Baseline screening for the presence of antimicrobial resistance in *E. coli* isolated from Kuwait's marine environment

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Abstract

Here we present the findings of a study where 598 isolates of Escherichia coli (351 derived from seawater; 247 derived from the Venus clam, Circenita callipyga) were obtained from Kuwait's marine environment. Isolates were screened for their potential resistance to an array of 23 commonly deployed frontline antibiotics. Results demonstrate the resistant was widespread across all sites with high-levels of resistance (seawater: summer 89–64%; winter 90–57% and biota: summer 77%; winter 88%) observed to at least 1 of the 23 antibiotics tested. Resistance to Ampicillin was by far the most widely observed profile in seawater and biota across both summer and winter seasons, with 55.9 to 70.9% isolates displaying resistance to this antibiotic. This study demonstrates the potential of AMR screening to be used in Kuwait to detect issues related to water quality and the consequences it may pose for human health.

Keywords: Kuwait; Marine waters; Antimicrobial resistance; Antibiotics Pollution; Bacteria

Baseline paper

Antimicrobial agents are widely used across health care and agricultural sectors to treat a range of disease conditions (Baquero et al. 2008; Martinez 2009). However, concern has been raised over their indiscriminate, or poorly regulated use and the adverse impact this may have on the environment (Kümmerer 2009; Taylor et al. 2011; Williams et al. 2016). Antimicrobial resistance (AMR) is known to span all classes of natural and synthetic antibiotics and the phenomenon is considered to be one of the foremost global health care problems (D'Costa et al. 2006; WHO 2005; O'Neill 2016). It has become increasingly clear that the diversity and abundance of antibiotic resistance in the environment has been underestimated, with potentially widespread ramifications (Williams et al. 2016). Recently, there has been an increasing amount of interest in the role that the marine environment plays in accentuating drug resistance (Taylor, Verner-Jeffreys, and Baker-Austin 2011). It has a high and diverse bacterial loading and is a known sink for a multitude of clinical bacteria and contaminants. Therefore, marine ecosystems are not only an important reservoir for AMR, but they also drive its emergence (Taylor et al. 2011; Williams et al. 2016).

Recent studies suggest that the Gulf Cooperative Council (GCC) region is susceptible to the emergence of AMR genes and bacteria (Balkhy et al. 2016). However, little attention has been paid to the role that the marine environment may play in acting as a sink, or promoting the emergence of AMR. Where available, studies have identified the presence of AMR bacteria in fish and seawater collected from locations close to sewage discharges (Al-Bahry et al. 2009) and within the effluent itself (Al-Bahry et al. 2011). The presence of AMR bacteria has also been used as an indicator to monitor the exposure of green turtles (*Chelonia mydas*) to different marine pollutants (Al-Bahry et al. 2012).

The marine environment of Kuwait is known to be heavily impacted by large volumes of partially or untreated sewage discharge (Lyons et al. 2015; Saeed et al. 2015; Al-Sarawi et al. 2015). An assessment of the degree of sewage contamination in Kuwait's marine environment used both microbial water quality data and concentrations of faecal sterols in sediment to reveal regular breaching of regional water quality guidelines, with clear pollution hot spots within Kuwait Bay and along the Arabian (Persian) Gulf coast (Devlin et al. 2015; Lyons et al. 2015; Saeed et al. 2015). It is also known that a wide array of chemical pollutants are associated with these effluents, some of which have antimicrobial modes of action (Smith et al. 2015; Saeed et al. 2017).

In Kuwait, research into AMR has been restricted to the clinical setting with high levels of resistance documented against a range of front line antibiotics (Jamal et al. 2013; Zhang et al. 2006). To date no studies have addressed the issue of AMR in marine systems, which represents a key data gap in Kuwait. Here data is presented from a baseline survey that is the first to obtain information on the prevalence of AMR within bacterial isolates collected from Kuwait's marine environment. In total 598 isolates of

Escherichia coli were isolated from seawater and biota (Venus clam, *Circenita callipyga*) and screened for resistance against a panel of 23 antibiotics.

