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Developing an integrated strategy for the assessment of hazardous substances in Kuwait's marine environment

by

Hanan Ahmad Al-Sarawi

A thesis submitted to Plymouth University in partial fulfilment for the degree of:

DOCTOR OF PHILOSOPHY

In collaboration with

Centre of Environment, Fisheries & Aquaculture science (Cefas)

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Author's declaration

At no time during the registration for the degree of *Doctor of Philosophy* has the author been registered for any other University award without prior agreement of the Graduate Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at any another establishment.

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Abstract

Developing an integrated strategy for the assessment of hazardous substances in Kuwait's marine environment

Hanan Ahmad Al-Sarawi

Kuwait is undergoing rapid economic growth involving substantial developments along its coastal shores and the marine environment. Many of the activities in the region are associated with oil industry, which can pollute the shores leading to contamination from oil residues, tar balls and trace metals. About 2 million barrels of oil are spilled annually from routine discharges into the Gulf, which derives mainly from dirty ballast waters and tank washing. The comprehensive literature review of hazardous substances in Kuwait's marine environment has concluded that for the majority of these pollutants, contamination is localized around industrialised areas, and elsewhere is generally below the permitted international standards. These finding have been supported by a fieldwork. This has been conducted to assess the use of biomarkers (bile metabolites and EROD activities) for 60 fish consisting of two native species Giant sea catfish (*Arius thalassinus*) and (*Pelates quadrilineatus*) to demonstrate the potential for the concentrations of oil based contaminants present to induce detectable levels of biological effects in fish species living in Kuwait's marine environment. Therefore, the focus of this research has shifted its attention to another anthropogenic source that chronically pollute Kuwait's marine environment. One of the main sources of contaminants is the continuous discharge of sewage, which impacts many locations around Kuwait. Sewage is known to contain wide array of substances that could pose an ecotoxicological impact at different levels of the ecosystem. One such threat is posed by antimicrobial agents that contribute to the growing global

concern surrounding the prevalence of antimicrobial resistant (AMR) bacteria. Therefore, a major theme of this research project was to conduct a novel survey of the prevalence of AMR bacteria isolated from Kuwait's marine environment. The AMR screening, including 598 *E.coli* isolated from seawater and bivalves samples during summer and winter seasons against 23 frontline antibiotics, revealed that resistance was observed from a number of locations (particularly associated with sewage outlets) for the majority of antibiotics (seawater: summer 89 - 64%; winter 90 - 57% and bivalves: summer 77%; winter 88%). A baseline screening for the class 1 integron which is known to be implicated in disseminating the antimicrobial resistance among bacteria was conducted for the isolated 598 *E.coli.* The findings highlighted the prevalence of such molecular genetic elements especially around the sewage outlets (36% of tested *E.coli*). The whole genome sequencing was conducted for a representative *E.coli* (26 *E.coli*) and it showed that *E.coli* derived from Kuwait's marine environment possessed a variety of genes implicated in antimicrobial resistance potential against wide spectrum of antibiotics and suggesting that genes are exchanged via the horizontal gene transfer. These observations and recording of antimicrobial resistance phenomenon support the notion that marine environment could act not only as a reservoir for antimicrobial resistance but could also play a significant role in driving it. The AMR bacteria is considered as an effective tool for monitoring the impacts of sewage pollution. Furthermore, it highlights one of the key human health risks sewage pollution poses and its assessments allows a fully integrated health assessment of Kuwait's marine environment to be undertaken. This will ultimately lead to recommendations outlining the future monitoring and remediation requirements required by Kuwait to tackle this issue including rational antibiotics consumption and stewardship; developing effective wastewater treatment processes

to improve removal efficiency of these pollutants in sewage treatment plants; more researches on this area will provide scientific information for responsible authorities to make up regulatory standards and guidelines to control environmental dissemination of these emerging contaminants.

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marine environment during summer and winter (2014-2016) from sewage-contaminated sites inside Kuwait Bay (Al-Salam, Al- Ghazali and Esherij) and outside Kuwait Bay (Abu-Al-Hasaniya), the reference sites was Khiran.

Table 5.2 The AMR profile for the 26 E.coli which were subjected to the whole genome sequencing. The AMR test was conducted against the following antibiotics were tested: 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). The antibiotic's classes were as follow: (A) Aminoglycosides, (B) Beta-lactams, (C) Aminoglycoside/beta-lactamase inhibitors, (D) Beta-lactam/beta-lactamase inhibitor, (E) Cephalosporins, (F) Fluoroquinolones, (G) Imipenem, (H) Quinolone and (I) Dihydrofolate reductase inhibitor/sulphonamide. The interpretive criteria depending on (CLSI, 2014) where notified as green for (susceptible), red for (resistant and white for intermediate.

Table 5.3 Antimicrobial resistance genes detected in *E.coli* isolates by the whole genome sequencing approach for Sc59 which is susceptible for the tested antibiotics and KHE11 which is resistant to 19 of these antibiotics.

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List of abbreviations

1-OH pyrene	1-Hydroxypyrene
A/S2	Ampicillin/Sulbactam
ACDP	Advisory Committee On Dangerous Pathogens
AMD	Advanced Molecular Detection technologies
AMI	Amikacin
AMP	Ampicillin
AMR	Antimicrobial resistance
ARGs	Antimicrobial resistance genes
ARI	Antibiotic resistance indexing
AXO	Ceftriaxone
AZT	Aztreonam
BSA	Bovine serum albumin standard
cBWD	Coastal Bathing Water Directive
CFU	Colony-forming unit
CIP	Ciprofloxacin
CLSI	Clinical Laboratory and Standard Institute
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EA	UK Environment Agency
EMA	European Medicines Agency
ERL	Effects Range Low
ERM	Effects Range Median
EROD	7-Ethoxyresorufin O-Deethylase
FAO	Food and Agricultural Organisation
FAZ	Cefazolin
FEP	Cefepime

FI	Fluorescence Intensity
FOX	Cefoxitin
FS	Faecal *streptococci*
FUR	Cefuroxime
GAT	Gatifloxacin
GCC	Gulf Co-operative Council
GCs	Gene cassettes
GEN	Gentamicin
HGT	Horizontal gene transfer
IMI	Imipenem
ISQGs	International sediment quality guidlines
KEPA	Kuwait Environment Public Authority
KISR	Kuwait Institute of Scientific Research
KU	Kuwait University
LCL	Lower control limits
LOD	Limits of detection
LWL	Lower warning limits
MDR	Multidrug resistant
MERO	Meropenem
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MOH	Ministry of Health
MRIs	Multi-resistance integrons
ND	Non-detectable
ng g^{-1}	Nano gram per gram
NIT	Nitrofurantoin
NOAA	National Oceanic and Atmospheric Administration
ORFs	Open Reading Frames

OSPAR	Oslo and Paris Convention
P/T4	Piperacillin/Tazobactam constant
PAAFR	Public Authority for Agriculture and Fish Resources
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-*p*-dioxins
PCDFs	polychlorinated dibenzofurans
PCR	polymerase chain reaction
PEC	predicted environmental concentrations
PIP	Piperacillin
PNEC	predicted no-effect concentrations
POD	Cefpodoxime
POPs	Persistent organic pollutants
PPCPs	Pharmaceuticals and personal care products
QA/QC	Quality assurance/quality control
QAC	Quaternary ammonium compound
RNA	Ribonucleic acid
ROPME	Regional Organisation for the Protection of the Marine Environment
SCP	Salt and chlorine plant
SFS	Synchronous Fluorescence Spectrometry
SIA	Shuaiba Industrial Area
SQGs	Sediment quality guidelines
STP	Sewage Treatment Plants
SXT	Trimethoprim/Sulfamethoxazole
TANS	Cefotetan
TAZ	Ceftazidime
TEQ	Toxic equivalency
THC	Total Hydrocarbon Content

TIM 2	Ticarcillin/Clavulanic acid constant
TOB	Tobramycin
TOC	Total organic carbon
TPHs	Total petroleum hydrocarbons
UCL	Upper control limits
UCM	Unresolved complex mixture
UNGA	United Nations General Assembly
UWL	Upper warning limits
VBNC	Viable but non-culturable
VLH	Volatile liquid hydrocarbon
WGS	Whole genome sequencing
WHO	World Health Organisation
WWTPs	Wastewater treatment plants
YES	Yeast Estrogen Screen
$\mu g\ g^{-1}$	Micro gram per gram

Conferences, workshops and modules

Conferences poster and presentation

- Assessment of anthropogenic sources of pollution in the marine environment of Kuwait. Postgraduate Society Conference, University of Plymouth, 24th March 2015 (poster).

- Assessment of anthropogenic sources of pollution in the marine environment of Kuwait. Group meeting, University of Plymouth, 25th March 2015 (oral).

- A Baseline survey for anti-microbial resistance (AMR) *Escherichia coli (E.coli)* in Kuwait's marine ecosystem. A new challenge in Kuwait's Marine Environment. SETAC Arabian Gulf 1st Annual meeting, Qatar, 21st and 22nd March 2017 (oral presentation).

Workshops attended at Plymouth University

- Keeping laboratory records 29th October 2013.
- Work place health safety risk management for research students 24th October 2013.
- Effective reading: productive, creative and reflexive reading 24th October 2013.
- Immersive academic writing 25th March 2015.
- Research methods 26th March 2015.
- Meet the editors 27th May 2016.
- GIS workshop 15th November 2016.
- SPSS workshop 12th July 2017.

Workshops attended at Cefas

- Introductory training course in quality assurance in the analytical chemistry laboratory 17th-21st November 2013.
- Training in the quantification of bile metabolites, determination of Ethoxyresorufin O-Deethylase (EROD) activities and protein content in marine fish tissue.
- A broad range of microbiological and molecular techniques related to investigate the presence of anti-microbial resistant strains of bacteria in environmental samples. Analysis 3rd - 15th Aug 2014.

Modules attended at Plymouth University 2015/2016

- Managements of coastal environments.
- Postgraduate research skills.
- Investigation and assessment of contaminated environments.

Publications

Paper 1 Al-Sarawi, H. A., Jha, A. N., Al-Sarawi, M. A. and Lyons, B. P. (2015) 'Historic and contemporary contamination in the marine environment of Kuwait: An overview', *Marine Pollution Bulletin,* 100(2), pp. 621-8.

Paper 2 Al-Zaidan, A. S., Al-Sarawi, H. A., Massoud, M. S., Al-Enezi, M., Smith, A. J., Bignell, J. P., Green, M. J., Askem, C., Bolam, T. P., Barber, J. L., Bersuder, P. and Lyons, B. P. (2015) 'Histopathology and contaminant concentrations in fish from Kuwait's marine environment', *Marine Pollution Bulletin,* 100(2), pp. 637-45.

Paper 3 Al-Sarawi, H. A., Jha, A. N., Baker-Austin, C., Al-Sarawi, M. A. and Lyons, B. P. (2017) 'Baseline screening for the presence of antimicrobial resistance in *E. coli* isolated from Kuwait's marine environment' *Marine Pollution Bulletin, in press.*

Paper 4 Le Quesne, W., Baker-Austin, C., Verner-Jeffreys, D.W., Al-Sarawi, H. A., and Lyons B.P. (2017) 'Antibiotic Resistance in the Gulf Cooperation Council Region: developing a framework to assess potential threats, impacts and mitigation measures associated with antimicrobial resistance in the aquatic and marine environment *Marine Pollution Bulletin, in draft.*

Chapter 1 General Introduction

A part of this chapter has been published in a Special Issue of Marine Pollution Bulletin

'Hanan A. Alsarawi, Awadhesh N. Jha, Mohammad A. Al-Sarawi Brett P. Lyons. (2015), Historic and contemporary contamination in the marine environment of Kuwait: an overview. Marine Pollution Bulletin.100:621-628.

1.1 Introduction

The State of Kuwait is situated at the north-western corner of the Arabian Gulf, which is considered among the highest anthropogenically impacted regions in the world (Halpern et al., 2008, Sheppard et al., 2010). Its territorial waters are characterised by shallow seas, high summer water temperatures (>30^{o}C), intense UV exposure and elevated salinities (average 41‰) (Sheppard, 1993 , Al-Rifaie et al., 2007). A main feature of its marine environment is Kuwait Bay (Figure 1.1), a 750 km^{2} semi-enclosed shallow body of water, around 35 km wide with a mean depth of 5m (Al-Sarawi et al., 1988, Al-Abdulghani et al., 2013). The area provides vital habitats and nursery grounds for many fish, shrimp and other ecologically important marine organisms. The waters around Kuwait contain some of the most northerly coral reef systems in the world and are internationally recognised for maintaining biodiversity, supporting fisheries and promoting recreational activities (Al-Ghadban et al., 2002, Al-Ghadban and El-Sammak, 2005, Al-Rifaie et al., 2007).

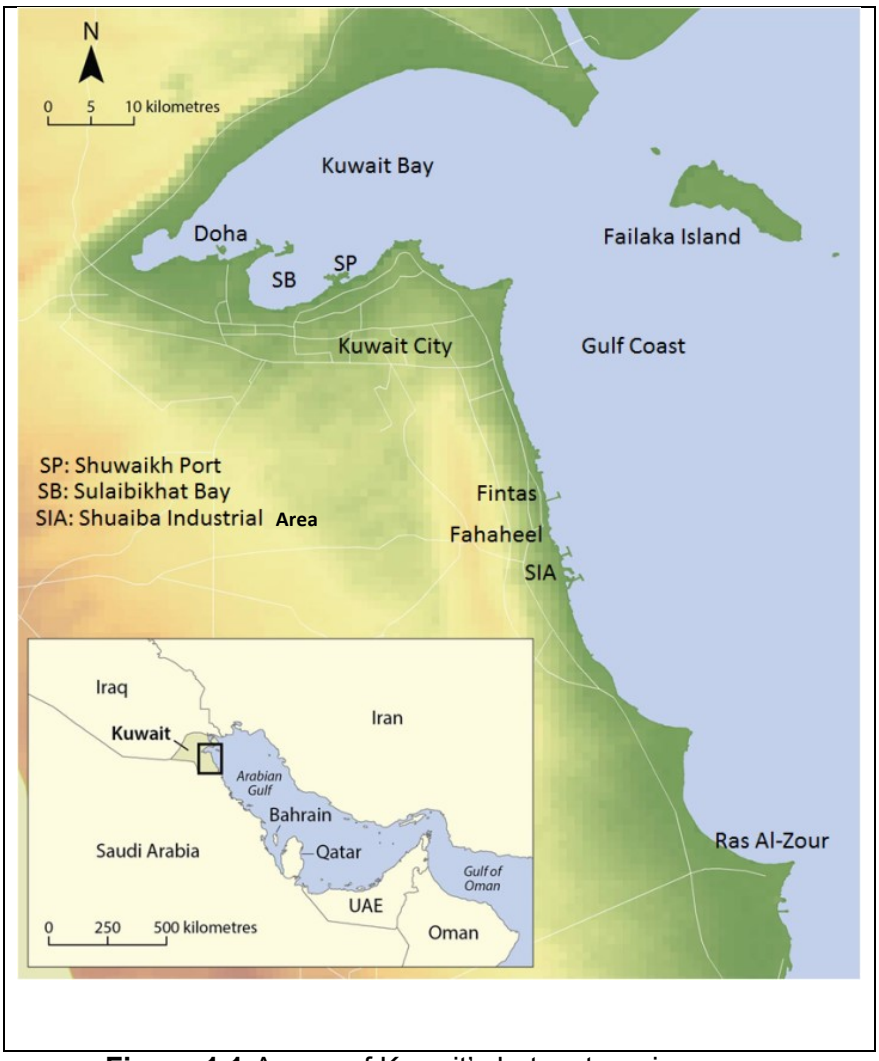

Figure 1.1 A map of Kuwait's hotspot marine areas

1.2 Sources of marine pollution in Kuwait

1.2.1 Oil exploration

Like many of the other countries that comprise the Gulf Co-operative Council (GCC), Kuwait has witnessed major economic, social and industrial development following the discovery and exploitation of its vast oil reserves (Al-Abdulghani et al., 2013). The Arabian Gulf is considered to contain the largest reserve of oil in the world (Literathy et al., 2002). Kuwait, along with other GCC states has expanded its industrial infrastructure to meet the demands of oil extraction, refining and transport leading to hot spots of marine degradation and contamination in areas associated with industrial activity (Al-Ghadban et al., 2002).

The rapid expansion of Kuwait's industrial sector has mainly occurred around its coastal margins. This has led to a variety of contaminants being discharged directly to the marine environment, including petroleum hydrocarbons, trace metals, nutrients (from domestic sewage), and contaminated brine from desalination plants, which are essential for the production of freshwater in the region (Readman et al., 1992, Al-Ghadban et al., 2002, Beg et al., 2003 , Al-Dousari, 2009). In addition, areas of natural oil seepage have been identified and are thought to be important point sources of contamination at various locations around the coast (Zarba et al., 1985, Massoud et al., 1996 , Massoud et al., 1998 , Al-Ghadban et al., 2002).

1.2.2 Impact of 1991 Gulf war

Historically, events such as the 1991 Gulf War, have intensified problems associated with rapid industrialization. During this period, it is estimated that 9 - 10.8 million barrels of oil, originating from sabotaged tankers and pipelines at the Al-Ahmadi terminal, were deliberately released into the coastal waters of Kuwait (Al-Abdali et al., 1996, Readman et al., 1996). As a result of the war, the environment was exposed to an array of contaminants which included pyrolytic petroleum hydrocarbons from burning oil wells; hazardous wastes from war damaged industries such as polychlorinated biphenyls (PCBs) and metals; along with spent or discarded munitions (Fowler et al., 1993 , Readman et al., 1996, Massoud et al., 1998). The impact of these activities are exacerbated by other sources of marine pollution that include atmospheric fallout from dust storms, natural oil seeps and particulate matter transported from the Shatt Al-Arab river that is formed by the confluence of the Euphrates and the Tigris in southern Iraq (Al-Ghadban et al., 2002, Al-Ghadban and El-Sammak, 2005). Given the environmental extremes of the region, many of Kuwait's marine species are likely to be functioning close to their physiological limits. Therefore, it is clear to see why concern has been raised by a number studies as to the additional role chemical

pollution may play in further stressing Kuwait's marine ecosystem (Al-Ghadban et al., 2002, Sheppard et al., 2010, Al-Abdulghani et al., 2013).

1.2.3 Sewage contamination

Kuwait is one of the Arabian Gulf region countries where the untreated, partially treated and treated wastewater is regularly discharged into the marine environment (Al-Ghadban et al., 2002, Al-Abdulghani et al., 2013, Lyons et al., 2015b). Reducing the input of sewage effluent into Kuwait's environment has been highlighted as one of the priority areas for management action (Saeed et al., 2015, Devlin et al., 2015a, Devlin et al., 2015b).

Unfortunately, the rapid urbanization of Kuwait, which has almost tripled since 1975, has not been matched by the corresponding development of wastewater treatment infrastructure. A large proportion of Kuwait's population situates along its coastal margin, 98% of Kuwait's 3.6 million inhabitants live within the 810 km^2 that covers the Kuwait Metropolitan Area (Figure 1.2). While Kuwait possesses modern tertiary and reverse osmosis treatment plants, they lack the capacity to meet the demand population growth has placed upon them (Saeed et al., 2012, Lyons et al., 2015b, Saeed et al., 2015). Currently, sewage is treated by 5 main Sewage Treatment Plants (STP), along with additional smaller facilities at Failaka Island, Al-Khiran and Al- Wafra. The STP network is currently being upgraded and it is expected that all of the produced sewage will received a tertiary or reverse osmosis treatment in the near future (Figure 1.3). However, until as recently as 2011 it was estimated the treatment network was receiving up to 100,000 m^3 day^{-1} of sewage above its design capacity (Lyons et al., 2015b).

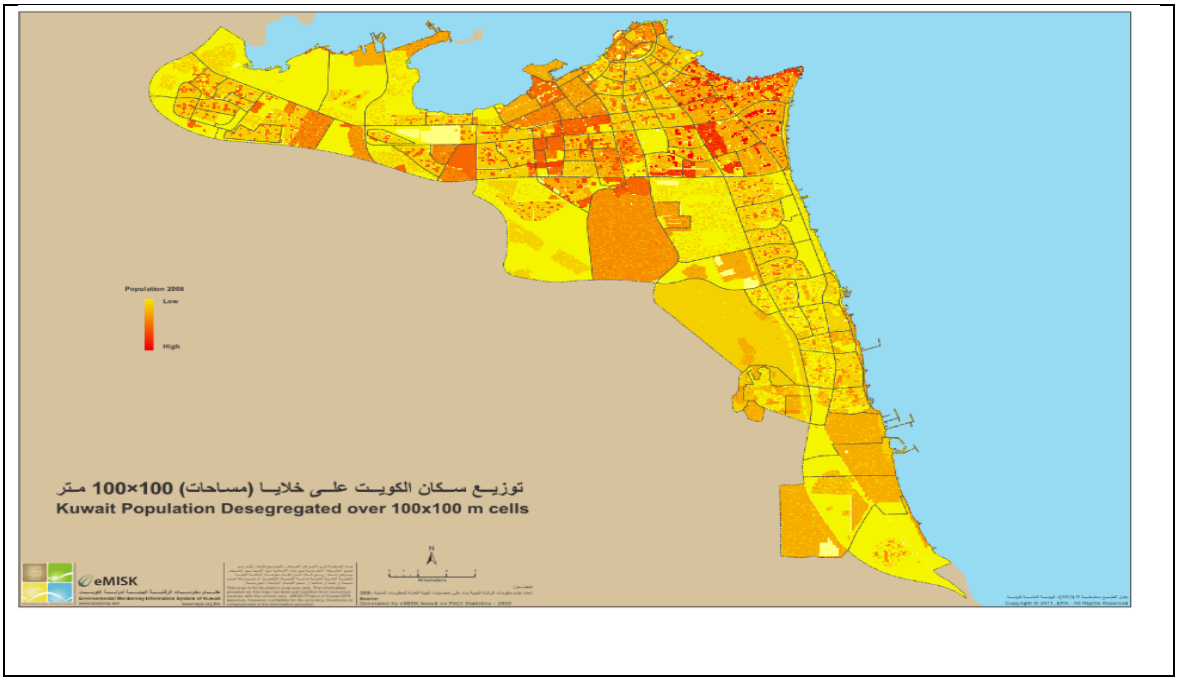

Figure 1.2 Kuwait population distribution. (Reproduced from www.bearona.net)

1.2.4 Mishref pumping station breakdown

In additional to the chronic sewage pollution issue, Kuwait has also suffered from sporadic environmental crises that increase the pressure on the environment. In recent years environmental disasters, such as the Mishref pumping station plant breakdown, have contributed to the degradation of Kuwait's marine environment (Saeed et al., 2012). The Mishref pumping station malfunctioned in August 2009, resulting in the discharge of around 150,000 m^3 day^{-1} of untreated sewage directly into the sea over a 24-month period (Lyons et al., 2015b). This vast amount of sewage discharge occurred via three main outfalls at Al-Bidda, Al-Khitabi and Al-Messela, and impacted beaches in a number of areas important for tourism and residential housing. Monitoring undertaken by Kuwait Environment Public Authority (KEPA) during this period indicated that approximately 20 km of coastline was affected, with a reduction in water quality parameters (e.g., ammonia and phosphate elevated) and bacterial indicators exceeding permitted guidelines (EPA, 2001). Such events further increase the pressure on an ecosystem that is chronically impacted with wastewater

and sewage discharges (Figure 1.3) (Saeed et al., 2012, Lyons et al., 2015b, Saeed et al., 2015).

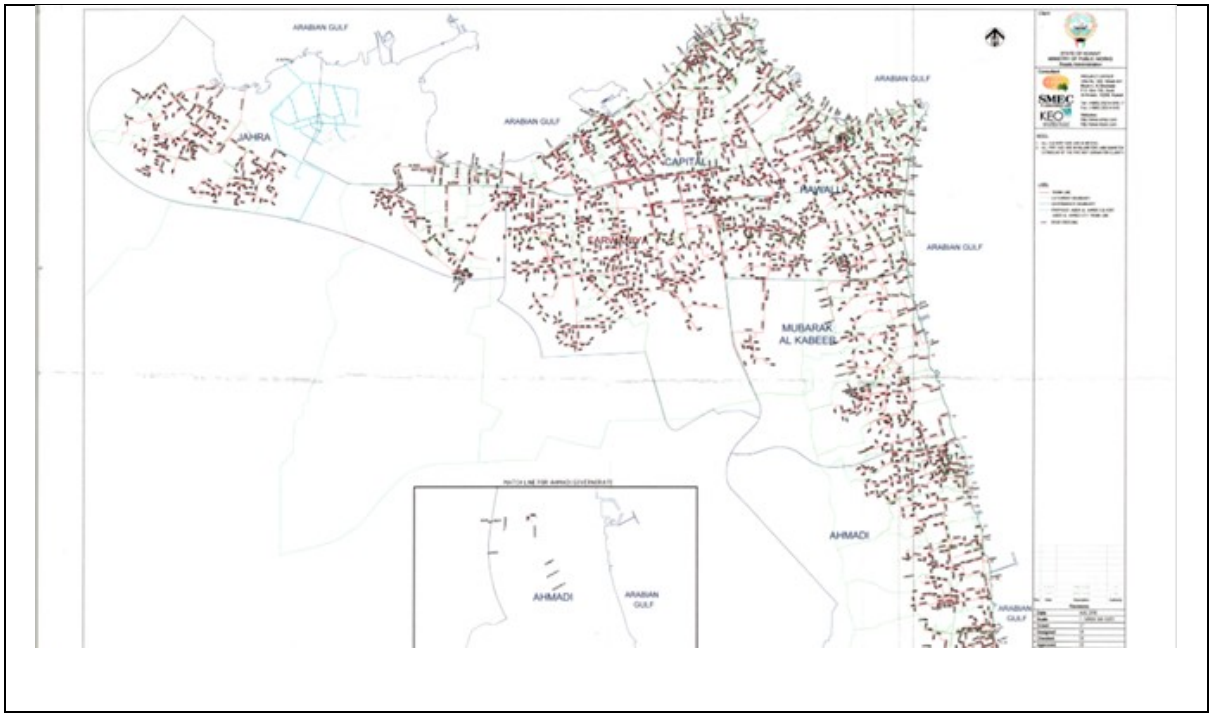

Figure 1.3 Kuwait sewage network outfalls reproduced from Ministry of Public works, Kuwait.

1.2.5 Desalination industries

The arid climatic conditions of Kuwait, associated with limited sources of natural fresh water, means that the human population depends heavily on desalinating waters collected from the Gulf as its major source of potable water (Al-Dousari, 2009, Freije, 2015). The combined seawater desalination capacity in the Gulf countries exceeds 11 million m^3 per day, which is 60% of the total world capacity (Purnama et al., 2005, Lattemann and Höpner, 2008). In 2002 Kuwait's annual production of desalinated water had reached 455 million m^3, representing over 93% of all the fresh water used in the country (Darwish and Al-Najem, 2005). While essential to the growth and prosperity of the region the desalination industries have an associated environmental cost. The regions desalination industries return over 7 km^3 per year of super saline water to the Gulf, which is usually warmer than receiving waters

and often containing pollutants such as biocides introduced to prevent biofouling (Hashim and Hajjaj, 2005, Sheppard et al., 2010). These can cause elevations of temperature (up to 5 °C) and salinity (up to 4‰) in the already warm and highly saline receiving waters (Al-Dousari, 2009).

1.3 Seawater contamination

Effluent discharges into Kuwait's marine environment come from a number of industrial and domestic sources and inputs span most of the country's 195km of coastline. The highest density of these point sources of pollution is associated with populated areas around the city or with industrial centres such as Doha and Shuaiba (Al-Ghadban et al., 2002). Sea water metal contamination, linked to the oil production/refining and desalination industries, have been the focus of a number of studies, including those conducted following the 1991 Gulf war. (Bu-Olayan et al., 1998b) ranked total mean seawater metal concentrations (Zn > V > Ni > Pb > M > C > Cr > Cd), with variation in contamination observed across sites spanning the whole coastline of Kuwait attributed to point source inputs. Similar seawater concentrations were reported by (Bu-Olayan et al., 2001a) who observed mean values (0.07 – 7.04 μg l^{-1}) of trace metals (Cu, Fe, Zn, Ni, Pb, Co) at a series of sites across Kuwait Bay and along the eastern coastline. (Al-Sarawi et al., 2002b) carried out a detailed survey of water and particulate samples collected from 48 locations within Sulaibikhat Bay. Seasonal differences were observed with elevated levels of trace metals detected in the summer months. Mean concentrations of dissolved phase trace metals were Cu 4.23 μg l^{-1}, Fe 100.0 μg l^{-1}, Zn 36.11 μg l^{-1}, Ni 0.8 μg l^{-1}, Pb 2.02 μg l^{-1}, V 14.5 μg l^{-1}, Cr 1.16 μg l^{-1}, and Mn 2.6 μg l^{-1}. Higher concentrations were found associated with particulate matter collected from the water column, with mean concentrations of Cu 90.5 μg g^{-1}, Fe 28,000 μg g^{-1}, Zn 351.4 μg g^{-1}, Ni 37.3 μg g^{-1}, Pb 70.4 μg g^{-1}, V 98.5 μg g^{-1}, Cr 152.3 μg g^{-1}, and Mn 54.8 μg g^{-1}. Similarly, (Bu-Olayan and Thomas, 2014) reported high Zn, Cr and Cu levels in wastewater

drain outfall samples, which were attributed to the effluents discharged from power plants, automobiles, paint industries, lubricants and domestic wastes from residential areas.

Apart from specific events related to the Gulf War the introduction of pollutants from the oil industry appear relatively low. The levels of total petroleum hydrocarbons (TPHs) were observed to remain relatively stable between 1988 and 1995 at a number of sites, which encompassed the whole of the Kuwait coastline. While values recorded peaked in 1991-1992 (8.3 – 22.1 μg l^{-1}) they were still considered to be within background levels (Al-Ghadban et al., 2002) (Al-Majed and Faraj, 1996). Higher values have been reported by (Bu-Olayan et al., 1998b) who, between 1993 -1994 observed PAHs (expressed as chrysene equivalents) ranging from 21.14 to 320.5 μg l^{-1} across a number of sites along the length of the Kuwait coastline. Direct comparison of these studies is difficult due to differences in the sites sampled and analytical methodologies employed. While reports on the concentrations of TPH and metals predominated the literature, only a few studies to date have focused on the toxic volatile liquid hydrocarbon (VLH) fractions of crude oil. VLH comprises hydrocarbon compounds such as benzene, xylenes and toluene, and can account to up to 40% of components of crude oil (Saeed et al., 1999a). Information on seawater contamination by VHL is particularly pertinent to the marine environment of Kuwait as they are known to co-distill with drinking water during the process of desalination (Ali and Riley, 1990). (Saeed et al., 1999a) reported concentrations in coastal seawater to be within the range 307 – 5017 ng l^{-1}, with benzenoids constituting 65% of the total VLH recorded. Work examining the contribution power and desalination plants make to the values detected was undertaken at Doha and Ras Al-Zor (Saeed et al., 1999b). Levels close to the intake and outfall pipes around the Doha power/desalination plant ranged from 307 to 7811 ng l^{-1}, while further south at Ras Al Zor values ranged from 465 to 4652 ng l^{-1}. Benzenoids (76-84%) dominated the makeup of the VHL, followed by *n*-alkanes (12-22%) and cycloalkanes (2-4%) (Saeed et al., 1999b).

1.4 Marine sediment contamination

In a comprehensive study undertaken to assess the impact of the 1991 Gulf War, attempts were made to develop estimates of natural background levels (upper permissible limits) of trace metal concentrations in the fine faction of Arabian Gulf sediments. Using data collected from both surface and sediments cores from 71 sites across the whole Arabian Gulf, guideline background levels were established for: Zn 30-60 μg g^{-1}, Pb 15-30 μg g^{-1}, Cd 1.2-2.0 μg g^{-1}, Ni 70-80 μg g^{-1}, Mn 300-600 μg g^{-1}, Fe 10,000-20,000 μg g^{-1}, V 20-30 μg g^{-1}, and Cu 15-30 μg g^{-1} (Al-Abdali et al., 1996). Studies conducted following the 1991 Gulf war indicated that metal contamination was restricted to a region extending 400km from the main sources of input (Fowler et al., 1993). The highest levels recorded were actually along the Northern coastal of Saudi Arabia, with levels recorded around Qaruh Island off the coast of Kuwait within ranges considered background for the region (Al-Abdali et al., 1996, Fowler et al., 1993). Further work to characterise heavy metal contamination was undertaken along a series of stations extending from Kuwait Bay and down along the coast south towards the border with Saudi Arabia (Metwally et al., 1997). The highest levels of Ni (range: 12.3 – 252.6 μg g^{-1}), Pb (range 71.55 -261.4 μg g^{-1}) and V (range: 24.8 – 179.41 μg g^{-1}) were observed close to industrial centres around Doha, Fintas and Shuaiba. Concentrations of all three of these metals levels fell considerably at sites removed from any known point sources of pollution (Metwally et al., 1997). Higher levels of metal contamination, which in some cases exceeded sediment quality guidelines, were reported by (Beg and Al-Ghadban, 2003). This was attributed to an influx of contamination following the draining of marshes in Iraqi to the north. Significantly these sediments also appeared to be intrinsically toxic when screened with a Microtox® solid phase bioassay test (Beg and Al-Ghadban, 2003). Al-Sarawi et al., 2002a, carried out an assessment of trace metal pollution in bottom sediments collected from of Sulaibikhat Bay, located in the south western corner of Kuwait Bay (Figure 1.1). Analysing samples collected over both summer and winter periods (Al-Sarawi et al.,

2002a) documented mean values of Cu 26.98 μg g^{-1}, Fe 24,900 μg g^{-1}, Zn 73.5 μg g^{-1}, Ni 12.95 μg g^{-1}, Pb 5.6 μg g^{-1}, V 67.87 μg g^{-1}, Cr 55.6 μg g^{-1}, and Mn 187.4 μg g^{-1}. The findings were similar to other data for the region and the concentrations of Cr, Pb, and Mn were all within their natural background levels (Al-Abdali et al., 1996). However, at certain locations within Sulaibikhat Bay the levels of Cu, Zn, Fe, and V were considered to be indicative of polluted sediment (Al-Sarawi et al., 2002a). The authors attributed this to the recent increase and diversification of both natural and anthropogenic inputs impacting this area that includes illegal sewage discharges and an outfall from a major desalination facility. A further detailed study of trace metal contamination in Sulaibikhat Bay has been recently undertaken, during which 34 sites were examined for grain size analysis, calcium carbonate and total organic carbon (TOC) contents, along with the determination of eight trace metals (Alshemmari et al., 2010). The levels recorded by (Alshemmari et al., 2010) varied across the Sulaibikhat Bay (Hg 0.1^{-1}.43 μg g^{-1}; As 1.13-6.63 μg g^{-1}; Cd 2.0-3.7 μg g^{-1}; Co 4.0-20 μg g^{-1}; Cr 65-190 μg g^{-1}; Cu 10-100 μg g^{-1}; Ni 25-130 μg g^{-1}; Zn 10-290 μg g^{-1}; PB 2-32 μg g^{-1}). While some of the concentrations recorded were clearly within the natural background levels previously reported by (Al-Abdali et al., 1996), the authors assessed the values obtained against a number of published sediment quality guidelines. Their analysis concluded that levels of As, Co and Pb were below any concentration thought to pose a toxicological threat to species living in Sulaibikhat Bay. Whereas at certain sites within Sulaibikhat Bay levels of Hg, Zn, Cu and Cr exceeded at least one of the sediment quality guidelines applied (Alshemmari et al., 2010). However, the validity of using sediment quality guidelines developed in other regions (such as Effects Range Low (ERL) and Effects Range Median (ERM) proposed by (Long and MacDonald, 1998) still need to be validated as in certain cases the background concentrations for the region as proposed by (Al-Abdali et al., 1996), actually exceed the established ERL/ERM criteria.

Attempts have been made to inventory total (T-) and methyl (Me) Hg contamination in Kuwait Bay. Work conducted by (Al-Majed Butayban and Preston, 2004) and (Bou-Rabee et al., 2006) investigated coastal sediment contamination resulting from inputs from a salt and chlorine plant (SCP) situated in Shuwaikh Port. It was estimated that during its operational life (1964-1985) approximately 20 tonnes of mercury was discharged into the coastal zone around the port area. In addition, there were airborne releases recorded from the plant (estimated to be 400kg) and other local sources of Hg pollution, such as untreated sewage, which discharged directly into Kuwait Bay. (Al-Majed Butayban and Preston, 2004) undertook an extensive spatial survey of surface and core sediment samples, which encompassed 103 different locations around Kuwait Bay. The study identified hot spots of T-Hg contamination (>180 ng g^{-1}) close to the former SCP outfall, with concentrations falling away to relatively low concentrations <30 ng g^{-1} at the northern side of the bay (Al-Majad and Preston, 2004). The presence of hot spots close to the former SCP outfalls in surface sediments was assumed to be due to remobilisation of historic contamination by dredging and mine sweeping activity (Bou-Rabee et al., 2006). Similar patterns were observed for MeHg, although the ratio of T-Hg to MeHg decreased with increasing distance from the SCP outfall. Analysis of several sediment cores indicated that historic contamination was mainly restricted to sites close to the Shuwaikh Port area, with the highest concentrations corresponding to peak production during the mid-1980s. Calculations made by the authors on the upper 40 cm of sediments from Kuwait Bay estimated a total of ~22.5 tonnes of T-Hg and ~80 kg MeHg to be present in the area studied, which correlated well with the known inputs over the life time of the plants operation (Al-Majed Butayban and Preston, 2004).

The analysis of total TPH and PAH contamination of sediments around the Kuwait coastline have been extensive, particularly in the period immediately following the 1991 Gulf War (Fowler et al., 1993 , Massoud et al., 1998 , Metwally et al., 1997 , Readman et al., 1996). Initial surveys conducted in 1991 indicated the TPH and PAH contamination was evident up

to approximately 400km from the sources of spilled oil (Readman et al., 1996). In subsequent surveys conducted between 1992 and 1993, these levels decreased by around 50%, with some small localised increases attributed to the resumption of industrial activity following the cessation of hostilities (Fowler et al., 1993 , Readman et al., 1996, Al‐Omran and Rao, 1997). Even at the heavily contaminated sites of Al-Bedaa and Az-Zor the concentrations of TPH (range: 40 - 240 μg g^{-1} dw) were relatively low when compared to contaminated sites in Europe and North America (Simpson et al., 1996, Woodhead et al., 1999). Metwally et al., 1997 reported the findings of a wider spatial survey which encompassed sites within Kuwait Bay and along the coastline towards the south of the country. Similar levels of TPH contamination were found with values ranging from 7.43 μg g^{-1} in reference sediments to 458.61 μg g^{-1} at sites close to point sources of industrial pollution around the Shuaiba Industrial Area (SIA). Apart from the elevated levels close to the SIA the majority of other sites were considered to contain concentrations of TPHs which reflected natural background values for the region (Metwally et al., 1997). This was supported by the findings of (Massoud et al., 1998) who undertook an extensive survey of the whole Arabian Gulf and categorised the majority of Kuwait's offshore sediments to be only slightly contaminated with TPHs (15-50 μg g^{-1}). While there has been some information pertaining to levels of chemical contamination, there has been limited studies to determine the environmental risk posed by these levels of contaminations. In this context, the environmental risk posed by sediments impacted by the oil spilled during the 1991 Gulf was assessed in an attempt to link TPH concentrations with mortality using a 96-hr marine amphipod toxicity test (Randolph et al., 1998). Thirty months after the spill sediment ecotoxicology tests using the phoxocephalid amphipod *Rhepoxynius abronius* indicated that only one out of 24 samples, collected from six different locations around Kuwait, was acutely toxic to the test organism. Analysis of the whole dataset (including samples taken along the Saudi Arabian coastline) demonstrated that *R. abronius* mortality was low when petroleum

hydrocarbons concentrations were below 1000 μg g^{-1} while those above this figure exhibited signs of acute toxicity (Randolph et al., 1998).

More recent studies conducted by (de Mora et al., 2010) reported hydrocarbon contamination from ten locations within Kuwaiti territorial waters. Conducted under the auspices of the Regional Organisation for the Protection of the Marine Environment (ROPME) and as part of a larger regional investigation, the study observed concentrations of TPH ranging from 2 to 251 μg g^{-1} dw, with hotspots of contamination documented in Sulaibikhat Bay, Ras Al Zour and Fahaheel. Applying the guidelines for TPH sediment pollution in the Gulf region proposed by (Massoud et al., 1996), where levels <15 μg g^{-1} dw chrysene equivalents are considered to represent natural background levels for the region, de Mora et al., (2010) characterised Sulaibikhat Bay (29 μg g^{-1} dw chrysene equivalents) as being slightly polluted. By investigating the unresolved complex mixture (UCM) aliphatics, (de Mora et al., 2010) was further able to gain a relative measure of chronic, degraded oil contamination at the sites investigated. A background concentration of 10 μg g^{-1} dw was exceeded only at Doha Bay, Ras Al Zour and Sulaibikhat Bay. Interestingly, further analysis of the high UCM/n-alkanes ratio (an oil weathering index) observed at Ras Al Zour suggested a more recent oil input when compared to other sites examined (de Mora et al., 2010). Similar levels of Total Hydrocarbon Content (THC), were reported from a series of sites within Kuwait Bay and along the coast towards Mina Abdulla (south of Shuaiba), with concentrations ranging from 4.2 to 744 mg kg^{-1}. Several studies have attempted to characterise the levels of sediment contamination close to the SIA, which is located along a 12.5 km stretch of coast south of Kuwait City (Beg et al., 2003 , Gevao et al., 2006a, Gevao et al., 2008, Gevao et al., 2009, Gevao et al., 2014). The SIA complex houses a number of significant industries including oil refineries, desalination facilities, fertilizer plants, a paper production company and a number of other small industrial units (Beg et al., 2003). The SIA is known to produce over 51,000 m^3 day^{-1} of effluent, 95% of which is discharged directly

into the sea (Al-Muzaini, 1998 , Gevao et al., 2006b, Gevao et al., 2009). The marine environment adjoining the industrial complex is known to be contaminated with a range of pollutants including PAHs, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polybrominated diphenyl ethers (PBDEs). In a detailed study of sediment PAH contamination (Beg et al., 2003) surveyed 21 locations covering a 15 km stretch of the coastline facing the SIA. The sampling regime included a number of transects moving in 0.5 km increments away from the shoreline. Particularly high levels of contamination were observed across a number of transects close to Shuaiba Harbour, with total PAH concentrations ranging from 201.27 – 1333.60 μg kg^{-1}. Such values are considerably higher than those reported at other locations around Kuwait (Saeed, 1999c, de Mora et al., 2010, Lyons et al., 2015a). Interestingly, this research noted that high molecular weight PAHs dominated the profiles at sites around the SIA, which differs when compared with the dominance of low molecular weight PAHs at sites in and around Kuwait Bay (Al‐Omran and Rao, 1997, Lyons et al., 2015a). At the most contaminated sites, located close inshore around the SIA, levels of individual PAHs including phenanthrene (maximum value recorded: 165.5 μg kg^{-1}), fluoranthene (maximum value recorded: 292.57 μg kg^{-1}) and benzo[a]pyrene (maximum value recorded: 94.75 μg kg^{-1}), exceeded international sediment quality guidelines (ISQGs) (CCME, 1999, Beg et al., 2003).

Wider spatial surveys of sediment PAH contamination have documented ΣPAH concentrations ranging from 12 to 1670 ng g^{-1} dw (de Mora et al., 2010) and 37 – 1332 ng g^{-1} dw (Lyons et al., 2015a). The study by de Mora et al., 2010 noted that the highest concentrations were observed at Ras Al Zour and Sulaibikhat Bay. The patterns of PAHs observed were indicative of petrogenic sources, though samples from Ras Al Zour and Sulaibikhat Bay exhibited a small contribution of combustion-derived PAHs (de Mora et al., 2010). In general, the levels reported by (de Mora et al., 2010) and (Lyons et al., 2015a) were still below international sediment quality guideline values, such as those established

for Effects Range Low (ERL: 4000 ng g^{-1} dw) by National Oceanic and Atmospheric Administration (NOAA), USA (Long and MacDonald, 1998, Long et al., 1995).

The marine area around the SIA has also been subject to a detailed survey of contamination by PCBs (Gevao et al., 2006b). The homologue distribution was observed to be dominated by penta-PCBs with the most abundant congeners being 138, 101, 110, 180, 153, 132, 149, and 118. The concentration of ΣPCB varied by two orders of magnitude across the survey location, ranging from 0.40 to 81.7 ng g^{-1} dw and were comparable to other international studies of industrialised coastal sediments reviewed by the authors. The highest values were associated with the port and sites close to waste water outlets, suggesting that atmospheric deposition of PCBs may not be a significant source of contamination in Kuwait (Gevao et al., 2006b). The depositional history of PCB sediment contamination has been studied in a number of cores collected from Sulaibikhat Bay. The data, which reflected sediment deposition over 37 ± 5 year period, demonstrated a peak in ΣPCB concentrations around 1991, after which values fall by 15x to the current sediment-water interface concentrations of around 2 ng g^{-1} dw (Gevao et al., 2012). The 1991 peak was attributed to the sudden input of PCBs following the destruction of a number of PCB-laden electrical transformers that occurred towards the end of the Gulf war. Two more recent studies have examined sediment PCB contamination at other locations within Kuwait's marine waters. ΣPCB concentrations of 96 – 5357 pg g^{-1} dw were reported by (de Mora et al., 2010) and these were considered to be generally low by global standards (Fowler, 1990). With respect to chlorinated hydrocarbons derived from industrial sources, relatively high levels of PCBs (based on the sum of Aroclors) were observed at only a few stations such as those near Doha (2.26 ng g^{-1}) and Sulaibikhat bay (7.84 ng g^{-1}). The amounts of PCBs in the sediments from all other locations were not exceptional, and comparable to levels reported elsewhere in the region (de Mora et al., 2004, Fowler, 2002).

