa* are well characterized (Livermore, Winstanley and Shannon 2001; Bonomo and Szabo 2006), while the combination with acquired mechanisms such as ESBLs may lead to complex resistant phenotypes (Fazeli *et al.* 2014); however, there is not much published information regarding aquatic *P. aeruginosa* isolates. In the present study, the majority of the resistant isolates presented non-enzymatic mechanisms with ATM-resistant isolates standing out (the R1 phenotype, Supplementary Table S2); high resistance to ATM has been previously reported in environmental isolates deriving from soil (Pitondo-Silva *et al.* 2014) or from hospital waste-water treatment (Santoro, Romão and Clementino 2012), but never in *P. aeruginosa* isolates deriving from aquatic ecosystems. The geographical dispersion of ATM-resistant isolates extends across all areas studied (Fig. 1). It seems that some of the integron positive isolates with R1 phenotype, presented intermediate resistance to some clinically relevant antibiotics such as CAZ, MEM and GM (Table 3). It has been previously stated that the presence of class 1 integron decreases the susceptibility to front-line antipseudomonal drugs (Cicek *et al.* 2013; Mano *et al.* 2015), and it should be taken into consideration for isolates deriving from aquatic environments (Zanetti *et al.* 2013; Tacao, Correia and Henriques 2015).

Mutational inactivation of oprD porin (the R3 phenotype) is known to occur in *P. aeruginosa* leading to carbapenem-resistant isolates (Ikonomidis *et al*. 2008). In our study the combination of the R2 and R3 phenotype led to multi-drug-resistant isolates (MDR) with resistant pattern CTX–ATM–IPM–MEM (isolate 263, Supplementary Table S2) and CTX–IPM–MEM (isolate 171b, Supplementary Table S2), indicating reduced outer-membrane permeability association with an increase in drug efflux, a mechanism that confers cross-resistance to many unrelated antibiotic classes leading to MDR isolates (Livermore 2002; Lee and Ko 2012).

The phenotypically ESBL and MBL positive isolates did not produce positive results when tested molecularly; these results were not surprising as a large number of resistance genes in *P. aeruginosa* have been described in the literature (Bonomo and Szabo 2006; Libisch *et al*. 2008). However, the presence of CTX-M group 9 β-lactamase in a small number of isolates (three isolates) highlights the emergence of ESBL genes in Greek aquatic environments. Until 2006 the CTX-M-type ESBLs had not been described in *P. aeruginosa* (Bonomo and Szabo 2006). Since then a number of studies have reported the presence of the CTX-M β-lactamases, and particularly the CTX-M group 9 in environmental isolates deriving from sites such as animal farms (Ma *et al*. 2012), rivers and lakes (Zurfluh *et al*. 2013), and waste-waters (Galvin *et al*. 2010). Among them a limited number concerned the presence of CTX-M group 9 in *P. aeruginosa* (Tacao, Correia and Henriques 2012). This result provides additional information on the acquired mechanisms of resistance circulating in many aquatic ecosystems in Greece. A complete characterization of the genetic environment of these genes is necessary in order to gain a more specific interpretation of the results. It is important to emphasize that CTX-M group 9 β-lactamase has been reported in hospital waste-waters in various countries around the world (Amaya *et al*. 2012; Korzeniewska and Hrnisz 2013; Bréchet *et al*. 2014), supporting hypotheses regarding increasing transmission of CTX-M genes via environmental vehicles (Woerther *et al*. 2003; Cantón and Coquehe 2006; Livermore *et al*. 2007). The CTX-M genes have been described or presumed to be natural and chromosome mediated in the species *K. cryocrescens*, *K. ascorbata*, and *K. georgiana*. In clinical strains, CTX-M-encoding genes have commonly been located on plasmids; Bonnet (2004) have reported the plasmid-mediated CTX-M group 9 gene inserted in complex class I integrons In60. However, in our study, the three CTX-M positive isolates were integron negative (Table 2). Additional experiments that include detection of plasmids and the characterization of the genetic environment of the CTX-M genes are necessary.

The isolates carrying integrons belonged to various resistant phenotypes deriving from diverse types of samples and from various geographical areas (Table 3). *aadA1* was present in four isolates and encodes for aminoglycoside adenyltransferase, conferring resistance to streptomycin and spectinomycin; although these two antibiotics were not used in our study, the presence of the gene cassette in the environmental isolates highlights the issue of the presence of mobile genetic units carrying resistant gene cassettes in aquatic habitats. Both are clinically relevant antibiotics, while spectinomycin is widely used as a growth promoter in food-producing animals (Juntunen *et al*. 2010). Integrons were present in various resistance patterns (Table 3 and Supplementary Table S2) indicating the probability of co-resistance to many isolates tested (i.e. 144, 193a; Table 3), leading to multi-resistant phenotypes. Wild isolates possessed integrons too, which is not surprising as wild and integron positive isolates coexist in the same aquatic habitats.

The dispersion in the water bodies of large amounts of antibiotics through fish farming and animal husbandry has been studied by many health organizations (European Centre for Disease Prevention and Control, ECDC; European Food Safety Authority, EFSA), highlighting the emergence of multi-drug-resistant isolates for widely used antibiotics. Also, in a recent scientific report of ECDC and EFSA, which was based on data from European countries including Greece, the extensive use of clinically relevant antibiotics, such as fluoroquinolones and third- and fourth-generation cephalosporins, in animal husbandry was stated (EFSA 2015).

In our study, limits on financial resources meant that the range of sampling sites was dependent on the schedule of CPHL, resulting in the uneven geographical distribution of the samples. The statistical analysis provided more trends than solid outcomes and conclusions due to the diversity of the water samples. However, an effort was made to present the correlation between the high resistance to some antibiotics and particular regions of Greece taking into consideration that Northern Greece is the main area of livestock farming in the country, while fish farms are active throughout most of the islands, where antibiotics are used extensively to prevent and cure diseases and to enhance growth rates (EFSA 2015). Thus, the presence of ATM- and CTX-resistant *P. aeruginosa* isolates in surface water samples in Northern Greece and IPM-resistant isolates in drilling water and water tanks in the Dodecanese and the Cycladic islands may provide an explanation for the spread of resistance in the water bodies. Lastly, the fact that according to the results of the study there is a wide geographical distribution of resistant phenotypes in variable water types may indicate that resistance mechanisms circulate in the environments regardless of the type of water sample and region (Fig. 1). The results of this study indicate that the high resistance of environmental *P. aeruginosa* isolates to clinically relevant antibiotics as well as the presence of conserved segments of integrons and CTX-M genes in these environments is a matter of concern and point to *P. aeruginosa* as a potential reservoir of resistance genes in aquatic environments in Greece.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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