Seawater samples for AMR screening were collected during both winter (December 2014-February 2015) and summer (July – August 2015) seasons from sites along the Kuwait coastline. Bivalves samples were collected across summer (July-August 2015) and winter (December 2015-February 2016) seasons (Figure 1). Physical-chemical parameters (pH, water temperature and salinity) were recorded at each sampling location using a portable multi-parameter water quality instrument (Hanna instruments model no. HI 9828, USA). Salinity (mean 36 ‰ ± 2.0) and pH (mean 8.2 ± 0.2) values were similar across sampling periods, whereas water temperatures varied between summer (34 °C ± 2.0) and winter (17.2 °C ± 2.0) seasons. Sites were selected to provide representative locations around known sewage outlets within Kuwait Bay (Al-Salam, Al-Ghazali) and along the Arabian Gulf Coast (Abu-Al-Hasaniya). At each site, seawater samples were collected close to known effluent discharge points. For comparison, seawater samples were collected at Khiran as a reference site. Samples of Venus clam were only available from Al-Salam. Multiple seawater samples at each location were taken into 1L sterile polyethylene bottles and stored on ice, before transporting to the laboratory. Bivalve samples were also collected by hand and stored in sterile plastic bags on ice before being transported to the laboratory.

At each location seawater samples were screened for both faecal coliform and *E. coli* to provide an overview of sewage contamination at the time of sampling (Table 1). This was achieved using a membrane filter technique as recommended by Regional Organization for the Protection of the Marine Environment (ROPME) (MOOPAM, 2010), Standard Methods for the Examination of Water and Waste water (Anon 2012) and Section 9222 D of Standard Methods for the Examination of Water and Waste water (APHA 1992). Briefly, under aseptic techniques, volumes 0.1, 1, 3, 10ml and serial dilutions (10^{-1}, 10^{-3}, 10^{-5}) using 0.1% peptone water for each seawater were filtered through sterile a 47-mm diameter, 0.45µm pore size filter paper. These filter papers were then placed separately on m-FC media with 10 ml 1% Rosolic acid in 0.2N NaOH, pH 7.4 plates. The plates were incubated at 37 °C for 24 h. For *E. coli* confirmation individual, green colonies (colour indicative of *E. coli*) were chosen from each sample and streaked onto Nutrient Agar with 4-methylumbelliferyl-β-D glucuronide (NA-MUG) (Villari, 1997). NA-MUG plates were incubated overnight (16–20 h) at 37 °C. Mean number of faecal bacteria and *E. coli* in seawater were expressed in colony-forming unities (CFU) per 100 ml (Table 1). To isolate *E. coli* in Venus clams, pooled samples were shelled under aseptic conditions using sterile knife. The flesh, as well as the liquor inside the shells, was then placed in sterile beakers. 20g of the tissue (pooled sample) was placed in sterile stomacher bag and homogenised using STOMACHER® 400 (Seward, UK) for 30 min. The resulting homogenate was placed into a sterile pestle and mortar and macerated under sterile conditions with 20 ml of sterile 0.1% peptone water. A serial dilution from each pooled

sample was performed (10^{-1}, 10^{-3}, 10^{-5}) using 0.1% peptone water. Filtration, incubation, counting, and confirmation procedures then followed the procedure outlined for seawater samples. Blank and positive control (*E. coli* ATCC25922) samples were analysed in parallel with those collected from the field for quality control purpose.

The process for AMR screening was conducted essentially following the guidelines of Clinical and laboratory Standards Institute {CLSI, 2015 #481}. Briefly, individual isolates were screened for susceptibility against a panel of 23 antibiotics. The minimal inhibitory concentrations (MICs) were determined by micro-dilution (48 h incubation) onto the custom dehydrated 96-well Sensititre™ GN2F panels (GN2F, Thermo Scientific, UK) using Cation Adjusted Muller Hinton (CAMHB) broth. The interpretation of results isolates were classified as resistant, intermediate (partially resistant) and susceptible (sensitive) according to the breakpoints recommended by Cefas that hybrid with the Clinical Laboratory and Standard Institute (CLSI, 2015) . The microbial agents used in the panels were selected based on their mode of action, history of use, resistance and the clinical relevance. The Sensititre® GN2F panels include nine main structural antibiotics groups [concentrations mg l^{-1}; ≥MIC]: Aminoglycosides: Amikacin (AMI) [8–64; ≥ 32], Gentamicin (GEN) [2–16; ≥8], Tobramycin (TOB) [4–8; ≥ 8]; Beta-lactams: Ampicillin (AMP) [4–32; ≥ 32], Aztreonam (AZT) [8–32; ≥ 32], Meropenem (MERO) [1–8; ≥ 8], Piperacillin (PIP) [16–128; ≥ 128]; Aminoglycoside/beta-lactamase inhibitors: Ampicillion/Sulbactam (A/S2) [4/2 to 32/16; ≥ 32/16]; Beta-lactam/beta-lactamase inhibitor: Piperacillin/Tazobactam constant (P/T4) [16/4–128/4; ≥ 128/4], Ticarcillin/Clavulanic acid constant (TIM 2) [16/2 –64/2; ≥ 64/2]; Cephalosporins: Cefazolin (FAZ) [4–32; ≥ 32], Cefepime (FEP) [4–32; ≥ 32], Cefotetan (TANS) [8–32; ≥ 32], Ceftriaxone (AXO) [1–64; ≥32], Ceftazidime (TAZ) [1–32; ≥ 32], Cefuroxime (FUR) [4–32; ≥ 32], Cefoxitin (FOX) [4–32; ≥ 32], Cefpodoxime (POD) [2–16; ≥ 8]; Fluoroquinolones: Ciprofloxacin (CIP) [0.5–4; ≥ 4], Gatifloxacin (GAT) [1–8; ≥ 8]; Imipenem: Imipenem (IMI) [2–16; ≥ 16]; Quinolone: Nitrofurantoin (NIT) [16–128; ≥128]; Dihydrofolate reductase inhibitor/sulfonamide: Trimethoprim/Sulfamethoxazole (SXT) [0.5/9.5–4/76; ≥ 4/76]. All concentrations used in this analysis were successive doublings from the range minimum to maximum. Strain of *E. coli* ATCC 25922 was used as control for the results following the criteria established by the Clinical Laboratory Standards institute (CLSI, 2015). Isolates were considered resistant according to standards suggested by (CLSI, 2015).