Comparable low levels of contamination were documented by (Lyons et al., 2015a) with the majority of PCB congeners less than the 0.1 ppb detection limit reported. Therefore, the published data available would indicate that the PCB contamination is low, by global standards, for near shore marine sediments with the majority of samples reported not exceeding the sediment quality guideline value of 23,000 pg g^{-1} dry weight proposed by NOAA (Long et al., 1995).

Data on sediment contamination by other emerging contaminants of concern such as polybrominated diphenyl ethers (PBDEs), Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are extremely scarce within Kuwait and the wider Gulf region. The data available in Kuwait is mainly restricted to a handful of studies (Gevao et al., 2006a, Gevao et al., 2009, Lyons et al., 2015a, Gevao et al., 2014). These compounds are of environmental concern due to their persistence, high lipophilicity and resistance to degradation (Allchin et al., 1999, Gevao et al., 2009). As such they quickly bind to suspended particles upon entering the marine environment and are transported to bottom sediments where they may enter the aquatic food chain and pose a toxicological risk (Gevao et al., 2006a, Gevao et al., 2009). PBDEs, which are a class of chemicals widely used as flame retardants, share many characteristics with other well-known persistent organic pollutants (POPs), including PCBs and PCDDs. The first study of its kind conducted in Kuwait was undertaken by (Gevao et al., 2006a). Focusing their studies on the SIA the authors investigated PBDE concentrations in a series of transects moving offshore from known point sources of pollution at different locations around the complex. Data indicated that BDE 153, 154 and 183 were most dominant forms of PBDEs detected with BDE 183 typically accounting for 60% of the congener mix. Values of PBDEs ranged from 0.08 to 3.8 ng g^{-1} dw. Studies conducted on a wider spatial scale, covering offshore sediment samples along with those within Kuwait Bay reported a generally lower level of PBDE contamination although the composition of PBDE differed with BDE 209 the dominant conger (Gevao et

al., 2014, Lyons et al., 2015a). While the mix of congeners analysed differed between all the studies reported the overall levels were similar with ΣPBDEs ranging from 0.06 to 0.44 ng g^{-1} (Gevao et al., 2014) and <0.02 to 0.35 ng g^{-1} dw (Lyons et al., 2015a). The analysis of core samples taken at the entrance to Kuwait Bay demonstrated that detectable levels of PBDE first appeared above background concentrations in the mid-1950s with peak inputs associated with periods of military conflict (Gevao et al., 2014). In global terms the concentrations of PBDEs reported for Kuwait's marine environment are low with values often an order of magnitude below those reported for locations in Northern Europe (Allchin et al., 1999), Japan (Watanabe and Sakai, 2003) and China (Luo et al., 2007).

Studies on the spatial distribution of the "dioxin like" compounds, PCDDs and PCDFs, which are anthropogenically introduced into the environment as by-products of industrial activities, have also been undertaken around the SIA (Gevao et al., 2009). Marine sediments were collected from ten locations around the port area and close to known effluent discharge points. PCDD/PCDFs concentrations varied widely across the sample locations, ranging from 0.40 to 313.4 pg g^{-1} dw, with the pattern of PCDD/PCDFs detected indicative of point sources of pollution rather than atmospheric deposition. (Gevao et al., 2009) converted the recorded values to their toxic equivalency (TEQ). The PCDF congeners contributed the majority of the TEQ (72%) calculated, with the highest values recorded close to an oil loading terminal and cement factory. When comparing the TEQs with those previous reported in the literature, values were generally low and representative of background concentrations from sites in New Zealand and Europe (Gevao et al., 2009).

1.5 Contaminants in marine biota

Primary producers are known to accumulate a wide range of chemical contaminants and are therefore considered important keystone species in the assessment of contaminant bioavailability and impact in marine ecosystems. A series of studies have attempted to

investigate the temporal and spatial distribution of metal contamination in phytoplankton collected from Kuwait coastal waters which were impacted by differing levels of industrialization (bu-Olayan et al., 2001b, Bu-Olayan et al., 2001a, Bu-Olayan and Thomas, 2004). The reported concentrations of metals differed across the sites examined, with samples collected close to the industrialised shoreline at Doha (Zn: 51.20 – 75.01; Cu: 48.51 – 67.10; Ni: 18.71 – 56.17; Pb: 33.40 – 61.80 μg g^{-1}); displaying the highest level of contamination (Bu-Olayan et al., 2001a). Toxicity tests undertaken in parallel with the field sampling demonstrated a decrease in oxygen rate with increasing metal burden (Bu-Olayan et al., 2001a). Seasonal differences were also observed with Fe and Co being statistically different between the four seasons sampled (Bu-Olayan et al., 2001b). Interestingly, analysis of phytoplankton abundance at Doha indicated a greater richness than that seen at other sites examined, which was attributed to the local environmental conditions and increased nutrient inputs in the area (Bu-Olayan et al., 2001b). Levels of Hg have also been assessed in zooplankton, collected in a series of transects across Kuwait Bay. Concentrations were generally low with Total Hg (T-Hg) ranging from 0.004 – 0.035 μg g^{-1} dw and methyl Hg (MeHg) accounting for <25% of T-Hg with authors noting concentrations observed were lower than those previously reported from North America (Al-Majed and Preston, 2000b).

Various invertebrate bio-monitoring species have been used to assess metal pollution in the marine environment around Kuwait. Studies conducted pre and post war investigated levels of metal contamination in the marine gastropod snail *Lunella coronatus* and Pearl oyster, *Pinctada radiate* (Bu-Olayan and Subrahmanyam, 1997). The concentrations of Cu, Ni and Zn all significantly increased in samples collected after the Gulf war when compared to those collected before the onset of hostilities. Work conducted by (Tarique et al., 2012) assessed the suitability of the bivalve clam *Amiantis umbonella* to monitor metal (Cd, Cr, Cu, Hg, Ni, Pb, V and Zn) contamination in coastal sediments collected from Kuwait Bay. An impacted

site and remote reference location was sampled in parallel with the impact site located close to a desalination and power plant in the Doha region of Kuwait Bay. The authors observed significant differences between sites for the majority of metals and tissues examined with clam kidney tissue the most reliable in reflecting external (water and sediment) metal contamination at the sites investigated (Tarique et al., 2012). Importantly this study also highlighted the inter-site differences that occur in bivalve soft tissues, related to both environmental and biological factors. Differences were observed in the size of the organs, with gonad weight five-fold lower at the contaminated site compared to those collected from the reference site, with authors suggesting the differences could have accounted for some of inter-site observed differences in metal contamination (Tarique et al., 2012). Further studies using *A. Umbonella*, along with the analysis of sediment and porewater samples, reported a decreasing trend in metal concentration in a 5km gradient moving away from a point source discharge associated with a power/desalination plant in Kuwait Bay (Tarique et al., 2013). The majority of the metals examined in the tissues of *A. Umbonella* were highly correlated with levels in the sediment than in porewater. The highest concentrations of Cd (7 mg kg^{-1} dw), Pb (3 mg kg^{-1} dw) and Hg (1-9 mg kg^{-1} dw) observed in Kuwait Bay breeched human consumption food safety limits as recommended by the Food and Agricultural Organisation (FAO) and World Health Organisation (WHO) (Tarique et al., 2013). However, this species is not a commercially exploited, so the risk to the human population via consumption is considered low. Elevated metal contamination was also detected in the gills of the gastropod *Cerithium scabridum* collected from around Doha with the authors attributing this to the influence of industrial effluents from the power and desalination industries in the area (Bu-Olayan and Thomas, 2001).

The effect of size and sex on the metal content of lobsters (*Thenus orientalis*) has been determined in animals caught from two locations between Fahaheel and Al-Kiran along the coastline of Kuwait (Bu-Olayan et al., 1998a). Significant differences between male and

female lobsters were observed for mean As, Se and Hg concentrations. Significantly, the authors reported that concentrations of As in lobster muscle tissue exceeded both regional (0.5 μg g^{-1}) and international (10 μg g^{-1}) food safety limits (Bu-Olayan et al., 1998a). Similar concentrations were also reported for Cu, Ni, Pb, V and Zn in the marine crabs, *Macrophthalmus depressus* (Bu-Olayan and Subrahmanyam, 1998) and *Portunus pelagicus* (Al-Mohanna and Subrahmanyam, 2001) with the highest concentrations associated with coastal sites close to industrialised areas of Kuwait Bay. Bu-Olayan and Al-Yakoob, (1998) reported assessments of Pb, Ni and V contamination in six fish and two shrimp species collected from Kuwait local fish markets. Data from demographic surveys of consumer eating habits showed there was no serious risk to the human population through the consumption of seafood from the three metals (Bu-Olayan and A.H., 1998). (Al-Zaidan et al., 2015) also reported levels of Hg, Cd and Pb to below concentrations thought to be of concern to human health in the Giant sea catfish (*Arius thalissinus*) Trace metals concentrations (Zn >Fe >Cu >Ni >Cr 24 >Pb >Hg) have been reported in sea bream (*Acanthopagrus latus*), with higher concentrations of metals found in the sequence of liver > gills > muscles, irrespective of the season and location of sampling (Bu-Olayan and Thomas, 2014). The accumulation of Hg through the aquatic food chain has been studied in zooplankton and commercially important fish species (Al-Majed and Preston, 2000a). T-Hg concentrations in zooplankton ranged from 0.004 -0.035 μg g^{-1} with MeHg accounting for <25% of the total. Values in fish were far higher (0.073 – 3.923 μg g^{-1}) and a significant difference in body burden was observed between species depending on their feeding habits. Assessments undertaken by the authors indicated that 20.6% of the fish sampled failed the WHO permissible limits for T-Hg (≥ 0.500 μg g^{-1}) and MeHg (≥ 0.300 μg g^{-1}). The relative proportion of MeHg to T-Hg also differed significantly between fish species, which was thought to have important health human implications. For the majority of fish examined a consumption rate of 200 g d^{-1}, which is common amongst some consumer groups in Kuwait,

approached or exceeded US-EPA limits set for MeHg (Al-Majed and Preston, 2000b, Al-Majed and Preston, 2000a).

As part of the large regional study undertaken by (de Mora et al., 2010) relatively minor TPH contamination have been reported for samples of Venus clam (*Circentia callipyga*) samples collected from Doha Bay, Al-Bida'a and Khiran, with values ranging from 15 – 40 μg g^{-1} dw (TPH equiv, ROPME oil). de Mora et al., 2010 noted these differences were observed between the sediment and clam PAH profiles collected from Doha Bay, suggesting that sediment bound contaminants were not the primary source of biota contamination. In the same study concentrations of TPH (2.3 – 2.7 μg g^{-1} dw TPH equiv, ROPME oil) and ΣPAH 2.9 - 3.5 ng g^{-1} dw were reported in Hamoor (*Epinephelus coioides*) and considered unexceptional and representative of uncontaminated fish by the authors. Concentrations of PAHs have been measured across both summer and winter months in the whole body of 3 fish species native to Kuwait waters (Beg et al., 2009). Seasonal differences in concentrations were observed both within and between the species examined, with the profile of high and low molecular weight PAHs indicating they were mainly from a petrogenic origin (Beg et al., 2009). Attempts have been made to estimate the risk PAH contamination of seafood caught from Kuwaiti waters pose to human consumers (Al-Yakoob et al., 1994, Saeed et al., 1995). Market based sampling of shrimp and fish from a range of trophic and ecological levels was matched to consumption figures for the key species examined (Saeed et al., 1995). Similar to the work of (Beg et al., 2009). the profile of detected PAHs indicated petrogenic sources of contamination with naphthalene (2.06 – 156.09 μg kg^{-1}) and phenanthrene (5.51 – 88.74 μg kg^{-1}) in particular the most abundant. Matching this data with consumer consumptions habits indicated that concentrations detected posed little if any public health concerns (Saeed et al., 1995).

There is little information available on the bioaccumulation of other POPs in marine biota from Kuwait. The comprehensive study by (de Mora et al., 2010), reported on levels of a range of chlorinated hydrocarbons, including PCBs, in the Venus clam and Hamoor. While levels reported were not indicative of high levels of pollution, they were still elevated compared to other samples collected from the Gulf region. Information on PBDEs, a class of chemicals commonly used as flame retardants, is limited to a handful of papers. Their accumulation in edible fillets from three commercially important fish species, yellow fin sea bream (*Acanthopagrus latus*), Klunzingers mullet (*Liza klunzingeri*) and tounguesole (*Cynoglossus arel*) has been reported in two areas, within and outside Kuwait Bay (Gevao et al., 2011). The dominant congeners detected were BDE 28, 47, 99 and 100, which accounted for >90% of the total PBDEs detected. The authors reported no significant difference between fish collected from inside or outside Kuwait Bay. Among the three species however differences were observed, with mullet (ΣPBDE range 11-160 ng g^{-1}) significantly higher than sea bream (ΣPBDE range 7.1 – 62 ng g^{-1}) (Gevao et al., 2011). Such differences between species were proposed to reflect a combination of factors including the ecological niche of the individual species and their average tissue lipid content (which is far higher in mullet). The authors under took a comparison exercise using lipid normalised concentrations of BDE 47. Values ranged between 1.8 – 48 ng g^{-1}, within fish collected from Kuwaiti waters, which were comparable to other international studies conducted in Greenland and the Baltic Sea. The values were however, an order of magnitude below those concentrations detected in fish close of a known production source in the UK and USA (Allchin et al., 1999, Gevao et al., 2011, Hall and Collis, 1998).

Conclusions The majority of studies would indicate that while Kuwait's marine environment has been subjected to a wide array of pollution events, the actual levels of contamination remains relatively low when compared to heavily industrialised regions elsewhere in the world. This finding is supported by a review study for metal and PAHs contaminants in the

Arabian Gulf (Freije, 2015). Studies to date have mainly focused in metal and petroleum/combustion hydrocarbon contamination, with only a limited of reports documenting other priority POPs (PCBs, PBDEs etc). Where studies have documented elevated level of contamination they have tended to be localised and associated with specific point sources of pollution, such as the major industrial facilities based at Doha, Sulaibikhat Bay and the SIA (Al-Ghadban et al., 2002, Al-Sarawi et al., 2002a, Beg et al., 2003 , Gevao et al., 2006a, Gevao et al., 2006b, Gevao et al., 2009, Tarique et al., 2012). The risk posed by such pollution hot spots remain to be fully determined and while attempts have been made to empirically evaluate risk (Beg and Al-Ghadban, 2003, Beg et al., 2003 , Alshemmari et al., 2010), they must be viewed with a degree of caution as natural background levels of certain contaminants within the Gulf may already exceed certain international sediment quality guidelines. A small number of studies attempted to evaluate the human health risks posed by the consumption of fish and crustaceans and results would suggest that PAHs (Beg et al., 2009, Saeed et al., 1995) pose little risk, while As in lobsters (Bu-Olayan et al., 1998a) and Hg in fish (Al-Majed and Preston, 2000a) have been reported to exceed human consumption limits. It is encouraging to see a growing number of studies attempt to linked toxicological endpoints to the study of Kuwait's marine contamination (Randolph et al., 1998, Beg and Al-Ghadban, 2003, Stentiford et al., 2014, Al-Zaidan et al., 2015, Smith et al., 2015). Such studies also highlight the need for research into so called emerging contaminants (e.g. pharmaceuticals) or the potential impact of chemical mixtures which to date have received little or no attention in Kuwait or the wider Gulf region.

1.6 Emerging contaminants

In the last decade, emerging contaminants have started to receive greater global attention, as researchers have investigated the risk pharmaceuticals and personal care products (PPCPs) pose to the marine environment (Crane et al., 2006, Ellis, 2006, Sui et al., 2012,

Bu et al., 2013, Liu and Wong, 2013). The term PPCPs covers a diverse collection of chemical substances, including human and veterinary drugs used to prevent or treat a wide variety of diseases (e.g. antibiotics), hormones, disinfectants and fragrances used in personal care products, ranging from lotions, body cleaning products to sun-screens; and domestic chemicals used to improve the quality of daily life (Kolpin et al., 2002, Thomas and Hilton, 2004, Richardson et al., 2005, Boxall et al., 2012, Liu and Wong, 2013, Bu et al., 2013).

1.6.1 Pharmaceuticals and personal care products PPCPs: Sources and Occurrence

PPCPs are a concern, as through global development and population expansion they are being produced and used in ever increasing amounts (Jones et al., 2002, Crane et al., 2006, Boxall et al., 2012, Liu and Wong, 2013, Bu et al., 2013, Wang et al., 2015b, Al-Khazrajy and Boxall, 2016). The worldwide annual production of PPCPs exceeds 1 x 10^6 tonnes (Daughton and Ternes, 1999, Richardson et al., 2005). For example, in China, which is the world's largest producer/ consumer of pharmaceutical products, with more than 25,000 tonnes of antibiotics administered annually (Bu et al., 2013). In Germany, over 600 tonnes of antibiotics are used annually and some 300 tonnes are used annually in each of France, Italy and Spain (Ellis, 2006). In the UK, over 3000 active substances are licensed for use, with over 2000 tonnes of paracetamol, 770 tonnes of acetylsalicylic acid and 106 tonnes of metformin being consumed annually (Thomas and Hilton, 2004, Ellis, 2006).

In general, PPCPs are non-degradable substances and are only slightly transformed during wastewater treatment plant process (WWTPs), which rely on conventional methods such as flocculation, sedimentation and active sludge treatment (Heberer, 2002, Boxall et al., 2012, Bu et al., 2013). This means many PPCPs enter to the aquatic environment unchanged and

it has been shown that concentrations can be in the μg l^{-1} range in sewage effluents and seawater downstream of municipal STPs (Heberer, 2002, Liu and Wong, 2013). Therefore, STPs and other facilities where they are directly applied (e.g. farms or aquaculture facilities) can act as major sources of PPCP input into the aquatic environment (Thomas and Hilton, 2004, Boxall et al., 2012, Bu et al., 2013, Liu and Wong, 2013).

Numerous studies have now identified PPCPs in aquatic environments in countries such as in USA, UK, Spain and Finland, Canada and Switzerland (Heberer, 2002, Crane et al., 2006, Boxall et al., 2012, Liu and Wong, 2013, Bu et al., 2013). Bu et al., 2013 reviewed the occurrence of 112 major PPCPs (including roxithromycin, ibuprofen and sulfamethoxazole) in waters and sediments around three major megacities in China. In most cases, levels were at ng l^{-1} (water) or ng g^{-1} (sediment) concentrations, which is comparable to other studies conducted elsewhere. Undertaking a risk assessment (Heberer, 2002, Crane et al., 2006). The same author named the top six PPCPs as erythromycin, roxithromycin (macrolide antibiotics), diclofenac, ibuprofen, salicylic acid (anti-inflammatory drugs) and sulfamethoxazole (a sulphonamide antibiotic). Hotspots of PPCPs were clearly associated with major urban areas, with major classes of emerging contaminants detected including antibiotics non-detectable (ND)-776 ng l^{-1} and hormones (ND-180 ng l^{-1}) (Bu et al., 2013).

In the UK, Thomas *et al*., (2004) conducted a study in order to establish the occurrence of pharmaceutical compounds in estuaries. Surface water samples collected from five UK estuaries were analysed by using liquid chromatography coupled to electrospray mass spectrometry for the presence of 14 pharmaceutical compounds selected from the priority lists of the UK Environment Agency (EA) and the Oslo and Paris Commission (OSPAR). These compounds included clofibric acid (herbicide), clotrimazole (anti-fungal), dextropropoxyphene, mefenamic acid (analgesic), diclofenac, ibuprofen, (anti-inflammatory drugs) , propranolol, tamoxifen (medications), and trimethoprim (antibiotic), which were

detected at measurable concentrations in the various samples collected. In contrast, the concentrations of other PPCPs including lofepramine (antidepressant), paracetamol (analgesic), erythromycin, sulfamethoxazole, acetyl-sulfamethoxazole (antibiotic) and were all below the limits of detection of the methods used (ranging between 4 - 20 ng l^{-1}). Clotrimazole (an anti-fungal agent) was the most frequently detected at a median concentration of 7 ng l^{-1} and the analgesic compound ibuprofen was detected at a median concentration of 48 ng l^{-1}. The other pharmaceutical compounds were detected at concentrations ranging between the limits of detection of the method used and 570 ng l^{-1} (Thomas and Hilton, 2004).

Worldwide, the contamination of surface water with antibiotics has been shown to be generally below µg l^{-1} with concentrations reported to be below limits of detection to 694 ng l^{-1} as reviewed by (Liu and Wong, 2013). However, studies have also shown that concentrations in impacted environments, such as those in China, can be as high as 6800 ng l^{-1} for norfloxacin (Zou et al., 2011). Similar studies from the US documented peak concentrations of 1900 ng l^{-1} of sulfamethoxazole in polluted streams (Kolpin et al., 2002). Generally, concentrations of hormones, which are one of the major PPCPs groups, do not exceed 20 ng l^{-1} according to most reports from various global studies (Ferguson, 2013). However, it has been shown that in polluted environments such as the US, they can reach 872 ng l^{-1} for 19-norethisterone which is an ovulation inhibitor (Kolpin et al., 2002, Liu and Wong, 2013).

1.6.2 Pharmaceutical toxicity

The ubiquity of PPCPs in all types of environment raises attention to the potential threat they pose to both the environment and human health (Boxall et al., 2012, Al-Khazrajy and Boxall, 2016). Therefore, there is clearly a need to monitor and assess the risk they pose to the marine environment (Crane et al., 2006, Boxall et al., 2012, Liu and Wong, 2013, Al-Khazrajy

and Boxall, 2016). The majority of PPCPs are persistent or pseudo- persistent (due to the continuous release of steady-state concentrations) in the environment (Ellis, 2006). They can be toxic to non-target organisms and have the potential to accumulate throughout the food chain to higher trophic levels (Crane et al., 2006, Bu et al., 2013, Liu and Wong, 2013). All these characteristics contribute to many PPCPs posing a significant threat to the environment from both ecological and human health perspectives (Crane et al., 2006, Ellis, 2006, Zou et al., 2011, Boxall et al., 2012, Bu et al., 2013, Liu and Wong, 2013).

There is a paucity of knowledge concerning the ecotoxicological effects of many pharmaceuticals, although the bioaccumulation potential of PPCPs may point towards their adverse effect on a longer-term scale. For example, in China, PPCPs (e.g. fluoroquinolone) have been found in concentrations up to 254.6 ng g^{-1} (w/w) in the liver tissue of fish from marine aquaculture regions of the Pearl River delta (He et al., 2012). However, until now little evidence is available on the adverse effect of antibiotics in vertebrates (fish) at realistic environmental concentrations (Crane et al., 2006).

In general, it is not thought that PPCPs pose a direct acute threat based on the ecotoxicological data available and the current concentrations measured in the environment. However, chronic effects are much more likely due to their persistent nature and specific modes of action (Crane et al., 2006, Ellis, 2006, Bu et al., 2013). One of the most widely studied impacts of PPCPs is that of the decline in wild vulture populations, which has been attributed to the residues of a veterinary medicine, diclofenac, being present in the carcasses of livestock. Studies such as those conducted in Pakistan document declines in vulture populations of up to 95% since 1990 (Oaks et al., 2004). While examples from the aquatic environment are not as common, the same drug was also implicated in causing vitellogenin induction in Japanese medaka (*Oryias latipes*) at concentrations in the environment measured at only 1 µg l^{-1} (Hong et al., 2007). From these examples, it is clear

that standard acute ecotoxicity data may not be suitable for addressing the risk posed by PPCPs, given their narrow scope of biological activity/ effect and potency (Thomas and Hilton, 2004, Crane et al., 2006). However, the chronic ecotoxicologial effects due to the continuous input of PPCPs, which potentially accumulate in biota could be significant (Bu et al., 2013, Liu and Wong, 2013). Chronic bio-toxicity assays had better reflect an organism's exposure to different concentrations of a chemical over a long or substantial part of their lifespan. These generally include measures of effects on reproduction and development, as PPCPs could pose, in long term, an irreversible negative effects on wildlife and human (Liu and Wong, 2013). Which means more attentions and efforts should be paid (Thomas and Hilton, 2004, Crane et al., 2006). Whereas acute tests tend to focus on mortality as the only measurable effect (Crane *et al*., 2006).

The best of our knowledge, very little data are available on the chronic ecotoxicological effects of PPCPs on aquatic organisms. The chronic effects of PPCPs are reported on representative organisms occupying different trophic levels, including microorganisms where substantial effects are recognised for several antibiotics on slow the reproduction of some bacteria assemblage e.g. *Vibrio fischeri* (Froehner et al., 2000). At higher trophic levels, studies have shown algae to be sensitive to several PPCPs classes, such as fluoroquinolone, sulfonamide antimicrobial agents and β-adrenergic receptor blockers (Liu and Wong, 2013, Crane et al., 2006). The sensitivity is varied to different PPCPs. For example, antibiotics such as roxithromycin, clarithromycin and tylosin caused growth inhibition at concentrations ranging from 40 - 64 μg l^{-1} for exposure span of three days in *Pseudokirchneriella subcapitata,* whereas triclosan and triclocarban caused the same effect with concentrations ranging from 0.4 - 10 μg l^{-1} over the same time span (Yang et al., 2008). There is a paucity of information on the PPCPs effects on aquatic benthic species, even though the sediment is a known to be a sink for many PPCPs that enter the environment

and consist a persist resource of these contaminants to the sediment dwelling organisms such as invertebrates (Heberer, 2002, Crane et al., 2006).

Hormones are the most studied PPCPs group, which have the clearest evidence for potential impacts related to the growing dataset demonstrating their adverse effects (Crane et al., 2006, Bu et al., 2013, Liu and Wong, 2013). Evidence has clearly linked hormones such as estrogen (E1), 17β- estradiol (E2) estriol (E3) and synthetic steroids that mostly are used in oral contraceptive such as ethinylestradiol (EE2), to a range of endocrine-disrupting related effects, such as reduced fertility, feminization of males and the intersex phenomena in aquatic species, even at ng l^{-1} concentration levels (Khanal et al., 2006, Liu and Wong, 2013). The oestrogenic potency of these hormones can be a great as 10,000 - 100,000 x higher than exogenous endocrine-disrupting chemicals, such as organochlorine aromatic compounds (Khanal et al., 2006, Liu and Wong, 2013). It is now recognised that many aquatic species are affected by the hormones that are being released into the environment, with impacts being observed in species ranging from turtles, crucian carp, trout and minnow (Khanal et al., 2006, Liu and Wong, 2013). For example, a wide range of therapeutic classes (including oestrogens and androgen methyltestosterone) can cause effects on invertebrates at concentration levels less than 1 mg l^{-1}. For example, ethinyloestradiol causes an adverse effect at very low concentrations (around 1 ng^{-1}) in invertebrates such as snails and amphibians (Belfroid and Leonards, 1996, Oetken et al., 2005). Globally, it is believed that aquatic vertebrates (e.g. fish) are highly sensitive to the endocrine modulation exposure by many PPCPs (Crane et al., 2006, Khanal et al., 2006). In goldfish (*Carassius auratus*), endocrine disruption and oxidation stress was caused by exposure to 2000 µg l^{-1} caffeine (a stimulant drug) for 7 days and 1.5 µg l^{-1} synthetic musk(fragrance) for 7 - 21 days, respectively (Li et al., 2012). In addition, fish are sensitive to other sex hormones such as methyl testosterone and β-adrenergic receptor blockers such as propranolol (β-blocker) that reduce the percentage of viable eggs of Japanese medaka (*Oryias latipes*) (Huggett et al.,

2002). At a concentration 5 ng l^{-1}, E2 induced the production of female-specific proteins in male Japanese medaka (Tabata et al., 2001). Moreover, personal care products have also been shown to have endocrine disrupting effects, including the antibacterial and antifungal agent triclosan and cosmetic preservatives including parabens that used as UV filters in sun creams (Liu and Wong, 2013, Crane et al., 2006).

These studies all identify the gaps in the ecotoxicological effects that may pose by the PPCPs in the environment to be addressed by researchers in order to fully understand the environmental impact of PPCPs on a long-term scale which occurs in the marine environment (Thomas and Hilton, 2004, Crane et al., 2006).

1.6.3 Risk assessments and prioritization of PPCPs

More than 4000 PPCPs are currently documented as being in use (Ellis, 2006, Khanal et al., 2006, Boxall et al., 2012). Prioritization and ranking approaches are important for focusing the screening, monitoring and research resources to target compounds that may pose an environmental risk (Boxall et al., 2012, Al-Khazrajy and Boxall, 2016). Many prioritization approaches have been developed, such as those based on the number of prescriptions, toxicity information, human metabolism and wastewater treatment removal information (Daughton and Ternes, 1999, Stuer-Lauridsen et al., 2000, Jones et al., 2002, Liu and Wong, 2013). Many studies which have developed risk assessments of PPCPs are based on the European Medicines Agency (EMA) guidelines, in which the risk quotient is calculated as the ratio between predicted environmental concentrations (PEC) and predicted no-effect concentrations (PNEC) (EMA, 2006). If the risk quotient is equal to or higher than one, it suggests that particular substances could cause potential adverse ecological effects (EMA, 2006 , Boxall et al., 2012, Liu and Wong, 2013). Many studies in England show that where the PEC/PNEC ratios is equal to or higher than one, PEC/PNEC ratios have been used to prioritise drugs in the aquatic environment (Jones et al., 2002). The study revealed that

PEC/PNEC ratios of paracetamol, amoxycillin, oxytetracycline, and mefenamic acid exceeded one. In Denmark (Stuer-Lauridsen et al., 2000), the top 25 pharmaceuticals were evaluated for the risk they posed to the aquatic environment using the PEC/PNEC ratio approach (Stuer-Lauridsen et al., 2000). This revealed that the PEC/PNEC ratios of ibuprofen, acetylsalicylic acid, and paracetamol were found to exceed one whereas the estrogens ratios reached one. Another prioritization study assessing the pharmaceutical consumption, removal performance in the wastewater treatment plants, and potential ecological effects was conducted in China (Sui et al., 2012). This study concluded that erythromycin, diclofenac, and ibuprofen had the ranked highest in terms of priority among the 39 pharmaceuticals studied. The authors suggested that these PPCPs should be closely monitored in China based on the prioritization criteria used (Sui et al., 2012). These studies support that those pollutants could pose a risk to organisms in the marine environment (Stuer-Lauridsen et al., 2000, Boxall et al., 2012, Liu and Wong, 2013).

In the Arabian Gulf region, little is currently known about the occurrence, effects and potential risks that pharmaceuticals pose in the environment. However, a recent study has attempted a risked- based prioritization of the top 99 PPCPs used in three of Iraq's major cities (Al-Khazrajy and Boxall, 2016). The study revealed that paracetamol, amoxicillin and metformin are the top PPCPs used in Iraq, with annual consumption exceeding 1000 tonnes $year^{-1}$. The pharmaceuticals were ranked based on the risk they posed to the receiving environments by investigating their individual PECs/PNECs ratios. The authors concluded that antibiotics, antidepressants and analgesics were the highest priority in surface water, sediment and terrestrial environments (Al-Khazrajy and Boxall, 2016).

1.6.4 Antimicrobial agents and emergence of antimicrobial resistance

AMR

Antimicrobial agents (e.g. antibiotics) are major pharmaceutical groups that are administered to both humans and animals on a vast scale globally. They are used as a therapeutic approach to control and treat infections in humans, aquaculture, agriculture (to promote the growth in animal farming) and are used in food preservation (Crane et al., 2006, Kümmerer, 2009a, Bu et al., 2013, Liu and Wong, 2013). 'Antimicrobial agent' is a broad terminology that includes agents which, in small amounts, can inhibit the growth of microbial organisms (biostatic agents) or kill them (biocidal agents) (Kümmerer, 2009a). Traditionally, these agents only included naturally occurring substances such as penicillin (produced by fungi in the genus *Penicillium*) and streptomycin from (*Streptomyces griseus)* bacteria (Martínez, 2008, Kümmerer, 2009a). However, recently developed antibiotics include substances produced by chemical synthesis, such as the sulfanilamide drugs (e.g. sulfamethoxazole) and chemically-modified compounds of natural origin such as aminopenicillins (Baquero et al., 2008, Al-Bahry et al., 2009b, Kümmerer, 2009a, Kümmerer, 2009b, Al-Bahry et al., 2011b, Taylor et al., 2011). Antibiotics can be classified based on their mode of action, chemical structure or spectrum of activities into different classes (Degnan, 1997, NCCLS, 2000b, Kümmerer, 2009a, Peach et al., 2013). Depending on their mode of action, antibiotics can be classified into β-lactam and non-β lactam agents. β-Lactam agents work on the inhibition of cell wall synthesis as the primary mode of action. While non-β lactams agents vary in their modes of action e.g. by inhibiting cell membrane synthesis, protein synthesis or the bacterial folate pathway. Non-β lactams also include agents that disrupt deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis by, for example, inhibiting DNA gyrase and DNA-dependent RNA polymerase Table 1.1 (Degnan, 1997, NCCLS, 2000b, Kümmerer, 2009a, Peach et al., 2013).

Table 1.1 Antimicrobial classes depending on the mode of actions (Baquero et al., 2008, Kümmerer, 2009a, Kümmerer, 2009b).

Antibiotics class	Examples	Mode of action	Targeted strains
β-Lactams:		Inhibition of cell wall synthesis	
penicillin	Aminopenicillins (ampicillin and amoxicillin) Carboxypenicillins (carbenicillin and ticarcillin) Ureidopenicillins (mezlocillin and piperacillin) Penicillinase-stable penicillins(cloxacillin,dicloxacillin,methicillin,nafcillin and oxacillin)		Non-β lactamase producing, aerobic, Gram-positive, some fastidious, aerobic, gram negative, hsome anaerobic bacteria
β-Lactam/β-lactamase inhibitor combinations	Clavulanic acid, sulbactum,tazobactam	Additionally, minimize bacterial activities	
cephems	Cephalosporin (1,2,3,4 generations) Cephamycin, oxacephem, carbacephems		gram-positive,gram-negative, aerobic/anaerobic bacteria
penems	Carbapenems	Additionally resistant to β-lactamase hydrolysis	Gram positive and gram negative

monobactams	Aztreonam		gram-negative, aerobic
Nonβ-Lactams:			
aminoglycosides		Inhibit bacterial protein synthesis at ribosomal level	Aerobic, Gram negative
Folate pathway inhibitors	Sulphonamides, trimethoprim	Inhibition of bacterial folate pathway	Gram positive,Gram negative
Glycopeptides	Vancomycin,teicoplanin	Inhibition of cell wall synthesis at different sites that that of the β-Lactams.	Aerobic, gram positive
lipopeptides	Polymyxin, daptomycin	Inhibition of cell membrane synthesis	Both gram positive and negative bacteria
macrolides	Azithromycin, clarithromycin, dirithromycin, ketolide telithromycin and fluroketolide solithromycin	Inhibit bacterial protein synthesis at ribosomal level	Fastidious, gram negative bacteria
nitroimidazoles	Metronidazole, tinidazole	Bactericidal: disrupt host deoxyribonucleic acid (DNA)	anaerobic bacteria

Oxazolidinones	Linezolid	Inhibit protein synthesis	Gram positive
quinolones	Quinolones, fluoroquinolones	Inhibit DNA-gyrase or topoisomerase activity	Both gram positive and negative bacteria
streptogramins	Quinupristin-dalfopristin, linopristin-flopristine	Inhibit protein synthesis	Gram positive bacteria
tertacyclines	Doxycycline, minocycline	Inhibit protein synthesis	Both Gram positive and negative
Single-drug classes:	Chloramphenicol (phenicol) Clindamycin (lincosamides) Fucidic acid (steroidal) Mupirocin (psedudomonic acid) Spectinomycin (aminocyclitols)	Inhibit protein synthesis	
	Rifampin (ansamycin) Fidaxomicin (macrocyclics)	Inhibit Ribonucleic acid (RNA) synthesis	

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Persistent environmental exposure to antibiotics can lead to the emergence of resistance in bacterial populations on a global scale (Crane et al., 2006, Zhang et al., 2006, Kümmerer, 2009a, Martinez, 2009, Al-Bahry et al., 2009b, Al-Bahry et al., 2012, Liu and Wong, 2013). Worldwide, it is thought that the indiscriminate usage and the fast development of antibiotics, from sulphonamide in the past to the wide powerful spectrum antibiotics of today, is exacerbating the antimicrobial resistance (AMR) potential in bacterial communities (Kümmerer, 2009a, Al-Bahry et al., 2011b). Such potential has been observed not only in clinical pathogens and normal enteric bacteria but also in environmental indigenous bacterial communities that are now showing increased resistance to antibiotics (Baquero et al., 2008, Al-Bahry et al., 2009b, Taylor et al., 2011, Moskot et al., 2012).This clearly poses a threat to the environment including both aquatic animals and ultimately human health (Crane et al., 2006, Wright et al., 2008, Kümmerer, 2009a, Martinez, 2009, Al-Bahry et al., 2009b, Taylor et al., 2011, Liu and Wong, 2013, McArthur et al., 2015). The emergence of resistance potential in bacterial communities is rapidly exacerbated in the environment and is now drawing serious attention worldwide (Baker-Austin et al., 2006, Al-Bahry et al., 2009b).

Aquatic environments can act a reservoir for resistance gene acquisition, due to accumulation of antimicrobials, biocides and other pollutants, along with abundance of different bacterial communities e.g. enteric bacteria and pathogens (Taylor et al., 2011). Bacterial communities acquire such potential when subjected to wide array of antibiotics, which are released into the environments via anthropogenic sources. Consequently, the resistance potential against these antibiotics develops as antimicrobial resistance genes (AGRs) are gained from other bacteria via horizontal gene transfer (HGT) mediated by different mobile genetic elements MGEs such as class 1 integron (Wright et al., 2008, Al-Bahry et al., 2009a, Al-Bahry et al., 2011b, Taylor et al., 2011, Di Cesare et al., 2016b). AMR bacteria have been detected in range different aquatic environmental matrices (Verner-Jeffreys et al., 2009, Wang et al., 2014). Genes conferring antibiotic resistance have been

detected in bacteria collected from many settings, including hospitals, livestock and water resources (Taylor et al., 2011, Al-Bahry et al., 2012, Moskot et al., 2012, Liu and Wong, 2013, Finley et al., 2013, Miranda et al., 2013, Fajardo et al., 2014, Domingues et al., 2015).

In the clinical setting, the issue of AMR has received considerable interest in China, where its prevalence has been examined in 128 hospitals (Liu and Wong, 2013). Over 270,000 bacterial isolates were screened and a resistance against wide range of used antibiotics rate of ~ 80% was observed, which was considered to pose a significant threat to human health. Wide spread consumption of antimicrobial agents to treat infectious diseases in both humans and animals has led to promoting (AMR) in aquatic environments (Crane et al., 2006, Taylor et al., 2011, Liu and Wong, 2013). This spread of environmental AMR has become a worldwide concern from both a human health and environmental perspective (Baker-Austin et al., 2006, Al-Bahry et al., 2009b, Taylor et al., 2011).

Environmentally, one of the few studies on AMR in the Arabian Gulf region reported the presence of resistant bacteria in marine fish and seawater collected from various locations in Oman impacted by sewage discharges (Al-Bahry et al., 2009b). The study screened 320 isolates, mainly comprising *E.coli* and *Enterobacter spp.*, for resistance against 14 different frontline antibiotics using the disk diffusion method. At the polluted locations, resistance was commonly seen for antimicrobial agents including ampicillin, tetracycline and amikacin (Al-Bahry et al., 2009b). Significantly, these antimicrobial agents are some of the most frequently prescribed antibiotics in Kuwait (Table 1.2). In a follow-on study, Al-Bahry et al., (2012) used AMR bacteria as a bioindicator to monitor the exposure of turtles (*Chelonia mydas*) to different marine pollutants to help assess factors contributing to the decline of this endangered species. Here, they isolated 42 different forms of antibiotic resistant bacteria (e.g. *E.coli*, and *Enterobacter spp.*) from oviductal fluid collected from 20 turtles during their

nesting period, with resistance to ampicillin the most common observation (Al-Bahry et al., 2012).

Table 1.2 Top 26 antibiotic consumption medicine in State of Kuwait depending on Kuwait Ministry of Health statistics for years 2010-2014.

RANK	Antibiotic	RANK	Antibiotic
1	Co-Trimoxazole	14	Cefuroxime
2	Clindamycin	15	Penicillin
3	Clarithromycin	16	Minocycline
4	Amoxicillin	17	Piperacil+Tazobactum (Tazocin)
5	Ciprofloxacin	18	pyrazinamide
6	Amox+clavulanic.A (Augmentin)	19	Ceftriaxone
7	Cephalexin	.20	EthambutolHCl (Mayambutol)
8	Nitrofurantoin	21	Dex+Neomycin (Maxitrol)
.9	Doxycycline (Vibramycin)	22	Lincomycin
10	Erythromycin	23	Meropenem
11	Isoniazide	24	Cefotaxime
12	Chloramphenicol	25	Ampicillin
.13	Rifampicin	26	Oxytetracyclin

There is also concern that metal contamination may also play a significant role in the maintenance and proliferation of AMR in environmental bacteria (Baker-Austin et al., 2006, Wright et al., 2006), with a growing body of evidence suggesting that metal contamination (e.g. As, Cu, Zn) of the environment is capable of enhancing the retention and proliferation of AMR and ARGs (McArthur and Tuckfield, 2000, Stepanauskas et al., 2005, Baker-Austin et al., 2006, Wright et al., 2006, Wright et al., 2008). This process is likely to be mediated via several molecular mechanisms, including co-resistance where antibiotic and metal resistance determinants physically present on the same genetic element (Liebert et al., 1997) and cross-resistance (the same genetic determinant being responsible for resistance to antibiotics and metals). Therefore, metals may play a pivotal role in co-selection associated with AMR because unlike antibiotics, metals are not subject to degradation, and can subsequently represent a chronic selection pressure (Stepanauskas et al., 2005). The marine environment in Kuwait is known to be chronically polluted with metals (Al-Sarawi et al., 2015, Lyons et al., 2015a). Therefore, it has been proposed that the metal contamination level in Kuwait's marine environment could act as an additional selective pressure, helping to maintain and proliferate antimicrobial resistance. Moreover, antimicrobial and metal resistance potential in bacteria isolated from seawater and biota inhabiting the marine environment could act as a bioindicator of the level of pollution and the subsequent risk it may pose to human health (Al-Bahry et al., 2009b, Taylor et al., 2011)

1.7 Emerging contaminants in Kuwait

Water quality is one of the major challenges facing the management of Kuwait's marine environment (Devlin et al., 2015a, Lyons et al., 2015b, Michelle J. Devlin et al., 2015, Saeed et al., 2015). Regular discharge of sewage from official networks and unregulated discharge points results in environmental impacts. These sewage discharges are reported to contain a wide variety of pollutants, such as PPCPs originating from many different sources including hospitals and industrial areas (Smith et al., 2015). In addition, due to the untreated sewage contained within these effluents, the levels of bacterial contamination (e.g. faecal coliforms and faecal streptococci) associated with discharges of domestic sewage are known to regularly breach international water quality standards (EPA, 2001, Lyons et al., 2015b). These discharges have been shown to contain other pollutants such as metals, with elevated concentrations detected close to known discharges of domestic sewage. The accumulation of these pollutants has been attributed to the closed, shallow nature of the receiving beaches, with outlets discharging within a few metres of the shoreline (Al-Ghadban et al., 2002, Bu-Olayan and Thomas, 2014).

Recently, sewage contamination of coastal area in Kuwait has been assessed (Lyons et al., 2015b, Saeed et al., 2015). Saeed *et al.,* 2015 determined the concentration of faecal sterols (e.g. coprostanol), measured as an indicator for sewage contamination. In total 112 sediment locations throughout Kuwait marine area were sampled, the results confirming that areas heavily contaminated with sewage were detectable close the vicinity of effluent outfalls. The greatest level of contamination was located in the western part of the Kuwait Bay and the contamination severity declined gradually towards the southern coastline (away from the major population areas). The coprostanol levels in Kuwait Bay ranging from 0-39,428 ng g^{-1} dry wt (Saeed et al., 2015). These findings are supported by a study which used both microbial water quality data and sediment faecal sterol concentrations to assess sewage

pollution around the Kuwait coast (Lyons et al., 2015b). The results identified spatial and temporal sources of pollution, with bacterial counts regularly breaching regional water quality guidelines. The coprostanol data revealed hot spot areas in the western part of Kuwait Bay, where both Doha and Sulaibikhat Bays are located, with concentrations ranging from 29-2420 ng g^{-1} dry wt. Both these studies identified inputs from the Al-Ghazali sewer as being a major point source of pollution in Kuwait Bay and these inputs of sewage are likely to be a key point source of PPCPs into the marine environment of Kuwait. Very few studies are available to indicate the range of emerging contaminants present, although recent research has detected both the presence of a range of PPCPs and associated biological effects at a series of locations around the Kuwait coast (Smith et al., 2015). In this study, the authors undertook extensive chemical screening of effluent waters and investigated potential toxicological and endocrine disrupting effects using a suite of bioassay tests. The results revealed that a wide range of industrial and domestic chemicals (including PPCPs) were present in effluent from discharge points including the Al Ghazali sewer. Oyster embryo (*Crassostrea gigas*) bioassays confirmed that, in certain locations, the waters were capable of completely inhibiting normal embryo development, while the application of the Yeast Estrogen Screen (YES) bioassay identified that the wastewaters contained chemicals with endocrine disrupting properties. Detectable levels of PPCPs in the wastewaters included triclosan, terpineol and chloroxylenol, which were recorded at concentrations >1µg l^{-1} (Smith et al., 2015). Significantly, these are known to have a range of toxicological properties including antimicrobial modes of action (Huang et al., 2016). Triclosan and triclocarban are typical antimicrobial agents used in personal care products and household chemicals such as insect repellents and are therefore likely to be widely used in Kuwait (Liu and Wong, 2013, Huang et al., 2016). Recently, the sources and levels of endocrine disrupting compounds in Kuwait's coastal areas were investigated (Saeed et al., 2017). Phthalates, alkylphenols and estrogens were measured in detectable levels in the seawater and sediment samples

collected from sewage impacted areas across Kuwait coastline with elevated levels of phthalates (ranging from 2145 to 15,722 µg/kg) and lower levels of alkylphenols (ranging from 2.49 to 15.14 µg/kg) estrogens (ranging from 4.1 to 214 µg/kg, dry wt in the sediment (Saeed et al., 2017). The sewage discharged into Kuwait's marine environment is likely to contain a range of pathogens such as the bacterium *Vibrio cholerae* and hepatitis viruses , *E.coli, Salmonella* spp. and *Shigella spp.* and therefore poses a direct human health risk (Saeed et al., 2015, Costa Andrade et al., 2015, Lyons et al., 2015b).