Results clearly indicate that seawater collected from sites known to be close to waste-water effluent discharge points fail a range of commonly applied international microbial water quality standards (Table 1). The highest counts for both microbial parameters were recorded in winter at the main sewage outlets in the Kuwait Bay (Al-Ghazali and Al-Salam sites, respectively). At these locations *E. coli* exceeded international water quality standards by almost 100x and the findings support previously published data that identified these sites as regularly being impacted by sewage effluent, resulting in persistent failures of microbial water quality standards (Lyons et al. 2015). Along with breaches in

microbial seawater quality, sediment at these locations has been shown to be contaminated with high concentrations of faecal sterols, indicative of a chronic sewage pollution problem at these locations (Lyons et al. 2015; Saeed et al. 2015).

In total 598 *E. coli* strains were isolated (351 seawater; 247 bivalves) and screened for their potential resistance to an array of commonly deployed frontline antibiotics. The isolates were collected across different seasons and overall the screening indicated a high percentage possessed some degree of resistance to antimicrobials. The % of isolates showing resistant to at least 1 of the 23 antibiotics tested is displayed in Table 2. Results demonstrate the resistant was widespread across all sites (seawater: summer 89 - 64%; winter 90-57% and biota: summer 77%; winter 88%). The level of resistance observed in this study was similar to that previously reported for *E. coli* isolated from sewage contaminated beaches in Brazil (67.5%) (Andrade et al. 2015), and from seawater collected close to an aquaculture facility in China (80%) (Wang et al. 2015). A full description of the resistance profile obtained for each isolate is provided in Supplementary file 1. Resistance to AMP was by far the most widely observed profile in seawater and biota across both summer and winter seasons, with 55.9 to 70.9% isolates displaying resistance (Supplementary file 1; Figure 2; Table 3). This supports previous studies that have documented wide spread resistance to this broad-spectrum beta-lactam antibiotic in the aquatic environment (Letchumanan et al. 2015; Watkinson et al. 2007; Al-Bahry et al. 2009). Ranking the resistance profiles for seawater and biota across both summer and winter periods suggests that the profile of resistance may be influenced by seasonal factors. For example, in strains of *E. coli* isolated from winter biota samples displayed a high-level of resistance to FOX (51.4%), which then dropped substantially in samples screened from the summer (7.7%) (Table 3). Seasonal differences in resistance profiles were also noted for AXO and TIM2 (seawater isolates) along with FUR and FAZ (biota isolates). Likewise, the resistance profiles between seawater and biota samples didn't always mirror each other and could point different drivers, within each matrix, being responsible for the promotion and maintenance of AMR. The dataset available doesn't allow for definitive statements to be made about either of these subjects, but does point to future research lines to follow.