Moreover, data obtained for Kuwait indicates that the consumption rate of antibiotics ranged from 2.7×10^6 - 4×10^6 prescription units/annually for the period 2010-2014 (personal communication, Kuwait Ministry of Health, 2015). These consumption values include many common front-line antibiotics used in high volumes (Table 1.2).

The level of antibiotic resistance in clinical settings in Kuwait has been compared to that in China and USA (Zhang et al., 2006). Kuwait was ranked second behind China with an average AMR rate of 17% between 1999 - 2003 (Zhang et al., 2006). The rise in the antibiotic resistance rate could be partially attributed to inappropriate prescribing in Kuwait, with almost four in ten prescriptions including antibiotics and widespread access, including self-medication, leading to overuse (Awad et al., 2010, Awad and Aboud, 2015).

Globally, little attention has been paid to date on emerging contaminants such as PPCPs, with the majority of research focusing on conventional contaminants. There is now a clear need to shift the research focus from some of the traditionally-studied chemical pollutants (PAHs, metals etc.) to also include PPCPs and the potential impact they may have on the marine environment (Ellis, 2006, Khanal et al., 2006, Sui et al., 2012).

To date, research into PPCPs has received little attention in Kuwait with the majority of research focused on conventional contaminants, related to the petrochemical industries (Readman et al., 1992, Al-Abdali et al., 1996, Massoud et al., 1996 , Metwally et al., 1997 ,

Allchin et al., 1999, Al-Sarawi et al., 2015). It is clear that these now need urgent attention, with evidence suggesting that plentiful of PPCPs were documented to be released into Kuwait's marine environment (Lyons et al., 2015, Smith et al., 2015; Saeed et al., 2015).

1.8 Hypothesis, aim and objectives

The main objective of this research work is to develop an integrated approach to the monitoring and assessing of hazard substances in Kuwait's marine ecosystem. To achieve this, a review of the current data available for hazardous substances in the marine environment (Chapter 1) has been undertaken. In support of this, studies were conducted to assess the use of biomarkers (bile metabolites and Ethoxyresorufin O-Deethylase (EROD) Activity) to investigate the potential for the concentrations of contaminants present in Kuwait's marine environment to induce any detectable levels of biological effects in biota (e.g. fish species) living in Kuwait's marine environment (Chapter 3). Finally, given the extensive issues identified in relation to wastewater and sewage pollution a research was conducted to investigate the potential threat emerging contaminants pose in relation to promoting the presence of AMR in environmental microbial communities in Kuwait marine environments. The survey of the most genetic elements (class 1 integron) that AMR dissemination relies upon was conducted with further molecular investigation into the pattern of AMR present in the Kuwait marine environment (Chapter 4).

Aim: Report the presence of AMR bacteria in seawater and biota collected from various locations impacted by sewage discharges across Kuwait marine environment

The main objectives are as follows:

1- Produce a desk review of marine chemical contaminated related research conducted in Kuwait.
2- Investigate the potential for the concentrations of contaminants present in Kuwait's marine environment to induce any detectable levels of biological effects in biota using biomarkers (biliary metabolites and EROD activities).
3- Initiate a Baseline survey to screen for antimicrobial resistance AMR potential in Kuwait's marine ecosystem.
4- Initiate a baseline molecular survey to study the prevalence of class 1 integron one of MGEs which is known to entitle with AMR and the pattern of AMR genes on integrons of isolated *E.coli* that used as bioindicators of sewage pollution of Kuwait marine environment.
5- Conclusions and recommendations: recommendations for the future monitoring for Kuwait's marine environment.

Chapter 2 Material and methods

2.1 Materials and methods

Methods presented in this chapter are those used in multiple chapters within this thesis. All biological and chemical field samples used in this thesis were obtained from Kuwait's marine environment. The main experimental facilities used to conduct the practical work undertaken in this research project included the laboratories associated with the Faculty of Science and Marine Centre of Kuwait University (for the chemical analysis for trace metals and bacterial isolation) and the laboratories of Plymouth University and the Lowestoft and Weymouth laboratories of the Centre for Environment Fisheries and Aquaculture Science, (Cefas, U.K.) for the fish biomarker analysis and the screening/molecular analysis of bacterial isolates.

2.2 Chemicals

Unless otherwise stated, all the chemicals used in this study were obtained from Sigma Aldrich Ltd. (Poole, U.K).

2.3. Baseline screening for polycyclic aromatic hydrocarbons (PAHs) exposure by the biomarkers: bile metabolites and 7-Ethoxyresorufin O-Deethylase (EROD) activities.

2.3.1 Sample collection

Fish were collected from five stations located within Kuwait Bay and along the cities eastern coastline during April 2014 (Figure 3.1). Sites were selected to provide representative locations of environments within and outside Kuwait Bay and at which fishing had been conducted successfully in the past by previous studies (e.g. free of bottom obstructions). In addition, due to the restriction of fishing within Kuwait Bay appropriate permissions were

obtaining from Public Authority for Agriculture Affairs and Fish Resources (PAAFR). Fishing at each location was undertaken using the Kuwait Institute of Scientific Research (KISR) vessel Bahith-II equipped with a stern otter trawl. Fishing tows were conducted for 20 min. A total of 60 samples of two local species, commonly found in Kuwait waters, the bottom feeding Giant sea catfish (*Arius thalassinus*) and the pelagic Fourlined terapon (*Pelates quadrilineatus*) were collected from sites.

An assessment of PAH bile metabolite concentrations and EROD activities were conducted in Kuwait for a total of 60 samples of two local species of fish, Giant sea catfish (*A. thalissinus*) and Fourlined terapon (*P. quadrilineatus*) sampled from 5 stations.

Immediately after capture, the fish were placed in tanks of well-aerated seawater tanks prior to sampling. On removal from the tank, the fish were wrapped in absorbent towelling for sure handling. The fish were humanely killed by a concussive blow to the cranium quickly followed by destruction of the brain. Before dissection, the size, total length and body weight, was recorded for each fish.

For bile analysis, the whole gall bladder from fish was carefully taken, ensuring that it was not ruptured during dissection. The liver was carefully moved aside, as the gall bladder lies beneath and attached to the liver, and bile withdrawn from the gall bladder into a one ml syringe to obtain as much bile as possible. The depending on the volume available either a sample of bile or whole gall bladder of each fish were placed into a uniquely labelled cryovial tube and immediately placed into a freezer at -20 °C. Once frozen, the sample vials were transferred to cryocanes and placed in a -80 °C freezer for long-term storage.

For 7-Ethoxyresorufin O-Deethylase (EROD) and protein analysis, the liver was removed from the fish as soon as possible after death. Care was taken to avoid contamination of the tissue by excess blood or bile. The liver was then placed in a uniquely labelled cryovial (or a section of it if it was too large) and immediately placed in a cryocane which was placed

into a freezer at -20 °C. Once frozen, the sample vials were transferred to -80 °C freezer for long-term storage to prevent enzyme activity diminishing.

All samples were shipped to Cefas, Lowestoft laboratory, where the analyses took place.

2.3.2 Synchronous Fluorescence Spectrometry (SFS) for determining biliary metabolites in fish.

The bile measurements followed standards that are published in the ICES Techniques (Davies and Vethaak, 2012) in Marine Environmental Sciences series:

(http://www.ices.dk/products/techniques.asp).

Bile samples were analyzed for fluorescent bile metabolites using synchronous fluorescence spectrometry (SFS) at a fixed wavelength, and were reported as 1-OH pyrene equivalents in ng g^{-1} wet weight as described by (Ariese et al., 1993, Cefas, 2007a, Davies and Vethaak, 2012).

As the bile metabolites were measured by using synchronous fluorescence spectroscopy to measure the amount of 1-Hydroxypyrene glucuronide, one of the major PAHs metabolites available in the bile of sampled fish, the amount can be quantified by the commercially obtained 1-Hydroxypyrene standard.

2.3.2.1 Samples and standard preparation

A surgical nick placed in each gall bladder membrane was make before defrosting the sample in an ultrasonic bath. 20 µl of sample was diluted 1:500 with (50/50 v/v) HPLC-grade ethanol/ nanopure water in a 10 ml volumetric flask. Each Sample was mixed well on a vortex or by hand before reading.

For the standard, 10 mg of 1-Hydroxypyrene (1-OH pyrene; Sigma Aldrich CAS number 5315-79-7) was added into 100 ml 50/50 HPLC ethanol/ nanopure water to give 100,000 ppb. This solution was diluted in 1: 100 with 50/50 HPLC ethanol/nanopure water to give 1000 ppb concentration. Further dilution for this solution to 1: 100 with 50/50 HPLC ethanol/nanopure water was performed to give 10ppb concentration.

A serial of dilutions of the standard (30%, 40%, 50%, 60%, 70%) was read at 349.72 nm in Fluorescence spectrophotometer (Perkin Elmer LS 50, UK) to create a standard curve against which the field samples of bile are compared (Figure 2.1).

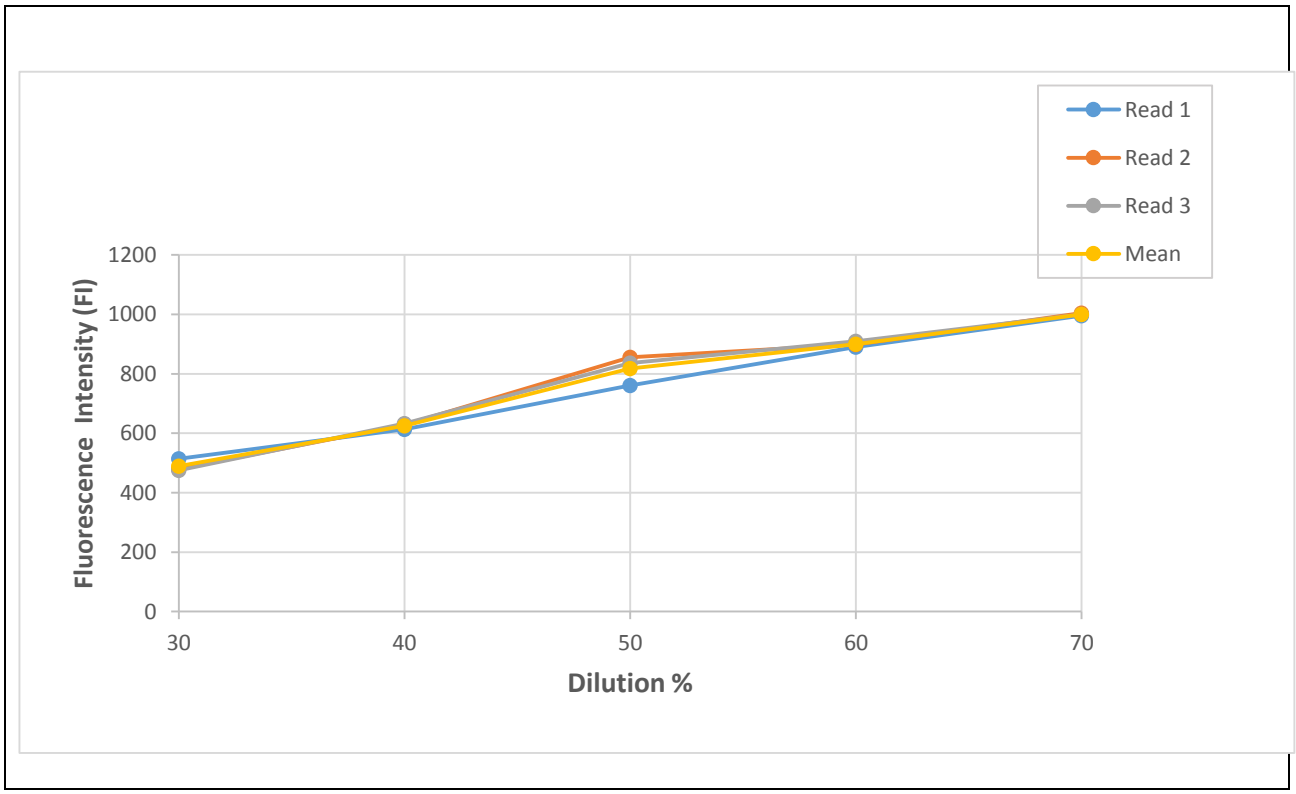

Figure 2.1 The standard curve for the bile analysis created by a serial of dilutions of the 1-OH pyrene standard1= (30%, 40%, 50%, 60%, 70%) was read at 349.72 nm in Fluorescence spectrophotometer (Perkin Elmer LS 50, UK).

2.3.2.2 Analysis of Bile Metabolites Using Synchronous Fluorescence Spectroscopy (SFS).

SFS spectra were measured in a 1 cm quartz cuvette using a synchronous fluorescence spectrophotometer (Perkin Elmer LS 50, UK; Figure two). The fluorescence between wavelength 323 and 423 nm was measured. Reading of blanks (50/50 HPLC ethanol/nanopure) was taken at 344.30nm for (sample blank) and at 349.75nm for (standard blank) (Figure 2.2).

For taking the standard (1-OH pyrene) reading, the peak height reading was taken at 349.75 nm and for each field sample, the reading of peak height was at 344.30 nm. The results were then expressed as ng g^{-1} of 1-OH pyrene equivalents.

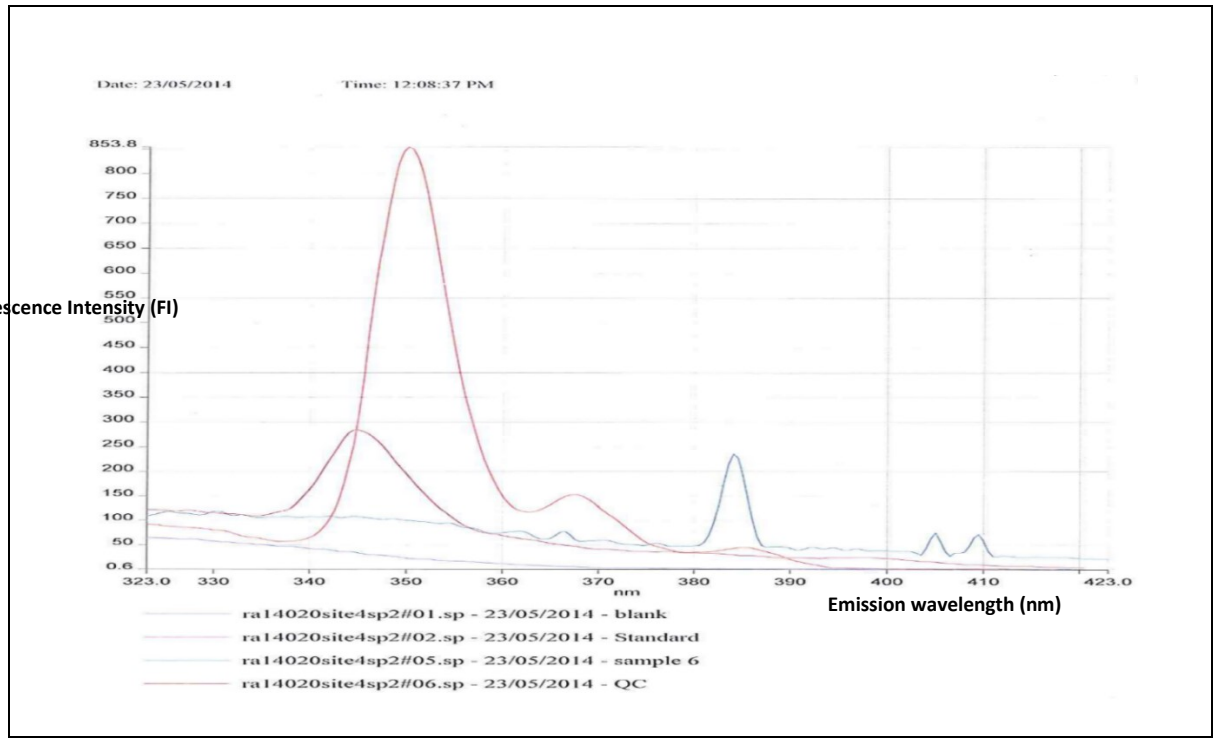

Figure 2.2 An example of the spectrophotometer (Perkin Elmer LS50) output. It shows the blank, standard, QC sample, and an example of field sample.

Calculation of the amounts of bile metabolites in the sample were done by applying the following formula:

(peak height sample – peak height blank 344.30 nm)÷(height standard – height blank 349.75 nm) x standard conc. x dilution factor, for example:

Date:21/05/2014		Sample ID: *A.thalassinus*, site1		
Blank: 33	Standard: 744	Standard-blank:715		
standard blank: 29	Standard ppb: 5			
Fish no.	**Peak Height**	**Peak height - blank**	**Dilution factor**	**Result ppb**
1	78	45	500	157.3427
2	74	41	500	143.3566
3	111	78	500	272.7273
QC	266	233	10000	16293.71

The results were then expressed as ng g^{-1} of 1-OH pyrene equivalents.

2.3.2.3 Quality assurance/quality control (QA/QC) procedure

QC samples were run with each batch of samples. The mean value and the standard deviation were calculated with the following values to be plotted in the QC chart:

Upper warning limits (UWL) = X+2s

Upper control limits (UCL) = X+3s

Lower warning limits (LWL) = X-2s

Lower control limits (LCL) = X-3s

Where, X= mean value, s= standard deviation (MOOPAM, 2010)

None of the samples run as part of this research project fell outside the limits of the assay. QC samples were all within (UCL) from the mean therefore associated analysis can be accepted (Figure 2.3). Only 1 sample was just over (UWL) but as the next sample was back within range the analysis was accepted (MOOPAM, 2010).

The QC samples used in this instance were bile samples that had previously been pooled from 10-20 fish (collected from the North Sea) and frozen at -80 °C in aliquots of 50-100 µl.

During each experimental run, a sample of this QC material was included, diluted and read in the same way as for samples of bile from fish collected from Kuwait (Figure 2.3).

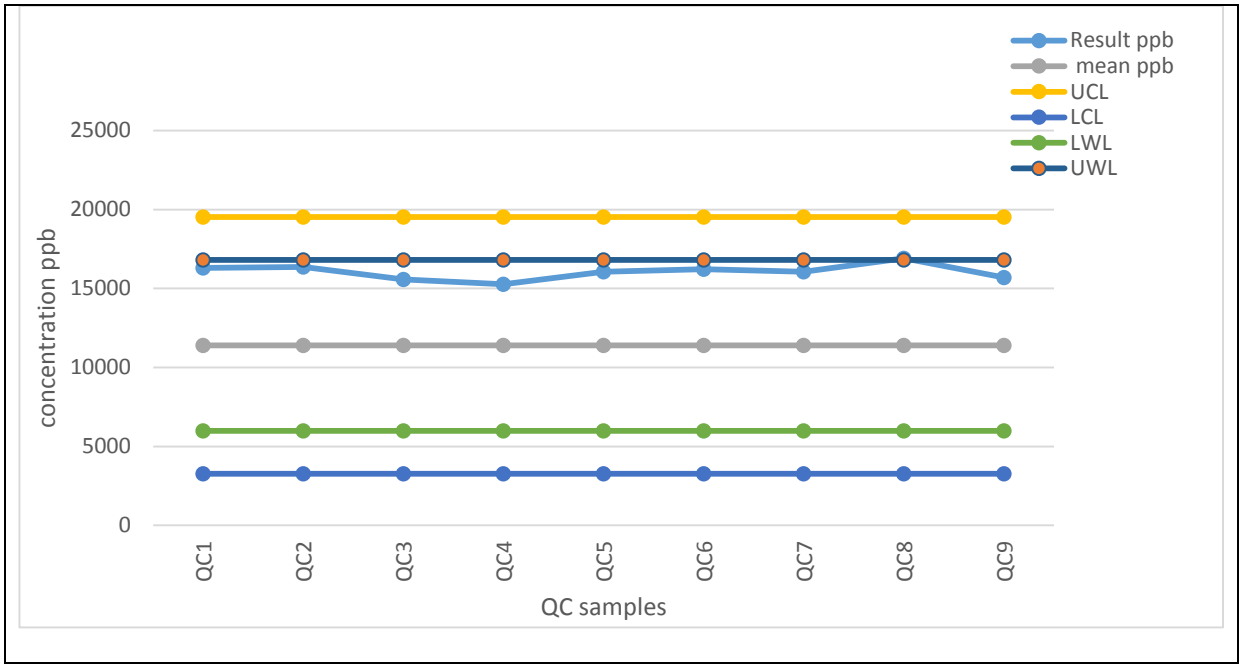

Figure 2.3 Control chart of QC samples used for bile assay for this study. The results were within the UCL except QC8 samples which reached the UWL. The QC chart limits, based off previous 30 QC sample runs.

2.3.3 7-Ethoxyresorufin O-Deethylase (EROD) activity determining by Spectrofluorometric analysis in Fish Livers

Procedures were performed according to ICES method no. 23 1998 (Cefas, 2007b). The levels of hepatic CYP1A were (EROD) activity. 7-Ethoxyresorufin was used as a substrate along with NADPH (a coenzyme) as the electron donor. Resorufin, which is the reaction product, was measured fluorimetrically. A known concentration of resorufin was used as a standard to calibrate the assay. Levels of reported EROD activity were normalised to the protein content of the liver supernatant as described in (Cefas, 2007c), and reported as nanomoles of Resorufin liberated in one minute per mg of protein (nmol min^{-1} mg $protein^{-1}$).

2.3.3.1 Sample and reagent Preparation

The homogenization medium and EROD assay procedure was according to (Stagg and Mcintosh, 1998). For sample preparation, a piece of liver tissue (200 mg ± 10 mg) was sliced from the sample while still frozen. The sample was placed in a homogeniser tube diluted with ice-cold homogenising buffer (100 mM K_2HPO_4 100 mM KH_2PO_4, 1 mM EDTA,1 mM Dithiothrietol, 150 mM KCl, pH 7.5). The tissue was homogenised with six strokes of a homogeniser (Potter-Elvehjem, USA) device set to 700 rpm. The homogenate was then spun at 10,000 x g for 20 minutes in a refrigerated (4 °C) centrifuge. After centrifugation, the supernatant was collected with a fine tip Pasteur pipette and divided into two labelled Eppendorf tubes (one used for EROD assay and other one for protein assay, as described in section 2.3.4) and stored 1-4°C until being assayed.

2.3.3.2 Performing the EROD assay

The Fluorescence spectrophotometer (LS55 Perkin Elmer, UK) was prepared with the excitation width slit set at 5.0 nm and the emission width slit set at 5.0 nm according to Resorufin standard strength which was 6.25 μm (Resorufin standard [CAS: R3257-5G] dissolved in pH 8 Buffer (100 mM K_2HPO_4, 100 mM KH_2PO_4. The actual Resorufin concentration in the cuvette was 0.03125 μm (both recorded in the results sheets; Figure 2.9). The excitation wavelength was 535 nm and the emission wavelength was 580 nm. The EROD assay mixture contained 100 mM assay buffer (100mM K_2HPO_4, 100mM KH_2PO_4,100mM KCl pH 7.5), 2 μM ethoxyresorufin (dissolved in Dimethyl sulfoxide**,** DMSO) and 50-100 μg ml^{-1} of sample was added to 2 ml of the assay mixture. In the fluorescence spectrophotometer (LS55, Perkin Elmer, UK), the baseline reading was taken before 25μM Resorufin standard was added to produce a calibration peak. The readings of the standard were taken at both the base and plateau of the peak. The peak was allowed to stabilise for

1 min (Figure 2.4). Then the reaction was started by adding 0.25mM (NADPH, CAS: N1630-100MG). Readings were taken at 0 and 60 secs from a linear section of the trace to establish the rate of the reaction. Each reaction was allowed to run at least 180 seconds. The results were recorded in results sheets (Figure 2.5). Total protein was determined in tissue homogenates according to (Bradford, 1976) with bovine serum albumin as the standard, as described in section 2.3.4.

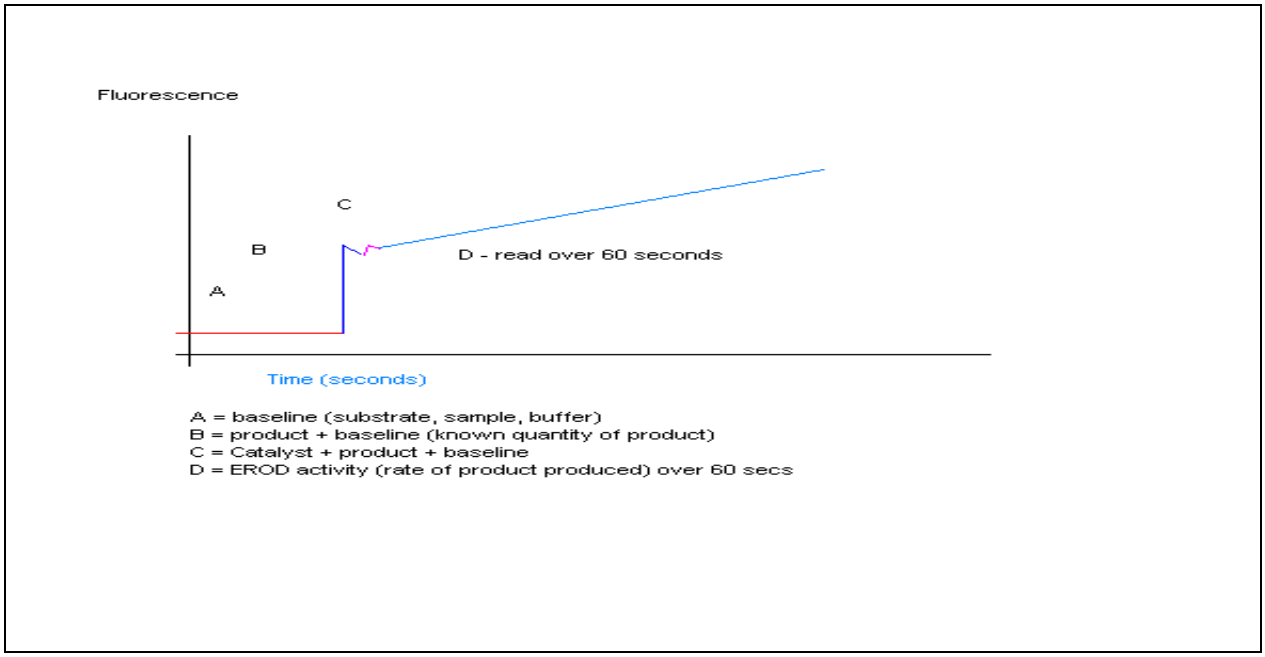

Figure 2.4 Describing the analysis steps and pathway of the reaction in the LS55 Spectrophotometer. The baseline reading (A) was taken before Resorufin standard was added (B) to produce a calibration peak. The readings of the standard were taken at both the base(c) and plateau of the peak(D) (Cefas, 2007b).

Sample ID:	Site 5 sp1	**A Actual concentration of absorbance adjusted resorufin:**						**23.34**	**D Protein standards slope:**					
Date :	29/5/14	**B Concentration of Resorufin working standard:**						**6.33**						
		C Concentration of Resorufin in cuvette:						**:0.031650**						
			E	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**
Fish no	**sex**	**mg liver**	**Mean Abs.**	**Protein mg/ml**	**Std in cuvette**	**Base Std.**	**Peak Std.**	**Peak height**	**0 secs**	**60 secs**	**Rate**	**Sample vol.**	**Activity**	**EROD pM/mg pro/min**
1		201	0.358	10.7	0.0316	38	144	106	135	140	5	20	0.075	6.95
2		197	0.298	8.9	0.0316	35	135	100	131	135	4	20	0.063	7.08
3		204	0.333	10.0	0.0316	36	138	102	133	132	-1	20	-0.016	-1.55
QC		200	0.457	13.7	0.0316	37	143	106	145	228	83	20	1.239	90.38

KEY

A	The concentration of resorufin standard before working dilution is made up (absorbance/0.0732).
B	Working concentration diluted dependant on species of fish used (divide A by 4).
C	10ul of resorufin was added to 2000ul volume in cuvette therefore concentration was 1 / 200.
D	R2 number from the protein assay printout on the standard curve.
E	Mean absorbance from the protein assay printout for each sample.
F	Mean absorbance x 30. 100ul of the 3000ul diluted supernatant is used in the assay 3000/100 = 30
G	Same as C
H	record from results sheet
I	record from results sheet
J	I - H to get the peak height without the baseline
K	record from results sheet
L	record from results sheet
M	L - K to get the rate for 60 secs
N	Sample volume added was 20ul
O	(std in cuvette/peak height) x rate x1000)/sample volume
P	O/F x1000

Figure 2.5 An example of the results template sheet and keys for EROD assay calculations. The results expressed as pico mol per mg of protein per one minute (pM^{-1} mg protein $^{-1}$ min^{-1}).

2.3.3.3 QA/QC Procedure for EROD analysis

Six QC samples were analysed alongside the samples. The QC samples were prepared by Cefas staff in 2010 in the Cefas laboratory at Lowestoft by pooling several Dab liver samples (*Limanda limanda*) which were then homogenised. The S9 fraction was separated and well mixed, then 250µl aliquots of these QC samples were kept in vials at -80 °C. One vial was defrosted on ice and analysed with every 10 samples as a QC check for each batch. The results of the QC samples were inputted into a control chart (Figure 2.6). None of the QC results were outside 3 standard deviation limits (UCL and LCL), only 1 QC sample breached the LWL for site 3 sp 2 which on investigation of the assay showed that the low EROD

activity could be due the high protein content (indicating a possible error with the protein assay QC), this would usually require the assays to be re-run. However, as the activity of all the samples for that site were below limits of detection it was deemed that re-running this data would not be useful. If the samples were re-analysed for protein, even if the protein values changed, there would still be no activity so no extra information would be gained for this site (Figure 2.6).

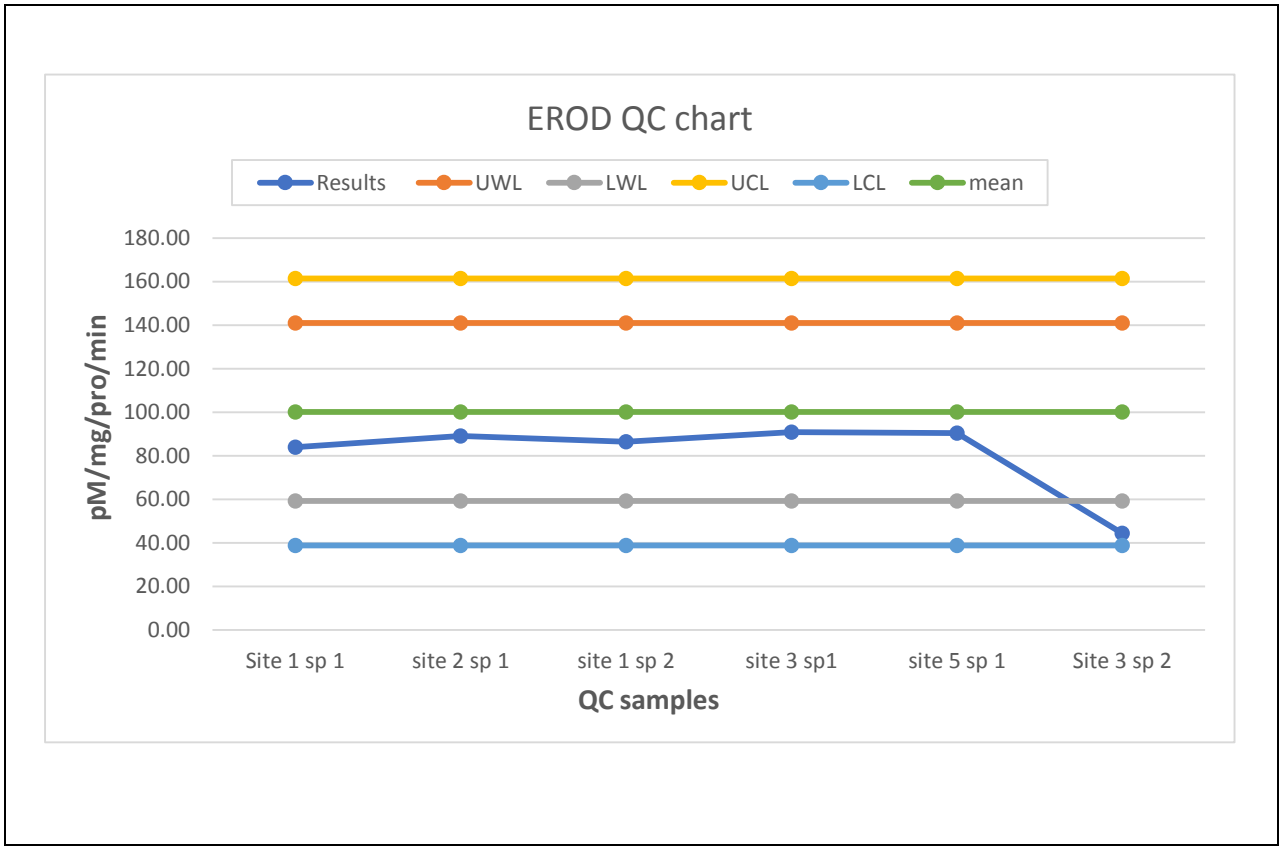

Figure 2.6 The control chart for EROD assay. None of the QC results were outside the deviation limits (UWL, UCL, LWL and LCL) except for the site 3 sp 2 sample that showed low EROD activity but still within the LCL. The QC samples were prepared by Cefas staff in 2010 in the Cefas laboratory at Lowestoft by measuring EROD activity in Dab *(Limanda limanda*).

2.3.4 Protein normalization for EROD assay

The amount of protein in a sample is a key step in the EROD assay that provide a good estimation for the enzymes available in the samples content (Bradford, 1976, Cefas, 2007c, Stagg and Mcintosh, 1998). In this study, the samples are normalised to protein content according to international standards (Stagg and Mcintosh, 1998). The Bradford assay is the method to quantify the protein and allow for the results to be expressed per amount of protein present as in the previous sheet at Figure 2.5 (Bradford, 1976, Cefas, 2007c).

The Bradford assay utilises a dye that changes colour when bound to protein. The bound form of the dye has an absorbance peak at 595 nm. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the concentration of protein present in the sample. A standard in the form of Bovine Serum Albumin (BSA) was run alongside the samples as a known quantity.

2.3.4.1 Preparation of Bio-rad assay dye reagent and BSA standard

Bio-rad assay dye reagent was diluted 1: 5 with nanopure water before use and then filtered through Whatman qualitative No.1 filter paper before storage in a glass container (250 ml) at room temperature.

Lyophilized bovine serum albumin standard (BSA) was prepared as standard for this assay. Nanopure water was directly added to the BSA standard bottle (Bio-Rad Protein Assay Standard II, catalogue # 500-0007) to obtain a concentration of 1.37 mg/ml. The solution was mixed thoroughly until dissolved. Eight dilutions of the stock BSA standard were prepared by diluting with homogenising buffer (8, 16, 24, 32, 40, 48, 56 and 64) as described in Table 2.1 to establish a precise standard curve. The linear range for BSA was 0.2 to 0.9 mg/ml (Figure 2.7).

Table 2.1 The 8 Dilutions of the stock BSA standard preparation by diluting with homogenising buffer in order to establish a precise standard curve.

% of stock	Volume of 100% BSA stock (µl)	nanopure water added (µl)	final volume (µl)	Protein concentration (mg/ml)
8	80	920	1000	0.110
16	160	840	1000	0.219
24	240	760	1000	0.329
32	320	680	1000	0.438
40	400	600	1000	0.548
48	480	520	1000	0.658
56	560	440	1000	0.767
64	640	360	1000	0.877

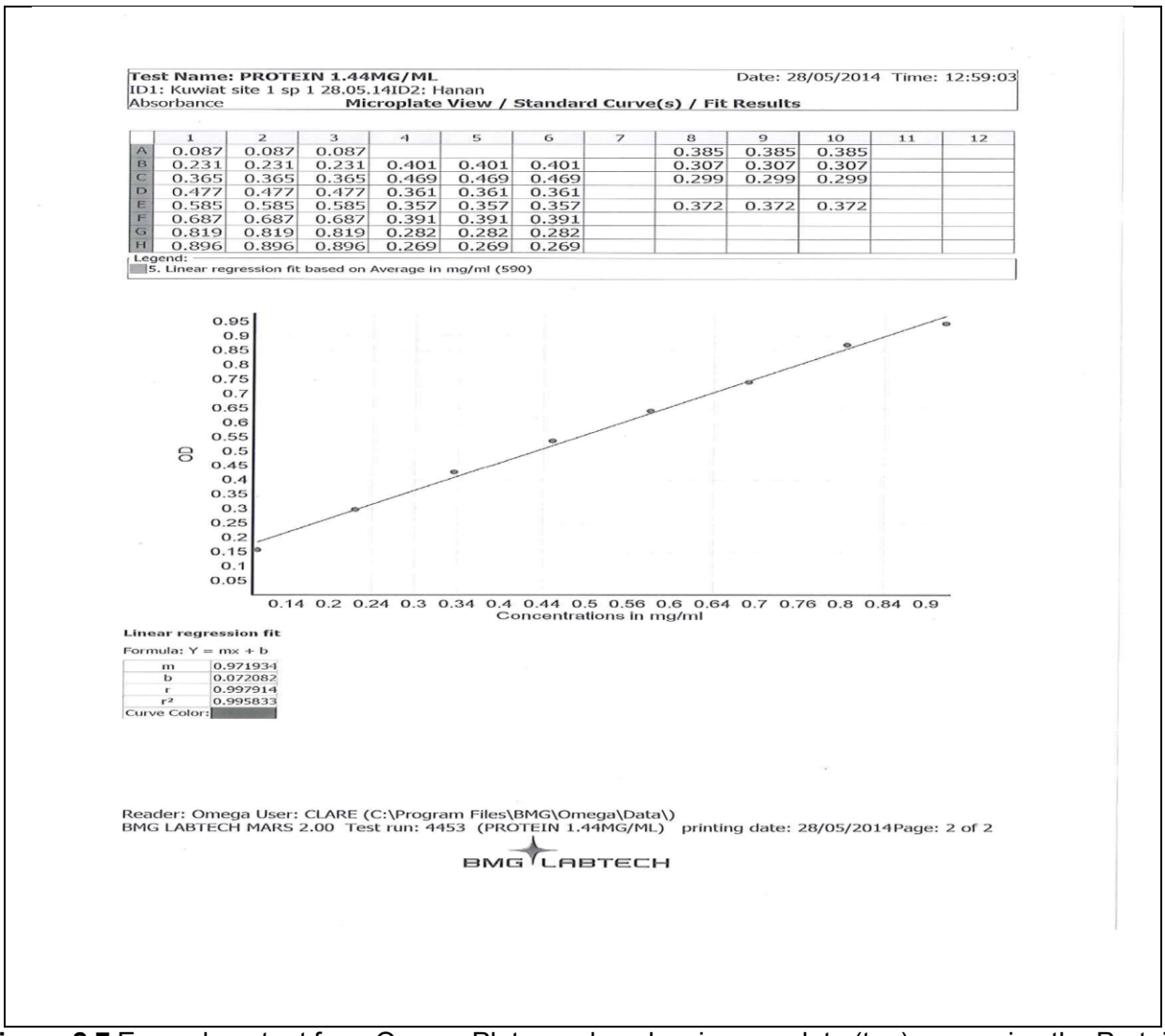

Figure 2.7 Example output from Omega Plate reader, showing raw data (top) measuring the Protein normalization for EROD assay and the BSA standard curve generated (below). The linear range for BSA was 0.2 to 0.9 mg/ml.

2.3.4.2 Protein Assay protocol

For each prepared sample as describe in section 2.3.3.1., one of the labelled Eppendorf tubes was used for this assay. Typically, as the EROD procedures for Dab were followed, the liver samples, the S9 fractions, were diluted by 1:30 (i.e., 2.9 ml nanopure to 100µl of liver S9 sample). The same procedure was followed for the QC samples. 100µl per tube of each Blank (homogenizing buffer) and standards were prepared, dilutions were not required. 100µl of diluted sample was transferred to a glass test tube. In the fume cupboard, 5ml of Bio-rad reagents was added to the sample tube as well as for blank and standards then all

tubes were mixed by vortexing for 30sec. The same procedure was followed for the QC samples. An aliquot of 380 µl of each sample was pipetted into each well of a 96 well, flat bottomed microplate using a multi-pipettor. Three replicates per sample were prepared.

Standards were pipetted first then blank followed by samples of Kuwaiti fish livers and finally the QC samples. All readings of Optical Density were taken at a wavelength of 590nm by using a microplate reader (SCI-TEK, UK). The final protein content of the original sample of fish liver tissue was calculated by using the plate reader software (Mars Omega). The results were adjusted to account for the dilution factor for this EROD assay and recorded in the same EROD calculation sheets (Figure 2.6).

2.3.4.3 QA/QC Procedure for Protien analysis

As for the EROD QC procedure, QC of known value were run alongside each sample batch. The QC result were recorded on the QC chart (Figure 2.8). There was slight variations in the protein content of the QC samples and the site 3 sp 2 was the highest with the protein levels however, this level was still within the UWL deviation limits.

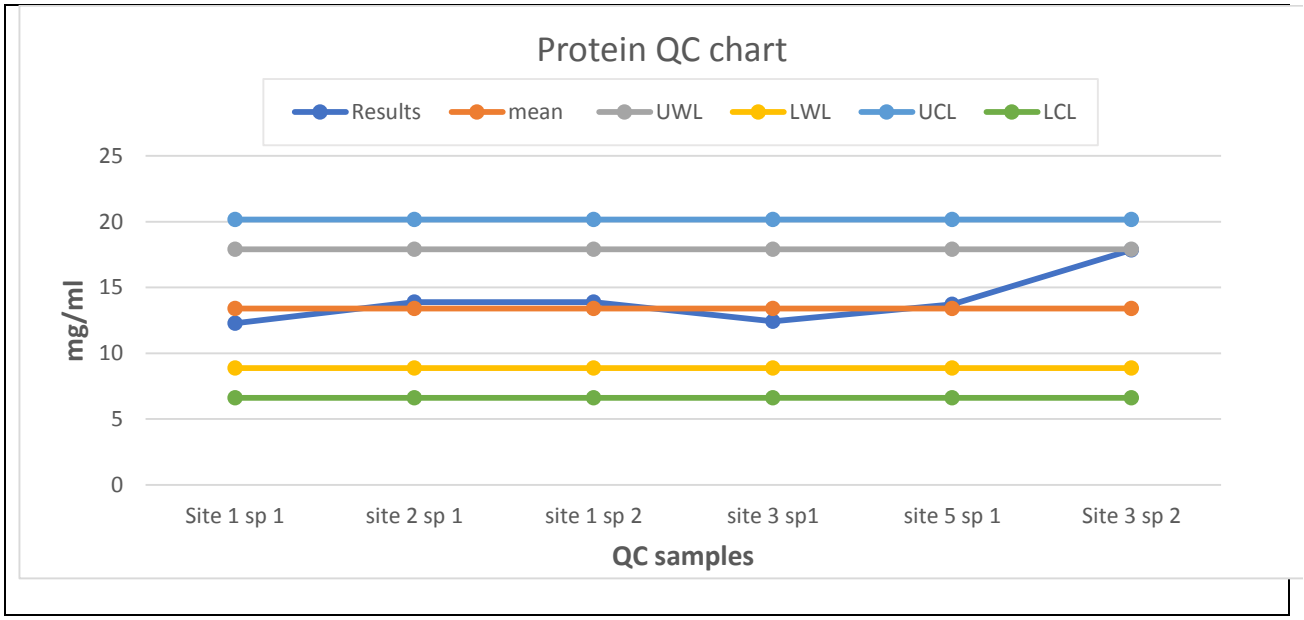

Figure 2.8 The QC chart of protein analysis for EROD assay. All the QC samples were within the UCL except for the site 3 sp 2 sample that showed elevated level of protein content. However, this level was fall within the UCL.

2.4 A baseline screening for Antimicrobial Resistance (AMR) in isolated *E.coli*

2.4.1 Sample collection and preparation

In the field, physico-chemical parameters (i.e. pH, water temperature and salinity) of seawater from each sampling site (see the map Figure 4.1) were measured *in situ* by using a portable calibrated multi-parameters water quality instrument (Hanna instruments model no. HI 9828, USA).

The average values for the major *in situ* physico-chemical parameters cross Kuwait marine sample sites showed that whilst salinity (mean 36 ‰ ± 2.0) and pH values (mean 8.4±0.2) remained stable during the year, the variability of temperature was significant (34 °C ± 2.0 and 17.2 °C ± 2.0) for summer and winter, respectively.