In Kuwait concern over AMR in a clinical setting has previously been raised due to a perceived lack of stewardship and irresponsible use of antimicrobials, which has seen a surge in the prescribing (and in some cases, open access over the counter) of a wide spectrum of antibiotics including 3rd and 4th generation cephalosporin and quinolone class of drugs (Awad & Aboud 2015). In this current survey, many isolates were resistance to multiple antibiotics and of the 598 isolates screened, 69% (n= 413) were resistant to two or more antibiotics, 52% (n= 313) were resistant to at least three antibiotics and one *E. coli* isolate obtained from an Al-Salam seawater sample displayed resistance to 22 out of the 23 antibiotics tested (Figure 3; Supplementary file 1). The *E. coli* screened displayed high-levels of resistance to older generation drugs, such as FAZ a 1st generation cephalosporin, which consistently ranked in the top 5 antibiotics for which resistance was observed (Table 3). Of some concern, a number

of isolates demonstrated resistance to important frontline classes of antimicrobial agents, such as the 3rd generation cephalosporin, AXO; 4th generation cephalosporin, FEP; and the 4th generation fluoroquinolone, GAT. This spread of observed resistances to older as well as new antibiotics, encompassing almost all tested classes and including antimicrobials used for a variety of clinical and veterinary applications is of some concern. Recently, the definition of multidrug-resistant (MDR) has been updated to incorporate any bacterial isolate that displays non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al. 2012). If we apply this definition to the isolates screened here 35% (206 isolates) would be classified as showing MDR (Supplementary file 2). The only antibiotic for which all 598 isolates were sensitive was IMI, a carbapenem class of drug used for the treatment of infections, known or suspected to be, caused by MDR bacteria. Their effectiveness is less affected by many common mechanisms of antibiotic resistance, so therefore it is not surprising to see isolates sensitive to this antimicrobial drug (Fuste et al. 2013).

To our knowledge this is the first study of this nature to be conducted in Kuwait. Based on the data presented here the marine environment is being exposed to antibiotic resistant bacteria likely to be originating from waste water effluent. Further information, such as antibiotic dosing statistics, wastewater discharge sources and both clinical and environmental microbiological data, would help to determine the risks posed to both human and ecosystem health of these bacteria entering marine systems.

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Table 1 Enumeration of faecal coliform (FC) and *E. coli* present in seawater samples and thresholds for microbiological measurements set from Kuwait Environmental Public Authority (KEPA), Brazilian legislation standards and current thresholds within the European coastal Bathing Water Directive (cBWD). Thresholds are presented here for a guide to the degree of sewage contamination and are not intended to be fully compliant with the overall process of the revised EU rBWD. Counts are calculated as colonies per 100ml (CFU/100 ml) for each of triplicate plates and reported as (mean values ±SD) over the total number of plates. *The values expressed to 10^3

Site	Season	Seawater % resistant to at least 1 antibiotic (number isolates screened)	Biota % resistant to at least 1 antibiotic (number isolates screened)
Al-Salam	Summer	80 (78)	77 (103)
Al-Salam	Winter	83 (155)	88 (144)
Abu-Al-Hasaniya	Summer	70 (79)	N/S
Al-Ghazali	Summer	89 (9)	N/S
Al- Ghazali	Winter	57 (14)	N/S
Khiran	Summer	64 (6)	N/S
Khiran	Winter	90 (10)	N/S

Table 2: The % of *E. coli* isolates resistant to at least 1 antibiotic in seawater and Venus clam samples collected from Kuwait's marine environment.

Site	Season	Seawater % resistant to at least 1 antibiotic (number isolates screened)	Biota % resistant to at least 1 antibiotic (number isolates screened)
Al-Salam	Summer	80 (78)	77 (103)
Al-Salam	Winter	83 (155)	88 (144)
Abu-Al-Hasaniya	Summer	70 (79)	N/S
Al-Ghazali	Summer	89 (9)	N/S
Al- Ghazali	Winter	57 (14)	N/S
Khiran	Summer	64 (6)	N/S
Khiran	Winter	90 (10)	N/S

Table 3 Seasonal antibiotic resistance ranking summary for E. coli (sites combined) isolated from seawater and biota (Venus clam) during both summer and winter periods. Antibiotics screened: Ampicillin (AMP), Tobramycin (TOB), Nitrofurantoin (NIT), Cefazolin (FAZ), Cefpodoxime(POD), Cefoxitin (FOX), Cefuroxime (FUR), Ceftriaxone (AXO), Aztreonam (AZT), Ampicillin/sulbactan (A/S2), Ticercillin/clavulanic acid (TIM2), Ciprofloxacin (CIP), Imipenam (IMI), Piperacillin (PIP), Cefepime (FEP), Trimethorprim/sulfamethoxazole (SXT), Cefotetan Na (TANS), Ceftazidime (TAZ), Meropenem (MERO), Piperacillin/tazobactam (P/T4), Gatifloxacin (GAT), Gentamycin (GEN) and Amikacin (AMI).