Samples were processed immediately (less than 2 hours after collection) in Kuwait University facilities (both in the laboratories of the Marine Centre and the Faculty of Science) to obtain the bacterial isolates for AMR testing. All bacterial isolates (640 *E.coli*) were stored in microbank vials (PRO-LAB Diagnostics, Canada) and shipped to Plymouth University (UK) for AMR analysis.

2.4.1.1 Surface seawater samples

Multiple seawater samples (4-6 per site, 200 m between each) were taken into 1L sterile polyethylene bottles and stored in a cooler with ice to limit further biological and chemical activities for no longer than 6h, before being returned to the laboratory for analysis at the Faculty of science in Kuwait University as described in detail in Section 2.4.2. A serial tenfold dilution from each pooled sample was performed (10^{-1}, 10^{-3}, 10^{-5}) using sterile 0.1% peptone water.

2.4.1.2 Bivalves samples

Bivalve's samples Venus clams (*Circenita callipyga*) were also collected by hands and stored in sterile plastic bags in a cooler with ice. More than 60 unit of each Venus clams (*Circenita callipyga*) were collected from site to prepare replicates pooled samples (20 g for each pooled sample). The average shell length was 3.25 cm for the Venus clams (*Circenita callipyga*). Samples were processed in the same day of sampling at Faculty of science in Kuwait University as described in section 2.4.2.

In the Laboratory, the bivalves were shelled under aseptic conditions using gloves and a sterile knife. The flesh as well as the liquor inside the shells was collected in sterile beakers. For homogenizing, 20 g of the bivalve's tissue (pooled sample of around 20 unit) was placed in a sterile stomacher bag and homogenised using a STOMACHER® 400 (Seward, UK) for 30 min. After that, the 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 tenfold dilution from each pooled sample was performed (10^{-1}, 10^{-3}, 10^{-5}) using sterile 0.1% peptone water.

2.4.2 *Escherichia coli (E.coli)* isolation and enumeration

The isolation and numeration of microbial quality parameters (faecal coliform and *E.coli*) was conducted at each site in both seawater and bivalve (where available) samples to get an estimation of the level of sewage contamination. This was undertaken using the membrane filter technique following the methods 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).

Under a septic technique, volumes 0.1, 1, 3, 10ml and serial dilution (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 cellulose filter paper. Filter papers were then placed separately on m-FC media (EMD, Germany) with 10 ml 1% Rosolic acid in 0.2*N* NaOH, pH 7.4 plates 4 (Atlas, 2004). The plates were incubated at 44°C for 24 h (Figure 2.9).

For *E.coli* confirmation individual green colonies (colour indicative of *E. coli*) were chosen from each sample and streaked onto Nutrient Agar (Difco, USA) with 4-methylumbelliferyl-β-D glucuronide (NA-MUG) (No. 18493-5VL, Sigma), which is a selective media for *E.coli* (Villari et al., 1997, McArthur et al., 2015). NA-MUG plates were incubated overnight (16–20 h) at 37 °C. Fluorescent colonies were considered to be *E.coli* and recorded (Figure 2.9) and sub-cultured on MUG-media in order to be used for AMR analysis.

To numerate and isolate faecal coliform and *E.coli* in bivalves, pooled samples were prepared for (Venus clams) as described in section 4.2.1.2. Filtration, incubation, counting, and confirmation procedures were the same as for the seawater samples. Blank and positive control samples were analysed in parallel with those collected from the field. Mean numeration values and standard deviation of bacteria in the seawater and bivalve's samples were obtained from bacterial counts.

Mean number of faecal bacteria and *E. coli* in seawater were expressed in colony-forming unities (CFU) per 100 ml and on bivalves in CFU per 100 g as the following equations:

Equation 1: (CFU) /100ml (seawater) =

(Number of colonies on m-FC media/volume of sample filtered) xdilution factor x100

Equation 2: CFU/100g (clams) =

(*X*) / total weight of clam's tissue in the pooled sample

Where (X) = (Number of colonies on m-FC media/volume of pooled sample filtered) x dilution factor x100

For verified *E.coli* counts, the initial count was adjusted based upon the positive percentage and was report as verified *E.coli* count/100 ml or count/100 g, as follow:

Percentage verified *E.coli* on NA-MUG media **=**

Number of verified colonies / total number of coliform colonies subjected to the verification X100.

After confirmation, *E.coli* strains were selected from seawater and bivalves in each site (around 640 *E.coli* isolates a cross Kuwait coastline during the year) and stored in Microbank vials (PRO.LAB Diagnostic, Canada) and shipped frozen to Plymouth University, (UK) where kept frozen at -20 °C prior to conducting the AMR screening and a subset of samples retained at −80 °C for longer term storage.

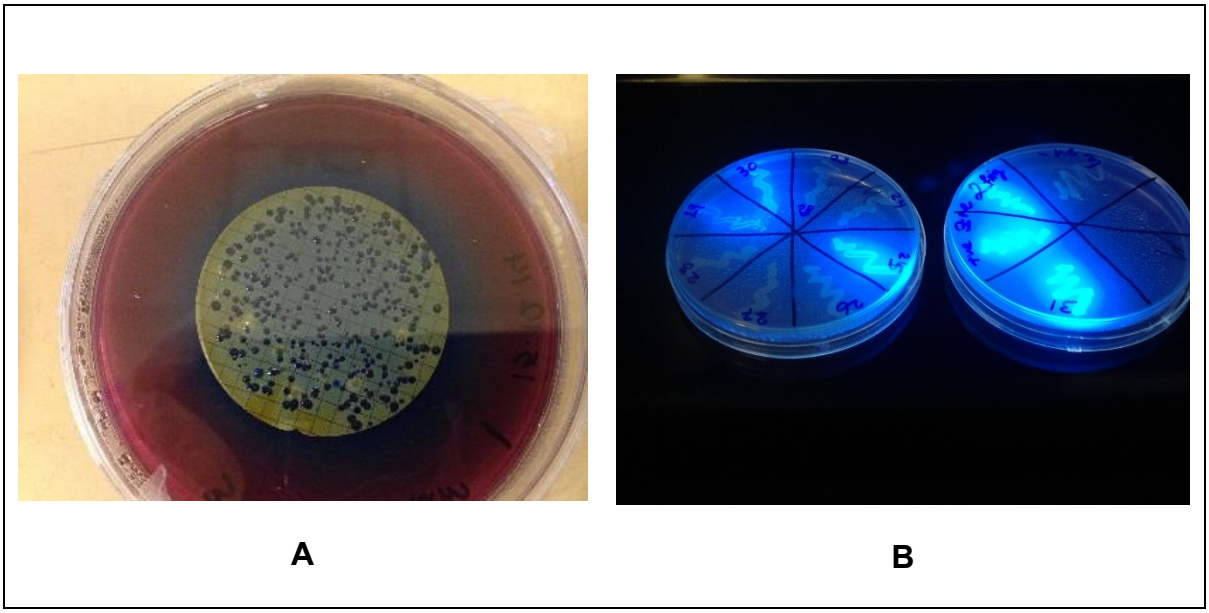

Figure 2.9 Faecal coliform/*Ecoli* culturing: (A) on m-FC media. (B) On NA-MUG media under UV with 2 different cultures: *Ecoli* (fluorescent)/another faecal coliform (non-fluorescent).

2.4.3 Optimisation *of E.coli* isolation and enumeration

For the purpose of *E.coli* isolation and enumeration, amended 3% NaCl m-FC media was prepared, thereby increasing the salinity of the original media. However, no results (colonies) were observed in comparison with the original media.

Along with *E.coil*, another bacterial species from seawater samples, faecal *streptococci* (FS), was isolated in order to choose the most suitable bacterial indicator for the AMR testing. The same procedures were followed as described in section 2.4.2. However, each filter paper was placed on Slanetz and Bartley media (Oxoid, CM0377, UK) and incubated for 48 h at 36 °C. Pink to red or concentrated red colonies were presumed to be FS. The membrane filters, with the colonies, were then aseptically transferred onto Esculin Iron Agar (Fluka, UK) for confirmation purpose (Atlas, 2004). Plates were incubated for 2h at 44°C and the black spots on the medium were considered to be FS and were counted. FS isolates were then sub-cultured on Brain heart infusion agar for 24 hr at 37 °C.

As the numbers of FS were considerably lower than those of *E.coli* for the same site, the focus was on *E.coli* to conduct the AMR testing during subsequent research.

For the bivalve's source of *E.coli*, rock oyster *(Saccosterea cucullate)* were also collected from sites (where available) to prepare replicates pooled samples (20 g for each pooled sample). The average shell length and 4.25 ± 2.25 cm for rock oyster *(Saccosterea cucullate)* respectively (Figure 2.10). Samples were processed in the same day of sampling at Faculty of science in Kuwait University as described in section 2.4.2. The same sample procedure was conducted to both the *(Saccosterea cucullate) and the (Circenita callipyga).* However, the rock oyster derived microbial water quality indicators were comparatively low (one isolate) when comparing with those derived from Venus clams within the same site. Therefore, the only biota source used in this thesis is the Venus clams.

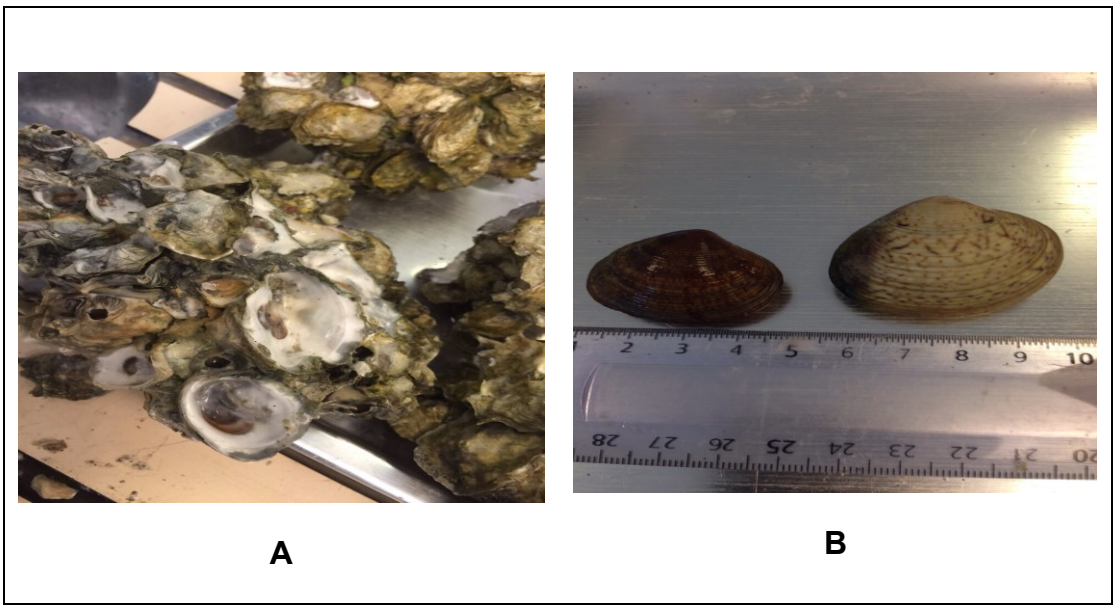

Figure 2.10 Bivalves used to provide the source material for the AMR baseline screening: (A) rock oyster (*Saccosterea cucullate*). (B) Venus clams (*Circenita callipyga*). Same number were collected from the bivalves within the site.

2.4.4 *E.coli* O157 screening test

Upon receiving the isolates which were (640 *E.coli*) collected as described in section 2.4.1.1 and 2.4.1.2 in Plymouth University, all isolates were screened for *E.coli* O157 presence as this is an obligatory protocol forced by the Advisory Committee On Dangerous Pathogens (ACDP) when dealing with hazard group 3 organisms. This is a routine protocol followed by Plymouth University because of the university policy that only allows handling of contaminant level (CL) 2 microorganisms in their laboratories. Suspected isolates were discarded immediately. The test was performed as follows:

E.coli isolates were cultured on M9 minimal (5x) media supplemented with 0.5% Sorbitol as a sole carbon source (g/L) (11.28 M9, 5 sorbitol, 2ml of 1M $MgSO_4$ (Fisher, USA), 0.1ml of 1M $CaCl_2$ (Fisher, USA), 12 agar). http://www.thelabrat.com/protocols/m9minimal.shtml. Plates were incubated at 37 °C for 24h. Isolates that failed to grow on the used media were presumed to be *E.coli* O157 and they were discarded.

2.4.5 Antimicrobial resistance testing (AMR)

E. coli isolates were tested for their antimicrobial resistance. The process for AMR screening was conducted following the guidelines of Clinical and laboratory Standards Institute (CLSI, 2014). 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 (CLSI, 2014).

A loopful of each isolate was inoculated into 10 ml CAMHB and incubated in a shaking incubator for 24 h at 37 °C. Turbidity was adjusted with 0.5 M MacFarland standard solution (0.5 ml of the 0.048 mol/l $BaCl_2$ solution (1.175% w/v $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.18 mol/l (0.36 N) H_2SO_4 (1% v/v) stock solution). The required inoculum suspension of the culture is approximately 5 x 10^5 CFU/ml. Within 15 minutes of preparation, the 0.5 MacFarland adjusted suspension was diluted 1:150 by adding 50µl of the suspension to 7.5ml (CAMHB) resulting in a approximately 1 x 10^6 CFU/ml. The prepared mixture was then poured into a reservoir to be ready to transfer into custom dehydrated 96-well Sensititre™ GN2F panels (GN2F, Thermo Scientific, UK) (Figure 2.11). The custom dehydrated 96-well Sensititre™ GN2F panel (GN2F, Thermo Scientific, UK) was composed of 23 different antimicrobial agents with dilution range concentrations as shown in Table 2.2. 50 µl of the mixture was added into each well by using a multi-micropipette. Each tray was sealed with a plastic self-adhesive cover before incubating it at 25°C for overnight to avoid mixture evaporation.

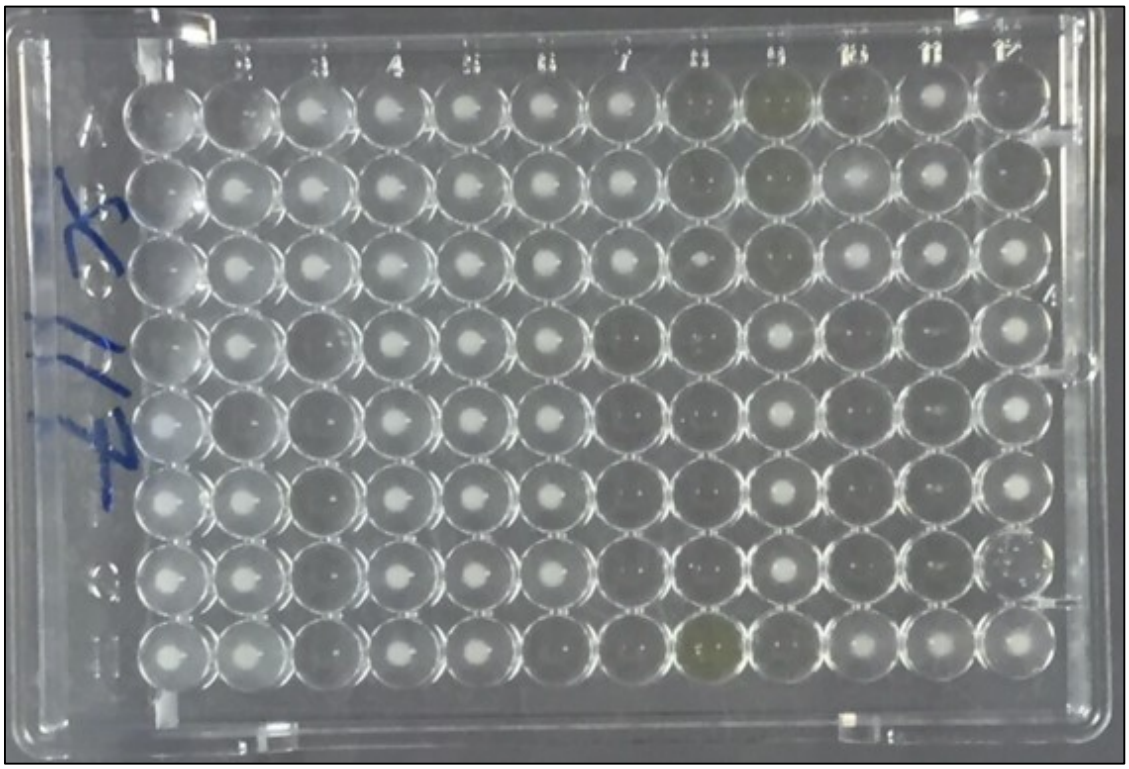

Figure 2.11 The inoculated dehydrated 96-well MicroScan panels GN2F with isolate under the name Sc117.

Table 2.2 The microbial agents with their belonged group, dilution range in the GN2F -Gram Negative MIC plate and the break points for susceptible, intermediate and resistant which are recommended by Cefas that hybrid with the Clinical Laboratory and Standard Institute (CLSI, 2014).

GN2F Abbreviation	Antimicrobial agent name	Antimicrobial group	Dilution range	Susceptible	intermediate	Break points resistant
AMI	Amikicin	Aminoglycoside	8-64	8	16	≥32
AMP	Ampicillin	Aminoglycoside	4-32	8	16	≥32
A/S2	Ampicillin/sulbactan	Aminoglycoside/beta-lactamase inhibitor	4/2-32/16	8	16	≥32/16
AZT	Aztreonam	Penicillin derivative	8-32	8	16	≥32
FAZ	Cefazolin	1st generation cephalosporin	4-32	8	16	≥32
FEP	Cefepime	4th generation cephalosporin	4-32	8	16	≥32
TANS	Cefotetan Na	2nd generation cephalosporin	8-32	8	16	≥32
AXO	Ceftriaxone	3rd generation cephalosporin	1-64	8	16	≥32
TAZ	Ceftazidime	3rd generation cephalosporin	1-32	8	16	≥32
FOX	cefoxitin	1st generation cephalosporin	4-32	8	16	≥32
FUR	Cefuroxime	1st generation cephalosporin	4-32	8	16	≥32
CIP	Ciprofloxacin	Flouroquinolone	0.5-4	1	2	≥4
GEN	Gentamycin	Aminoglycoside	2-16	4	-	≥8
IMI	Imipenam	Imipenam	2-16	4	8	≥16
GAT	Gatifloxacin	Fluoroquinolone	1-8	2	4	≥8
MERO	Meropenem	Carbapenams	1-8	4	-	≥4
PIP	Piperacillin	ESBL class	16-128	16	32-64	≥128
NIT	Nitrofurantoin	Quinolone	16-128	32	64	≥128
P/T4	Piperacillin/tazobactam	beta-lactam/beta-lactamase inhibitor	16/4-128/4	16	32-64	≥128/4
TIM2	Ticercillin/clavulanic acid	beta-lactam/beta-lactamase inhibitor	16/2-64/2	16	32	≥128/2
TOB	Tobramycin	Aminoglycoside	4-8	4	-	≥8
POD	Cefpodoxime	3rd generation cephalosporin	2-16	2	4	≥8
SXT	Trimethorprim/sulfamethoxazole	Dihydrofolate reductase inhibitor/sulfonamide	0.5/9.5-4/76	2	-	≥8/152

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The resulted data was inputted into a spread excel sheet format, generated by Cefas laboratory, for analysis as in Figure 2.12. The bacterial growth at different concentration of the tested antimicrobial agents was represented by 1 where the blank shows no growth at certain concentrations (Figure 2.12 A). the excel spread sheets were formatting to translate the growth notifications whether 1 or blank to the exact concentration values and count the break point depending on the values added for resistant, intermediate (partially resistant) and susceptible (sensitive) which are recommended by Cefas that hybrid with the Clinical Laboratory and Standard Institute (CLSI, 2014) (Figure 2.12 B).

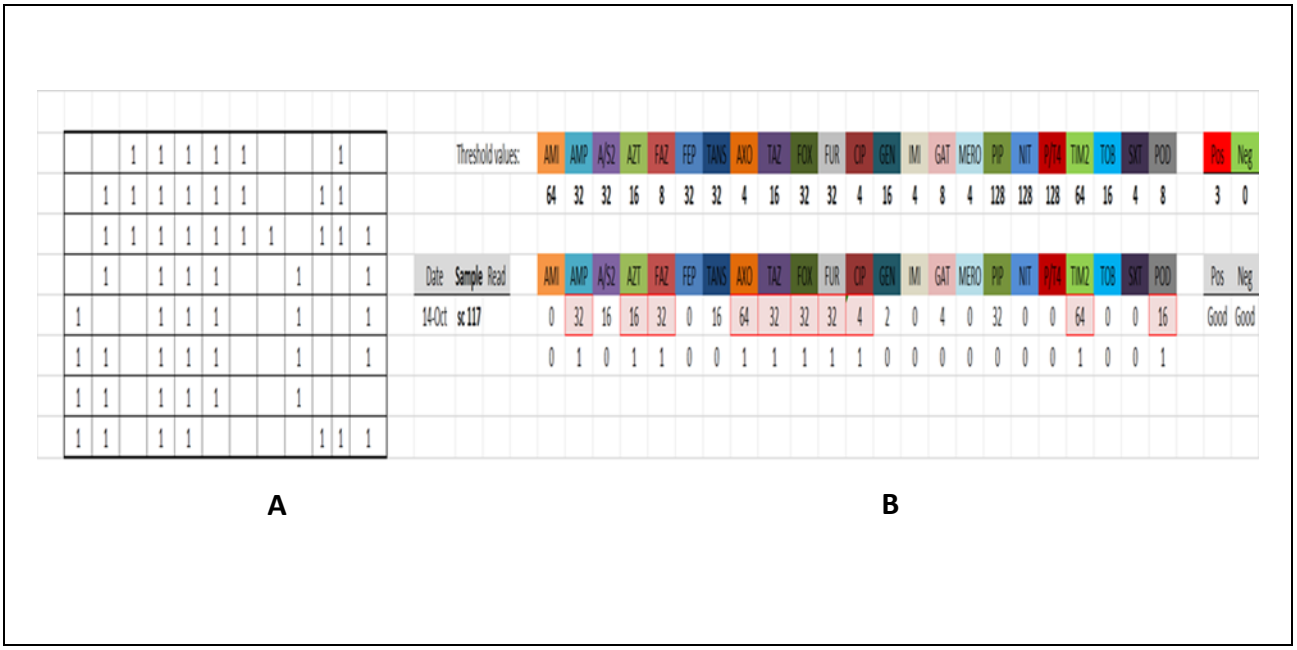


Figure 2.12 An example of the excel spread sheet format for the same *E.cpli* isolate (Sc. 117) illustrating the resistant break points for each tested antimicrobial agent. (A) Typical panel to the tested MicroScan panels GN2F for each isolate. (B) The translating format for the growth in different concentrations for each tested antimicrobial agent and counting the break points for the classifications: resistant, intermediate and susceptible depending on the provided values.

2.5 Class 1 Integrons Screening test for the isolated *E.coli*

2.5.1 DNA extraction

A loopful of each pure isolate was added to 100µl Tris-EDTA (TE) buffer (10 Mm Tris, 1mm EDTA, pH 8.0) in a sterile Eppendorf tube and was incubated for 10 min in a 95 °C heat block. After centrifuging for 3 min at high speed (13,000 rpm), the supernatant was transferred to a sterile Eppendorf tube and kept at -20 °C for short term storage or -80 °C for long term storage.

2.5.2 Amplification of integrase genes by PCR

Integrase genes, which are specific for the class 1 integron, were amplified from the extracted *E.coli* DNA with the int1.F/R, while gene cassettes were amplified with the HS286 and HS287 primer set as described in Table 2.3 (Nemergut et al., 2004). A master mix was prepared depending on the number of samples processed, composed of 0.5 µl from each primer, 9.5 µl sterile molecular water and 12.5 µl of MangoMix (Bioline, UK). The master mix (23 ul was added to 2 µl of extracted DNA in a sterile 0.2 ml PCR tube, modified protocol of (Nemergut et al., 2004). The PCR was performed in a Gene Amp PCR system 9700 thermocycler (Applied Biosystem, USA) and the PCR program used was as follows: initial denaturation step at 94 °C for 1 min, followed by 35 cycles of 94°C for 1 min, 58 °C for 30 s and 72 °C for 2.5 min with a terminal 10 min extension at 72 °C (Nemergut et al., 2004). The reference positive control isolates from Cefas (control code: 08019, 08021 and 08022) were run with every PCR.

Table 2.3 Primers used in class 1 integrons screening for the *E.coli* isolates seawater and bivalves samples following (Nemergut et al., 2004) after optimisation.

Primer	Target	Sequence (degree of degeneracy)
int1.F	Integron integrases	GGGTCAAGGATCTGGATTTCG
int1.R	Integron integrases	ACATGCGTGTAAATCATCGTCG
intltdF	Integron integrase	CTNYTNTAYGGNWCNGG
intltdR	Integron integrase	TCYTGNACNGWNCKDATRTC
HS286	Gene cassettes	GGGATCCTCSGCTKGARCGAMTTGTTAGVC
HS287	Gene cassettes	GGGATCCGCSGCTKANCTCVRRCGTTAGSC

2.5.3 Optimization for Amplification of integrase genes by PCR

(Nemergut et al., 2004) amplified the integrase genes with int1.F/R and intltd F/R, and gene cassettes were amplified with HS286 and HS287 primers Table 2.3. The master mix was prepared depending on number of samples as follows: 2µl from each primer with 6.5µl sterile molecular water, 12.5 µl of Mango Mix (Bioline, UK). 23µl of master mix was added to each sterile 0.2ml PCR tubes where 2µl of sample's DNA was added. After an initial denaturation step at 94°C for 1 min, 35 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 2.5 min with a terminal 10-min extension at 72°C were performed in thermocycler (Gene Amp PCR system 9700, Applied Biosystem, USA). The reaction conditions consisted of a 400 nM (intI1.F/R) or 4 µM (intltdF/intltdR and HS286/HS287) concentration of each primer.

As the results bands were too condense and the primer dimers were also present, the primer's amount was decreased to 0.5µl to give more clear results.

The primer intltd F/R, Table 2.4, do not give any positive results (including the positive control: 08019, 08021 and 08022). The modified annealing/ denature temperature and time were tried several times, i.e. 5 min denaturation at 96 °C, followed by 40 cycles of 30 s,

95 °C, 90 s at 56 °C and 72 °C for 20 s with a final extension step of 72 C for 5 min; 3 min denaturation at 96 °C, followed by 50 cycles of 30 s, 95 °C, 90 s at 56 °C and 72 °C for 30 s with a final extension step of 72 °C for 3 min. However, no successful results were obtained for this intltd F/R primers. Therefore, the focus to amplify the integrase genes by intI1 F/R and the cassettes were amplified with HS286 and HS287 primers.

Table 2.4 The sequence of intltdF/R set of primers which were not give any positive results in current study derived *E.coli* from seawater and bivalves.

Primer	Target	Sequence (degree of degeneracy)
intltdF	Integron integrase	CTNYTNTAYGGNWCNGG
intltdR	Integron integrase	TCYTGNACNGWNCKDATRTC

2.5.4 The agarose gel (1.5%) electrophoresis

Agarose gel electrophoresis was used to investigate the amplified PCR products (Figure 2.13). 8 µl of each sample, was run alongside a 50 bp genomic DNA Hyper ladder (bioline, USA) on a 1.5% (w/v) agarose gel (Fisher, USA) in deionized water supplemented with 10 µl SYBR ® Safe DNA gel stain (10,000X in DMSO) (Invitrogen, USA). The run was carried out for 40 mins at 95 volts in an agarose gel electrophoresis unit (Alpha labs, UK). The gel was then visualized under UV by using a GelDoc XR gel imaging system (Bio-Rad, UK).

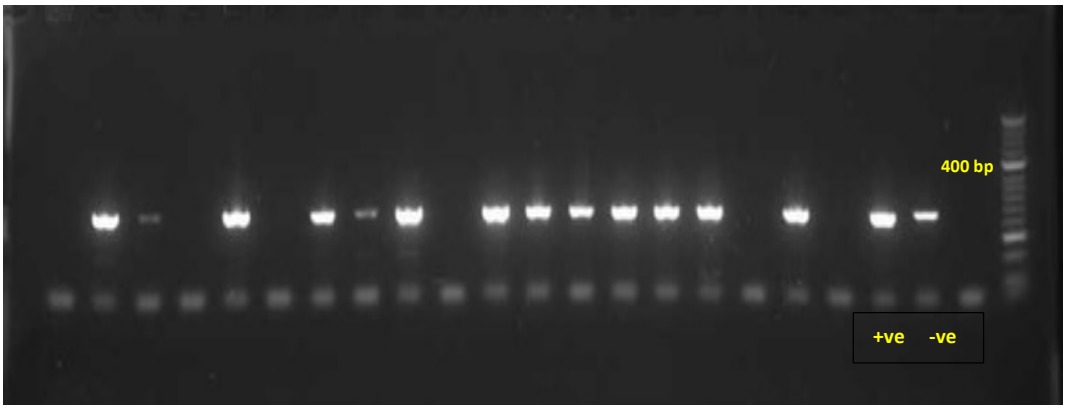


Figure 2.13 An Integrase gene(intI1) was amplified with int1.F/R in different isolates with running positive (+ve) Cefas 08019 and negative (-ve) controls with each PCR run.

2.6 Whole genome sequencing for representative *E.coli* strains derived from the Kuwait marine environment

A representative *E.coli* isolates with different AMR pattern (i.e. some are resistant to (2, 3, or more tested antibiotics and other with susceptible pattern) were subjected to whole genome sequencing using Illumina Nextera XT library preparation and Illumina Miseq V3 chemistry (Illumina, USA). The sequencing was performed at the Cefas laboratories (Weymouth, U.K.) as follows:

2.6.1 DNA extraction/ quantification

2.6.1.1 DNA extraction

Each of the representative *E. coli* Isolates were cultured in 15ml Tryptic soy broth (TSB, Oxoid) overnight static at 37°C prior to DNA extraction. One milliliter of culture was pelleted by centrifugation at 13,000 - 16.000 x g for 2min for extraction and DNA was purified using the Promega Wizard ®Genomic DNA extraction Kit (Promega, UK) protocol for Gram negative bacteria, according to the manufacturer's instructions.

2.6.1.2 DNA quantification

DNA was quantified fluorometrically using a Quantus fluorimeter machine (Promega, UK) with the DNAOne reagent system following manufacturer's protocol. Following quantification, DNA was diluted to 0.2 ng/µl with molecular grade water and was stored in -20 °C.

2.6.2 DNA sequencing

2.6.2.1 Tagmentation and Amplification

Tagmentation and amplification were carried out using a Nextera XT DNA sample preparation kit (Illumina, USA) with an adapted manufactures protocol; the adapted protocol used 2.5 µl of 0.2 ng/µl DNA and half the manufacturer recommended quantity of each reagent up to the DNA clean-up stage. DNA was then diluted 2-fold in molecular grade water prior to clean-up.

2.6.2.2 Library clean up

This step was conducted using 30µl AgenCourt® AMPure® XP (BeckMan Coulter, USA) beads according to the recommended Illumina protocol (Nextera XT DNA Sample prep).

2.6.2.3 Library normalization, pooling and sequencing

This process was undertaken according to the manufacturer's protocol (Nextera XT DNA Sample prep). After normalization DNA was pooled, diluted in HT1 hybridization buffer (Illumina, USA) and then denatured at 98C for 2 minutes. DNA was sequenced on the MiSeq platform with a V3600 Illumina Sequencing Cartridge.

2.6.3 Quality Check, Assembly

Sequences were trimmed using version 0.36 of Trimmomatic, with options recommended on the website, for paired end sequencing (Bolger, Lohse, & Usadel, 2014). FastQC version 0.11.5 was used to check the quality of trimmed reads, and to ensure there were no large contaminants (Babraham Bioinformatics- FastQC A Quality Control tool for High Throughput Sequence Data. (n.d.), Retrieved May 17,2017).

2.6.4 Assembly and Identifying Open Reading Frames (ORFs)

Spades version 3.10.1 was used for assembly, with default options (Bankevich et al., 2012). Assembled genomes were annotated using version 1.11 of Prokka, with default options (Seemann, 2014).

2.6.5 Multi-locus sequence typing (MLST) Analysis

Gene models for the from the Pfam database (Finn et al., 2014). The hmmscan tool, included as part of version 3.1 of the hmmer toolkit (Finn et al., 2011) was then used to identify the probably of each open reading frame in the assembled genomes actually corresponding with any one of the *adk*, *fumC*, *gyrB*, *icd*, *mdh, purA* or recA genes. Finally, once each of the seven genes had been identified in each of the sequenced isolates they were aligned using version 7.305 of the mafft software, with default options (Katoh et al., 2002). After aligning sequenced genes, each gene alignment was processed using version 1.9.8 of the UGENE software (Okonechnikov et al., 2012) to exclude unaligned regions at the 5' and 3' end of the alignment, before concatenating the alignments. Finally, FastTree version 2.1.8 was used to carry out a maximum-likelihood phylogenetic analysis (Price et al., 2010) and FigTree version 1.4.2 was used to draw the tree (FigTree., Retrieved May 17, 2017).

Chapter 3 Baseline levels of biliary PAH's metabolites and EROD activities in fish collected from Kuwait's marine environment

This work was included as part of a wider survey of fish health which was published in a Special Issue of Marine Pollution Bulletin.

Al-Zaidan, A., Al-Sarawi, H.A., Al-Enezi, M., Smith, A.J., Bignell, J.P., Green, M.J., Askem, C., Bolam, T.P.C., Barber, J.L., Bersuder, P., Lyons, B.P. (2015) Histopathology and contaminant concentrations in fish from Kuwait's marine environment. Marine Pollution Bulletin.100:637-645.

3.1 Introduction

In recent decades, the Gulf Cooperative Council (GCC) countries have witnessed a rapid development in urban centers and industries related to the petrochemical industry production (Al-Sarawi et al., 2015, de Mora et al., 2010, Sheppard et al., 2010). It has been estimated that the Arabian Gulf region receives 2 to 4 million barrels of crude oil each year, mainly due to exploratory drilling, refinery operations, effluents from petroleum industries, municipal waste discharges and transportation of oil (Dauabul and Al-Saad, 1985, Literathy et al., 2002, de Mora et al., 2010, Sheppard et al., 2010).

As such, Kuwait's marine and coastal environments have witnessed rapid urban and industrial development after the discovery of oil, which has led to increasing pressures on its natural environment (Al-Sarawi et al., 2015, Beg et al., 2015b). In addition, the northern marine area of Kuwait's is the receiving basin for the influx of around 48 tonnes of oil residue $year^{-1}$ and 2×10^{10} m^3 nutrient rich water $year^{-1}$ from Shatt Al-Arab estuary, along with 50 – 100×10^6 tonnes $year^{-1}$ suspended sediment from Khor Al-Zubair estuary in southern Iraq (Beg et al., 2015b). Consequently, there is the potential for concentrations of petroleum contaminants to be significantly increased, particularly in marine coastal areas (Al-Sarawi et al., 2015, Al-Zaidan et al., 2015). For example, the Kuwait Bay area is impacted by local municipal and industrial discharges with resuspension of sediment-deposited pollutants (Al-Sarawi et al., 2015, Al-Zaidan et al., 2015, Devlin et al., 2015a, Lyons et al., 2015a, Lyons et al., 2015b, Saeed et al., 2015, Saeed et al., 2017). Studies have also shown that Kuwait Bay area is influenced more by petroleum hydrocarbons compared to the areas around Kuwait territorial waters (de Mora et al., 2010, Al-Sarawi et al., 2015).

In the marine environment, petroleum products are a major and wide spread class of contaminants (Trisciani et al., 2011, Bozcaarmutlu et al., 2015). These pollutants have drawn serious attention worldwide due to their persistence in the sediment and their

associated toxicity and carcinogenicity (Trisciani et al., 2011). Polycyclic aromatic hydrocarbons (PAHs) are one of the most toxic components of petroleum products (Trisciani et al., 2011, Tairova et al., 2012). They contain two to six fused aromatic rings and are characterized by low water and high lipid solubility and some are known mutagens/ carcinogens such as Benzo [a] pyrene (Bozcaarmutlu et al., 2015). These types of contaminants are viewed as a high priority for environmental regulations and ecological risk assessments for industrial and urban effluent discharges, such as those undertaken by the US Environmental Protection Agency (USEPA) that prioritize regular monitoring for PAHs (Beyer et al., 2010, Bozcaarmutlu et al., 2015).

The anthropogenic sources have significantly contributed to the increasing of PAH levels in the marine environment, which is in addition to natural sources such as oil seeps and forest fires (Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012). The anthropogenic sources are categorized as pyrogenic (incomplete combustion of organic matter and fuels), or petrogenic (derived from the crude oil or petroleum products release (Ariese et al., 1993, Tairova et al., 2009, Trisciani et al., 2011, van der Oost et al., 2003). Consequently, PAHs can be found ubiquitously in the marine environment and in elevated concentrations in harbours and coastal areas which are close to industrial and urban discharges (Bozcaarmutlu et al., 2015).

In water, the hydrophobic PAHs are mostly resistant to the bacterial degradation due to the adsorption to organic particles which consequently leads to sinking to the sediment (Ariese et al., 1993, Tairova et al., 2009, Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012). Therefore, measuring PAH concentrations in the sediments can provide valuable information about the PAH load in specific areas where the adverse biological effects on the aquatic biota are observed or suspected (Beyer et al., 2010, de Mora et al., 2010). A recent survey of PAH concentrations in Kuwait sediment found concentrations ranging from 37 –

1332 ng g^{-1} dw (∑PAH) (Lyons et al., 2015a) which is considered relatively low when compared to other industrial areas wide world (Lyons et al., 2015a). These are also below international sediment quality guideline values, such as those established for Effects Range Low (ERL: 4000 ng g^{-1} dw) by National Oceanic and Atmospheric Administration (NOAA), USA (Long et al., 1995, Long and MacDonald, 1998). However, in the marine environment, the toxicity of PAHs and their metabolites have received serious attention due to their ability to persist for a long time in the sediment and their ability to accumulate in the tissue of marine organisms due to their hydrophobic characteristics (Fuentes-Rios et al., 2005, Beyer et al., 2010, Bozcaarmutlu et al., 2015). Some marine organisms (e.g. fish) have the potential to metabolize PAHs by activating enzymatic biotransformation (bioactivation) systems (Ariese et al., 1993, Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012, Kammann et al., 2013, Liu et al., 2016). Studies have shown that up to 99% of PAHs can be rapidly metabolized in fish and converted to metabolites (i.e. glucuronide, sulfate or glutathione conjugates) within 24h of uptake (Ariese et al., 1993, Trisciani et al., 2011). The induction of these systems by different pollutants (e.g. PAHs) is considered to be part of the organism's detoxification and metabolism pathway. Fish possess well-developed enzyme systems which efficiently convert PAHs to epoxides and hydroxylated derivatives during phase I metabolism. These products are subjected to further conversion to highly soluble conjugates to facilitate their excretion (e.g. glucuronide or sulfate) during phase II metabolism and then secreted into the gall bladder, where they are concentrated and stored prior to be excreted outside the body (Aas et al., 1998, Aas and Klungsøyr, 1998, da Silva et al., 2006, Beyer et al., 2010, Bozcaarmutlu et al., 2015, Al-Zaidan et al., 2015). Thus, the bile of PAH- exposed fish contains a multitude of oxygenated PAH derivatives and represents the major excretion route for bio-transformed PAH metabolites especially in fish (Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012).

Some of the active metabolites can act as a carcinogenic, mutagenic and cytotoxic agent that covalently bind to the macromolecules in the organisms such as DNA, RNA and protein causing serious ecologically relevant threats to the well-being of humans such as lung, skin and bladder cancer (Fuentes-Rios et al., 2005, Beyer et al., 2010, Bozcaarmutlu et al., 2015, Al-Zaidan et al., 2015, Abdel-Shafy and Mansour, 2016). The adverse effects of PAH exposure in fish include liver neoplasms and liver tumours, delayed growth rate and reduced survival (Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012).

As a consequence of the biotransformation process, the actual concentrations of PAHs or their residues in the tissues of expose fish tends to be low and is likely to be poorly correlated with real exposure, leading to an underestimation of exposure (Ariese et al., 1993, Aas et al., 1998, Aas and Klungsøyr, 1998, Aas et al., 2000, Trisciani et al., 2011, Tairova et al., 2012, Kammann et al., 2013). For example, Budzinski *et al*., 2004 investigated PAH levels of sole (*Solea solea*) after the Erika oil spill in 1999. The results showed no increase in muscle PAH levels whereas the biliary metabolites of the same fish indicated significant PAH exposure levels (Budzinski et al., 2004). Therefore, PAH monitoring by determination of exposure biomarkers in organisms serves as a convenient ecotoxicological tool to assess the exposure of fish to pollutants such as PAHs (Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012). One of the most commonly used biomarkers used in the biomonitoring of marine pollutants is by measuring hepatic cytochrome P450 1A (CYP1A) via the measurement of 7-ethoxyresorufin-*O*-deethylase (EROD) catalytic activity (Shailaja and D'Silva, 2003, Fuentes-Rios et al., 2005, Sturve et al., 2006, Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012, Bozcaarmutlu et al., 2015). In fish, the primary biotransformation system for detoxifying/bioactivating PAHs is cytochrome P450 monooxygenase (CYP) (Trisciani et al., 2011, Tairova et al., 2012). Several CYP enzymes have been identified, however, the CYP1A, phase I biotransformation enzyme is the hydrocarbon-inducible isoform enzyme that is most suitable for use as a biomarker for PAH

exposure among the other identified CYPs in fish (Trisciani et al., 2011, Bozcaarmutlu et al., 2015). EROD delivers a reasonably rapid indication of toxic compound uptake by organisms (Beg et al., 2015b). Extensive validation of Cytochrome P450 1A as a biomarker has led to its incorporation into several contaminant monitoring programs worldwide e.g., North Sea Task Force (Stagg, 1991, Pluta, 1995), French National Observation Network (Godefroy et al., 1996), Swan–Canning Estuary, Australia (Webb and Gagnon, 2002); Baltic Sea, Sweden (Hannson et al., 2006), and Columbia River Basin, USA (Hinck et al., 2006).

The measurement of EROD acts as a general biological indicator of exposure, but it does not reveal the specific class identity of the inducible pollutant (it can be induced by a range of pollutants in addition to PAHs (Fuentes-Rios et al., 2005, Bozcaarmutlu et al., 2015). Therefore, determining the concentration of PAH metabolites as fluorescent aromatic compounds in bile fluid of fish has become a complementary method to assess and identify the PAH exposure (Fuentes-Rios et al., 2005, Bozcaarmutlu et al., 2015). For environmental screening purposes, one of the simple, cheap and quick methods for determining the total PAH exposure (biliary PAH concentrations) is by synchronous fluorescence screening (SFS) of 1-Hydroxypyrene (1-OH pyrene) in fish bile (Ariese et al., 1993, Giessing et al., 2003, Ariese et al., 2005, Fuentes-Rios et al., 2005, Beyer et al., 2010, Trisciani et al., 2011, Bozcaarmutlu et al., 2015). It reflects the sum of certain PAH groups rather than a single compound, which is more environmental relevant, and expressed as an equivalent of pyrene (Ariese et al., 1993). Pyrene is the most ubiquitous PAH form due to its relative stability and the oxidised form 1-OH pyrene is one of the main metabolites of pyrene presents in the bile of fish that have been exposed to PAH-contaminated sediment (Ariese et al., 1993, Ruddock et al., 2002, Ruddock et al., 2003, Beyer et al., 2010). The identification of 1-OH pyrene has been recommended by OSPAR and ICES as a biomarker for exposure and metabolism of PAHs, and has been successfully integrated into international environmental monitoring schemes, such as the Oslo Paris Convention Coordinated Environmental Monitoring

Programme (OSPAR CEMP), which is designed for the North Sea and northeast Atlantic regions (Ariese et al., 2005, Beyer et al., 2010, Trisciani et al., 2011, Tairova et al., 2012, Kammann et al., 2013, Koenig et al., 2013). In addition, a diverse number of studies have used the measurement of biliary PAH metabolites to monitor PAH exposure in different marine fish species such as Atlantic cod **(***Gadus morhua)*, flounder *(Platichthys flesus)* (Beyer et al., 1996), eelpout (*Zoarces viviparous)* (Tairova et al., 2012) and plaice *(Pleuronectes platessa) (*Richardson et al., 2004). In recent years, a number of studies have combined the measurement of hepatic enzymes and the florescence biliary PAHs metabolites as exposure biomarkers for pollutants in environmental monitoring programmes (Fuentes-Rios et al., 2005, Kammann et al., 2013, Koenig et al., 2013).

3.2 Aims and objectives

This study looked to further investigate and confirm the findings of the previous researches conducted by other groups and reported in the literature review in Chapter 1, which indicated that negligible biological risk was posed by the reported levels of PAH contamination.

1- A baseline screening survey of PAHs exposure in fish inhabit Kuwait marine environment by biomarkers: biliary metabolites and hepatic EROD activity which were examined to investigate if the levels of PAH concentrations found in various locations around the Kuwait marine environment could pose any adverse biological effects in resident fish species.

3.2 Material and methods

3.2.1 Sample collection and site information

Fish were collected from five stations located within Kuwait Bay and along the cities eastern coastline during April 2014 (Figure 3.1). Sites were selected to provide representative locations of environments within and outside Kuwait Bay and at which fishing had been conducted successfully in previous studies (e.g. free of obstructions). In addition, due to the restriction of fishing within Kuwait Bay appropriate permissions were obtaining from Public Authority for Agriculture Affairs and Fish Resources (PAAFR). Fishing at each location was undertaken using the Kuwait Institute of Scientific Research (KISR) vessel Bahith-II, equipped with a stern otter trawl. Fishing tows were conducted for 20 min. A total of 60 samples of two local species, commonly found in Kuwait waters. Bottom-feeding Giant sea catfish (*Arius thalassinus*), were collected from site 1 (n=10), site 2 (n=10), site 3 (n=10) and site 5 (n=10). The pelagic Four-lined terapon (*Pelates quadrilineatus*) were collected from site 1 (n=10) and site 3 (n=10; Figure 3.1). An assessment of PAH bile metabolite concentrations and EROD activities was conducted in Kuwait for a total of 60 samples of the two species, sampled from 5 stations Figure 3.1. Forty-six were successfully used analysis of bile metabolites and 60 were used for the EROD bioassay.