	Seawater						**Biota**				
Rank	**Antibiotic**	**Summer % resistance *E. coli* (n=172)**	**Rank**	**Antibiotic**	**Winter % resistance *E. coli* (n= 179)**	**Rank**	**Antibiotic**	**Summer % resistance *E. coli* (n=103)**	**Rank**	**Antibiotic**	**Winter % resistance *E. coli* (n= 144)**
1	AMP	70.9	**1**	AMP	55.9	**1**	AMP	67.9	**1**	AMP	69.4
2	SXT	49.4	**2**	SXT	52.5	**2**	PIP	47.5	**2**	FAZ	55.6
3	PIP	42.4	**3**	FAZ	34.6	**3**	SXT	30	**3**	FOX	51.4
4	FAZ	25	**4**	PIP	31.8	**4**	FAZ	12.6	**4**	SXT	49.3
5	POD	23.8	**5**	CIP	26.8	**5**	CIP	10.7	**5**	PIP	25.7
6	FUR	22.7	**6**	POD	25.1	**6**	POD	9.7	**6**	POD	23.6
7	CIP	22.7	**7**	FOX	24	**7**	AXO	9.7	**7**	FUR	20.8
8	AXO	17.4	**8**	FUR	18.9	**8**	FOX	7.7	**8**	CIP	14.6
9	AZT	12.2	**9**	TIM2	18.4	**9**	TIM2	5.8	**9**	A/S2	11.1
10	A/S2	10.5	**10**	A/S2	16.8	**10**	AZT	4.8	**10**	TANS	11.1
11	TIM2	8.7	**11**	GEN	15.1	**11**	TAZ	3.9	**11**	TIM2	10
12	GAT	8.1	**12**	GAT	8.4	**12**	A/S2	2.9	**12**	AXO	8.33
13	GEN	8.1	**13**	TOB	7.8	**13**	GAT	2.9	**13**	AZT	3.47
14	FOX	4.7	**14**	AZT	7.8	**14**	TANS	2.9	**14**	GEN	2.8

	Seawater					Biota					
Rank	**Antibiotic**	**Summer % resistance *E. coli* (n=172)**	**Rank**	**Antibiotic**	**Winter % resistance *E. coli* (n= 179)**	**Rank**	**Antibiotic**	**Summer % resistance *E. coli* (n=103)**	**Rank**	**Antibiotic**	**Winter % resistance *E. coli* (n= 144)**
15	FEP	4.1	**15**	TANS	6.7	**15**	FEP	1.9	**15**	FEP	2.8
16	TOB	3.4	**16**	AXO	6.7	**16**	GEN	1.9	**16**	GAT	2.8
17	TANS	1.7	**17**	TAZ	5	**17**	P/T4	1.9	**17**	TOB	2.1
18	TAZ	1.7	**18**	P/T4	3.4	**18**	MERO	0.9	**18**	NIT	1.4
19	NIT	1.2	**19**	FEP	2.2	**19**	TOB	0.9	**19**	TAZ	0.7
20	P/T4	0.6	**20**	NIT	1.1	**20**	AMI	0.9	**20**	MERO	0.7
21	MERO	0	**21**	MERO	1.1	**21**	IMI	0	**21**	P/T4	0.7
22	IMI	0	**22**	AMI	0.6	**22**	NIT	0	**22**	AMI	0
23	AMI	0	**23**	IMI	0	**23**	FUR	0	**23**	IMI	0

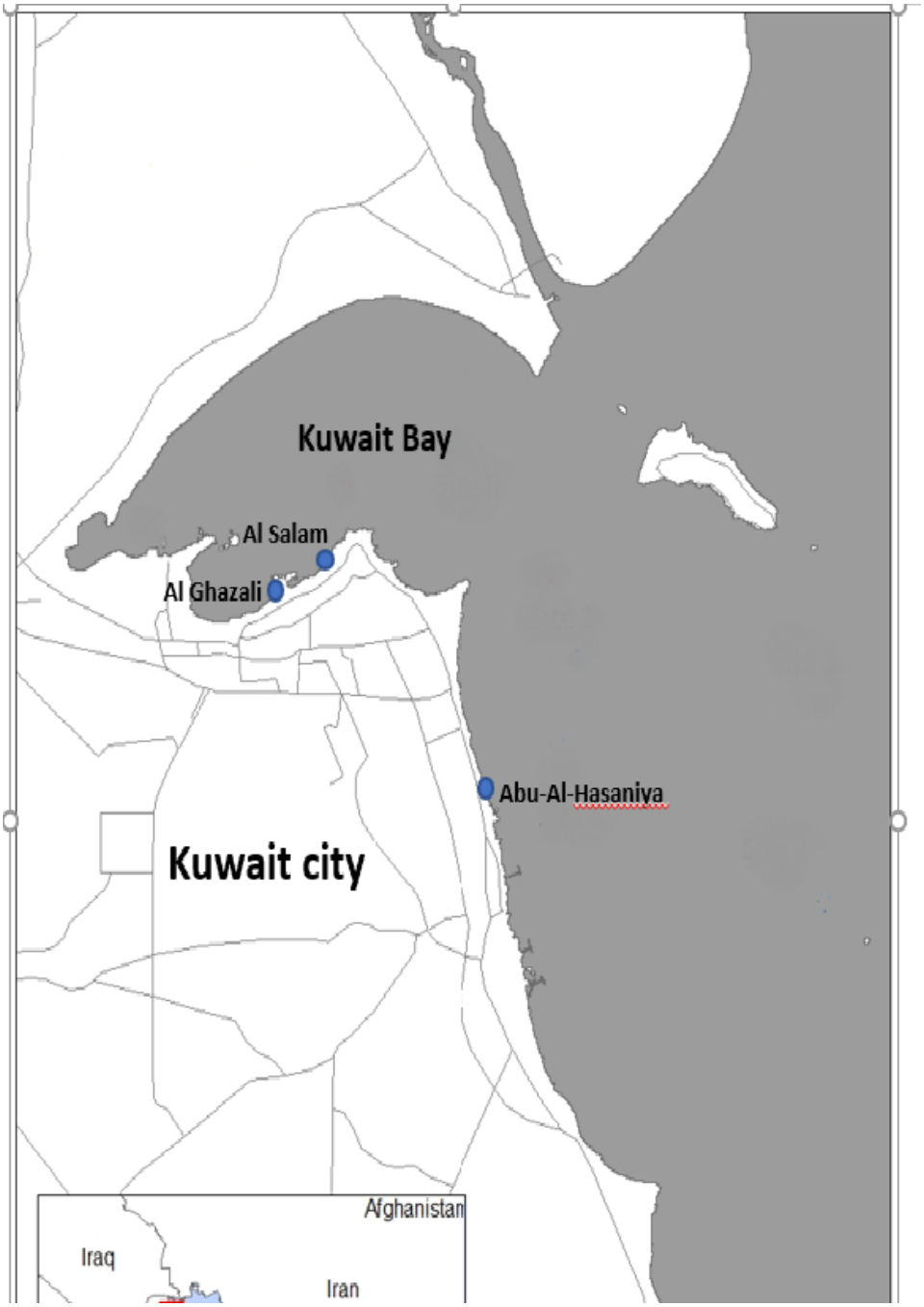
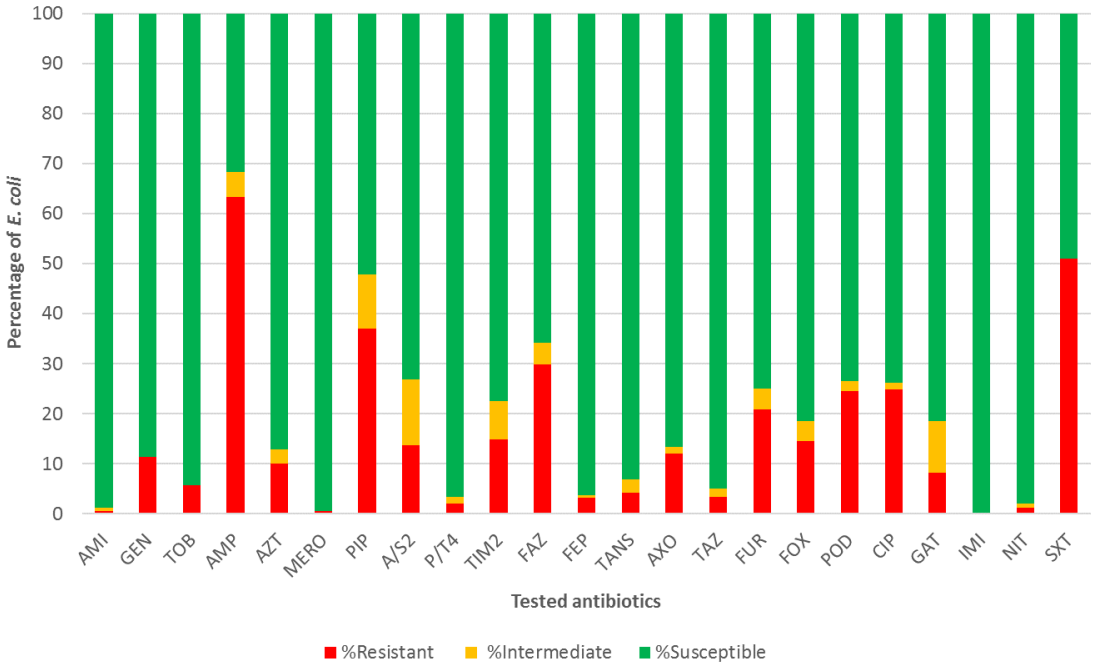