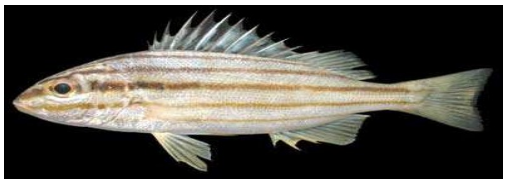
The coefficient of condition of each fish was represented by the condition factor (CF) and was calculated according to the following formula;

$CF = W \times 100 / L^3$

Where, W is the fish weight in grams; L is its length in centimetres (Beg et al., 2015b).

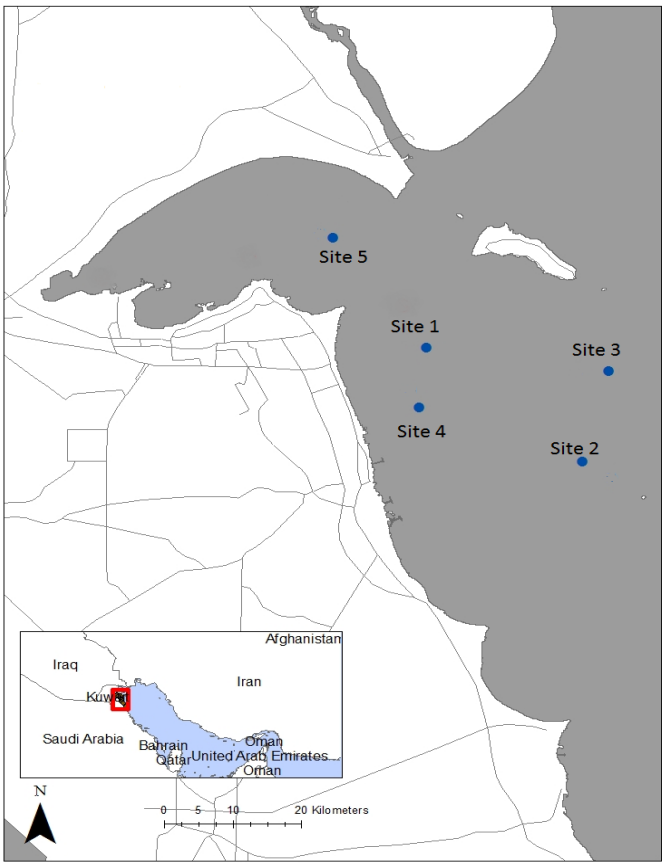
Giant sea catfish (*A. thalissinus*)

(A)



Four-lined terapon (*P. quadrilineatus*)

(B)



(C)

Figure 3.1 (A) Giant sea catfish (*A. thalissinus*) and (B) the Four-lined terapon (*P. quadrilineatus*) (*www.google.com*) are the two native fish species used in this survey. (C) Map of the Kuwait coastline showing the sampling sites where the *A. thalissinus* were collected from sites 1, 2, 3 and 5, whereas *P. quadrilineatus* were collected from sites 1 and 3.

3.2.2 Bile metabolite measurement in fish by synchronous fluorescence spectrometry (SFS) analysis

Bile samples were analysed for fluorescent bile metabolites using direct synchronous fluorescence spectrometry (SFS), as described in section 2.3.2 (Ariese et al., 1993, Ariese et al., 2005). Briefly, following euthanasia, the gallbladders from both Giant catfish (*A. thalassinus)* and Four-line terapon (*P. quabrilineatus)* were removed and immediately placed in a −20 °C freezer. Bile samples were thawed in an ultrasonic bath and diluted with

1:500 ethanol/water (50:50 v/v). SFS spectra were measured in a 1 cm quartz cuvette using a Perkin Elmer LS50 spectrofluorimeter. For quantification, the height peak area from excitation wavelength 323–423 nm was measured and expressed as ng g^{-1} wet weight (1-OH pyrene) equivalents. The results were standardised by the commercially obtained 1-Hydroxypyrene standard (Sigma Aldrich CAS number 5315-79-7).

2.2.3 Determination of Ethoxyresorufin O-Deethylase (EROD) activity by Spectrofluorometric analysis

CYP1A-associated 7-ethoxyresorufin-O-deethylase (EROD) activities of sampled fish livers were determined according to procedures described in section 2.3.3 (Stagg and Mcintosh, 1998). Fluorimetrically measuring of resorufin formation (a reaction product) were performed using 7-ethoxyresorufin as a substrate along with NADPH (a coenzyme) as the electron donor Figure 3.2. Assay conditions were optimized for Dab (*Limanda limanda*) liver and have previously been shown to be suitable for other fish species (Cefas, 2007b). A typical reaction medium contained reagent volumes and concentrations as listed in Table 3.1, which resulted in test solutions (final volume 2 ml; pH 7.8) which was pipetted into a fluorometer cuvette. EROD activity was determined by the addition of the sample in a fluorescence spectrophotometer (Perkin-Elmer LS55, UK) set at 535 nm (excitation) and 580 nm (emission) wave lengths. The known amount of resorufin standard was added to the reaction mixture to produce a calibration peak. Readings were taken at the base and plateau of the peak to establish the height. The peak was allowed to stabilise. Ten μl NADPH solution was added and readings were taken at 0 and 60 secs from a linear section of the trace, to establish the rate of the reaction. Each reaction was allowed to run for at least 180 seconds. A Quality control (QC) sample was run twice during the assay batch. A known product

(resorufin) concentration was added and the enzyme activity of the sample deduced by relating resorufin production back to this standard.

Levels of reported EROD activity were normalised to the protein content of the sampled liver supernatant. The total protein was determined in tissue homogenates as described in section 2.3.4 (Bradford, 1976) with bovine serum albumin (BSA) as a standard and expressed as picomoles resorufin formed per minute per milligram of protein (pmol min^{-1} mg protein $^{-1}$).

Table 3.1 Reagent volumes and concentrations used in the determination of 7-ethoxyresorufin-O-deethylase (EROD) activities.

Reagent	**Reagent concentration**	**Volumes**	**Final Concentration**
Buffer	102.0 mM	1.96 ml	100 mM
7-ethoxyresorufin in DMSO	0.4 mM	10 µl	2.0 µM
NADPH	100.0 mM	10 µl	0.25 mM
Sample	5-10 mg protien ml^{-1}	20 µl	50-100 µg ml^{-1}

3.2. Statistical analysis

The data were statistically analysed using IBM SPSS statistics 23. The data are presented as mean ± standard deviation (SD). The data were normally distributed according to the Shapiro-Wilk test for data less than 20. The t-test was run for the species within sites and no violation of the normality and homogeneity assumptions. A one-way ANOVA test was

used for comparisons among sites and a *post-hoc* test (Tukey) was used to check the statistical difference between each pair of sites. Only $p < 0.05$ was accepted as significant.

3.3 Results

The Morphometric measurements (length, weight and condition factor) of the collected *A. thalassinus* and *P. quadrilineatus* samples from Kuwait marine environment are presented in Table 3.2. Sex of fish was determined. The ratio of male to female was 1:1.4 in *A. thalassinus* and 1:1.3 in *P. quadrilineatus*.

The average length of each fish collected from the sampling sites were almost the same for each species. The average weight for both fish species were varied affecting the K. The highest K in *A. thalassinus* was 1.4 ± 0.52 at site 5. For *P. quadrilineatus,* the K in site 1 (2.89 ± 0.85) was higher than site 3 (1.45 ± 0.25). However, no significant difference was observed.

Table 3.2 The Morphometric measurements of fish collected from Kuwait marine environment. Information of length, weight, condition factor (CF) and numbers of collected *A. thalassinus* and *P. quadrilineatus* samples from each site. NS=no sample.

Site	Fish species (n)	Length (cm)	Body weight (g)	Condition factor, K
1	*A. thalassinus* (n=10)	28.4± 2.7	290.0 ± 82.0	1.26 ± 0.12
1	*P. quadrilineatus (n=10)*	19.6 ± 2.5	218.2 ± 30.0	2.89 ± 0.85
2	*A. thalassinus* (n=10)	34.7 ± 3.8	334.5 ± 158.2	0.8 ± 1.12
3	*A. thalassinus* (n=10)	38.3 ± 10.1	500.5 ± 300.3	1.34 ± 1.23
3	*P. quadrilineatus (n=10)*	19.0 ± 1.1	100.0 ± 20.1	1.45 ± 0.25
4	NS	NS	NS	NS
5	*A. thalassinus* (n=10)	29.3 ± 1.4	289.5± 56.8	1.4± 0.52

3.3.1 Measurements of PAHs biliary metabolites

A direct fluorescence detection technique was used for estimating relative concentrations of the metabolites in the bile of two representative fish collected from five locations in the Kuwait marine environment. The appropriate QC samples were run with each analysed batch of samples. The levels of PAH biliary metabolites (expressed as 1-OH equivalents) for both Giant sea catfish (*A. thalassinus*) and Four-lined terapon (*P. quadrilineatus)* in the individual locations are displayed in Table 3.3.

A significant difference in the biliary metabolites between *(A. thalassinus*) and (*P. quadrilineatus*) was observed within site one (p = 0.018), and difference was highly significant at site 3 (p < 0.000). Moreover, there is a highly significant difference in the *(A. thalassinus*) among all sites with (p<0.000), nevertheless; according to *post hoc* test site 2 shows the only highly significant difference (477.5 ± 290.7 µg kg^{-1} wet weight 1-OH pyrene equivalents) (p<0.000). The highest biliary metabolites in Four-lined terapon (*P. quadrilineatus*) was documented in site 3, however, there was no significant difference in biliary metabolites from site 1 (312.8 ± 108.1 µg kg^{-1} wet weight 1-OH pyrene equivalents) and site 3 (417.2 ±167.8 µg kg^{-1} wet weight 1-OH pyrene equivalents; p=0.124).

Table 3.3 Mean ± Standard Deviation concentrations (µg kg^{-1} wet weight 1-OH pyrene equivalents) of biliary PAH metabolites as a biomarker of exposure in the (*A. thalassinus*) and (*P. quadrilineatus*) and EROD activity measurements in sampled fish species (*A. thalissinus*) and (*P. quadrilineatus*) from different sites of Kuwait marine environment expressed as Mean ± Standard Deviation concentrations $pM^{-1}mg\ pro^{-1}min$. NS, no sample. * t-test = significant difference $p<0.05$; ** ANOVA test among the five sites. Depending on *post-hoc* significant difference $p<0.000$, [a] showed no significant and [b] the significant difference site. LOD= limits of detection.

Site	Species	Mean bile metabolites (µg kg^{-1} wet weight 1-OH pyrene equivalents)	Mean EROD (pM^{-1} mg pro^{-1} min)
1	[a]**(*A. thalissinus*) *	185.3 ± 51.8 (n=7)	< LOD
	(*P. quadrilineatus*) *	312.8 ± 108.1(n=6)	< LOD
2	[b]**(*A. thalissinus*)	477.5 ± 290.7** (n=8)	< LOD
	(*P. quadrilineatus*)	NS	NS
3	[a]**(*A. thalissinus*) *	129.0 ± 50.7(n=7)	< LOD
	(*P. quadrilineatus*) *	417.2 ±167.8(n=10)	< LOD
4	[a]**(*A. thalissinus*)	NS	NS
	(*P. quadrilineatus*)	NS	NS
5	[a]**(*A. thalissinus*)	163.2 ± 77.4 (n=7)	2.04±3.57 (n=10)
	(*P. quadrilineatus*)	NS	NS

3.3.2 Measurements of EROD activity in the liver of collected fish

The results of the EROD activity obtained from collected fish samples from different sites are present in Table 3.3, the data showing very low levels to be detected even though the QC samples were run parallel to each batch of samples with indicate that the bioassay was correctly run. However, the samples of Giant sea catfish (*A. thalissinus*) at site 5 are given positive values which are relatively showed and indicate the low level of EROD activities.

3.4 Discussion

Marine pollution by hydrocarbons is one of the major challenges facing oil producing countries, which surround the Arabian Gulf region as they rely on this environment for both their water and food security (Beg et al., 2001, de Mora et al., 2010, Sheppard et al., 2010 Al-Sarawi et al., 2015). Frequent oil spills and oil seepage can place an additional pressure to the discharges from oil refining and other industrial facilities located in the region (Al-Sarawi et al., 2015, Beg et al., 2015b, Lyons et al., 2015a). The main concern expressed over the hydrocarbon contamination is due to their persistence, their ability to bioaccumulate through the food chain and to pose both a toxic and carcinogenic threat to marine biota (Aas et al., 2000, da Silva et al., 2006, Beyer et al., 2010, Kammann et al., 2013, Koenig et al., 2013, Beg et al., 2015b, Bozcaarmutlu et al., 2015). Hence the use of a battery of biomarkers such as the induction of EROD activity in hepatic tissue and monitoring of biliary PAH metabolites, both of which have been widely used in biomonitoring studies to investigate the risk that hydrocarbon contamination poses to the marine environment (Ariese et al., 1993, Beyer et al., 2010). However, to date only a few studies have investigated their use in Kuwaiti (or indeed wider Arabian Gulf) waters (Beg et al., 2003 , Beg et al., 2009, Al-Zaidan et al., 2015, Beg et al., 2015a).

In this study, both the determination of biliary PAHs concentrations by SFS and EROD activities were used as PAHs exposure biomarkers to assess the detectable levels of PAHs pollutants that could pose biological effects.

The levels of bile metabolites (expressed as 1-OH equivalents) for both *A. thalassinus* and *P. quadrilineatus* were significantly differed among sites and between species. There was a significant difference in the biliary metabolites between species 1 and 2 within site one and the difference was highly significant in site 3 with values 129.0 ± 50.7, 417.2 ±167.8 μg kg^{-1} wet weight 1-OH pyrene equivalents, respectively.

Moreover, there is a highly significant difference in the (*A. thalassinus)* among all sites, nevertheless; according to post hoc test, site 2 showed the only highly significant difference and the bile metabolites value (477.5 ± 290.7 μg kg^{-1} wet weight 1-OH pyrene equivalents) than other sites. Site 2 is an off-shore site and many studies have shown there to be offshore seeps of oil which may naturally expose the fish to hydrocarbons. Natural oil seepage, accidental damage to pipelines, accidental spillage from tankers and tanker ballasting have been identified and are thought to be important point sources of contamination at various locations including the area close to site 2 (Zarba et al., 1985, Massoud et al., 1996, Massoud et al., 1998; Al-Ghadban et al., 2002, Al-Sarawi et al., 2015).

Results for the *(P. quadrilineatus),* indicated that the highest level of bile metabolites were observed at site 3 (417.2 ±167.8 μg kg^{-1} wet weight 1-OH pyrene equivalents) although it was not significant to other sites (p=0.124). In site 3, the level of biliary metabolites in *A. thalassinus* was comparatively low (129.0 ± 50.7 μg kg^{-1} wet weight 1-OH pyrene equivalents). This may indicate differences in exposure history between the fish collected. As the ability of (Four-lined *P. quadrilineatus*) being a free moving pelagic fish, to efficiently escape from the polluted locations in comparison to demersal catfish *(A. thalassinus)*. However, it is difficult to determine the biological significance of the data as previously

conducted analysis of sediment at all the 29 locations across Kuwait marine environment, including sites where the fish were collected in this study, indicated very low levels of PAH contamination (ΣPAH 40.7–97.7 μg kg^{-1} dw) (Lyons et al., 2015a). In the Kuwait marine sediment, the known PAH inducers of Cytochrome P450 1A activity in fish were detected in many sites such as low molecular weight PAHs like acenaphthylene, fluorene, phenanthrene and pyrene; among the high molecular compounds benzo(a)anthracene, chrysene, benzo(b)fluoranthene were present frequently in most of the Kuwait sediment samples (Beg et al., 2003, Beg et al., 2009, Al-Sarawi et al., 2015, Beg et al., 2015b, Lyons et al., 2015a). Recently, the low levels of PAHs were detected in almost the entire marine area ranging from 37-1332 ng g^{-1} and are localized to the industrial zone areas such as Shuaiba industrial area (Lyons et al., 2015a, Al-Sarawi et al., 2015). Wider spatial surveys of sediment PAH contamination have documented ΣPAH concentrations ranging from 12 to 1670 ng g^{-1} dw (de Mora et al., 2010). Some of low molecular weight (LMW) and high molecular weight (HMW) PAHs compound were measured such as pyrene which were detected in levels ranged from 0.26-67.4 μg kg^{-1} dw (Lyons et al., 2015a). This level was low than the Canadian ISQG 153 μg kg^{-1} dw and probable effect level (PEL) 1398 μg kg^{-1} dw (Canadian Council of Ministers of the Environment, 1999). Other PAHs were measured in sediments, such as fluorene ranged from <0.1-0.80 μg kg^{-1}, Benz*(a)*anthracene 0.1- 43.0 μg kg^{-1} and chrysene 0.1-23 μg kg^{-1}. The high values of these compounds were detected around the industrial area south of Kuwait coastline. However, the levels were below the Canadian quality guidelines and PEL for sediment (fluorene: 21.2 - 144 μg kg^{-1}, Benz*(a)*anthracene: 74.8 - 693 μg kg^{-1}, chrysene:108-846 μg kg^{-1}). The levels of these compounds in the sediments were support the levels of bile metabolites raised in this study. The PAHs levels were low and generally below commonly applied internationally recognised sediment quality guidelines such as those proposed by NOAA: ERLs and ERMs (Long et al., 1995, Long and MacDonald, 1998) where sediments containing PAHs <100 ng g^{-1}

indicates non-pollution, 250–500 ng g^{-1} as low to medium pollution, and >5000 ng g^{-1} as high pollution (Long et al., 1995, Long and MacDonald, 1998).

The levels of bile metabolites in this study are comparable to the findings of (Beg et al., 2015b), which used a fixed-wavelength fluorescence (FF) method for biliary metabolites measurement as exposure biomarkers in two fish species, yellow fin seabream (*Acanthopagrus latus*) and tongue sole (*Cynoglossus arel*) captured from inside and outside of Kuwait Bay. The results indicated predominant exposure to low molecular weight, naphthalene rich petroleum products (375 ± 91.0 pg ml^{-1}) in both species.

Both SFS and FF are used as a rapid and cost-effective biomonitoring tools for detecting PAH contamination (Dissanayake and Galloway, 2004, Beyer et al., 2010). Although both SFS and FF measure the sum total of PAH contamination rather than individual compounds, SFS is more selective than FF and the concentrations of predominant metabolites such as conjugated 1-OH pyrene can be detected with relative accuracy (Ariese et al., 1993, Dissanayake and Galloway, 2004, Beyer et al., 2010), as the fluorescence interference from other metabolite groups is reduced, resulting in a simpler spectrum that varies according to which PAH metabolites are present in the bile mixture. Therefore, SFS analysis is considered a quick screening assay useful in differentiating between varied PAH contaminated sites (Dissanayake and Galloway, 2004, Beyer et al., 2010). FF is a technique that does not differentiate PAH metabolites from other compounds due to the fluorescence overlap contributed by different PAH compounds, which may occur in field situations. At specified wave lengths the types of compounds which fluoresce include the parent compound and its metabolites and alkylated derivatives of the parent and its subsequent metabolites (Dissanayake and Galloway, 2004, Beyer et al., 2010). Therefore, FF analysis is considered as a qualitative measure of exposure to various PAH groups (Aas et al., 2000, Dissanayake and Galloway, 2004, Beyer et al., 2010).

When comparing the levels of bile metabolites detected in this current study to others conducted in other regions the levels of bile metabolites measures are actually very low (Ariese et al., 1993, Beyer et al., 2010), and the concentrations of 1-OH pyrene are similar or lower than other studies known reference (unpolluted) sites (Ariese et al., 1993, Beyer et al., 2010). For examples, biliary PAH metabolites as a 1-OH pyrene equivalents were detected in flounder (*Platichthys flesus*) captured in the Wadden Sea in Rotterdam (Ariese et al., 1993). The values at polluted sites ranged from 13600 ± 5000 μg kg^{-1} bile, the value was 820±540 μg kg^{-1} in the control site (Ariese et al., 1993). Lyons et al., 1999, reported levels of biliary PAH metabolites between 14,572 and 22,247 μg kg^{-1} wet weight 1-OH pyrene equivalents in flatfish caught from contaminated estuaries in the UK whereas in the control site it was 632 ± 56 μg kg^{-1} wet weight 1-OH pyrene equivalents. In contrast, the values of PAH biliary metabolites in this study were also higher than the baseline levels documented from reference locations such as biliary PAH metabolites in cod (*Gadus morhua*) at the baseline polluted area of Statfjord in UK (0.9 μg ml^{-1}) whereas it was 2.4 μg ml^{-1} in caged fish taken from the North Sea (Davies and Vethaak, 2012). Moreover, baseline levels of PAH metabolites have been established for many of the species relevant to monitoring in Norwegian coastal and offshore waters (Ruus et al., 2003). Bile values for the relevant species are: Atlantic cod (*Gadus morhua)*, 0.6 – 4 μg kg^{-1} bile; flounder (*Platichthys flesus*) 27– 89 μg kg^{-1} bile, dab 3.1– 34 μg kg^{-1} bile, plaice (*Pleuronectes platessa*) 0.4 – 3 μg kg^{-1} (Ruus et al., 2003, Davies and Vethaak, 2012).These baseline levels are lower than those detected in this study, where PAH metabolites ranged from 129 - 477.5 μg kg^{-1} for pelagic (*A. thalissinus*) and ranged 225 - 417 μg kg^{-1} for Four-lined (*P. quadrilineatus*), which could mean that these fishes were exposed to detectable levels of PAHs.

The adverse biological effects that could be posed by the exposure for PAHs are assessed against the criteria for biological effects measurements values of bile metabolites which are proposed by OSPAR as assessment criteria for contaminant concentration data such as

background assessment criteria (BAC) and environmental assessment criteria (EAC) (Davies and Vethaak, 2012). When apply these criteria which are available for flatfish and pelagic fish species as follow: BAC values for dab, cod and flounder are for bile metabolites (pyrene) are 150, 1100 and 1300 µg kg^{-1} respectively. Whereas the EAC values are 22000 , 35000 and 29000 µg kg^{-1} respectively. The levels detected in this current study are an order of magnitude below those concentrations thought to pose a toxicological threat (Davies and Vethaak, 2012, Al-Zaidan et al., 2015). The spatial variation in (*A. thalassinus)* suggest it to be used as sentinel models for biliary metabolites biomarker in Kuwait marine environment. The giant sea catfish (*A. thalissinus)* is a demersal fish which may be more exposed to the sediment which is known to be a sink for pollutants whereas *P. quadrilineatus,* being a free moving pelagic fish, is more capable of efficiently escaping from the polluted locations (Beg et al., 2015b). However, seasonal variations in bile metabolites were recognized in the different fish species (Davies and Vethaak, 2012). Therefore, more study considering the seasonal variations would be worth for more investigations in Kuwait.

The contamination of PAHs studied in the sediment documented that (LMW) PAHs such as acenaphthylene, acenaphthene, fluorene, phenanthrene and pyrene were present frequently in the sediment samples from Kuwait marine environment (Al-Sarawi et al., 2015, Beg et al., 2015b, Lyons et al., 2015a). Among the HMW compounds benzo(a)anthracene, chrysene, benzo(b)fluoranthene, and benzo(k)fluoranthene which are also the known inducers of Cytochrome P450 1A activity in fish and the ΣPAHs in sediment was higher in winter than summer time (Beg et al., 2015b).

In this study, the EROD results gave negative results (too low to detect) for both fish species, except in site 5 where the mean EROD activity was 2.04 ± 3.57 pmol min^{-1} mg^{-1} protein for the *A. thalassinus.* The QC samples, which were run in parallel with each batch of analyzed samples, gave positive results suggesting than the bioassay was correctly conducted. This

could be due to the samples being compromised during the shipping or could be due to long-term storage with variations in temperature, although every effort was taken to ensure the samples were frozen immediately on collection and were kept in such a state until they were processed in the laboratory (Stagg and Mcintosh, 1998). The other possible explanations is that the Resorufin fluorescence could vary among different fish species (Vehniäinen et al., 2012). Resorufin fluorescence could be affected by the S9 liver fractions of varied species resulting in that using the Resorufin curve without the S9 could leads to under estimating the actual levels of EROD activates (Vehniäinen et al., 2012). Therefore, more research and laboratory works are recommended to be done in this area to optimise the best procedure setting to measure the EROD activities for these specific fish types inhabiting Kuwait's marine environment. The procedures followed in this current study were optimised for the European flatfish species Dab (*Limanda limanda*) fish. Addressing such information gaps may provide a better optimised assay for assessing the EROD activity levels in fish specific for Kuwait's marine environment. Though it shouldn't be discounted that the levels of EROD activity were in fact indicative of low levels of chemical contamination as supported by the concentrations of bile metabolites and previous studies of PAH contamination (Lyons et al., 2015a)

The level of PAHs in fish inhabiting the Kuwait marine environment has been documented in the last decade. Beg et al., 2009 reported the concentrations of PAHs in three native fish species collected from the Kuwait Bay area: mullet (*Liza klunzingeri*), sea bream *(Acanthopagrus latus*) and tongue sole (*Lesan althour)*. Whole fish analysis revealed that the LMW PAHs (pyrene, fluoranthene and phenanthrene) were available in all the three varieties of fish in considerably higher amounts. Also, benzo (a) anthracene and chrysene was high and were documented in high concentrations. The contamination of PAHs was higher in the winter season in mullet (*Liza klunzingeri*), sea bream *(Acanthopagrus latus*) (7.02, 5.7 μg g^{-1}, respectively), whereas in tongue sole (*Lesan althour)* a reverse pattern

was found (15.02 μg g^{-1}). These have indicated that in general PAHs levels are generally low. However, from a toxicological perspective, flux information is more environmentally applicable to approximating the actual biotic stress caused by PAH exposure than the body burden data (Davies and Vethaak, 2012). Therefore, the PAH exposure biomarker were used in this study.

A 2015 study by Beg has reported the levels of EROD as a measure of exposure biomarkers in yellow fin seabream (*Acanthopagrus latus*) and tongue sole (*Cynoglossus arel*) captured from the Kuwait environment. In *A. latus*, median values of EROD were 8.64 and 6.34 pmol min^{-1} mg^{-1} protein in summer and winter months respectively from the Auha area, whereas in fish captured from Kuwait Bay area in the winter season, the median value was 14.87 pmol min^{-1} mg^{-1} proteins compared to summer median value 7.32 pmol min^{-1} mg^{-1} proteins. The median values of EROD activity in tongue sole (*Cynoglossus arel*) fish from Auha and Kuwait Bay area were documented (Beg et al., 2015b). In summer, the median value was 1.94 and 1.79 pmol min^{-1} mg^{-1} protein and in winter these values were 3.23 and 3.42 pmol min^{-1} mg^{-1} protein from Auha and Kuwait Bay area, respectively. The study concluded that although the non-seasonal effects on the seabream EROD activities shift the attention for the tongue sole, a significant difference in EROD activity was observed in summer and winter, to be one of the sentinel model used for the EROD assay. However, further work is now required to expand these approaches to involve other species which inhabit Kuwait marine environment during different seasons and to link specific findings with those of allied disciplines.

The spatial and temporal changes in contamination with PAHs in marine water which were observed via regular monitoring of Kuwait EPA (Devlin et al., 2015b, Lyons et al., 2015b) could play a significant role in the fluctuation of exposure in fish (Devlin et al., 2015b). Since EROD is induced by various components of PAHs, some fish exposed/ unexposed from distant areas might have migrated to the site and been captured along with other fish that

lived in the sampling area for a longer period. This could be also due to long residence of PAHs in water column at low temperature in winter time (Beg et al., 2015b).

Moreover, EROD activity is affected by seasonal changes such as pH values with the optimum value being 8 (Beg et al., 2001). Temperature as well is affecting the EROD activities where at low water temperature area the high EROD activity were observed (Ruus et al., 2003, Davies and Vethaak, 2012). Reproductive seasonal fluctuations were observed in different fish species such as Dab and flounder before and after the spawning period for male and female, suggesting that this needs to be considered in sampling for monitoring programmes (Eggens et al., 1996 , Wunderlich et al., 2015). The protein level is known to be influenced by the CF of fish (Sleiderink et al., 1995). The reason for the low EROD levels observed, could be due to deterioration in the CF of fish. The data obtained from this study was not sufficient to determine the impact of these factors. More studies are suggested to study the level of PAH ecotoxicological effects in Kuwait marine environment in more depth, including sampling a wide variety of residing pelagic and demersal fish with different reproductive status in different seasons and more representative locations as well as the fish from the wholesale markets in Kuwait.

When comparing the available EROD activities values for the fish taken from the Kuwait marine environment and the only finding value of this study (2.04±3.57 pmol min^{-1} mg^{-1} protein) with values ranges of EROD in fish collected at reference sites (Ruus et al., 2003) for the relevant species are (pmol min^{-1} mg^{-1} protein): Atlantic cod (*Gadus morhua*) 9–95, flounder *(Platichthys flesus)* 10–43, dab (*Limanda limanda*) 123-529 and plaice (*Pleuronectes plantessa*) 33–146. The levels of EROD activity in the Kuwait marine environment are within the range values that in reference sites and are below the levels of the BAC initiated for assessing the EROD activities (pmol min^{-1} mg^{-1} protein): Dab (Male/Female) 680, Flounder (Male) 24, Plaice 255 (Male/Female) that could pose an ecotoxicological effects as it has been generally thought that the activity of P4501A system

would not be limiting to the metabolism of pyrene but is induced by PAHs, which are considered “strong” inducers, elicit a greater than 100-fold induction (Whyte et al., 2000, Davies and Vethaak, 2012). Indeed, the results may indicate the low level of PAHs intake and bioavailable.

In attempts to develop integrated monitoring programmes that aim to assess the health of fish and shellfish in relation to chemical contamination in the Kuwait marine environment; a histopathology baseline survey in a range of pathologies, liver and gonadal histopathology in oriental sole (*Synaptura orientalis*) and the large-toothed flounder (*Pseudorhombus arsius*), which are two potential sentinel flatfish species present in the Arabian Gulf has previously been conducted (Stentiford et al., 2014). The results documented in the Kuwait marine environment were similar to those previously observed in European and American pollution effects surveys (including pathology markers indicative of possible carcinogenesis and endocrine disruption such as Hepatocellular foci and pre-neoplastic lesions). Some of these pathologies could represent the biological endpoint for the contaminants (Stentiford et al., 2014). However, the low prevalence of these biomarkers indicates that the levels of potential carcinogenic or genotoxic contaminants are below the internationally recognized risk threshold (de Mora et al., 2010, Stentiford et al., 2014,). These findings have been supported by a histopathology baseline survey in two other potential sentinel species, (*A. thalassinus*) and the Four-lined (*P. quadrilineatus*) to assess the health of biota inhabiting Kuwait’s marine environment. Histological analysis revealed several lesion types in both species, although the prevalence was generally considered low with no discernible differences between sampling locations (Al-Zaidan et al., 2015). The histopathology analysis results strengthen the findings of the current study in that the fish inhabit Kuwait marine environment are exposed to negligible levels of contaminants.

The findings of this study where both the PAHs exposure biomarkers the EROD activities vales with those of bile metabolites obtained are comparable with other studies as well for

Kuwait environment including the histopathology and sediment data (Beg et al., 2003 , Beg et al., 2009, Stentiford et al., 2014, Al-Sarawi et al., 2015, Al-Zaidan et al., 2015, Beg et al., 2015b). All are supported that a negligible biological risk could posed by the reported levels of PAHs contamination in Kuwait marine environment. These findings support the literature review presented in Chapter one, which reports that Kuwait marine environment has been subjected to a wide array of pollutants. However, the concentration of many of the reported pollutants are considered to be below international quality standards for water, sediment or biota. In addition, where levels of certain contaminants were elevated (e.g. PAHs, PCBs or PBDEs) they tended to be localized and restricted to the point source of pollution, such as the major industrial facilities based at Doha, Sulaibikhat Bay and the Shuaiba Industrial area (SIA). In common with many countries, Kuwait has developed marine monitoring programmes in response to environmental events, proposed constructions in the coastal zone, or suspected risks to human health. Such monitoring programmes provide data on the state of the environment in terms of water, sediment, marine organism health, bathing beach quality and harmful algal blooms and may allow conclusions to be drawn about trends in environmental quality over time. The current study has attempted to build upon this capacity by introducing the potential for pollution effects studies using selected natural biota present within the Kuwait marine environment to be assessed as well. It has been supported by a growing number of studies attempting to linked toxicological endpoints to the study of Kuwait's marine contamination (Al-Zaidan et al., 2015, Beg et al., 2015a, Beg et al., 2015b). Such studies also highlight the need for researches into so-called emerging contaminants (e.g. pharmaceuticals) or the potential impact of chemical mixtures, which to date have received little or no attention in Kuwait or the wider Gulf region. Therefore, attention was paid to another anthropogenic source of pollution that chronically put pressure on Kuwait marine environment, namely sewage effluent that is known to be discharged into Kuwait's marine environment in large volumes (Lyons et al., 2015b). Significantly, these effluents are

known to contain emerging contaminants with a range of toxicological properties including antimicrobial modes of action which have been detected at different locations around Kuwait marine environment (Lyons et al., 2015a, Lyons et al., 2015b).

Chapter 4 Baseline survey to screen for antimicrobial resistance potential in Kuwait marine ecosystem

A part of this chapter has been submitted for publication in Marine Pollution Bulletin

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4.1 Introduction

Antimicrobial agents are one of the powerful drug types administered to combat a range of infections and are widely used across sectors including human health, agriculture and animal farming (Al-Bahry et al., 2009b, Baquero et al., 2008, Martinez, 2009, Zou et al., 2012, Al-Bahry et al., 2012). However, concern has been raised over their indiscriminate use and the adverse impacts this may have on the environment (Baquero et al., 2008, Martínez, 2008, Martinez, 2009, Taylor et al., 2011). Antimicrobial agents are often only partially metabolized by humans and animals after administration, with up to 70% of the original active compound being discharged into the wastewater effluent network (Kümmerer and Henninger, 2003, Wang et al., 2014, Wang et al., 2015a). Unfortunately, antimicrobial agents are only partially eliminated from sewage treatment plants (STPs), and even in those systems, which include purification technologies such as coagulation, activated carbon filtration, ionic treatment and micelle-clay systems, some of the active agents remain after treatment (Watkinson et al., 2007a, Kümmerer, 2009a, Michael et al., 2013). In treated effluents, the antimicrobial residues concentrations can vary from ng l^{-1} up to µg l^{-1} (Watkinson et al., 2007a, Finley et al., 2013). Nevertheless, these very low concentrations are still pharmalogically potent and have the potential to drive the emergence of antimicrobial resistant (AMR) in bacterial communities that come into contact with the effluent (Kümmerer, 2009b, Martinez, 2009, Finley et al., 2013).

In recent decades, it has become apparent that antimicrobial agents should be considered an important class of aquatic contaminants and pose an emerging threat to the environment via the proliferation of AMR in bacterial communities (Baquero et al., 2008, Martínez, 2008, Martinez, 2009, Michael et al., 2013). The exacerbation of the AMR phenomenon is now receiving serious attention worldwide and it is considered an important ecological and environmental problem impacting both environmental and human health (Baker-Austin et al., 2006, Al-Bahry et al., 2009b, Finley et al., 2013, Wang et al., 2015a).

4.1.1 Role of the aquatic environment in maintaining AMR

Globally, aquatic environments act as a repository and reservoir for sewage discharges that are known to contain antimicrobials, metals, biocides and other pollutants that can act as a selective pressure driving AMR (Baquero et al., 2008, Kümmerer, 2009a, Taylor et al., 2011, Di Cesare et al., 2013). All these pollutants accumulate in the aquatic systems and are particularly prevalent in the riverine, near shore and coastal environment close to sewage outlets (Rooklidge, 2004, Baquero et al., 2008, Martínez, 2008, Martinez, 2009, Taylor et al., 2011, Al-Bahry et al., 2012, Di Cesare et al., 2013, Wang et al., 2014). Therefore, the aquatic environment can play a significant role in driving AMR emergence, acquisition, maintenance and proliferation among bacterial communities (Baker-Austin et al., 2006, Wright et al., 2008, Taylor et al., 2011, Wang et al., 2014, Wang et al., 2015a). This also poses a significant risk to human health, either by direct contact with AMR bacteria in the water (e.g. recreation water users) or through the consumption of contaminated foodstuffs (e.g. shellfish) (Taylor et al., 2011, Costa Andrade et al., 2015).

4.1.2 Use of AMR as a biomarker for monitoring pollution in the marine environment

Antimicrobial resistant (AMR) bacteria have now been reported from a range of environmental matrices including the biota (Miranda, 2001, Al-Bahry et al., 2009a, Al-Bahry et al., 2011b, Zhang et al., 2011, Al-Bahry et al., 2012, Wang et al., 2014, Wang et al., 2015a), sediment (Mazel et al., 2000, Costa Andrade et al., 2015), natural waters (Parveen et al., 1997, McArthur and Tuckfield, 2000, Baker-Austin et al., 2008, Al-Bahry et al., 2009a, McArthur et al., 2015) and sewage effluent (Galvin et al., 2010, Al-Bahry et al., 2011a). Thus, it has been proposed that monitoring for the presence of AMR bacteria in aquatic

environments can be used as a biomonitoring tool for detecting the presence of antibiotic pollution in the marine environment (Baquero et al., 2008, Martínez, 2008). The biomonitoring of AMR in the marine environment would help to provide evidence to direct and inform environmental management programmes aimed at mitigating and restricting the release of antibiotics into the marine environment (Martínez, 2008, Kümmerer, 2009b, Martinez, 2009, Al-Bahry et al., 2012, Finley et al., 2013, Stentiford et al., 2014). Many Studies worldwide have demonstrated the use of AMR in the marine environment as a biomarker for monitoring pollution (Miranda, 2001, Baquero et al., 2008, Baker-Austin et al., 2008, Al-Bahry et al., 2009b, Al-Bahry et al., 2011b, Al-Bahry et al., 2012, ,Moskot et al., 2012, Miranda et al., 2013, Baliere et al., 2015). For example, the prevalence of AMR strains of *E.coli* in surface water samples has been used to differentiate point and non-point sources of pollution in the Apalachicola National Estuarine Research Reserve (Parveen et al., 1997). The AMR profile of a total 765 *E.coli* were determined against 10 antimicrobial agents and results indicated higher frequencies of multi-resistant bacteria associated with point source samples compared to those collected from non-point source locations. Similar studies in Brazil observed differences in AMR potential in *E.coli* isolated from both sand and recreational waters from different level of polluted beaches depending on the local bathing water quality (Costa Andrade et al., 2015). Beaches with the higher degree of pollution were shown to contain a greater prevalence (67.5% seawater; 71.9% wet sand) of AMR bacteria compared to those isolated from a location known to be with less polluted (50% seawater; 53.8% wet sand). Another study looked at a range of water, sediment and shrimp samples collected from industrially polluted Iskenderun Bay, on Turkey's southern coast (Matyar et al., 2008). Here, over 50% of the *E.coli* screened were resistant to more than 7 of the antibiotics tested and over 93% displayed resistance to ampicillin. A similar level of resistance was also observed in *E. coli* isolates collected from multiple locations along nine streams that drain into the US Department of Energy's Savannah River Site (McArthur et al.,

2015). Here isolates were screened against a panel of five antibiotics. At the majority of sites, higher levels of resistance were found in waterborne *E. coli* isolates although at 3 locations the greatest level of resistance was observed in sediment samples. In recent years, it has also been proposed marine bivalves could act as an important reservoir and source for AMR bacteria in the marine environment (Taylor et al., 2011). This was attributed to their wide geographic distribution, tolerance of a wide variety environmental conditions, mode of feeding, sedentary nature, relative sensitivity to contaminants in sediments and water and their potential to bioaccumulate contaminants when compared to background environmental concentrations (Zorba et al., 1992, Tarique et al., 2012). Bivalve molluscs are likely to contain a high concentration of taxonomically diverse communities of microorganisms, which thrive within the bivalve's environment. Indeed, bacterial replication rates may be enhanced by temperature cycling as bivalves are exposed to daily tidal cycles via (Taylor et al., 2011) and filter feeding organisms, such as these, can play a significant role in evolution of AMR in aquatic systems (Taylor et al., 2011). Consequently, many studies have used bivalves as a sentinel model matrix to study the presence of AMR in aquatic systems (Ryu et al., 2012, Wang et al., 2014, Wang et al., 2015a, Olalemi et al., 2016). For example, a recent study from China showed that bacteria collected from the intestine of the oyster (*Crassostrea hongkongensis*) had a higher level of resistance to 7 out of the 10 antibiotics tested than seawater collected from the rearing cages used to house the oysters (Wang et al., 2014).

4.1.3 Potential AMR risk to the marine environments of the Arabian Gulf and Kuwait

In the Arabian Gulf region, the issue of AMR in the marine environment has received very little attention to date and has mainly focused on a handful of studies conducted in Oman. These have included reports identifying the presence of AMR bacteria in fish and seawater collected from locations close to sewage discharges (Al-Bahry et al., 2009b) and in the effluent itself (Al-Bahry et al., 2011a). Moreover, the presence of AMR bacteria has been used as an indicator to monitor the exposure of Green sea turtles (*Chelonia mydas*) to different marine pollutants (Al-Bahry et al., 2012). As has been previous described (see Chapter 1) the marine environment of Kuwait is known to be heavily impacted by large volumes of partially or untreated sewage discharge (Saeed et al., 2012, Lyons et al., 2015b, Saeed et al., 2015). A recent assessment of the degree of sewage contamination degree 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 spot areas within Kuwait Bay and along the Arabian Gulf coast. (Devlin et al., 2015a, Lyons et al., 2015b). In addition to this, a wide array of chemical pollutants has been identified in these effluents, some of which are known to have antimicrobial modes of action (Smith et al., 2015). This is hardly surprising as it is known that in recent years that the prescription and usage of antibiotics within Kuwait's population is significant (3.6×10^6 prescription unit/ annum as an average for 2010- 2014, personal communication Kuwait Ministry of Health, 2015).

In Kuwait, sewage discharge also contains other pollutants such as metals (Devlin et al., 2015b, Lyons et al., 2015a) that are believed to favor co-selection for AMR in the marine environment (Di Cesare et al., 2016a, Di Cesare et al., 2016b). Therefore, it is clear that the potential exists for large volumes of antibiotics and/ or their metabolites to be released into Kuwait's marine environment and these discharges may also contain other contaminants that are known to act as an additional selective pressure driving AMR emergence in the marine environment.

Along with other countries in the Gulf region, Kuwait has developed marine monitoring programmes in response to environmental disasters, rapid industrialisation and development of the coastal zone, or suspected risks to human health from the consumption of sea food (Stentiford et al., 2014, Devlin et al., 2015a). The majority of these programmes have focussed on providing data on environmental status in terms of chemical contamination, bathing beach water quality and eutrophication and there is currently no active environmental surveillance programme monitoring for the prevalence of environmental AMR in Kuwait, where research into AMR has been restricted to the clinical setting with high levels of resistance documented against a range of front line antibiotics (Zhang, Eggleston, Rotimi, & Zeckhauser, 2006, Jamal et al., 2013). 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.

4.2 Aims and Objectives

This study aimed to investigate the prevalence of AMR bacteria derived from Kuwait's marine environment.

1- A baseline AMR screening was conducted using the micro-dilution (48 h incubation) onto the custom dehydrated 96-well Sensititre™ GN2F panels for bacteria isolated from seawater and bivalves) across Kuwait coastal environment during different seasons.

4.3 Material and methods

4.3.1 Site and sampling information

Samples (seawater) for AMR screening were collected during both winter (December 2014 - February 2015) and summer (July – August 2015) from sites along the Kuwait coastline. Bivalves samples were collected a cross both seasons summer (July-August 2015) and winter (December 2015-February 2016).

As shown in Figure 4.1, sites were selected to provide representative locations around the main sewage outlets within Kuwait Bay (Al-Salam and AL-Ghazali) and along the Arabian Gulf Coast (Abu-Al-Hasaniya). At each site, where possible, the seawater samples were collected from beaches close to known sewage discharge locations. For comparison, seawater samples were collected from the Khiran site as reference samples, as no bivalves were available here.

Multiple seawater samples from the most upper (4-6 per site, 200 m towards each) were taken into 1L sterile polyethylene bottles and stored in a cooler with ice for no longer than 6h, to reduce any further biological activities, before being returned to the laboratory for analysis at the Faculty of science in Kuwait University as described in detail in Section 2.4.1.1.

Bivalve samples were also collected by hand and stored in sterile plastic bags in a cooler with ice. Species differed depending on availability with the Venus Clams (*Circenita callipyga*) collected from Al-Salam. More than 60 Venus clams were collected to prepare replicates pooled samples (20g for each pooled sample). Samples were processed in the same day of sampling at Faculty of science in Kuwait University as described in section 2.4.1.2.

In the field, physical-chemical parameters (i.e. pH, water temperature and salinity) of seawater from each sampling site during both sampling period (summer and winter) were measured *in situ* by using a portable multi-parameters water quality instrument (Hanna instruments model no. HI 9828, USA).The average values for the major *in situ* physical-

chemical parameters across Kuwait marine sample sites were as follows: salinity (mean 36 ‰ ± 2.0) and the pH values (mean 8.2 ± 0.2) were stable during both sampling periods and within the Kuwait EPA water Quality standards (EPA, 2001), whereas water temperatures varied substantially (summer: 34 °C ± 2.0; and winter: 17.2 °C ± 2.0).