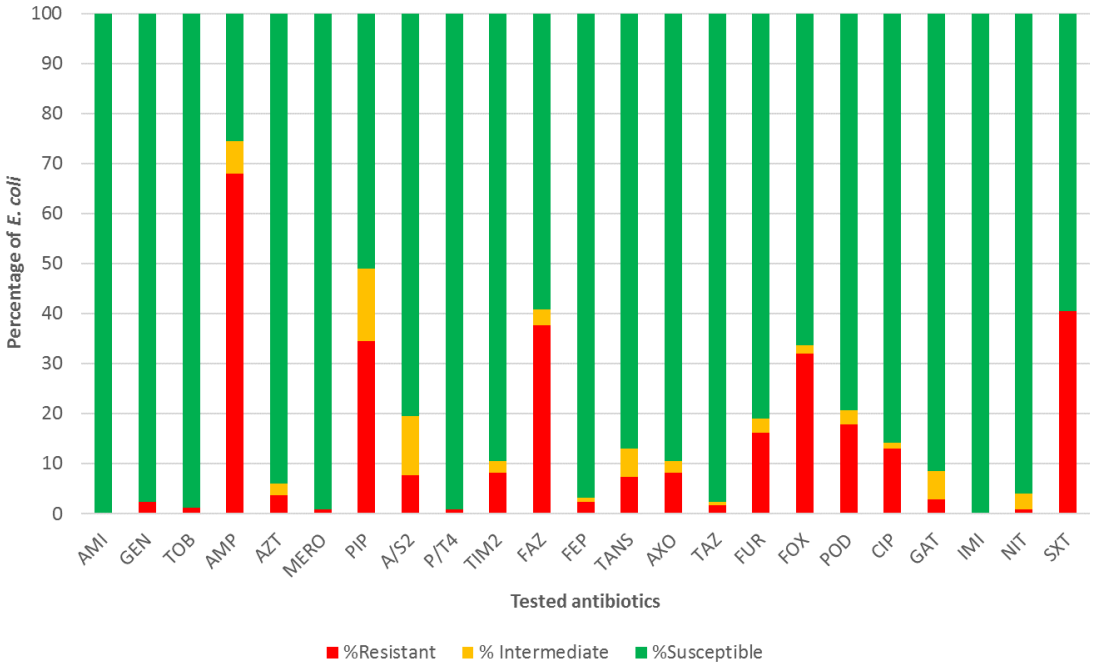