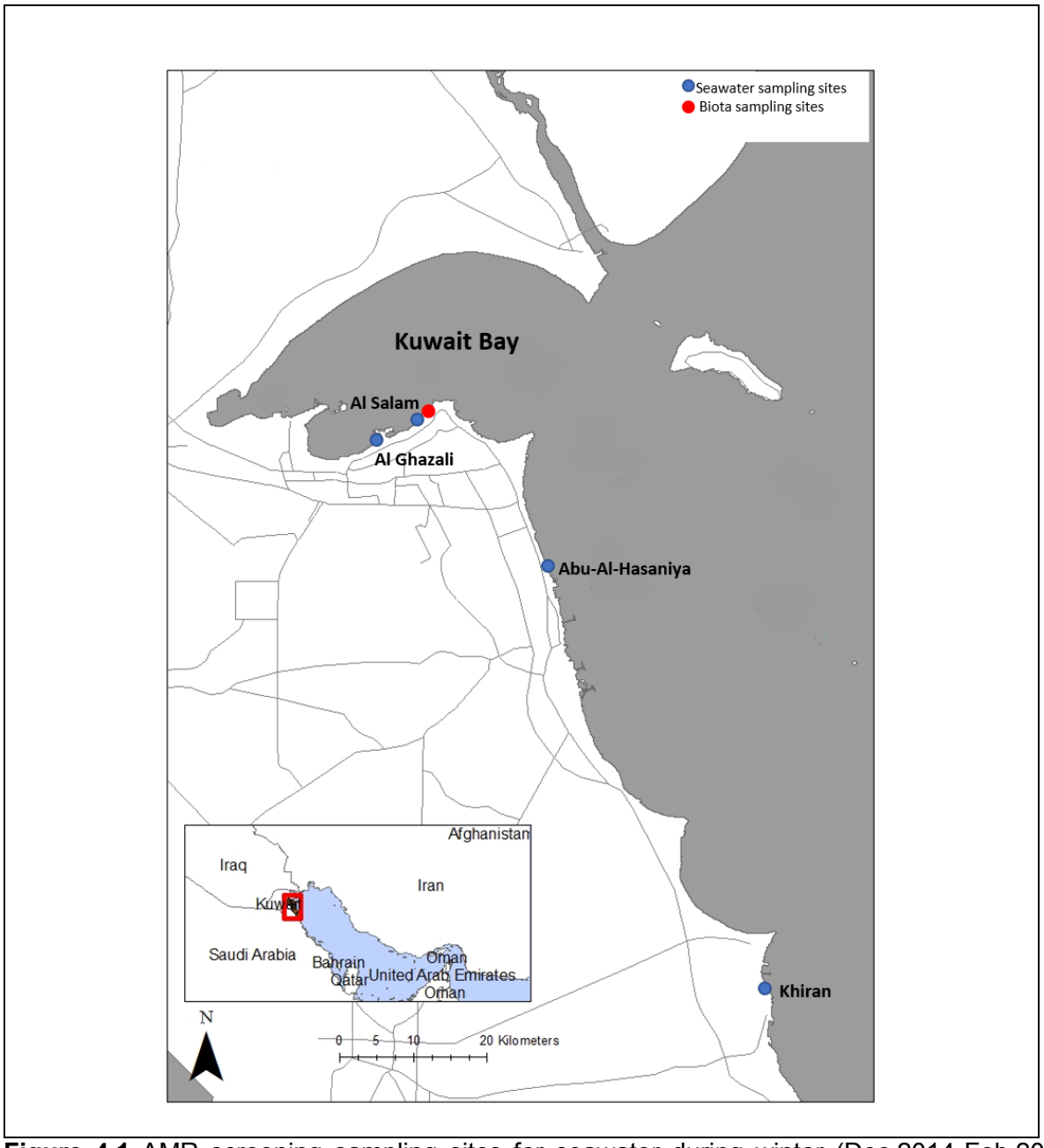

Figure 4.1 AMR screening sampling sites for seawater during winter (Dec.2014-Feb.2015) and summer (Jul.-Aug. 2015) from inside Kuwait Bay sites: Al-Salam and Al-Ghazali and outside the Kuwait Bay sites: Abu-Al-Hasaniya. Khiran was as a seawater reference site. Bivalve samples were collected (as available) from Al-Salam, during summer (Jul.-Aug. 2015) and winter (Dec.2015-Feb.2016).

4.3.2 Isolation and enumeration of microbial water quality indicators (faecal coliforms and *E.coli*)

The enumeration and isolation of microbial quality parameters (faecal coliforms,FC,and *E.coli*) was conducted at each site in both seawater and bivalve samples to get an estimation of the level of sewage contamination. This was undertaken using the membrane filter technique as described in section 2.4.2. following the methods 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 technique, 0.1, 1, 3, 10 ml volumes and tenfold serially diluted volumes 10^{-1}, $10^{-3}$and 10^{-5} dilutions using 0.1% peptone water for each seawater samples were prepared. These were filtered through sterile a 47 mm diameter, 0.45 µm pore size membrane filters (Durapore®, Mereck Millipore, USA), which were then placed separately onto m-FC media with 10 ml 1% Rosolic acid in 0.2*N* 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 et al., 1997, McArthur et al., 2015). NA-MUG plates were incubated overnight (16–20 h) at 37 °C. Fluorescent colonies were assumed to be *E.coli* and sub-cultured on MUC-media in order to be used for AMR analysis.

To enumerate and isolate faecal coliform and *E.coli* in bivalves, pooled samples were prepared for (Venus clams) as described in section 4.2.1.2. Filtration, incubation, counting, and confirmation procedures were the same as for the seawater samples. Blank and positive control samples were analysed in parallel with those collected from the field. The mean ±

SD (six replicate samples) of FC in seawater was expressed in colony-forming units (CFU) per 100 ml and per 100 g bivalve tissue as per the following equations:

Equation to calculate FC numbers in seawater: count (CFU) /100ml (seawater) =

(Number of colonies on m-FC media/volume of sample filtered) x dilution factor x100

Equation to calculate FC numbers in bivalve's tissue: count CFU/100g (clams) =

(*X*) / total weight of clam's tissue in the pool

Where (X) = (Number of colonies on m-FC media/volume of pooled sample filtered) x dilution factor x100

For verified *E.coli* counts, the initial count was adjusted based upon the positive percentage and was reported as verified *E.coli* count/ 100 ml or count/ 100g for seawater and tissue, respectively, as follows:

Percentage verified *E.coli* on NA-MUG media **=**

Number of verified colonies / total number of coliform colonies subjected to the verification X100.

After confirmation, *E.coli* isolates were selected from seawater and bivalves in each site (around 640 *E.coli* isolates across Kuwait coastline during the year) and stored in Microbank vials (PRO-LAB Diagnostics, Canada) and shipped frozen to Plymouth University (UK), where they were kept frozen at -20 °C prior to conducting the AMR screening. A subset of samples was retained at −80 °C for longer term storage.

4.3.3 Antimicrobial resistant (AMR) screening in *E.coli*

Before conducting the AMR screening, all isolates were subjected to *E.coli* O157 screening, as this is an obligatory protocol forced by the Advisory Committee On Dangerous Pathogens (ACDP) when dealing with hazards group 3 organisms. This is a routine protocol followed

by Plymouth University as the local biosecurity regulations only allow for the handling contaminant level 2 microorganisms within their laboratories. The isolates were screened as described in section 2.4.3. Briefly*, E.coli* isolates were cultured on M9 minimal (5X) media supplemented with 0.5% Sorbitol as a carbon source (Sigma-Aldrich- Co, UK). Plates were incubated at 37 °C for 24 h. Suspected 0157 isolate, which are showing no growth, were discarded immediately into appropriate clinical waste streams. The total number of *E.coli* isolates after O157 *E.coli* screening was 598 *E.coli* which were then successfully subjected to the following AMR screening.

The process for AMR screening was conducted following the guidelines of Clinical and laboratory Standards Institute (CLSI, 2014). 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. Isolates were classified as either resistant, intermediate (partially resistant) and susceptible (sensitive), according to the breakpoints recommended by Cefas that hybrid with the Clinical Laboratory and Standard Institute (CLSI, 2014).

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 antibiotic 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]; Carbapenems: 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. *E. coli* ATCC 25922 was used as control for the results following the criteria established by the Clinical Laboratory Standards institute (CLSI, 2014).

Interpretive criteria are the MIC values used to indicate susceptible, intermediate, and resistant breakpoints (CLSI, 2014). For example, for antimicrobial agent FUR with interpretive criteria in the Table 4.1 below, the susceptibility breakpoint is 4 µg/ ml, the intermediate breakpoints are 8-16 µg/ml and the resistant breakpoint is 32 µg/ml. A fully description of the resistance profile obtained for each isolate against each tested antibiotics are provided in Appendix 1.

Table 4.1 The interpretive criteria used to determine the susceptible (S), intermediate (I) and resistant *E,coli* depending on the growth on define concentrations according to the breakpoints recommended by Cefas that hybrid with the Clinical Laboratory and Standard Institute (CLSI, 2014) .

Antimicrobial agents	**MIC interpretive criteria, µg/ml**		
	S	**I**	**R**
FUR	≤ 4	8-16	≥ 32

4.3.4 Statistical analysis

Data on all *E.coli* isolates from seawater and bivalves, including their responses to the different tested antibiotics in AMR test (%), were entered into an Excel worksheet (Microsoft office professional, 2016). The formula used to calculate the percentage of resistant strains was as follows:

Percentage of resistant strains to a single antibiotic (%) = (quantity of resistant strains to the single antibiotic/ total strains for the test) × 100.

The t-test was calculated using SPSS version 23 statistical software (Microsoft office professional 2016) to determine whether there were any significant differences in AMR levels during summer and winter or between seawater and bivalve-derived *E.coli*.

4.4 Results

4.4.1 Microbial water quality in the Kuwait marine environment

The mean number of faecal coliforms and *E.coli* present in 100 ml of seawater samples is presented in Table 4.2. The results were compared with various international microbial water quality standards, such as the coastal Bathing Water Directive (cBWD) adopted in the European Union (original Directive 76/160/EEC) (Anon, 2006) which sets out a number of microbiological and physiochemical standards that bathing waters must either comply with Table 4.2. One of the standards used to assess the quality of bathing water, in addition to faecal coliforms, is *E. coli*, as it is one of the main bacteria commonly found in the guts of humans and other warm-blooded animals. Thus, *E.coli* is used as an indicator of sewage related pollution (Titilawo et al., 2015). Kuwait Environment Public Authority (KEPA) have established thresholds for the seawater microbial quality parameters including those used in this study (faecal coliform and *E.coli)* (EPA, 2001) which are compliant with the current European Bathing Water Directive (cBWD) (Anon, 2006) (Table 4.2) and have been

developed to protect bathing water sites by setting thresholds for the main microbiological organisms to avoid impact on human health. Results for seawater were also compared against the Brazilian legislation for bathing water quality. According to the Brazilian legislation, *E. coli* concentrations of more than 800 CFU /100 ml in at least two samples during a period of 5 weeks or values above 2000 /CFU 100 ml at the preceding sampling classified the beach as unsuitable for primary contact recreation (CONAMA and Ambiente., 2000).

Table 4.2 Enumeration of (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 expressed as colony forming units per 100ml (CFU/100 ml) for each of six replicate plates and reported as (mean values ±SD) over the total number of plates. * The values expressed to 10^3.

				International thresholds (CFU/100 ml)	
Site	**Mean ±SD CFU/100 ml (FC)**	**Mean ±SD CFU/100 ml (*E. coli*)**	**KEPA Max limit (FC) 200**	**Brazilian legislation Max limit *(E. coli)* 2000**	**EU cBWD (*E. coli)* Good <500; Excellent <250**
Al-Salam (summer)	226.21 ± 46.05*	111.88 ± 16.01*	Exceed	Exceed	Exceed
Al-Salam (winter)	380.54 ± 51.21*	184.25 ± 11.89*	Exceed	Exceed	Exceed
Abu-Al-Hasaniya (summer)	179.14 ± 9.00*	91.04 ± 15.78*	Exceed	Exceed	Exceed
Al-Ghazali (summer)	125.25 ± 11.75*	64.95 ± 12.23*	Exceed	Exceed	Exceed
Al- Ghazali (winter)	391.29 ± 57.56*	200.65 ± 35.48*	Exceed	Exceed	Exceed
Khiran (summer)	36.57 ± 5.63	18.12 ± 6.32	Below	Below	Below
Khiran (winter)	75.00 ± 8.20	36.21 ± 7.36	Below	Below	Below

Results quite clearly indicate poor water quality i.e. sewage contamination, in the replicate seawater samples, collected from most of sampling sites across the Kuwait coastline during both summer and winter seasons. The faecal coliform and *E.coli* counts exceeded the water quality guidelines most of time, especially at sites Al-Salam, Abu-Al-Hasaniya and Al-Ghazali where wastewater effluent pipes are known to discharge (Table 4.2). The highest counts for both microbial parameters were recorded in winter at the main sewage outlets in Kuwait Bay region (Al-Ghazali and Al-Salam sites, respectively). The standards exceedance of *E.coli* counts at these locations were a further order of magnitude above the thresholds. In winter, the faecal coliform and *E.coli* counts derived from seawater samples ranked as follows: Al-Ghazali > Al-Salam > Khiran. In summer, the ranking was slightly differed among sites as follows: Al-Salam > Abu-Al-Hasaniya > Al-Ghazali > Khiran. The failure to comply the guide standards by up to 200-fold in water samples collected from Al-Salam site during the summer. The site located at Khiran is considered as a reference site as no known major sewage discharge points are thought to be located in this area. However, the microbial water quality parameters used in this study (faecal coliform bacteria and *E.coli*) were detected in seawater samples collected from Khiran site as well. This may be due to number of private villas constructed along the coastline at this location contributing small amounts of sewage to the coastal environment. Nevertheless, the counts were low and compliant with the most stringent microbial water quality standards during both sampling periods (Table 4.2).

In this study, sampling sites were chosen to represent a point source of sewage pollution areas around Kuwait coastline such as Al-Salam and Al-Ghazali and are where some well-known sewage discharge outlets are located. However, the number of faecal coliform and *E.coli* derived (isolated) from Al-Ghazali was unexpected and low comparing to other sites. This could be due to the actual distance from the point source of sewage discharge as some of the sites are not easy to access. The number of Venus clams, collected from Al-Salam site was 60. The CFU/ 100g for the FC and *E.coli* are presented in Table. 4.3.

Table 4.3 Enumeration of *(FC) and* E.coli present in Venus clam samples collected from different locations across the Kuwait marine environment during summer and winter. *The values expressed to 10^3.

sites	Faecal coliforms (CFU/ 100g)	(*E.coli*) (CFU/ 100g)
Al-Salam (summer)	39.8 ± 6.8*	19.3 ± 6.4*
Al-Salam (winter)	60.2 ± 7.7*	27.6 ± 4.3*

4.4.2 AMR Screening for *E.coli* derived from the Kuwait marine environment

From Kuwait marine environment, the number of *E.coli* isolates after *E.co*li O157screening was 598 (351 from seawater; 247 from bivalves). These were 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 percentage of isolates showing resistance to at least 1 of the 23 antibiotics tested is displayed in Table 4.4. Results demonstrate that resistance was widespread across all sites (seawater: summer 64- 89%; winter 57-90 % and bivalves: summer 77%; winter 88%). A full description of the resistance profile obtained for each isolate is provided in Appendix 1.

Table 4.4 The % of *E. coli* isolates resistant to at least 1 of the 23 antibiotics tested in seawater and Venus clam samples collected from Kuwait's marine environment during summer and winter. N/S: no sample.

Site	Season	Seawater	Bivalves
		% resistant to at least 1 antibiotic (number isolates screened)	% 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

4.4.3 Seasonality variation of AMR in *E.coli* derived from the Kuwait marine environment

The percentage of AMR *E.coli* isolated during different seasons from both seawater and bivalves presented by Figure 4.2. The levels of AMR *E.coli* were significantly higher in winter for both seawater and bivalves (81% and 88%, respectively) than summer (73% and 76% respectively; $p <$ 001). Moreover, no significant differences were found when comparing the AMR from seawater and bivalves within the same season as the *p* values were 0.817 for winter and p=0.304 for summer.

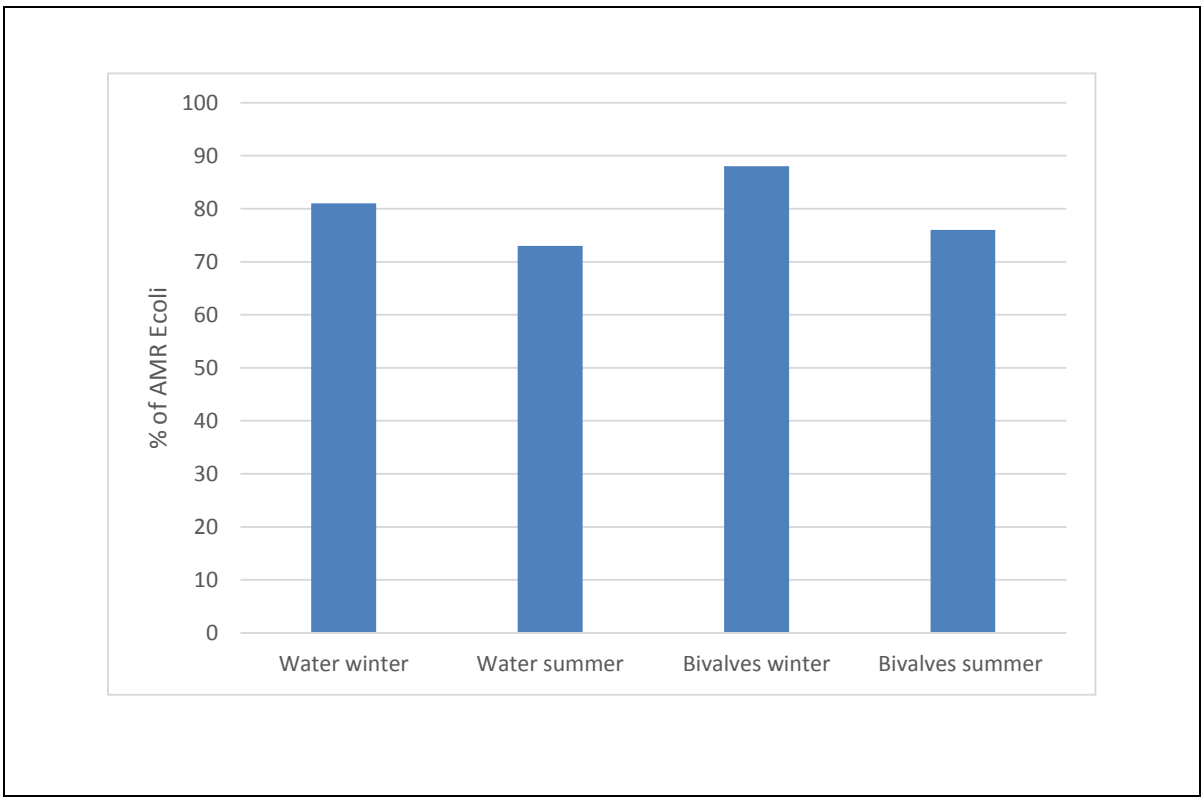

Figure 4.2 AMR *E.coli* derived from seawater and bivalves in Kuwait marine environment during summer and winter seasons.

In this current survey, out 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 (Appendix 1; Figure 4.3). No single isolates showed resistance to all tested

antibiotics. In general, *E.coli* isolated during winter season showed higher AMR levels than summer in terms of the number of resistant isolates $p < 001$, and the wider variety of antibiotics to be resistant. In winter, the AMR *E.coli* were against 21 out of 23 tested antimicrobial agents were in summer the resistance was against 19 antimicrobial agents (Appendix 1).

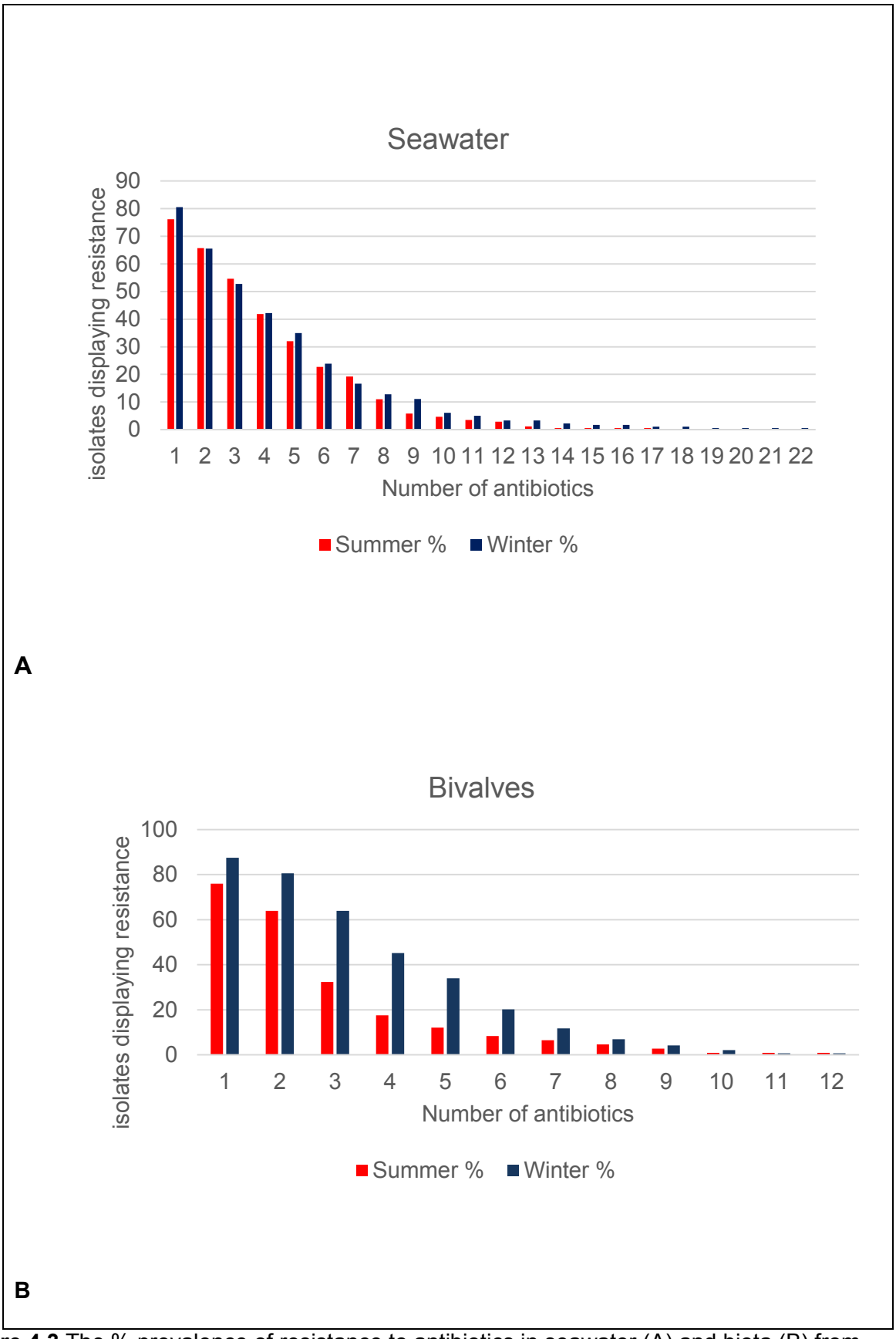

Figure 4.3 The % prevalence of resistance to antibiotics in seawater (A) and biota (B) from summer (red) and winter (black) sampling periods.

4.4.4 Top resistant antibiotics in Kuwait marine environment

Resistance against the majority of the tested antibiotics was detected in *E. coli* derived from the Kuwait marine environment during winter and summer seasons although there was a degree of difference in terms of frequency (Appendix1; Table 4.5 and Figure 4.4). Resistance to (AMP) was by far the most widely observed in both seawater and bivalves across both summer and winter seasons, with 55.9 to 70.9% isolates displaying resistance in *E.coli* derived from seawater during both summer and winter, respectively, and 67.9% 69.4% in biota derived *E.coli* both seasons, respectively (Appendix 1; Table 4.5). Following the AMP in the five-highest resistant rank, were (SXT), (PIP), (CIP) and (FAZ). These are belonging to different classes of antibiotics indicating the wide spectrum resistance level by isolated *E.coli* from Kuwait marine environment. Within the 3rd generation cephalosporins, isolates exhibited high resistance to (POD), (AXO) than (TAZ). The least resistance was observed for the 4th generation of Cephalosporins (FEP). The ranking of resistance against these antibiotics was varied between bivalves and seawater during summer and winter (Table 4.5). The only antibiotic to which all 598 isolates were sensitive to was Imipenem (IMI), a carbapenem class of antibiotic. Resistance to Amikacin (AMI) was also poor, with only 0.6% of seawater *E.coli* (winter) among all isolates were resistant to AMI, an aminoglycoside class of antibiotic (Appendix1; Table 4.5 and Figure 4.4).

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, strains of *E. coli* isolated from winter seawater and bivalve's samples displayed a high-level of resistance to (FOX; 24% and 51.4%, respectively) which then dropped substantially in samples screened from the summer (4.7% and 5.8%, respectively; Table 4.5). Similar seasonal differences in resistance profiles were also noted for (AXO) and (TIM2) (seawater isolates) along with (FUR), (PIP) and (FAZ) bivalve's isolates as shown in Table 4.5.

Table 4.5 Seasonal antibiotic resistance ranking summary for *E. coli* (sites combined) isolated from seawater and Venus clam.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						bivalves				
Rank	**Antibiotic**	**Summer % resistance of *E. coli* (n=172)**	**Rank**	**Antibiotic**	**Winter % resistance *E. coli* (n= 179)**	**Rank**	**Antibiotic**	**Summer % resistance *E. coli* (n=103)**	**Rank**	**Antibiotic**	**Winter bivalves % resistance of *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	42.44	**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	22.67	**7**	FOX	24	**7**	FUR	9.7	**7**	FUR	20.8
8	AXO	17.4	**8**	FUR	18.9	**8**	AXO	7.7	**8**	CIP	14.6
9	AZT	12.2	**9**	TIM2	18.4	**9**	FOX	5.8	**9**	A/S2	11.1
10	A/S2	10.5	**10**	A/S2	16.8	**10**	TIM2	4.8	**10**	TANS	11.1
11	TIM2	8.7	**11**	GEN	15.1	**11**	AZT	3.9	**11**	TIM2	10
12	GAT	8.1	**12**	GAT	8.4	**12**	TAZ	2.9	**12**	AXO	8.33

	Seawater						bivalves				
Rank	**Antibiotic**	**Summer % resistance of *E. coli* (n=172)**	**Rank**	**Antibiotic**	**Winter % resistance *E. coli* (n= 179)**	**Rank**	**Antibiotic**	**Summer % resistance *E. coli* (n=103)**	**Rank**	**Antibiotic**	**Winter bivalves % resistance of *E. coli* (n= 144)**
13	GEN	8.1	**13**	TOB	7.8	**13**	A/S2	2.9	**13**	AZT	3.47
14	FOX	4.7	**14**	AZT	7.8	**14**	GAT	2.9	**14**	GEN	2.8
15	FEP	4.1	**15**	TANS	6.7	**15**	TANS	1.9	**15**	FEP	2.8
16	TOB	3.4	**16**	AXO	6.7	**16**	FEP	1.9	**16**	GAT	2.8
17	TANS	1.7	**17**	TAZ	5	**17**	GEN	1.9	**17**	TOB	2.1
18	TAZ	1.7	**18**	P/T4	3.4	**18**	P/T4	0.9	**18**	NIT	1.4
19	NIT	1.2	**19**	FEP	2.2	**19**	MERO	0.9	**19**	TAZ	0.7
20	P/T4	0.6	**20**	NIT	1.1	**20**	TOB	0.9	**20**	MERO	0.7
21	MERO	0	**21**	MERO	1.1	**21**	AMI	0	**21**	P/T4	0.7
22	IMI	0	**22**	AMI	0.6	**22**	IMI	0	**22**	AMI	0
23	AMI	0	**23**	IMI	0	**23**	NIT	0	**23**	IMI	0

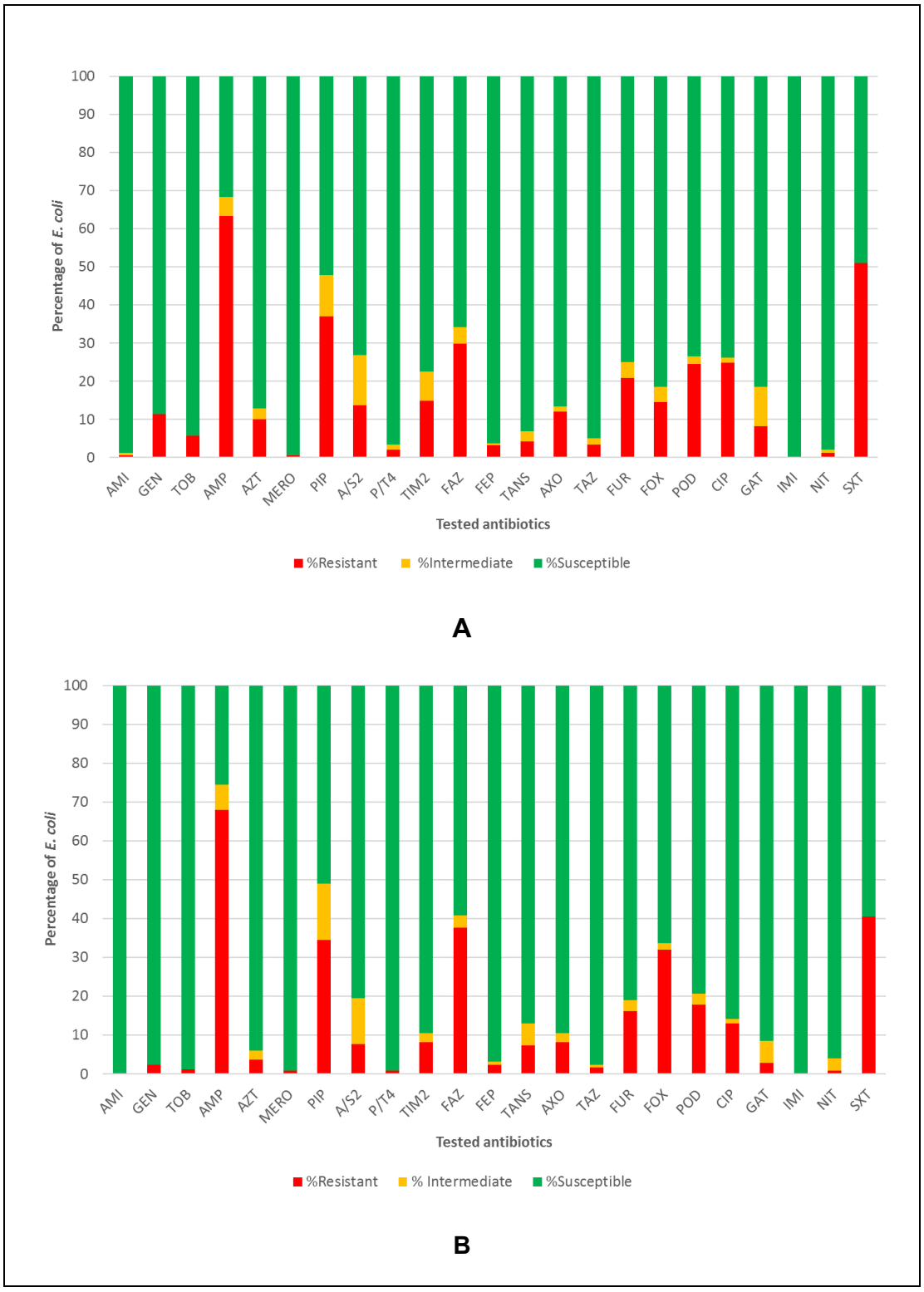

Figure 4.4 Profile of resistance patterns (Resistant = red; Intermediate = amber; Susceptible = green) according to the breakpoints recommended by Cefas that hybrid with the Clinical Laboratory and Standard Institute (CLSI, 2014) to antibiotics in seawater (A) and biota (B). Antibiotics screened: Amikacin (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).

4.4.5 Antibiotic resistance index (ARI) and multi-drug resistant (MDR) indexing

Antibiotic resistance indexing (ARI) is an effective tool that enables one to determine the dissemination and prevalence of bacterial resistance in a given population at a specified location in order to evaluate the impact of urbanization among the locations (Mohanta and Goel, 2014, Titilawo et al., 2015).

Isolates from water sources contaminated with antibiotics often give an ARI value > 0.2, which is an indication of high-risk source of contamination (Krumperman, 1983). However, when antibiotics are seldom or never used, an ARI value < 0.2 is observed (Krumperman 1983). In this study, (ARI) index was used for analysing the prevalence of bacterial resistance in a given population at a specific location during different seasons to evaluate the level of resistance seen at each site to allow comparison with other studies that have used this index. The ARI of each sampling site was determined using the formula:

$$(\text{ARI}) = A/NY$$

Where A is the total number of resistant determinants recorded in a population of size N (number of isolates) for the specified location and Y is the total number of antibiotics tested in the sensitivity test (Mohanta and Goel, 2014).

Moreover, the definition of a multidrug-resistant (MDR) bacteria has recently 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 as displayed in Table 4.6.

Table 4.6 displays the ARI index of isolates screened in this current study and provides information on the MDR classification to provide an insight on the levels of resistance

observed at these locations. For this study, the ARI values ranged from 0.08 - 0.17. In the case of most of the sampling sites, ARI values were marginally around the threshold of 0.2. The higher ARI values were observed in known polluted sites along with the higher MDR, for example at Al-Salam and Abu-Al-Hasaniya as shown in Table 4.6. Variations in ARI were observed during the summer and winter seasons, with winter ARI values higher than summer as described in Table 4.6.

Table 4.6 Number of resistant antibiotic classes including the MDR and the ARI for seawater and bivalve's samples from sites across Kuwait marine environment during different seasons. MDR defined as bacterial isolate that displays non-susceptibility to at least one agent in three or more antimicrobial categories; whereas the ARI is showing the degree of sewage contamination from each site during summer and winter seasons. The tested antibiotics were: Aminoglycosides, Beta-lactams, Aminoglycoside/beta-lactamase inhibitors, Beta-lactam/beta-lactamase inhibitor, Cephalosporin, Fluoroquinolones, Imipenem, Quinolone, Dihydrofolate reductase inhibitor/sulfonamid.

Source	**Bivalves**		**Seawater**						
Seasons	**winter (n=144)**	**summer(n=103)**	**winter (n=179)**			**summer(n=172)**			
sites	**AL-Salam (n=144)**	**AL-Salam (n=103)**	**AL-Salam (n=155)**	**Al-Ghazali (n=14)**	**Khiran (n=10)**	**AL-Salam (n=78)**	**Abu-Al-Hasaniya (n=79)**	**Al-Ghazali (n=9)**	**Khiran (n=6)**
no. of resistant antibiotic class									
0	20	25	28	6	1	14	24	1	2
1	21	42	28	4	6	13	10	2	2
2	52	20	30	0	0	25	14	3	3
3	27	9	23	3	2	18	9	2	2
4	13	7	19	0	0	5	14	0	0
5	10	0	19	1	0	3	5	1	1
6	1	0	7	0	0	0	1	0	0
7	0	0	0	0	0	0	2	0	0
8	0	0	1	0	1	0	0	0	0
9	0	0	0	0	0	0	0	0	0
ARI index	0.10	0.16	0.17	0.08	0.17	0.14	0.15	0.14	0.14

4.5 Discussion

Sewage contamination is one of the key challenges in the management of water quality in Kuwait's marine waters (Al-Ghadban et al., 2002, Al-Abdulghani et al., 2013, Lyons et al., 2015b, Saeed et al., 2015). The major sources of these pollutants in the marine areas are the discharges from Shatt Al Arab, untreated domestic sewage, untreated industrial effluents, petroleum production, refining and transportation, in addition to atmospheric deposition (Smith et al., 2015). Over the past few decades, the marine environment has unfortunately been subjected to continuous raw or partially treated sewage contamination (Lyons et al., 2015b, Saeed et al., 2015). Sewage effluent is considered an important source of large inputs of anthropogenic compounds that may pose a significant risk to the ecosystem in the coastal areas (Lyons et al., 2015b, Saeed et al., 2015, Saeed et al., 2017). Emergent contaminants have been documented to be present in Kuwait marine environment including those with antimicrobial modes of action (Smith et al., 2015). In addition to the trace metals and oil-related chemicals which have been detected close to known point sources of sewage effluent, within a few metres of the shoreline (Al-Ghadban et al., 2002; Al-Sarawi et al., 2015, Lyons et al., 2015b, Saeed et al., 2015). It has been frequently suggested that these could pose a range of ecotoxicological threats including that of driving the emergence of AMR (Baker-Austin et al., 2006, Wright et al., 2006, Blasco et al., 2008, Wright, 2010, Al-Bahry et al., 2012).

Results clearly indicate that seawater collected from sites known to be close to waste-water effluent discharge points failed a range of commonly applied international microbial water quality standards. 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* numbers exceeded international water quality standards by multiple orders of magnitude. In this study, the highest exceedance was observed in Al-Salam sites for both seawater and bivalves derived faecal coliform counts. This may also indicate that the polluted sites have been heavily subjected to continuous sewage contamination that may leads to a steady deterioration of the habitats and the marine wild life of these areas in the long term (Devlin et al., 2015a).

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., 2015b). The work of Lyons et al. assessed the degree of sewage contamination impacting in Kuwait's marine environment and revealed widespread microbial water quality failures across number of sites along the coastline of Kuwait, including those examined in this study (Al-Salam) which were sampled under the KEPA national monitoring programme. Such studies indicate that sewage contamination has been a chronic problem for many years in Kuwait (Lyons et al., 2015b). 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 chronic sewage pollution problem at these locations from previous studies (e.g. the Al Ghazali site in this current study), with data indicating untreated sewage is being discharged in significant quantities (Lyons et al., 2015b, Saeed et al., 2015). Sewage pollution in these areas are thought to result from illegal connections and discharges from storm drains, such as that sited at Al-Ghazali and the failure of the sewage treatment network to keep pace with demands for capacity driven by rapid population growth that has almost tripled since 1975 (Al-Zaidan et al., 2013). This assumption is supported by the other faecal sterol contamination data available for

Kuwait, which suggests wastewater discharge regimes did not significantly change between 1998 and 2012 (Al-Omran, 1998; Saeed et al., 2012; Saeed et al., 2015). These data support the hypothesis that emerging contaminants available together with AMR bacteria derived from different sources such as human and warm-blooded animal guts could be entered the sewage effluent which may already contained AMR bacteria. This may pose an ecotoxicological threat and may act to promoting the AMR in the isolated *E.coli* from Kuwait marine environment.

Worldwide, AMR surveillance data suggests that resistance in *E. coli* is consistently high for those antimicrobials that have been in use for a long time in human and veterinary medicine (Titilawo et al., 2015). The findings of this study confirmed the prevalence of AMR *E.coli* isolated from Kuwait's marine environment. Results demonstrate the resistant was widespread across all sites (seawater: summer 64-89 %; winter 57- 90% and biota: summer 77%; winter 88%). As to the best of our knowledge this is the first survey in Kuwait reporting AMR in bacteria isolated from the marine environment. Significantly, AMR could be found in bacteria isolated from reference sites (where no recognisable waste-water effluent), indicating that this phenomenon is not restricted to areas heavily impacted by sewage.

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%) (Costa Andrade et al., 2015), from seawater collected close to an aquaculture facility in China (80%) (Wang et al., 2015a) and fish farming seawater *E.coli* in China (76.7%) (Wang et al., 2014). Moreover, the AMR bacterial strains have been recorded in biota such as oysters (66%) (Wang et al., 2014) and of abalone (61%) (Wang et al., 2015a).

In Kuwait, the observation of AMR prevalence in environmental samples mirrors the elevated levels of AMR documented in a clinical setting (Jamal et al., 2015, Aly and Balkhy, 2012). In a comprehensive review of a number of studies across the Gulf Collaborating Council (GCC countries), which included Kuwait, the screening the AMR bacteria in the clinical setting revealed that *E.coli* was the dominant (44%) resistant species among a total of 37,295 isolates displaying resistance. Of these GCC- tested *E.coli,* 77% were documented in Kuwait's main hospitals and classified as AMR bacteria (Aly and Balkhy, 2012). Other clinical studies support these findings and the analysis of another long-term dataset (2002-2007 and 2008-2012) for anaerobic bacteria isolated from the main four hospitals in Kuwait indicated the increase of AMR level in Gram negative bacteria between 2008-2012 for penicillin, clindamycin, pipercillin and amoxicillin-clavulanic acid, when compared to data collected between 2002-2007 (Jamal et al., 2015, Jamal et al., 2010).

In the current study, the 598 *E.coli* isolated from Kuwait's marine environment were screened against 23 of the frontline antibiotics, which represented nine different antibiotics group commonly used worldwide, including those widely prescribed in Kuwait (see Table 1.2 in Chapter 1; personal communication from the Ministry of health in Kuwait, 2014). In the current dataset, resistance to (AMP) was by far the most widely observed profile in seawater and bivalves across both summer and winter seasons, with 55.9 to 70.9% isolates displaying resistance. This supports previous studies that have documented wide spread resistance to this broad-spectrum beta-lactam antibiotic in the aquatic environment (Watkinson et al., 2007b, Al-Bahry et al., 2009b, Letchumanan et al., 2015). In the Arabian gulf region, this finding is similar to that in Oman where AMP was one of the top resistant antibiotics in isolated bacteria from seawater and fish (Al-Bahry et al., 2009b), turtles' eggs (Al-Bahry et al., 2009a),

oviductal fluid (Al-Bahry et al., 2011b), tertiary treated sewage effluent (Al-Bahry et al., 2011a) snails, soil and pond water (Mahmoud et al., 2013).

Worldwide, many studies have reported AMP as the highest antibiotic to be resistant. Miranda (2001), reported that AMP was the highest resistant antibiotic in bacteria derived from fish, with the lowest resistance being observed for AMI (Miranda, 2001). In Korea, this finding was also supported where AMP is among the most resistible antibiotics and no resistance against AMI was displayed in *E.coli* isolated from commercial fish and seafood (Ryu et al., 2012). AMP dominated the resistant profiles displayed by *E.coli* derived from sand and seawater of São Vicente beaches in Brazil (Costa Andrade et al., 2015). Moreover, (Matyar et al., 2008) documented the highest resistance incidence against AMP for isolates from seawater, shrimp and sediment of Iskenderun Bay in Turkey. The AMP also dominate the resistance antibiotics in AMR bacteria inhabit shrimp hatchery water in China (Zhang et al., 2011).

The *E. coli* screened displayed high-levels of resistance to older generation drugs, such as FAZ, a 1st generation cephalosporin, SXT a dihydrofolate inhibitor/sulphonamide and PIP, one of the widespread β-lactams. These antibiotics are consistently ranked in the top five antibiotics for which resistance was observed and are among the most widely used antibiotics classes in Kuwait (personal communications with Kuwait Ministry of health, 2014; Table 1.2). Of some concern, a number of isolates demonstrated resistance to important frontline classes of antimicrobial agents, such as the 3rd generation cephalosporin, AXO, the 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. 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 (Aly and Balkhy Hanan, 2012, Awad and Aboud, 2015).

These findings were quite similar to the AMR profile documented in the clinical field in Kuwait, where the highest resistance rates were against Penicillin (100%), Clindamycin (44.2%), PIP (42.7%) and Amoxicillin-clavulanic acid (14%) in an anaerobic bacteria dataset between 2002-2012, isolated from Kuwait's main hospitals (Jamal et al., 2015). These antibiotics belongs to the β-lactams except the clindamycin that classified as protein synthesis inhibitor (non-β-lactam).

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%). Seasonal differences in resistance profiles were also noted for AXO and TIM2 (seawater isolates) along with FUR and FAZ (bivalve's 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 general, for this study, the level of AMR and the wider range of antibiotics resistance was higher in winter than summer. These findings could be due to high temperature in summer that plays a significant role in

bacteria proliferation and dissemination (Wang et al., 2014) and rain run off that could increase AMR bacteria in aquatic systems (Taylor et al., 2011, Mohanta and Goel, 2014). 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). Both IMI and AMI are considered as the drugs of choice to treat *E.coli* infections as none or few isolates were found to be resistant to these antibiotics (Titilawo et al., 2015). This finding was supported with a study of AMR in *Vibrio parahaemolyticus* isolated in Malaysia, where the isolates showed high susceptibility (90%) to the IMI (Letchumanan et al., 2015) and 100% of *E.coli* were susceptible to the IMI isolated from rivers in Osun State in Nigeria (Titilawo et al., 2015) .

In this current survey, many isolates were showed resistance to multiple antibiotics, 69% were resistant to two or more antibiotics, 52% 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. 35% of isolates were classified as showing MDR, that displays non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012).

The higher MDR% was observed in highly polluted sites with sewage contamination such as Al-Salam. The increasing in MDR bacteria in the aquatic environment could generate a selective pressure in natural bacterial strains and could be an indication for contamination with antimicrobial agents (Al-Bahry et al., 2009a, Titilawo et al., 2015).

ARI provides an insight on the dissemination of bacterial resistance in specific populations for particular location (Manjusha et al., 2005, Mohanta and Goel, 2014, Titilawo et al., 2015). ARI is an excellent tool that could show the degree of contaminants with sewage pollutant in certain areas and distinguish the high-risk contamination sites in aquatic environment (Krumperman, 1983, Manjusha et al., 2005, Chitanand et al., 2010, Magiorakos et al., 2012, Mohanta and Goel, 2014, Titilawo et al., 2015). The ARI values of this study were calculated and showed a degree of sewage contaminations as the values were ranged from 0.08-0.17 and where were observed isolates also identified as displaying MDR. Although, the ARI values in this study were lower than those determined for river and ground water through different seasons in west Bengal where the ARI were far higher than 0.2 in the polluted sites when they tested against similar antimicrobial agents (Mohanta and Goel, 2014), the trend of ARI and MDR for seasons showed the higher values in winter than summer. This finding support the finding of this study. The seasonality variation could be attributed to the runoff of the terrestrial side (Mohanta and Goel, 2014). The ARI values were recorded along the bank of the Godavari River in India to assess the contamination level along the river. The ARI values at upstream point was 0.15 whereas the downstream was 0.43 (Chitanand et al., 2010).

The rise of antibiotic resistance rate in Kuwait could be attributed to many factors, the lack of legislation and stewardship on the prudent and responsible use of antimicrobials, inappropriate prescribing, with almost four in ten prescriptions including antibiotics, overuse of antibiotics including self-medications, especially in the community pharmacies which frequently could dispense antimicrobial agents with no prescription (Awad et al., 2010, Al-Mousa and Aly, 2011, Aly and Balkhy Hanan, 2012,

Awad and Aboud, 2015, Balkhy et al., 2016). In a wider image in GCC, the AMR could be as a consequence of the heavy international travel of a large population of expatriate workers in the region. In addition, there is thriving tourism in several of the GCC countries, such as the holy cities, which host of over 4 million pilgrims throughout the year and travel is a known risk factor for acquiring and transmitting AMR bacteria. Furthermore, the use of antibiotics is prevalent and often without supervision or guidelines in the treatment of animals; in some of the GCC countries there is a growing evidence that antibiotics are used as growth promoters in agriculture (Balkhy et al., 2016, Aly and Balkhay,2012) . In the Arabian Gulf region, antimicrobial agents have been classified as highly prioritized pharmaceuticals that should be assessed and monitored and monitored in the seawater, sediment and terrestrial areas (Al-Khazrajy and Boxall, 2016).

Recently, the GCC countries have placed the emergence of AMR at the top of their agenda for the past four years (Balkhy et al., 2016). The initial draft for the GCC strategic plan for combating AMR were developed in 2014. The strategic plan stems from the world health organisation (WHO) mandate to combat AMR at all levels. The first workshop was conducted in Riyadh in January 2015 and included, for the first time, representation of relevant ministries and agencies as well as international experts in the field. One of the major strategic plans for each of the GCC members is to implement their own AMR tailored national action plan for improving adherence to stringent infection control measures, applying regulations controlling antibiotic use in both human and animal sectors, implementing proactive surveillance of resistance, and enhance awareness and education about AMR among the specialist such as healthcare workers and public (Balkhy et al., 2016). Depending on that, in Kuwait, the Directorate of Infection Control in Ministry of health organized and conducted the first

National Campaign for the Proper Use of Antibiotics during the period 19–26 March, 2009. The campaign vision was to rationalize antibiotic use in healthcare settings by 2014 while its mission was to promote this concept through awareness, education and adoption of best practices (Al-Mousa and Aly, 2011). The success and effectiveness of this campaign was reflected by the drop in the amount of antimicrobial agents used in Kuwait per year, that decreased from 4 x 10^6 during 2010 to reach 2.7 x10^6 in 2014 (personal communication with Ministry of health, 2015). The environmental perspective for the AMR issue worth to receive more attention to end up with an effective addressing to the AMR in Kuwait.

Along with other countries in the Gulf region, Kuwait has developed marine monitoring programmes in response to environmental disasters, industrialisation, development of the coastal zone and suspected risks to human health (Al-Ghadban et al., 2002; Al-Abdulghani et al., 2013). The majority of these programmes focus on providing data on environmental status in terms of chemical contamination, bathing beach water quality and eutrophication (Al-Ghadban et al., 2002). However, there is no active environmental surveillance programme monitoring AMR or any other emerging contaminants in the Kuwait marine environment, and to date very few studies have attempted to assess the health of organisms residing in Kuwait's marine environment (Stentiford et al., 2014) and other adverse ecotoxicological threats posed by present concentrations of emergence contaminants. Antimicrobial agents and their metabolites, which act to increase the AMR potential in microbial communities, become highly diluted upon entering the aquatic environment and therefore detection of these compounds becomes extremely difficult (Al-Bahry et al., 2009a, Kümmerer, 2009b) Hence, the AMR bacteria have been used as bio-indicators of polluted effluents, since AMR bacteria can be easily isolated and detected (Al-Bahry et al.,

2009). The current study reports baseline AMR data from seawater and bivalve species (e.g. Venus clams) from across the Kuwait coastline. This current study has attempted to build upon the current capacity by introducing the potential for pollution effects studies using a member species of the natural biota present within Kuwait to be a part of the national hazards monitoring programme. This is the first such attempt to apply marine species and compartments from Kuwait and potentially heralds an era of improved understanding of anthropogenic environmental interaction especially in Kuwait Bay using biological endpoints of historical exposure. Such studies will help facilitate the use of AMR as part of a suite of endpoints for assessing the health status of marine biota.

For example, in this baseline study, *E.coli* was used as a bio indicator for AMR. It is a common inhabitant of the intestinal tract of humans and animals and can be easily disseminated in different ecosystems through the food chain and water. *E. coli* has been shown to exchange genetic material with other bacterial species and this organism may pass antibiotic resistance genes to transient bacterial pathogens that cause disease in humans. Further investigation to study more about the molecular facilities beyond the prominent level of AMR dissemination in bacterial communities that inhabit Kuwait marine environment is covered by conducting a baseline survey for the integrons class 1 prevalence in Kuwait marine environment, as reported in the next chapter (Chapter 5). Integrons are one of the mobile genetic elements that, mediated by horizontal gene transfer to pass different genes among bacterial strains (Wright et al., 2008, Aminov, 2011, Taylor et al., 2011).

Nevertheless, other microbial species could be examined for the AMR potential in Kuwait marine environment such as *Vibrio* spp. (Wang et al., 2015a). Different types of bacteria could be obtained from different marine compartments, such as coastal

sediment (wet sand), where AMR bacteria are known to be concentrated at high concentrations (Costa Andrade et al., 2015); treated sewage effluents such those documented (Al-Bahry et al., 2011a) and (Galvin et al., 2010), also in different biota species such as fish and shrimp as the Kuwaiti population is highly reliant on seafood for their diet (Miranda, 2001) and aquaculture (Wang et al., 2014).

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.

The survey concludes that *E. coli* isolated from Kuwait's marine environment display a range of AMR. The patterns observed revealed resistance to a large number of antibiotics in both seawater and biota derived *E.coli*. The similarity in the level and the variety of AMR across all sites in Kuwait suggest that the source of such pollution is the sewage effluent that is known to be a wide spread pollutant. Ampicillin is the most resistant antibiotic in Kuwait, and mirrors the findings of other studies conducted elsewhere. However, a varied degree of resistance against the tested antibiotics was also documented. Thus, research could be conducted to investigate in more depth this phenomenon as an adverse effect of chronic exposure of emergence contaminants that is receiving global attention.

Chapter 5 Baseline survey of class 1 integrons prevalence in the Kuwait marine environment and exploring the potential of whole genome sequencing for applied environmental science

5.1 Introduction

The overuse of antimicrobial agents in human and veterinary medicine, pastoral agriculture, and industry, and their subsequent release in wastewater treatment plants (WWTPs) have contributed to a global concern regarding the emergence and dissemination of antimicrobial resistant bacteria into the environment, including bacteria which cause infections in both humans and animals (Zhang et al., 2006, Zhang et al., 2009, Stalder et al., 2012, Liu and Wong, 2013). Although antibiotic resistance has become a major threat to human health worldwide, until recently this phenomenon has not been widely studied from an environmental perspective (Martínez, 2008, Marti et al., 2014). The environment represents a sink where bacteria from different origins, as well as a wide variety of antibiotics, disinfectants, and heavy metals are mixed, and this has contributed to the emergence and dissemination of antibiotic resistance (AMR) in the environment (Baquero et al., 2008). AMR in most environmental bacterial communities occurs due to horizontal gene transfer (HGT) mediated by mobile genetic elements MGEs (Wright et al., 2008, Zhang et al., 2009, Taylor et al., 2011, Finley et al., 2013, Domingues et al., 2015). Aquatic environments could be an ideal setting for the acquisition and dissemination of AMR, as they are frequently impacted by anthropogenic activities and provide a suitable environment for the horizontal genetic exchange of MGEs encoding antibiotic resistance genes (ARGs) (Richardson et al., 2004, Zhang et al., 2009, Taylor et al., 2011, Stalder et al., 2012, Marti et al., 2014).

5.1.1 Horizontal gene transfer (HGT)

Previously susceptible bacteria may become resistant to antibiotics through acquisition of one or more complex mechanisms, such as (i) exclusion of the antibiotic by the cell membrane, (ii) intracellular modification and/or deactivation of the antibiotic, (iii) reduction in sensitivity of the cellular target, (iv) extrusion from the cell and (v) intracellular sequestration (Taylor et al., 2011, Marti et al., 2014). These mechanisms can develop via an adaptive mutation, due to exposure to an antimicrobial agent, or due to inheritance of a genetic trait during the cell division process (intrinsic resistance or vertical gene transfer) (Martinez, 2009, Zhang et al., 2009). The bacteria can also acquire genes (e.g. ARGs) by HGT which is the flow of genetic material across bacterial communities mediated by the MGEs that transfer the genetic material throughout bacterial communities (whether the same or different strains) (Taylor et al., 2011). HGT contributes to acquisition and recombination of foreign DNA in bacteria, leading to genetically diverse bacterial communities with a high evolutionary rate which can adapt rapidly to environmental change; especially in environments that are recognized as having been impacted by human activities that could be considered as selective pressures (Mazel et al., 2000, Nemergut et al., 2004, Wright et al., 2008, Zhang et al., 2009, Stalder et al., 2012).

The HGT of exogenous DNA can confer AMR independently of the genetic relatedness of the bacterial species involved through three main mechanisms: (1) conjugations by mobile genetic elements MGEs such as plasmid, transposons and integrons on plasmids or transposons, (2) transduction by bacteriophages and (3) transformation of DNA in bacteria existing either in a naturally competent state bacteria or in an environmentally-induced competency state by substances such as calcium (Wright et al., 2008, Taylor et al., 2011, Finley et al., 2013). These mechanisms have

the potential to occur in the aquatic environment (Wright et al., 2008, Zhang et al., 2009, Taylor et al., 2011, Finley et al., 2013, Domingues et al., 2015).

Recently, it has become common understanding that the mobile genetic elements (MGEs) which play a significant role in horizontally transferring ARGs are the integrons, particularly class 1 integrons (Nemergut et al., 2004, Verner-Jeffreys et al., 2009, Gaze et al., 2011).

5.1.2 Integrons

Integrons are genetic elements that incorporate exogenous sources of mobile DNA called gene cassettes (GCs) into the recipient genome and convert it into functional genes (Nemergut et al., 2004, Stalder et al., 2012). Integrons are characterized by a specific-recombination system that can capture, express and exchange specific DNA elements, notably those encoding AMR within the GCs (Stokes et al., 2001, Nemergut et al., 2004, Wright et al., 2008, Zhang et al., 2009, Stalder et al., 2012, Domingues et al., 2015) (Figure 5.1).

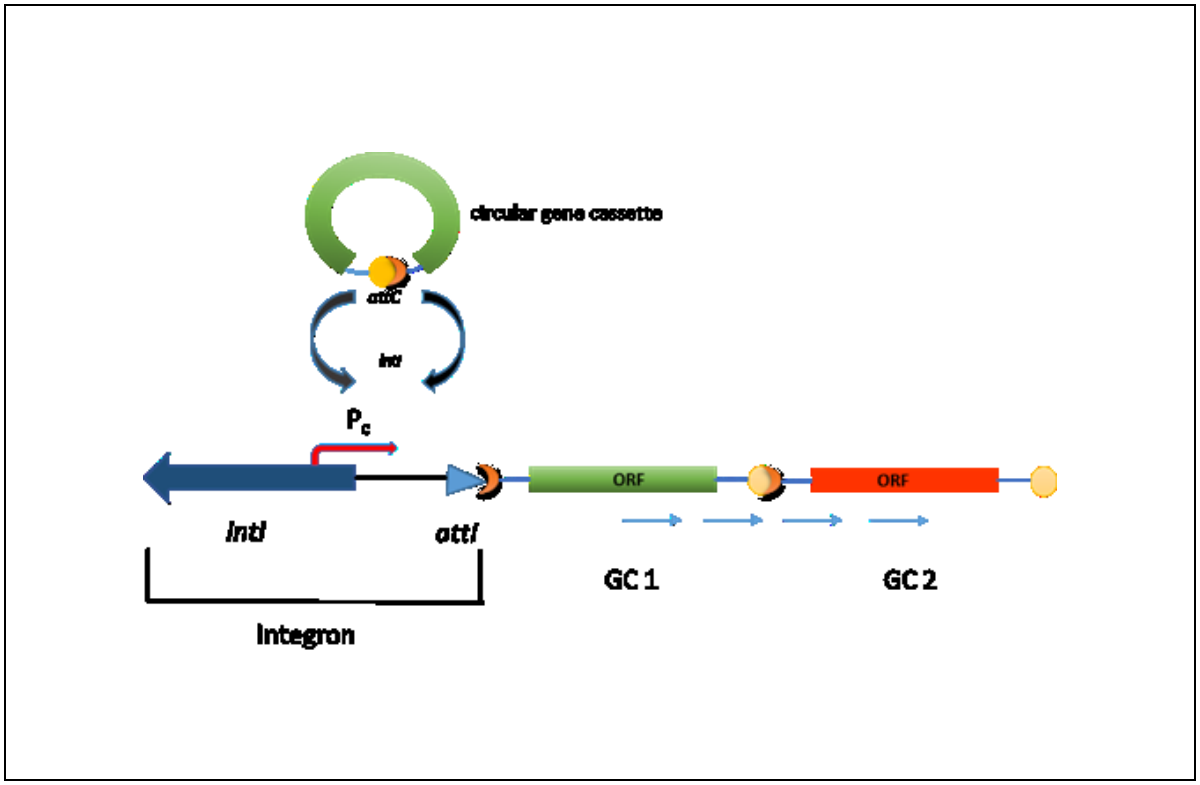

Figure 5.1 Integron/gene cassette system general structure including *intI*1 genes encode *intI* protein, attI integron recombination site and the cassette-associated promoter, Pc.

5.1.2.1 Integron Structure

There are five different classes of integrons, based on the amino acid sequence of the *intI* protein (Stalder et al., 2012, Domingues et al., 2015). The most commonly detected classes are 1, 2, and 3, whereas 4 and 5 are rare (Stalder et al., 2012). All classes include the same two necessary elements for site-specific recombination and expression of foreign DNA: (i) stationary integron platform (ii) mobile gene cassettes (GCs) as shown in Figure 5.1 (Nemergut et al., 2004, Wright et al., 2008, Aminov, 2011).

The stationary platform consists of the integrase gene (*intI*), which is highly conserved within the integron class, a strong promoter P_C (previously called P_{ANT}) that ensures the correct expression of genes and a recombination site (*attI*) that is highly conserved

within the integron class and varies between different classes (Mazel et al., 2000) (Figure 5.1).

The *IntI* gene encodes for an integrase enzyme, belonging to the tyrosine recombinase family, which catalyzes a site-specific recombination process between the *attI* and *attC* sites. This process either integrates gene cassettes between the *attI* and *attC* sites, resulting in the insertion of gene cassettes at the *attI* site or it excises gene cassettes between two *attC* sites, leading to the excision of the GC (Mazel et al., 2000, Nemergut et al., 2004, Zhang et al., 2009) (Figure 5.1).

Gene cassettes (GCs) consist of a promoterless single open reading frame (ORF) and a cassette-associated recombination site, designated a 59-base element or (*attC*), which is unique for each GC and possesses a common feature that includes a conserved sequence of approximately 25 bp at each end to form imperfect inverted repeats (Stokes et al., 2001). GCs are expressed through transcription by the promoter p_{ANT} then converted to functional genes (Mazel et al., 2000, Baquero et al., 2008, Wright et al., 2008). The last integrated cassette is the closest to the promoter which means it has the highest level of expression in the integron (Stalder et al., 2012). Variable numbers and types of GCs can be found in the central variable region resulting in integrons with diverse compositions of gene cassette arrays (Zhang et al., 2009, Stalder et al., 2012). The CGs could include novel sequences that are not related to any known ARGs which support the integrons to be a potential resource for bacteria to adapt to their environments through the acquisition of new MGEs (Stokes et al., 2001, Wright et al., 2008).

5.1.2.2 Integron Distribution

Integrons are ubiquitous elements in environmental bacteria (Domingues et al., 2015). They can be found in wide range of lineages in both pathogenic and environmental communities associated with aquatic and terrestrial ecosystems. They are commonly associated with disseminating multiple AMR genes, particularly in Enterobacteriaceae, Gram-positive and Gram-negative bacteria (Baquero et al., 2008, Wright et al., 2008, Stalder et al., 2012). Their association with MGEs carrying ARGs facilitates their dispersal among bacterial communities (Boucher et al., 2007, Guangchao et al., 2013). Integrons are more prevalent in polluted environments, than unpolluted environments (Stokes et al., 2001, Nemergut et al., 2004, Wright et al., 2008, Stalder et al., 2012). In particular this includes with aquatic regions affected by urbanization, industrial effluents, agriculture and aquaculture activities (Stalder et al., 2012). For example, Wright et al. 2008, quantified the abundance of the class 1 integrase (*intI1*) gene in total community DNA extracted from contaminated and reference riverine and estuarine microhabitats and in metal- or antibiotic-amended freshwater microcosms. The authors found that the *intI1* gene was more abundant in all contaminant-exposed bacterial communities, indicating that gene transfer potential is higher in these communities (Wright et al., 2008). Moreover, Rosewarne et al. 2010, demonstrated that the abundance of *intI1* increased with ecosystem perturbation, as indicated by a strong positive correlation with heavy metals such as zinc, mercury, lead, and copper (Rosewarne et al., 2010). Both studies suggested that the presence of some pollutants, such as heavy metals, could co-select for antibiotic resistance.

5.1.3 Detection of class 1 integrons and associated GCs and ARGs

The global threat of antimicrobial resistance is growing at an alarming rate (Zhang et al., 2006, Wright et al., 2008, Zhang et al., 2009, Rowe et al., 2015, Rowe et al., 2016, Roderova et al., 2017). The lack of systematic analysis of AMR is disproportional to its importance and imparts a burden to monitoring the emergence of new pollutants that lead to the sustainable use of marine resources and development (Al-Bahry et al., 2009; Taylor et al., 2011; Wang et al., 2015). This has led to the conclusion that more must be done to monitor and combat the occurrence and spread of AMR (Rowe et al., 2015, Rowe et al., 2016).

Currently, conventional analytical laboratory approaches for the detection of AMR relies on isolate culturing, followed by growth inhibition assays for the identification of resistant phenotypes and determination of minimum inhibitory concentration (MIC) against a range of antimicrobials (NCCLS, 2000a, Port et al., 2014, Rowe et al., 2015, Rowe et al., 2016).

Alternatively, the MGEs (e.g. integrons) and ARGs may be identified using polymerase chain reaction (PCR) requiring specific primers for the amplification of target sequences (Mazel et al., 2000, Nemergut et al., 2004, Rowe et al., 2015, Rowe et al., 2016).

However, these approaches have disadvantages in terms of time and resources, in addition to the limitations that may result from relevant resistances being undetected e.g. missing the viable but non-culturable (VBNC) bacteria and non-expressed ARGs, whereas limitations of multiplex composition and size in molecular testing complicates the detection of ARGs and integrons (Rowe et al., 2015, Rowe et al., 2016). Advanced Molecular Detection (AMD) technologies, such as the whole genome sequencing of bacterial isolates as well as uncultured bacteria (metagenomics sequencing), have the

potential to identify AMR more quickly and effectively than conventional laboratory assays (Port et al., 2014, Rowe et al., 2015, Rowe et al., 2016). Consequently, AMD technologies offer an alternative screening tool for the AMR in environmental monitoring (Port et al., 2014). However, little is understood about the limitations and complexities of these novel techniques and it is worth exploring how relevant molecular testing is for the analysis of AMR in environmental bacteria.

5.2 Aims and objectives

This baseline survey aimed to firstly estimate the prevalence of class 1 integrons, that have been implicated in the spread and dissemination of ARGs among Gram-negative bacteria, in *E.coli* derived from Kuwait marine environment and provide an insight on the role of class 1 integrons conferring of AMR potential in Kuwait marine environment which was confirmed by the previous phenotypic baseline survey (Chapter 4).

1) Screening the total number of isolated samples (598 *E.coli*) from seawater and bivalves for the integron class 1 using PCR to target conserved regions and gene cassettes of class 1 integrons.
2) Use whole genome sequencing of representative *E.coli* isolates to gain an insight about how this technique can be used to understand the complex relationships between ARGs, MGEs and phenotypic resistance profiles in isolates from different seasons and sites across the Kuwait coastal environment.

5.3 Materials and methods

5.3.1 Samples collections

The *E.coli* strains tested in this study were collected and isolated from Kuwait marine environment seawater and biota during different seasons as described in section 2.5.

5.3.2 Class 1 Integron screening of *E.coli* isolates

Screening for the key integrons (Class 1) known to be associated with antimicrobial resistance was conducted for all sampled isolates (598 *E.coli* strains). DNA was extracted from these isolates as described in Chapter 2 section 2.5.1. Integrase genes were amplified from the extracted *E.coli* DNA with int1.F/R and *intltd* F/R primer set, while gene cassettes were amplified with the HS286 and HS287 primer set following (Mazel et al., 2000, Nemergut et al., 2004) as described in Chapter 2, section 2.5.2. Gel electrophoresis was used to investigate the amplified products as described in section 2.5.3.

5.3.2 Whole genome sequencing of representative *E.coli* strains from the Kuwait marine environment

A number of *E.coli* isolates (26 *E.coli*) were selected to representing the total 598 *E.coli* derived from seawater and bivalves during winter and summer seasons from different sites across Kuwait marine environment (Chapter 4). These representative *E.coli* are resistant to different combinations of antimicrobials (i.e. some are resistant to 1,2, 3, or more tested antibiotics and others were susceptible). Whole genomes were

sequenced on Illumina Miseq with V3 chemistry (Illumina, USA). Sequencing was performed at the Cefas laboratories (Weymouth, U.K.) as follows:

5.3.2.1 DNA extraction/ quantification

5.3.2.1.1. DNA extraction

Each of the twenty-six *E. coli* Isolates were cultured in 15ml Tryptic soy broth (TSB, Oxoid) overnight static at 37°C prior to DNA extraction. One milliliter of culture was pelleted by centrifugation at 13,000 - 16.000 x g for 2min for extraction and DNA was purified using the Promega Wizard ®Genomic DNA extraction Kit (Promega, UK) protocol for Gram negative bacteria, according to the manufacturer's instructions.

5.3.2.1.2 DNA quantification

DNA was quantified fluorometrically using a Quantus fluorometer (Promega, UK) with the DNAOne reagent system following manufacturer's protocol.

5.3.2.2 DNA sequencing

5.3.2.2.1 Tagmentation and Amplification

Tagmentation and amplification were carried out using a Nextera XT DNA sample preparation kit (Illumina, USA) with an adapted manufactures protocol; the adapted protocol used 2.5 µl of 0.2 ng/µl DNA and half the manufacturer recommended quantity of each reagent up to the DNA clean-up stage. DNA was then diluted 2-fold in molecular grade water prior to clean-up.

5.3.2.2.2 Library clean up

This step was done with 30µl AgenCourt® AMPure® XP (BeckMan Coulter, USA) beads according to the recommended Illumina protocol (Nextera XT DNA).

5.3.2.2.3 Library normalization, pooling and sequencing

Library normalization was done by bead based system according to (Nextera XT DNA Sample prep) protocol. After normalization DNA was pooled, diluted in HT1 hybridization buffer (Illumina, USA) and then denatured at 98C for 2 minutes. DNA was sequenced on the MiSeq platform with a V3600 Illumina Sequencing Cartridge.

5.3.2.2.4 Quality Check, Assembly

Sequences were trimmed using version 0.36 of Trimmomatic, with options recommended on the website, for paired end sequencing (Bolger, Lohse, & Usadel, 2014). FastQC version 0.11.5 was used to check the quality of trimmed reads, and to ensure there were no large contaminants (Babraham Bioinformatics- FastQC A Quality Control tool for High Throughput Sequence Data. (n.d.), Retrieved May 17,2017).

5.3.2.2.5 Assembly and Identifying Open Reading Frames (ORFs)

Spades version 3.10.1 was used for assembly, with default options (Bankevich et al., 2012). Assembled genomes were annotated using version 1.11 of Prokka, with default options (Seemann, 2014).

5.3.2.2.6 Multi-locus sequence typing (MLST) Analysis

Gene models for the from the Pfam database (Finn et al., 2014). The hmmscan tool, included as part of version 3.1 of the hmmer toolkit (Finn et al., 2011) was then used to identify the probably of each open reading frame in the assembled genomes actually

corresponding with any one of the *adk*, *fumC*, *gyrB*, *icd*, *mdh, purA* or recA genes. Finally, once each of the seven genes had been identified in each of the sequenced isolates they were aligned using version 7.305 of the mafft software, with default options (Katoh et al., 2002). After aligning sequenced genes, each gene alignment was processed using version 1.9.8 of the UGENE software (Okonechnikov et al., 2012) to exclude unaligned regions at the 5' and 3' end of the alignment, before concatenating the alignments. Finally, FastTree version 2.1.8 was used to carry out a maximum-likelihood phylogenetic analysis (Price et al., 2010) and FigTree version 1.4.2 was used to draw the tree (FigTree., Retrieved May 17, 2017).

5.3.3. Statistical analysis

Correlation coefficient was calculated using Spearman in SPSS statistics 23(Microsoft office professional plus, 2016) to find out the relations between the variables

T-test was done to compare the prevalence of intI1 and the GC during different seasons for biota and seawater. Multiple regression was used to assess the influence of the *intI1* and GC on the AMR.

5.4. Results

5.4.1. Prevalence of class 1 integron in the Kuwait marine environment

Figure 5.2 (below) shows the abundance of class 1 integron (*intI1*) and the associated (GC) in the *E.coli* derived from seawater and bivalves of Kuwait marine environment. These *E.coli* were previously subjected to the AMR screening. The abundance of *intI1*

gene in the tested *E.coli* isolates was 36% (n=598) whereas the associated GCs was 33% (n=598).

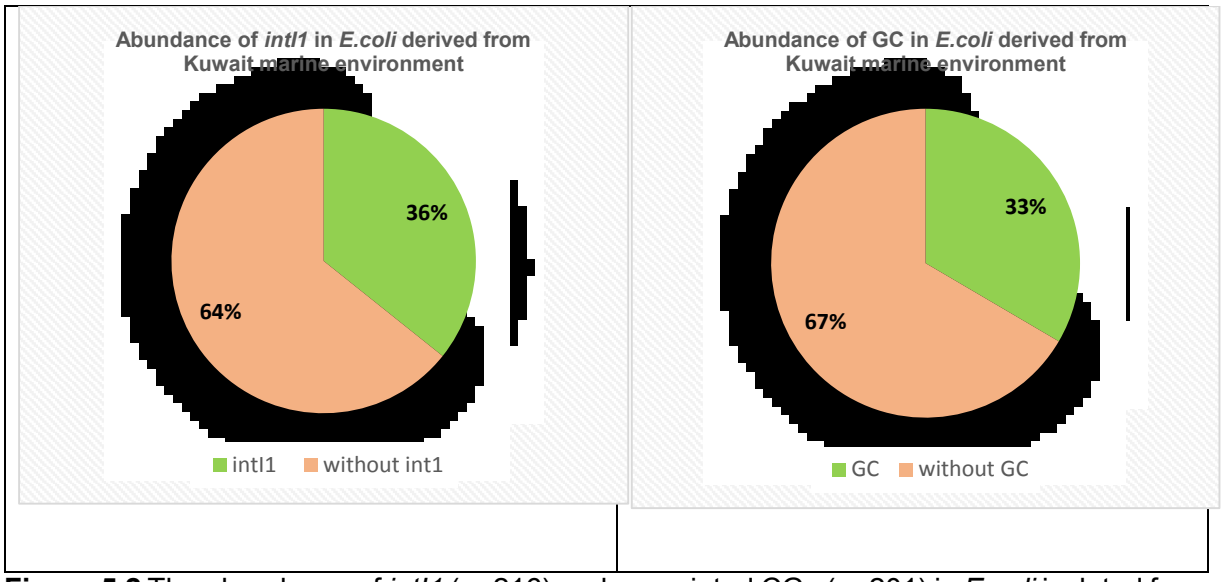

Figure 5.2 The abundance of *intI1* (n=216) and associated CGs (n=201) in *E.coli* isolated from Kuwait marine environment during winter and summer season (n=598).

95% (n=204) of the *E.coli* that possess the *intI1* genes are classified as AMR *E.coli* whereas only 5% (n=12) are antimicrobial susceptible (depending on the AMR test conducted in chapter 4) (Figure 5.3). The AMR *E.coli* which do not possess the *intI1* gene consist 49% of the total isolated E.coli (n=598) (Figure 5.3).

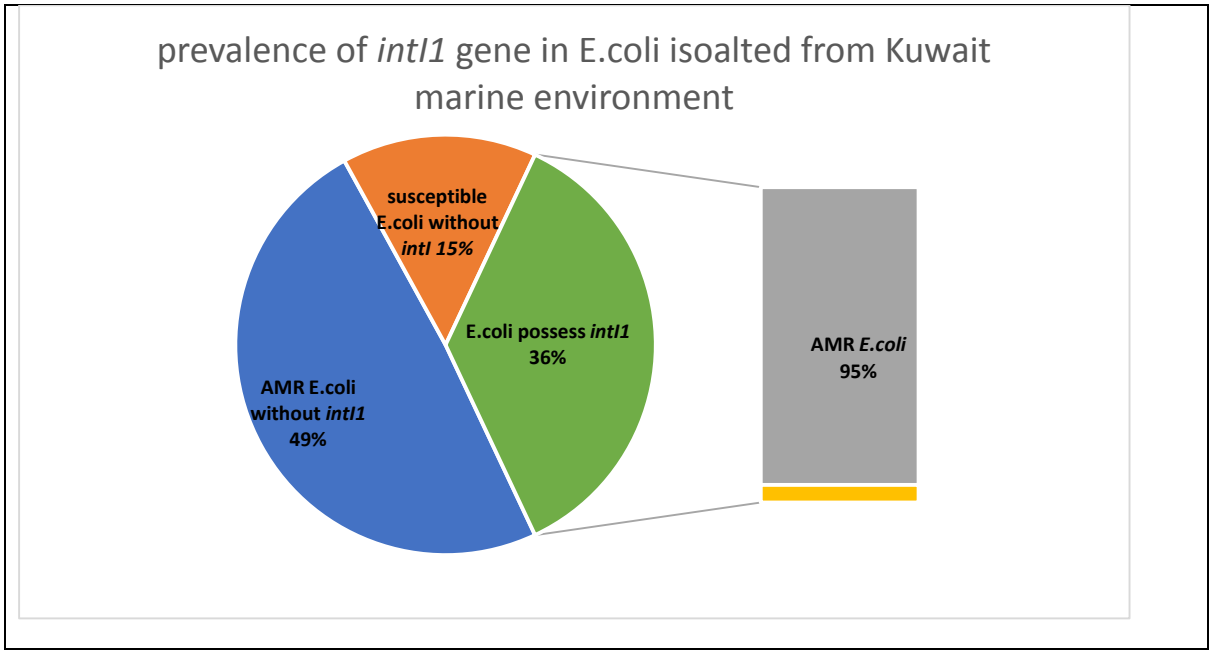

Figure 5.3 The prevalence of *intI1* gene in the *E.coli* isolated from seawater and biota across the Kuwait marine environment during summer and winter (n=598).

In this study of *E.coli* isolates (n=598) there was a significant correlation between the AMR and *intI1* gene (r= 0.311) and weak significant correlation between GC gene (r=0.188) (p< 0.000) (the full data available in appendix 1).

There was a positive but weak correlation between *intI1* gene and the GC (r=0.122) (p=0.003).

Multiple Linear regression was used to explore the relationship between presence of class 1 integron and GCs and AMR in *E.coli* isolated from Kuwait marine environment. Preliminary analyses were conducted to ensure no violation of the assumptions of normality, linearity, multicollinearity and homoscedasticity. The *intI1* and GC explaining 7.4% of the variance in AMR (R^2=0.074), *F* (24.28) = 604, *p* < .001.The measures were statistically significant, with the *intI1* recording a higher beta value (*beta* = 0.231, *p* < .001) than the GC (*beta* =0.120, *p* < .001).

5.4.2. Class 1 Integrons prevalence in high and low anthropogenic source sites in the Kuwait marine environment

Table 5.1 illustrates the abundance of the *intI1* and GC genes during both summer and winter for *the E.coli* derived from seawater and bivalves across the Kuwait marine environment.

The prevalence of *intI1* found in seawater at the different sites ranked as follows: Al-Salam > Abu-Al-Hasaniya > Al-Ghazali >Khiran. On the other hand, the associated GC ranked as follows: Al-Salam> Al-Ghazali > Abu-Al-Hasaniya > Khiran as shown in Table 5.1.

Seasonality could play a significant role in the abundance of the *IntI1and* the GC genes. There was a significant difference ($p<0.000$) in *intI1* prevalence in bivalves-derived *E.coli* during summer and winter. However, there was no significant difference ($p=0.452$) in seawater-derived *E.coli* during summer and winter. For the GC content, there was a significant difference for GC in summer and winter for both bivalves-derived *E.coli* ($p<0.000$) and seawater *E.coli* ($p<0.000$).

In Al-Salam, during summer, 45% (n=35) of seawater-derived *E.coli* possessed *IntI1,* and in winter, 39% (n=60) of the *E.coli* isolates possessed the *intI1* (Table 5.1). For the bivalve-derived *E.coli* isolates, seasonality could play a significant role in the abundance of the *IntI1* genes as these significantly decreased from 44% in winter to 18% in summer ($p<0.000$), although it is difficult to prove causality. The abundance for the associated GC in Al-Salam was significantly higher in both seawater and bivalve-derived *E.coli* during the winter (39% and 83% respectively) than in the summer *E.coli* (4% and 11%, respectively) ($p<0.000$).

On the other hand, the levels of *intI1* in Al-Ghazali was variable between seasons, with *intI1* was detected in 27% (n=3) of the isolates in summer, while only 14% (n=2) of the winter isolates were positive for the presence of *intI1*. However, it should be noted limited numbers of isolates were collected from this site.

Table 5.1 Abundance of class 1 integrons (*intI1*) and associated gene cassettes (GC) in *E.coli* isolated from seawater and bivalves across the Kuwait marine environment during summer and winter (2014-2016) from sewage-contaminated sites inside Kuwait Bay Al-Salam, Al- Ghazali and outside Kuwait Bay in Abu-Al-Hasaniya, the reference sites was Khiran.

Site	Season	Seawater % *intI1* (number isolates screened)	Seawater % GC (number isolates screened)
Al-Salam	Summer	45 (78)	4 (78)
Al-Salam	Winter	39 (155)	39 (155)
Abu-Al-Hasaniya	Summer	38 (79)	4 (79)
Al-Ghazali	Summer	22 (9)	0 (9)
Al- Ghazali	Winter	36 (14)	7 (14)
Khiran	Summer	50 (6)	0(6)
Khiran	Winter	0 (10)	0 (10)
		bivalves **% *intI1* (number isolates screened)**	**bivalves** **% GC (number isolates screened)**
Al-Salam	Summer	18 (103)	11 (103)
Al-Salam	Winter	44 (144)	83 (144)

5.4.5 The ARG profile of representative *E.coli* derived from the Kuwait marine environment

Whole bacterial genomes were sequenced on Illumina Miseq with V3 chemistry (Illumina, USA). In total, 26 representative *E.coli* which had previously been subjected to the AMR analysis, as discussed in Chapter 4, were sequenced. Table 5.2 below shows the AMR profile for the 26 representative *E.coli* against the tested antimicrobial agents as described previously in Chapter 4. These *E.coli* were chosen to represent different AMR phenotypes (i.e. some are susceptible and others are resistant to different number of antibiotics) in different season, locations and sources of isolates as displayed in Table 5.2.

Table 5.3 shows some of the ARGs determined by the Whole genome sequencing (WGS) profile for one of the strains phenotypically, identified as susceptible (as per AMR phenotyping described in Chapter 4). *E.coli* (SC 59) was collected from Al-Salam during summer. SC 59 *E.coli* was susceptible to the all 23 tested antibiotics. Likewise, Table 5.3, shows the ARGs for one of the most resistant *E.coli*, a multi-drug resistant (MDR) *E.coli* (KHE11) which was isolated from the reference site (Khiran) during winter time and documented as resistant to 19 of tested antimicrobial agents as described in Table 5.2.

The results of the ARGs for these two strains showed that the SC 59 *E.coli* strain could possess antimicrobial genes likely to be responsible for resistance to different antibiotcs classes such as aminoglucosides and fluoroquinolone such as *acrB and gadX* although SC 59 phenotypically classified as a susceptible for the tested antibiotics . On the other hand, in KHE11 isolate, some genes that are known to be implicated mainly in resistance to aminoglycosides, β-lactams, and trimethoprim such as *dfr7* and aadA5 (Zhang et al., 2009, Domingues et al., 2015) both were not recognized in this strain (KHE11) although it was

phenotypically showed the resistant against 19 of the tested antibiotics belonged to different antibiotic class (Table 5.3).

Table 5.2 The AMR profile for the 26 *E.coli* which were subjected to the whole genome sequencing. The AMR test was conducted against the following antibiotics were tested: 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). The antibiotic's classes were as follow: (A) Aminoglycosides, (B) Beta-lactams, (C) Aminoglycoside/beta-lactamase inhibitors, (D) Beta-lactam/beta-lactamase inhibitor, (E) Cephalosporins, (F) Fluoroquinolones, (G) Imipenem, (H) Quinolone and (I) Dihydrofolate reductase inhibitor/sulphonamide. The interpretive criteria depending on (CLSI, 2014) where notified as green for (susceptible), red for (resistant and white for intermediate.

				A			B				C	D		E								F		G	H	I
Isolate ID	Season	Source	site	AMI	GEN	TOB	AMP	AZT	MERO	PIP	A/S2	P/T4	TIM2	FAZ	FEP	TANS	AXO	TAZ	FOX	POD	FUR	CIP	GAT	IMI	NIT	SXT
Sc 59	Summer	Venus clam	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
8KHE1	Summer	Seawater	Khiran	0	2	0	16	8	0	32	32	0	64	32	0	0	16	2	4	0	32	0	0	0	0	4
HE 40	Summer	Seawater	Al-Salam	0	8	4	32	32	0	128	32	128	64	32	8	32	64	32	32	16	32	4	8	0	0	4
SC63	Summer	Venus clam	Al-Salam	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	4	0	0	4	8	0	0	4
SC70	Summer	Venus clam	Al-Salam	0	0	0	32	0	0	128	4	0	0	8	0	0	0	0	4	0	0	1	1	0	0	4
SC117	Summer	Venus clam	Al-Salam	0	2	0	32	16	0	32	16	0	64	32	0	16	64	32	32	16	32	4	4	0	0	0
SC118	Summer	Venus clam	Al-Salam	0	0	0	32	32	0	128	16	0	64	32	0	32	64	32	32	16	32	4	4	0	0	0
HE 73	Summer	Seawater	Abu-Al-Hasaniya	0	8	0	32	32	0	128	32	0	64	32	4	0	32	8	16	16	32	4	8	0	0	4
HE 33	Summer	Seawater	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SC89	Summer	Venus clam	Al-Salam	0	0	0	32	0	0	64	4	0	0	4	0	0	0	0	4	0	0	0	0	0	0	4
SE25	Summer	Seawater	Al-Salam	0	0	0	32	32	0	128	8	0	0	32	32	0	64	4	4	16	32	0	0	0	0	0
7SE60	Summer	Seawater	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SE 181	Winter	Seawater	Al-Salam	0	0	0	32	16	0	128	32	0	64	32	4	32	32	4	32	16	32	4	8	0	32	4
SE 19	Winter	Seawater	Al-Salam	8	16	8	32	32	8	128	32	0	64	32	16	32	4	32	32	16	32	4	8	0	32	0
SE 51	Winter	Seawater	Al-Salam	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	4	0	4	0	0	0	0	0
KHE11	Winter	Seawater	Khiran	64	16	8	32	32	1	128	32	128	64	32	4	32	64	32	4	16	32	4	8	0	128	8
SE 109	Winter	Seawater	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	4
SE 124	Winter	Seawater	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	0	0	0	0	0
SE 158	Winter	Seawater	Al-Salam	0	8	0	32	32	2	128	32	128	64	32	0	32	1	32	8	16	32	0	0	0	16	4
SE 138	Winter	Seawater	Al-Salam	0	16	4	16	8	0	64	4	0	0	32	0	0	32	2	4	16	8	4	1	0	0	4
SE 111	Winter	Seawater	Al-Salam	0	0	8	32	0	0	128	16	0	64	32	0	16	0	4	32	16	32	0	0	0	0	4
CW 138	Winter	Venus clam	Al-Salam	0	0	0	32	0	0	128	8	0	0	32	0	0	0	0	32	0	0	4	2	0	0	8
CW140	Winter	Venus clam	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
CW 121	Winter	Venus clam	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
Cw 141	Winter	Venus clam	Al-Salam	0	0	0	16	0	0	128	16	0	16	4	0	0	0	0	4	0	0	0	0	0	0	8
Cw 106	Winter	Venus clam	Al-Salam	0	0	0	32	0	0	64	32	0	64	32	0	32	2	1	32	8	4	4	8	0	16	8

Table 5.3 Antimicrobial resistance genes detected in *E.coli* isolates by the whole genome sequencing approach for Sc59 which is susceptible for the tested antibiotics and KHE11 which is resistant to 19 of these antibiotics.

Gene Name	Antimicrobial resistance function	SC59	KHE11
aadA15	**aminoglycoside**	0	0
aadA23	**aminoglycoside**	0	0
aadA5	**aminoglycoside**	0	0
OXA-48	**beta-lactam**	0	0
TEM-1	**beta-lactam**	0	0
dfrA7	**diaminopyrimidine, beta-lactam, aminoglycoside**	0	0
acrB	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	1	1
emrR	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	1	1
acrA	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	1	1
mfd	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	1	1
msbA	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	1	1
msrB	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	0	1
patA	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	1	1
YojI	**sulfonamide, aminoglycoside, triclosan, fluoroquinolone**	1	1
AcrE	**polymyxin, fluoroquinolone**	0	1
AcrF	**polymyxin, fluoroquinolone**	0	0
mdtD	**polymyxin, fluoroquinolone**	1	1
mdtF	**polymyxin, fluoroquinolone**	1	0
mdtH	**polymyxin, fluoroquinolone**	1	1
mdtL	**polymyxin, fluoroquinolone**	1	1
mdtM	**polymyxin, fluoroquinolone**	1	1
acrD	**sulfonamide, fluoroquinolone, beta-lactam**	1	0
baeR	**sulfonamide, fluoroquinolone, beta-lactam**	1	0
gadX	**sulfonamide, fluoroquinolone, beta-lactam**	1	1

.

The phylogenic relationship of the representative 26 *E.coli* strains is shown in Figure 5.4. The phylogeny tree demonstrates several interesting aspects of biology about the *E.coli* isolates. For example, some isolates from different locations across Kuwait marine environment and different source substrates (seawater or bivalves) were found to be very closely related according to the MLST analysis. Isolate SE19 and Sc118 are closely related, but originated from winter seawater, or summer Venus clam respectively.

In addition, for these high related isolates (according to MLST), AMR profiles varied. For example, isolate SE19 and Sc118 differ in their susceptibility to Aminoglycosides, Beta-lactams and Aminoglycoside/beta-lactamase inhibitors as display in Table 5.2.