Figure 1 MAP of sampling sites for AMR analysis

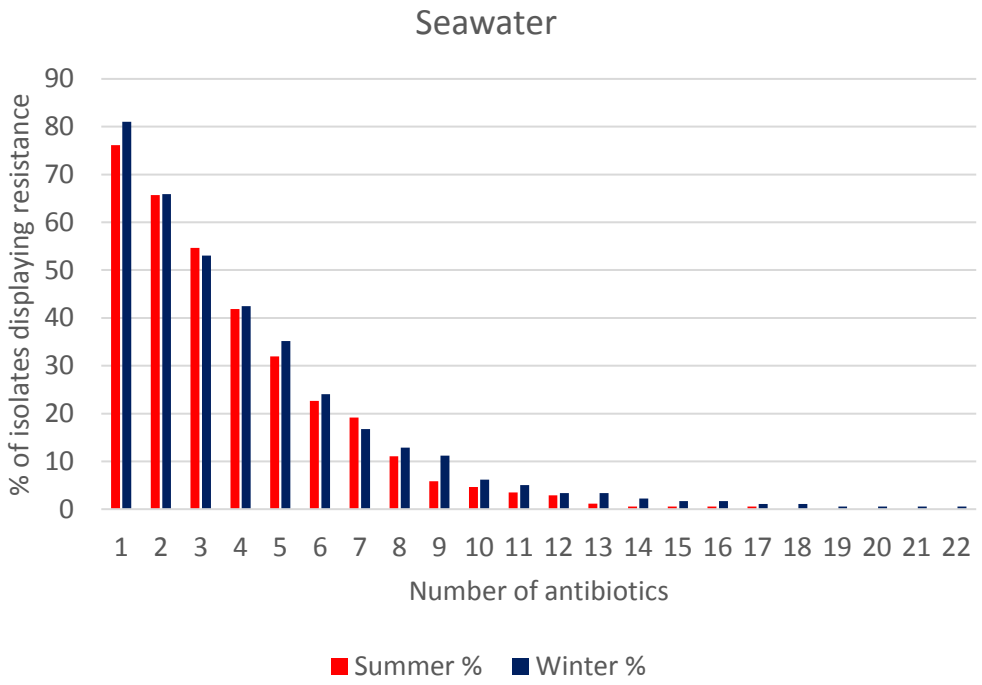

100
90
80
70
60
50
40
30
20
10
0
Percentage of E. coli
AMI
GEN
TOB
AMP
AZT
MERO
PIP
A/S2
P/T4
TIM2
FAZ
FEP
TANS
AXO
TAZ
FUR
FOX
POD
CIP
GAT
IMI
NIT
SXT
Tested antibiotics
%Resistant
%Intermediate
%Susceptible

A

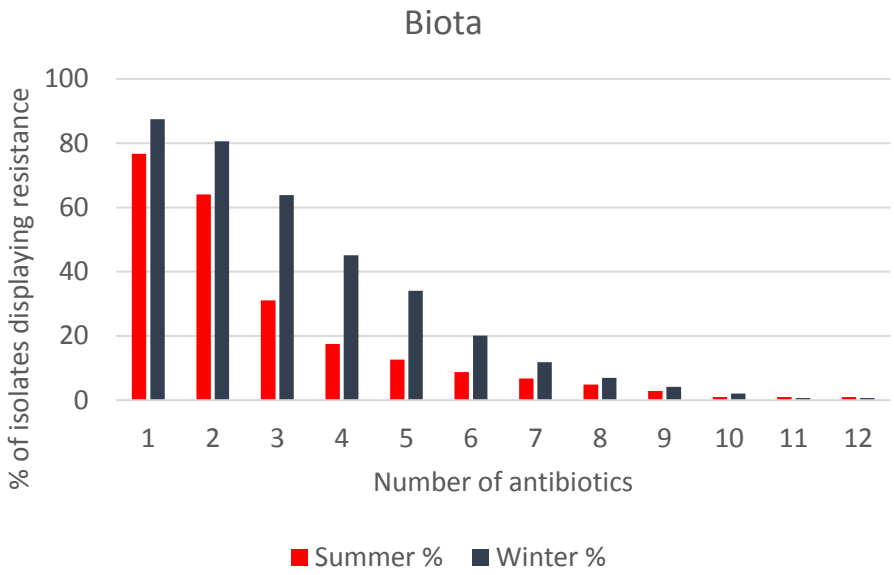


B

Figure 2: Profile of resistance patterns (Resistant = red; Intermediate = amber; Susceptible = green) to antibiotics in seawater (A) and biota (B). Antibiotics screened: Amikacin (AMI), Gentamycin (GEN), Tobramycin (TOB), Ampicillin (AMP), Aztreonam (AZT), Meropenem (MERO), Piperacillin (PIP), Ampicillin/sulbactan (A/S2), Piperacillin/tazobactam (P/T4), Ticercillin/clavulanic acid (TIM2), Cefazolin (FAZ), Cefepime (FEP), Cefotetan Na (TANS), Ceftriaxone (AXO), Ceftazidime (TAZ), Cefuroxime (FUR), Cefoxitin (FOX), Cefpodoxime(POD), Ciprofloxacin (CIP), Gatifloxacin (GAT), Imipenam (IMI), Nitrofurantoin (NIT) and Trimethorprim/sulfamethoxazole (SXT).


Seawater
% of isolates displaying resistance
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Number of antibiotics
Summer %
Winter %

A



B

Figure 3: The % prevalence of resistance to antibiotics in seawater (A) and biota (B) from summer (red) and winter (black) sampling periods.