The tree also shows another aspect of biology that is unexpected according to previous metabolic profiling. As shown in Figure 5.4, SE111, SE181 and SE158 isolates were less related to the other *E.coli* in this study. Further interrogation of the data helps to affiliate these isolates as a different, although closely related species of *Enterobacter cloacae,* suggesting previous metabolic tests may not be able to distinguish between *E. coli* and *E. cloacae.*

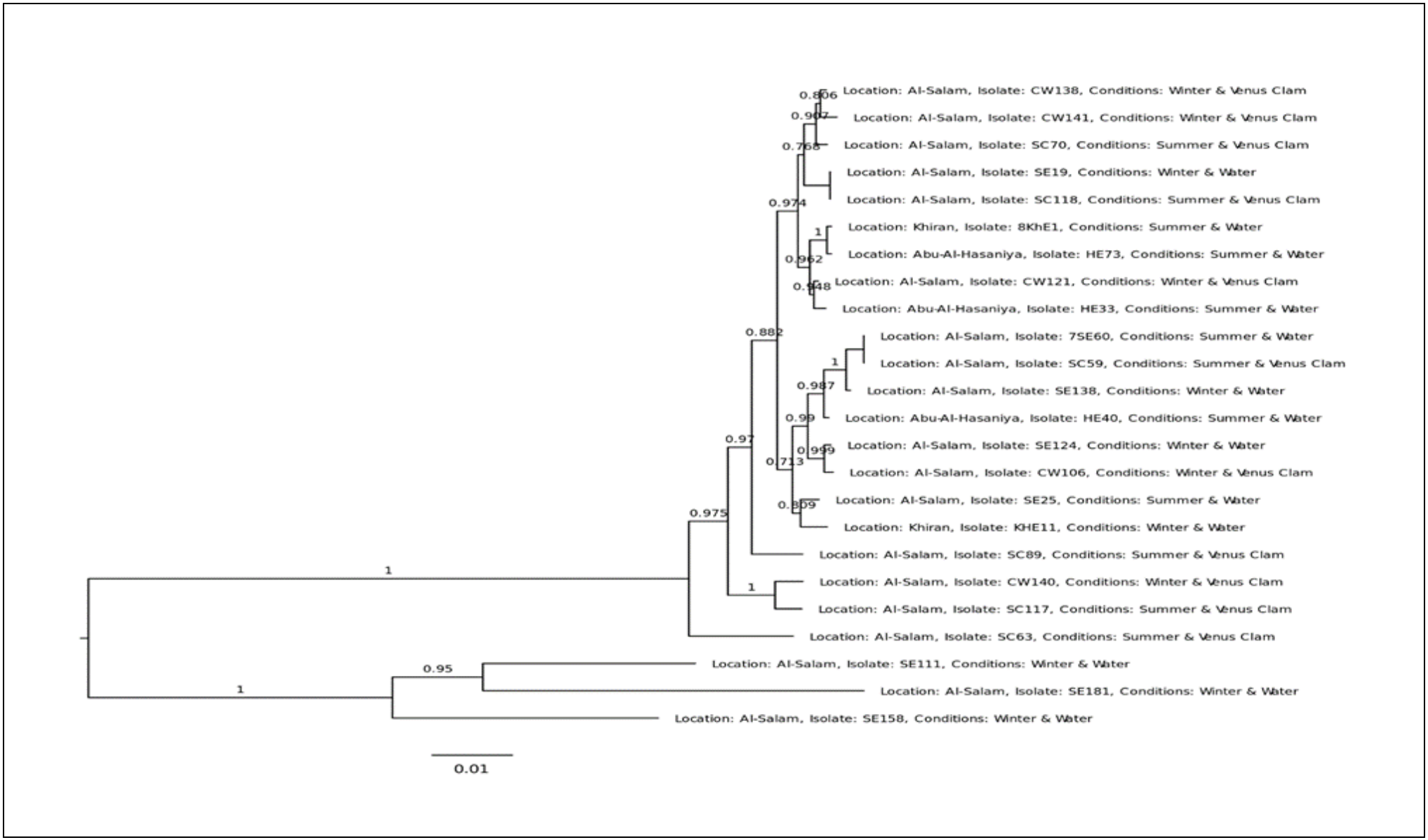

Figure 5.4 The phytogenic tree for the 26 *E.coli* isolates derived from Kuwait marine environment.

5.5 Discussion

Aquatic systems represent a vital environmental sink for the accumulation of antimicrobials and other pollutants. This leads to persistence and dissemination of AMR bacteria and the spread of resistance genes associated with MGEs (Wright et al., 2008, Zhang et al., 2009, Taylor et al., 2011). The action of integrons is one of the important contributory factors in the dissemination of AMR among Gram-negative bacteria (Ryu et al., 2012). Integrons are genetic structures able to capture, excise and express genes, frequently included into MGEs as plasmids, that allow their dissemination among environmental bacteria (Wright et al., 2008, Taylor et al., 2011, Ryu et al., 2012). To the best of our knowledge this is the first study to screen the prevalence of the class 1 integrons in the Kuwait marine environment which has suffered chronically from deteriorated water quality due, in particular, to sewage contaminations (Lyons et al., 2015b, Saeed et al., 2015).

In this study, the prevalence of class 1 integrons in the Kuwait marine environment (36%) was in a similar range to that observed in *Enterobacteriaceae* found elsewhere e.g. fish and seafood derived *E.coli* (41.4%) in Korea (Ryu et al., 2012), and 12.1% of *Enterobacteriaceae* stains isolated from municipal wastewater treatment plant in Poland (Mokracka et al., 2012).

The abundance of class 1 integrons in *E.coli* isolated from the Kuwait marine environment was greatest in the vicinity of the sewage outlets, regardless of whether their locations were inside or outside of Kuwait Bay. This finding suggests that the source of these MGEs may be the sewage contamination. However, there is also the potential that the contamination may provide adaptive pressure in the marine environment which increases gene transfer rate. Indeed, gene transfer potential is known to be higher in bacterial communities, from highly anthropogenic impacted areas (Stokes et al., 2001, Nemergut et al., 2004, Wright et al., 2008, Rosewarne et al., 2010, Gaze et al., 2011, Stalder et al., 2012, Marti et al., 2014).

The abundance of *intI1* was much higher in Al-Salam than Ghazali, both major sewage outlets. This could be due to many reasons but it should not be ruled out that this could be due to the distance of sampling from the sewage outlet; closer in Al-Salam and further in AL-Ghazali (see the map in Chapter 4). The variation in quantity of bacteria from different locations that possess class1 integrons could be due to different factors: (1) distance to an enriched source of class 1 integrons (e.g. sewage treatment plant); (2) integron dispersal ability (e.g. mobilization of the integron via physical linkage to a plasmid or transposon); (3) the taxonomic composition of the community; (4) HGT potential in a given habitat; and (5) intensity of selective pressure favoring the maintenance of genes contained within the integron (Wright et al., 2008). The prevalence of the class 1 integron in *E.coli* isolated from Khiran site which is further down south in the Kuwait coastline, supports the theory that class 1 integrons are ubiquitous and abundant in environmental bacterial communities and indicates that this group of MGEs can play a substantial role in the acquisition of a diverse array of gene cassettes beyond their demonstrated impact in AMR in clinical bacteria (Wright et al., 2008). Further environmental factors could also affect this variation. For example, temperature and UV irradiation may affect the degree of HGT within bacterial communities and thereby may delineate community exchange boundaries (Wright et al., 2008, Zhang et al., 2009, Domingues et al., 2015). Seasonality could play a significant role in disseminating the *intI1* and GCs in the Kuwait marine environment. The finding of this study could shed lights to this role, however, more research is needed to fully characterise the impact of season.

In this study, the class 1 integron genes were amplified from the extracted *E.coli* DNA using the *intI1*.F/R and *intItd* F/R primer set (Nemergut et al., 2004). The *intItd* F/R primer set was tested but did not give any positive results including the positive control samples suggesting

technical issues. More work could be address the optimal conditions and primes to amplified required genes to identify such MGEs.

The effectiveness (relationship) of *intI1* and GCs on the AMR in *E.coli* was predicted by regression (results show 7.4%). Accordingly, the presence of *intI1* gene and GCs were positively correlated to an AMR phenotype. This suggest class 1 integron may be a factor in transmitting AMR potential among bacterial communities (Wright et al., 2008). However, factors other than *intI1* and GC will no doubt also influence the presence and spread of AMR in bacterial communities in marine environment such as metal contamination and other MGEs (Baquero et al., 2008, Wright et al., 2008, Zhang et al., 2009, Aminov, 2011, Domingues et al., 2015, Di Cesare et al., 2016a).

In this study, some *E.coli* isolates (5%, n=93) possessed the class 1 integron, but were susceptible to antimicrobial agents. This could be due to this molecular toolbox playing a different role in theses isolates. For example, they could be resistant to other pollutants, carrying genes such as *qacEΔ1*, that encodes resistance to quaternary ammonium salts (Guangchao et al., 2013, Domingues et al., 2015). In this study, 49% of isolated *E.coli* were classified as AMR without possession of *intI1*. This may suggest to that the AMR genes were carried by other genetic mobile elements (MGEs) such as plasmid or transposons, or be integral parts of the genome in these isolates (Boucher et al., 2007, Aminov, 2011).

Whole genome sequencing has been done on a representative *E.coil* (26 isolates) isolated from the Kuwait marine environment. These *E.coli* isolates were previously examined for the antimicrobial susceptibility test, and were selected for sequencing due to their differing, and representative, phenotypes. The WGS analysis revealed that the phenotypically susceptible isolates to the tested antimicrobial actually possess ARGs. These ARGs are found in both the highly resistant and susceptible isolates suggesting a complex relationship between

genotype and phenotype, which cannot be completely explained with current knowledge. This may be a biological complexity, such as susceptible isolates which carry some ARGs not having a complete set of the required molecular machinery to be resistant; or a technical complexity, such as errors in homology based characterisation of ARGs. Either way the data suggests the widespread presence of large numbers of ARGs which could be implicated in the antimicrobial resistance potential of a population of *E.coli,* even if the AMR phenotypes of individuals cannot be fully explained by current genotype knowledge (Zhang et al., 2009).

E.coli isolated from different seasons and sources could be identified as phylogenetically highly related according to the (MLST) analysis. This suggests that the isolates share an ancestor, and therefore most likely originate from the same source. If this is the case it is plausible to suggest that some isolates of *E. coli* in the Kuwait marine environment may originate from point source contamination. A large scale study of the phylogenetics of *E.coli* populations from Kuwait waters, and indeed from potential point sources of contamination, may help to resolve this hypothesis. Moreover, the phenotypes of AMR between highly related isolates (according to MLST) are not always in concordance. This suggests that resistance may be acquired through horizontal gene transfer rather than through vertical inheritance (Wright et al., 2006, Aminov and Mackie, 2007, Wright et al., 2008, Zhang et al., 2009, Aminov, 2011).

Similar to resistance genes for other pollutants such as heavy metals it's widely believed that ARGs emerge in the environment and persistent for long-term (Aminov and Mackie, 2007). This may be one explanation for common detection of ARGs in antibiotic-free environments (Aminov and Mackie, 2007, Zhang et al., 2009).

Whole genome sequencing (WGS) of these isolates highlighted that some were not *E. coli* as expected, but were in fact the closely related *Enterobacter cloacae.* Although the conventional method to identify the bacterial species strains is widely used to confirm the

species (as in Chapter 4), molecular approaches are considered as a more precise method with which to distinguish between the highly related species such *E.coil* and *Enterobacter cloacae.* The occurrence of *Enterobacter cloacae* Bacteria is common in water, sewage, soil, food, and as commensal microflora in the intestinal tracts of humans and animals (Sanders Jr. and Sanders, 1997). However, the phenotypic identification of this species is usually difficult and not reliable; therefore, molecular methods are highly recommended (Krzymińska et al., 2010).

The WGS data has been used to do several different types of analyses that help us to understand the biology of AMR in the environment (Zhang et al., 2009, Rowe et al., 2015, Rowe et al., 2016). However, it is worth remarking that these analyses vary in usefulness of output and are not necessarily able to replace traditional, phenotypic, analysis (such as AMR phenotyping). The advantages and disadvantages of diverse types of analysis have become particularly obvious when trying to draw firm conclusions that are useful for applied environmental science and regulation, as opposed to studies exploring the mechanistic aspects of AMR (Zhang et al., 2009, Rowe et al., 2015).

For example, WGS can be used to do phylogenetic analyses of *E. coli* and attempt to understand, or even designate, their origins (such as point source pollution, or commensal environmental bacteria).

As observed in this study, where WGS data has been used to identify genes required for AMR, it has proved difficult to resolve differences between genes present and phenotypes observed. Further work is required to understand the differences between genotype and phenotype.

This work suggests that there is a very useful for sequencing within applied environmental science, but this has not yet reached its full potential.

In summary, this study supports theories that the marine environment can act as a reservoir for bacteria with ARGs, from which AMR can be proliferated and disseminated among the bacterial communities. This may be especially significant in those inhabit the anthropogenically impacted areas. In Kuwait, the prevalence of the class 1 integron was higher in those areas which means the high transmission of the ARGs may occur regardless of direct exposure to antibiotics. Moreover, other MGEs, known to mediate ARGs were detected by the whole genome sequencing.

Chapter 6 General discussion

From this research study, the main findings are:

- The comprehensive literature review (covering more than 60 papers) of hazardous substances in Kuwait's marine environment concluded that while Kuwait's marine environment has been subjected to a wide array of pollution events, the actual levels of contamination remains relatively low when compared to heavily industrialised regions elsewhere in the world and below the regularly applied international standards. Industrial contamination is known to be localized to specific areas such as the SIA or embayment's within Kuwait Bay.

- The findings of this literature review were supported with field work to assess the use of biological effects or biomarkers (bile metabolites and EROD activity) in fish collected across Kuwait's marine environment. A total of 60 samples of two local species Giant sea catfish (*Arius thalassinus*) and Fourlined terapon (*Pelates quadrilineatus*), were studied to demonstrate the potential for the concentrations of oil based contaminants present to induce detectable levels of biological effects. In general, the findings demonstrated that the concentration of bile metabolites and the activity of EROD was low and below any internationally recognized environmental quality guidelines and were very low compared to other studies that have also applied this method for biomonitoring studies. Therefore, the data indicated that the concentrations of contaminants responsible for the induction of these biomarkers is low and not thought to pose any toxicological threat to resident marine species.

- The baseline survey for the AMR bacteria across Kuwait's marine environment firstly revealed breaches in microbial seawater quality at sewage outlets locations (e.g. Al-Salam) where *(E. coli* and faecal coliform) exceeded international water quality standards by almost 100x. These findings support previously published data that

identified these sites as regularly being impacted by sewage effluents and resulting in persistent failures of microbial water quality standards. The AMR baseline survey for 598 isolated *E.coli,* demonstrated the resistant for most of the frontline tested antibiotics and was widespread across all sites (seawater: summer 64- 89 %; winter 57-90% 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 worldwide.

- The baseline survey for the class 1 integron that is known to be implicated in the HGT for the ARGS was conducted for the 598 *E.coil.* The findings show that 36% of these *E.coil* possess the class 1 integron in a similar range to that observed in *Enterobacteriaceae* found elsewhere. The abundance of class 1 integrons in *E.coli* isolated from the Kuwait's marine environment was greatest in the vicinity of the sewage outlets, regardless of whether their locations were inside or outside of Kuwait Bay. This finding suggests that the source of these MGEs may be the sewage contamination.

- Whole genome sequencing has been done on a representative *E.coil* (26 isolates) isolated from the Kuwait marine environment. The data suggests the widespread presence of large numbers of ARGs which could be implicated in the antimicrobial resistance potential of a population of *E.coli,* even if the AMR phenotypes of individuals cannot be fully explained by current genotype knowledge. The phenotypes of AMR between highly genetically related isolates (according to MLST) are not always in concordance. This suggests that resistance may be acquired through horizontal gene transfer rather than through vertical inheritance.

AMR issue could be considered as new challenge for Kuwait's marine environment. It supports and gives an evidence that this marine environment has chronically and still being impacted by a volume of sewage effluent, the impacts of which are a priority for management.

In Kuwait, the additional of AMR screening of environmental samples could be used to improve and develop the existing chemical focused marine monitoring preprogramme. To date they are focused on petrochemical associated pollutants, this now need to address the impacts of the chemical and biological pollutants that may be part of the sewage effluent entering the marine environment in large volumes. This would be in line with the development of marine monitoring programmes as out lined in the recently published Kuwait environmental law No. 42, which came into effect in 2014 and has a clear goal to improve the governance and management of Kuwait's marine and coastal ecosystem.

The State of Kuwait is situated at the north-western corner of the Arabian Gulf, which is considered among the highest anthropogenically impacted regions in the world (Halpern et al., 2008, Sheppard et al., 2010). Its territorial waters are characterised by shallow seas, high summer water temperatures (>30^{o}C), intense UV exposure and elevated salinities (average 41‰) (Sheppard, 1993 , Al-Rifaie et al., 2007). A main feature of its marine environment is Kuwait Bay, a 750 km^2 semi-enclosed shallow body of water, around 35 km wide with a mean depth of 5 m (Al-Sarawi et al., 1988, Al-Abdulghani et al., 2013). The area provides vital habitats and nursery grounds for many fish, shrimp and other ecologically important marine organisms. The waters around Kuwait contain some of the most northerly coral reef systems in the world and are internationally recognised for maintaining biodiversity, supporting fisheries and promoting recreational activities (Al-Ghadban et al., 2002, Al-Ghadban and El-Sammak, 2005, Al-Rifaie et al., 2007).

Kuwait is undergoing rapid economic growth involving substantial construction along its coastal shores and marine environment (Al-Sarawi et al., 2015, Al-Zaidan et al., 2015, Devlin et al., 2015a, Devlin et al., 2015b). The rapid expansion of Kuwait's industrial sector has mainly occurred around its coastal margins. This has led to a variety of contaminants being discharged directly to the marine environment, including petroleum hydrocarbons, trace metals, nutrients (from domestic sewage), and contaminated brine from desalination plants, which are essential for the production of freshwater in the region (Readman et al., 1992, Al-Ghadban et al., 2002, Beg et al., 2003 , Al-Dousari, 2009). In addition, areas of natural oil seepage have been identified and are thought to be important point sources of contamination at various locations around the coast (Zarba et al., 1985, Massoud et al., 1996 , Massoud et al., 1998 , Al-Ghadban et al., 2002).

As part of this thesis a comprehensive literature review of hazardous substances in Kuwait's marine environment was conducted. This concluded that while Kuwait's marine environment has been subjected to a wide array of pollution events, the actual levels of contamination remains relatively low when compared to heavily industrialised regions elsewhere in the world and and below the regularly applied international standards. Industrial contamination is known to be localized to specific areas such as the SIA or embayment's within Kuwait Bay. The finding of this literature review (covering more than 60 papers), as presented in Chapter 1, has been supported by field work that has been conducted to assess the use of biological effects markers (so called biomarkers including bile metabolites and EROD activity) in fish collected from Kuwait's marine environment to demonstrate the potential for the concentrations of oil based contaminants present to induce detectable levels of biological effects (Chapter 3). For this field work, a total of 60 samples of two local species, the bottom feeding Giant sea catfish (*Arius thalassinus*) and the pelagic Fourlined terapon (*Pelates quadrilineatus*) were collected from different off shore sites across Kuwait's marine

environment. In general, the findings demonstrated that the concentration of bile metabolites and the activity of EROD was low and below any internationally recognized environmental quality guidelines (Davies and Vethaak, 2012, Al-Zaidan et al., 2015). In fact, results were very low compared to other studies that have also applied this method in biomonitoring studies (Ariese et al., 1993, Aas and Klungsøyr, 1998, Lyons et al., 1999, Davies and Vethaak, 2012). Therefore, the data indicated that the concentrations of contaminants responsible for the induction of these biomarkers is low and not thought to pose any toxicological threat to resident marine species. However, the risk posed by such pollution hot spots remain to be fully determined and while attempts have been made to empirically evaluate risk very little work has been conducted to link chemical exposure with adverse biological effects in marine species (Beg and Al-Ghadban, 2003, Beg et al., 2003 , Alshemmari et al., 2010). Therefore, it is encouraging to see a small, but growing number of studies attempting to link toxicological endpoints to the study of Kuwait's marine contamination (Randolph et al., 1998, Beg and Al-Ghadban, 2003, Stentiford et al., 2014, Al-Zaidan et al., 2015, Smith et al., 2015, Saeed et al., 2017). Such studies also highlight the need for research into so called emerging contaminants (e.g. pharmaceuticals) or the potential impact of chemical mixtures, which to date have received little or no attention in Kuwait or the wider Gulf region. Therefore, the focus of this research thesis incorporated other anthropogenic sources that chronically pollute Kuwait's marine environment. The main one being the discharge of sewage effluent, which impacts many locations around Kuwait, and is known to contain wide array of substances that could pose an ecotoxicological threat to different levels of the marine ecosystem.

Water quality is one of the major challenges facing the management of Kuwait's marine environment (Devlin et al., 2015b, Lyons et al., 2015b, Saeed et al., 2015). Regular discharge of sewage from official networks and unregulated discharge points results in

environmental impacts across many areas of Kuwait's coastline (Lyons et al., 2015b, Saeed et al., 2015). In addition major pollution incidents have also been reported, such as the failure of the Mishref pumping station in Kuwait, which resulted in 180,000–200,000 m^3/day of raw sewage being discharged over a period of months into the sea (Saeed et al., 2015). Available historic microbial datasets for the Kuwait marine environment also indicate wide spread breaches in water quality, suggestive of long-term pollution problems in certain regions (Lyons et al., 2015b). These sewage discharges are reported to contain a wide variety of pollutants, such as PPCPs originating from many various sources including hospitals and industrial areas (Smith et al., 2015). In addition, due to the untreated sewage contained within these effluents, the levels of bacterial contamination (e.g. faecal coliforms and faecal streptococci) associated with discharges of domestic sewage are known to regularly breach international water quality standards (EPA, 2001, Lyons et al., 2015b). These discharges have been shown to contain other pollutants such as metals, with elevated concentrations detected close to known discharges of domestic sewage. The accumulation of these pollutants has been attributed to the closed, shallow nature of the receiving beaches, with outlets discharging within a few metres of the shoreline (Al-Ghadban et al., 2002, Bu-Olayan and Thomas, 2014).

In common with many countries, Kuwait has developed marine monitoring programmes in response to environmental events, proposed constructions in the coastal zone, or suspected risks to human health. Such monitoring programmes provide data on the state of the environment in terms of water, bottom sediment, marine organism health, bathing beach quality and harmful algal blooms and may allow conclusions to be drawn about trends in environmental quality over time.

To date, research into pharmaceuticals and personal care products (PPCPs) has received little attention in Kuwait with the majority of research focused on conventional contaminants

related to the petrochemical industries (Readman et al., 1992, Al-Abdali et al., 1996, Massoud et al., 1996 , Metwally et al., 1997 , Allchin et al., 1999, Al-Sarawi et al., 2015). It is clear that these now need urgent attention, with evidence suggesting that plentiful supply of PPCPs may be released into Kuwait's marine environment (Lyons et al., 2015b, Saeed et al., 2015, Smith et al., 2015, Saeed et al., 2017). Some of which are known to have antimicrobial modes of action (Smith et al., 2015, Saeed et al., 2017). 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, O'Neill, 2016, WHO, 2005). 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). Significantly, data obtained for Kuwait indicates that the consumption rate of antibiotics ranged from 2.7×10^6 - 4×10^6 prescription units/annually for the period 2010-2014 (personal communication, Kuwait Ministry of Health, 2015). These consumption values include many common front-line antibiotics used in high volumes.

The World Health Organisation (WHO) now considers antibiotic resistance as one of the most pressing global issues which poses a fundamental threat to human health, development, and security (WHO 2016). Such is the concern over the speed bacteria are gaining resistance to all major frontline antibiotics that the United Nations General Assembly (UNGA) held a special session on the issue in September 2016 (WHO 2016). This is only the 4th time a health issue has been taken up by the UNGA, the others were HIV, non-

communicable diseases, and Ebola. Underlining the importance of antibiotic resistance on human health the FAO/OIE/WHO recently established a tripartite alliance in this area (OIE 2017).

Recently, there has been an increasing amount of interest in the role that the marine environment plays in accentuating drug resistance (Taylor et al., 2011). It has a high and diverse bacterial loading and is a known sink for a multitude of clinical bacteria and contaminants. There is clearly a number of routes through which these resistance genes and associated bacteria can transfer back into contact with humans, through a myriad of potential routes, such as through seafood, fisheries products and recreational exposure (D'Costa et al., 2006, Le Quesne et al., 2017). 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., 2011b). 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).

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). 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 a wider image, the GCC region is characterized by a strong inward migration pattern, and has increased in population size by 17 million over the last 2 decades, largely attributed to an influx of foreign labor (Abdul Salam et al., 2015). The high population density in coastal regions surrounding the Arabian Gulf is also an important consideration.

To date no studies have addressed the issue of AMR in marine systems, which represents a key data gap in Kuwait. Within this thesis 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 *E. coli* (351 seawater; 247 bivalves) were isolated from seawater and biota (Venus clam, *Circenita callipyga*) across Kuwait coastline and screened for their potential for resistance against an array of commonly deployed frontline antibiotics frontline (23 antibiotics) by micro-dilution (48 h incubation) onto the custom dehydrated 96-well Sensititre™ GN2F panels (GN2F, Thermo Scientific, UK). Results demonstrate the resistant was widespread across all sites (seawater: summer 64-89%; winter 57-90% 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 polluted sites (Al-Bahry et al., 2009b, Zhang et al., 2011, Wang et al., 2014, Costa Andrade et al., 2015, Wang et al., 2015a). 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%). 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 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. 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. 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. 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. 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). 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.

In this research project, a baseline survey aimed to estimate the prevalence of class 1 integrons, that have been implicated in the spread and dissemination of antimicrobial resistance genes among Gram-negative bacteria, in the same *E.coli* derived from Kuwait marine environment was conducted to have an insight on the role of class 1 integron underlying the surveillance and conferring of AMR potential in Kuwait marine environment which was confirmed by the previous AMR baseline survey.

The whole genome sequencing (next generation DNA sequencing) using Illumina Nextera XT library preparation and Illumina Miseq V3 chemistry (Illumina, USA) was conducted for representative *E.coli* to gain an insight regarding the ARGs and the MGEs in isolates from different seasons and sites across the Kuwait coastal environment. 36% of derived *E.coli* showed to possess the class 1 integron.The abundance of class 1 integrons in *E.coli* isolated from the Kuwait marine environment was greatest in the vicinity of the sewage outlets. This study supports the view that the marine environment acts as a reservoir where AMR potential could be proliferating and disseminate among the bacterial communities, especially those inhabit the anthropogenic impacted areas. The prevalence of the class 1 integron was higher in those areas which could lead to high transmission of the ARGs without the need for direct selective pressure from antibiotics. This could help play a role in the propagation of AMR in Kuwait marine environment.

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.

Alongside these issues, high background levels of metals – which have long been implicated as a co-selection factor in AMR emergence (Baker-Austin et al. 2006) are present in marine sediments, water and biota samples surrounding the Gulf (Naser, 2013, Al Rashdi et al., 2015, Lyons et al., 2015a, Williams et al., 2016, Al-Hammad and Abd El-Salam, 2017, Alharbi et al., 2017, OIE, 2017, Youssef et al., 2015). The highest levels of metal contamination are frequently found in areas impacted most severely by anthropogenic activities, with localized hotspots of chronic metal pollution in areas influenced by industrial

facilities, desalination plants, and oil refineries (Naser, 2013). To date there are no published studies that have assessed the phenomenon of metal-AMR co-selection this GCC region, which is surprising given the history of metal contamination in the area. However, our study offers a baseline study for a cohesive programme to assess the prevalence and extent of AMR bacteria and antimicrobial contaminants in environmental settings such as marine and aquatic systems. Such information is likely needed to help contain the development of antimicrobial resistance, and could be incorporated into existing efforts such as regional strategic action plans to combating the AMR parallely with the clinical efforts which have been recently established including a stewardship programme (Enani, 2016) and a GCC strategic plan (Balkhy et al., 2016) to tackle the emerging of AMR. The recently published GCC wide strategic plan has highlighted the need to develop research efforts in this area of AMR, augmented by academic and strategic funding partners and conduct research that measures the effectiveness of interventions such as drug restriction, guidelines and other management strategies (Balkhy et al., 2016).

Recommendations

Five core areas of work have been highlighted to be tackled to provide a risk assessment framework in marine and aquatic systems as described in Figure 6.1 (Le Quesne et al., 2017). First and foremost, a centralised mechanism to share data, funding and research efforts in region should be established. Gathering data from source to sink, including prescription and dosage information (including from agriculture and aquaculture sectors), routes of chemicals into the environment along with bacteria and associated residues into aquatic and marine systems should be initiated (Figure 6.1). This would serve the purpose of ascertaining the extent antimicrobial usage and associated pollution in region, and provide a starting point for further (downstream) environmental and clinical risk assessment work.

Data generated on the associated residence of antimicrobials and AMR bacteria (e.g. bacteria of risk to public and animal health) in aquatic and marine systems could then be determined, perhaps using “worst case scenario” e.g. most impacted sites, such as adjacent to large municipal wastewater systems and industrial sites. The exchange and assessment of this information between GCC state countries could then be utilised to make broad assessments on the extent of AMR from such environmental sources. A key component of a risk assessment framework would be in the sharing of data such as the prevalence of AMR strains and detection of unusual or clinically-relevant environmentally-derived strains (e.g. NDM-1 bacteria, strains possessing multi-antibiotic resistances etc, Figure 6.1). This information could then be compared against clinically-derived information to determine linkages between the two. Assessing links between the environment and clinical systems is imperative. Obviously, the emergence of new or novel resistant strains in the GCC region would be a topic of priority tackled by both clinical, environmental and epidemiological studies. A centralised repository for data storage, collection and sharing should be established in the GCC region to help facilitate these efforts.

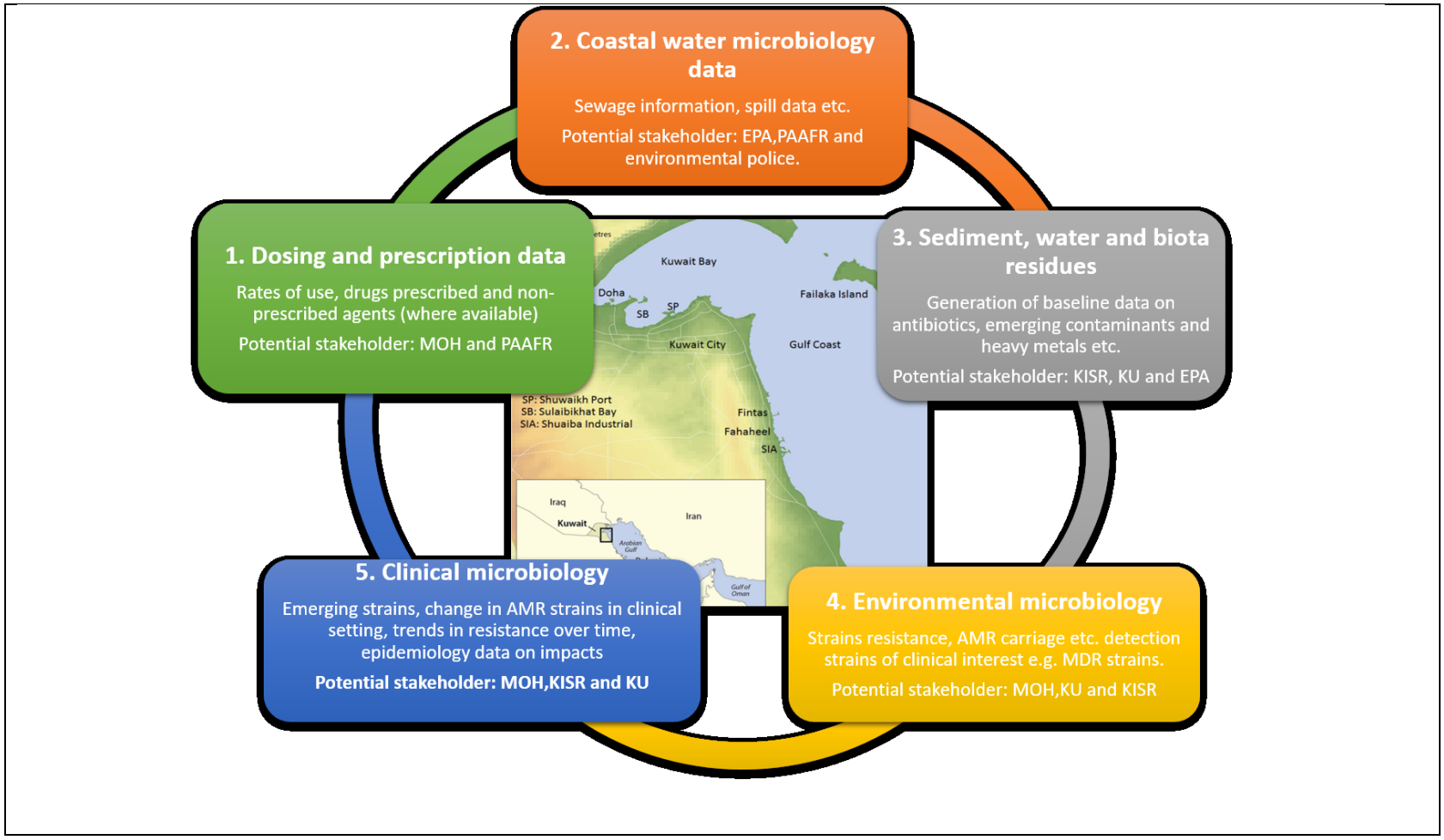

Figure 6.1 The main Five core areas of work have been highlighted to be tackled to provide a risk assessment framework in marine and aquatic systems for the AMR. The potential stakeholders in Kuwait for each core area were proposed; MOH= Ministry of health; KISR=Kuwait Institute for scientific Research; EPA= Environment Public Authority; PAAFR= Public Authority for Agriculture and Fish Resources; KU= Kuwait University.

There is a pressing need for combining disparate data sources for risk assessment purposes, because currently, there is little systematic data gathered that answers even the most fundamental questions regarding AMR risk. A basic framework to determine these risks, combining dosing information, monitoring and risk assessment of wastewater discharge sources, coupled to clinical and environmental microbiological information to determine the risks posed by these bacteria and antimicrobials entering the aquatic and marine environment is urgently required.

Summary

It is clear from the findings of this research project that there is a requirement to update and improve the current marine environment monitoring programs in Kuwait. This includes building upon the current capacity, which includes data for the environmental status in terms of petrochemical pollutants, eutrophication and the microbial water quality of bathing beaches and evolve to collect excotoxicology and biological effects endpoints for native species inhabit Kuwait's marine environment. This is especially required close to those areas identified as being highly polluted, such as Shuaiba industrial area and both Doha and Sulabikhat embayments in Kuwait Bay.

Work in this thesis started to examine the application of biological effects endpoints in two native fish species Giant Catfish (*A. thalissinus*) and Four-lined Terapon (*P. quadrillineatus*) for assessment of PAH exposure biomarkers (biliary PAHs and EROD activities). Further research is required to validate the assays employed, starting with preliminary experiments to determine the baseline levels of these biomarkers and study more in depth the influence of other factors such as seasonality and sex. The suitability of other species should also be investigated to provide greater geographical coverage (e.g. to ensure sentinel species are available at impacted locations).

This study supported previous findings that Kuwait's coastline is impacted by sewage pollution. A wide variety of emerging contaminants have previously been documented and some of these are known to have antimicrobial modes of action. Thus, a prioritization should be to conduct further analytical studies to gain a greater understanding of these contaminants, their sources, fate and effects on the marine environment.

Antimicrobial resistance (AMR) in bacterial communities is an adverse biological effect of widespread and abuse of the pharmaceuticals and personal care products (PPCPs) including the microbial agents. Thus, they could be used as a bio indicator (index) for the degree of sewage contaminations among sites, as an endpoint. This approach may be particularly useful as antibiotics and other emerging contaminants are likely to be highly diluted when entering the waters and thus difficult to detect. Therefore it is recommend to introduce the AMR monitoring screening into the exiting microbial water quality programs. The sites monitored such include areas such as Doha and the Sulibikhat bay, where major effluent outlets are known to exist.

It's widely believed that aquatic environment could play a significant role in the emerging, disseminating, proliferating and maintenance of the AMR in microbial communities. Recently, WHO considered the AMR as a global pressing issue that could pose a fundamental threat to the human health, development and security. The WHO launched the General Action Plan (GAP) for AMR control and combat. The WHO calls for the member nations to tailor their own national aligned with the GAP. In Kuwait, there are a disparate efforts which mainly focus on the clinical setting. The research present in this thesis provides a baseline study for the environmental AMR in bacterial communities (*E.coli*). However, a number of additional studies should be conducted to fully understanding the AMR in Kuwait marine environment. This will require a combination of stakeholders, including the Environment Public Authority and Ministry of Health (Public Health Department) along with researchers based at institutions such as Kuwait University and Kuwait Institute of Scientific Research (KISR). Work will also be required to highlight the issue with funding bodies within Kuwait and the findings should be presented to Kuwait Foundation for the Advancement of Science (KFAS). Key areas for further work include:

- Different bacterial species should be examined for their AMR potential, with samples collected from a range of matrices including seawater, sediments, bivalves and other species such as crabs, shrimps or different fish species.
- The seasonality could play a significant role. Thus, more in-depth work should be carried out to determine the influence of such factors to fully understand the extent of AMR potential in Kuwait's marine environment.
- The role of other selective pressures (such as metals) should be studied to examine their influence on the degree of AMR in Kuwait's marine environment.
- The baseline screening for class 1 integron in Kuwait marine environment revealed that their presence was higher in the sewage polluted sites. Their presence in other bacterial species at different sites, during different seasons could be useful to fully assess the resistance disseminating, proliferating and maintenance of AMR.
- The whole genome sequencing approaches requires further study. This should focus on validating and optimizing the analysis to develop a clearer understanding of the molecular mechanisms conferring resistance to bacterial isolates. This may then help to resolve the mismatch observed between the phenotype and genotype of *E.coli* isolates observed in the current study.
- This information should be gathered with other data, such as those obtained from the clinical setting and maintained on a centralized data hub. It is recommended that this should be led by KEPA, the authority mandated to implement the new Kuwait environmental law No.42 for 2014 in the state of Kuwait. KEPA should then work with the Ministry of Health to tailor a national plan for AMR and share the official data with other counties in the region under GCC and ROPME umbrella.

Appendices

Appendix 1: AMR profile and class 1 integron for the 598 isolated *E.coli* from Kuwait marine environment

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
Smple ID #	source	Season	Location	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	intI1	GC
	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
1	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	8	0	32	0	0	0	0	0	4	4	0	4	8	0	0	4	1	0
2	Seawater	winter	Al-Salam	0	0	0	4	0	0	0	4	0	0	32	0	0	0	0	4	32	16	0	0	0	16	0	0	1
3	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	1	1
4	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	32	0	0	0	0	0	4	0	0	0	0	0	4	1	1
5	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	64	64	32	0	0	0	0	8	16	4	0	0	0	0	4	0	0
6	Seawater	winter	Al-Salam	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
7	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	32	0	16	0	4	32	32	16	0.5	0	0	0	4	1	1
8	Seawater	winter	Al-Salam	16	0	8	32	32	1	0	4	0	0	8	4	32	1	32	32	8	16	0	0	4	0	0	1	0
9	Seawater	winter	Al-Salam	0	2	0	32	8	0	32	4	0	0	32	0	0	32	2	32	0	8	4	1	0	0	4	0	1
10	Seawater	winter	Al-Salam	0	0	0	32	0	0	64	8	16	0	0	0	0	0	0	4	0	0	0	0	0	16	0	0	0
11	Seawater	winter	Al-Salam	0	16	8	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	1
12	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	Seawater	winter	Al-Salam	8	16	8	32	32	8	128	32	0	64	32	16	32	4	32	32	32	16	4	8	0	32	0	0	0
14	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	8	0	0	0	0	0	0	0	0	0	0	0	4	1	1
15	Seawater	winter	Al-Salam	0	0	0	32	0	0	64	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
16	Seawater	winter	Al-Salam	16	4	8	0	32	4	0	0	16	16	32	16	32	64	32	32	32	16	2	0	0	32	0.5	0	0
17	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	8	0	0	0	0	0
18	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	8	16	0	0	0	0	0	0	0	0
19	Seawater	winter	Al-Salam	0	16	4	32	0	0	0	4	0	0	4	0	0	0	0	16	32	0	4	2	0	16	4	0	0
20	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	32	0	32	1	32	32	32	16	0	0	0	0	4	1	1
21	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0.5	0	0	0	4	1	0
22	Seawater	winter	Al-Salam	8	4	4	16	32	2	0	4	0	0	16	0	32	2	32	16	16	16	4	0	0	32	4	0	1
23	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	8	0	0	0	0	0	0	0	0	0	0	0	4	0	0
24	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	16	32	0	0	0	0	8	16	8	0.5	0	0	0	4	1	0
25	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1
26	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	1
27	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	32	32	0	0	0	0	8	8	8	0	0	0	0	4	1	0
28	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	32	0	0	0	4	16	32	8	0	0	0	0	4	1	1
29	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	64	32	0	8	0	0	32	32	16	4	1	0	0	4	1	1
30	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
31	Seawater	winter	Al-Salam	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	4	0	0	4	0	0
32	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	16	0	0	32	0	16	0	0	0	32	0	0	0	0	0	4	0	0
33	Seawater	winter	Al-Salam	0	0	0	32	0	0	64	16	0	64	32	0	0	0	4	32	32	4	0	0	0	0	4	0	0
34	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
35	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
36	Seawater	winter	Al-Salam	0	0	0	32	32	0	0	0	0	0	32	0	0	0	4	16	32	8	0	0	0	0	4	0	0
37	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	16	0	0	32	0	0	8	16	32	32	8	4	2	0	0	4	1	1
38	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	32	0	64	32	0	16	0	0	32	32	16	4	2	0	0	0	0	1
39	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	0	4	0	1
40	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	32	16	0	0	0	0	0	32	0	0	0	0	16	0	1	1
41	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0	32	0	4	2	0	0	0	0	1
42	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	32	4	0	0	0	0	4	0	0	0	0	0	0	0	1	0
43	Seawater	winter	Al-Salam	64	16	8	32	32	8	128	32	128	64	32	32	32	64	32	32	32	16	4	8	2	128	4	1	1
44	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	128	64	32	0	16	1	2	32	32	16	0	0	0	0	4	1	1
45	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	4	4	0	0	4	0	1
46	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	8	0	16	32	0	32	0	0	0	32	2	0	0	0	16	0	0	1
47	Seawater	winter	Al-Salam	0	8	0	32	0	0	0	16	0	0	0	0	0	1	0	16	4	2	4	0	0	0	4	1	0

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	AMI	GENTOB		AMP	AZT	MERO	PIP	AIS2	PIT4	TIM2	FAZ	FEP	TANS	AXO	TAZ	FUR	FOX	POD	CIP	GAT	IMI	NIT	SXT	intI1	GC
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID ●	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
48	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
49	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	16	2	0	0	0	0	0	0	1
50	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	4	0	1
51	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
52	Seawater	winter	Al-Salam	0	0	0	32	0	0	64	32	0	64	32	0	32	0	8	32	32	16	0	0	0	0	4	1	1
53	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	0	0	0	0	0	0	4	0	0	0	0	32	0	0	1
54	Seawater	winter	Al-Salam	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	1	1
55	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	32	0	0	0	0	4	32	0	0	0	0	0	0	0	1
56	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	1
57	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0.5	0	1
58	Seawater	winter	Al-Salam	0	0	8	32	32	0	128	16	0	32	32	4	0	64	4	32	8	16	4	8	0	0	4	1	1
59	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	4	0	0
60	Seawater	winter	Al-Salam	0	16	0	32	0	0	0	0	128	0	32	0	0	0	0	4	32	4	2	1	0	0	0	0	0
61	Seawater	winter	Al-Salam	0	0	8	32	0	0	128	16	0	64	32	0	16	0	4	32	32	16	0	0	0	0	4	1	0
62	Seawater	winter	Al-Salam	0	0	0	8	0	0	0	4	0	0	32	0	0	0	0	0	32	8	0	0	0	0	4	1	1
63	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	32	0	32	1	32	32	32	16	0	0	0	0	4	1	0
64	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	64	32	0	8	0	8	32	32	16	0	0	0	0	4	1	0
65	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	16	0	0	0	0	8	32	4	4	4	0	0	4	0	0
66	Seawater	winter	Al-Salam	0	0	0	32	8	0	128	16	0	16	32	0	0	64	2	32	8	16	0	1	0	0	4	0	0
67	Seawater	winter	Al-Salam	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
68	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	32	0	16	0	4	32	32	16	0	0	0	0	4	1	1
69	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	1
70	Seawater	winter	Al-Salam	0	2	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	16	4	0	0
71	Seawater	winter	Al-Salam	0	0	0	32	0	4	64	16	0	16	32	0	8	0	0	0	0	16	0	0	0	0	4	1	0
72	Seawater	winter	Al-Salam	0	16	0	32	0	0	16	0	0	0	0	0	0	0	0	4	8	0	0	0	0	32	0	0	0
73	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	4	0	4	2	0	0	0	0	0
74	Seawater	winter	Al-Salam	0	16	4	16	8	0	64	4	0	0	32	0	0	32	2	8	4	16	4	1	0	0	4	0	1
75	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	0	1
76	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	32	0	0	0	0	4	16	0	0	0	0	0	0	0	0
77	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	32	0	0	1	0	32	0	2	0	0	0	0	0	0	1
78	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	16	0	32	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
79	Seawater	winter	Al-Salam	0	0	0	0	32	0	0	0	0	0	0	0	0	0	0	4	4	0	0.5	2	0	64	0	0	0
80	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	32	0	64	32	0	0	16	16	32	32	8	4	4	0	0	4	1	0
81	Seawater	winter	Al-Salam	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
82	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
83	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	8	0	0	0	0	0	32	0	0	0	0	0	4	1	1
84	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
85	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	32	64	32	0	0	0	0	8	0	0	0	0	0	16	4	0	0
86	Seawater	winter	Al-Salam	0	8	0	32	32	2	128	32	128	64	32	0	32	1	32	32	8	16	0	0	0	16	4	0	1
87	Seawater	winter	Al-Salam	0	16	8	32	0	0	128	8	0	0	8	0	0	0	0	4	4	0	4	4	0	16	0	1	0
88	Seawater	winter	Al-Salam	0	8	0	32	0	0	0	16	0	0	32	0	0	0	0	4	0	2	0	0	0	0	4	1	0
89	Seawater	winter	Al-Salam	0	16	0	32	0	0	128	32	0	64	32	0	16	2	4	32	32	16	0	0	0	0	4	1	1
90	Seawater	winter	Al-Salam	0	16	8	0	0	0	0	8	0	0	0	0	0	0	0	4	4	0	0	0	0	0	4	1	1
91	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
92	Seawater	winter	Al-Salam	0	16	8	16	0	0	0	0	0	16	32	0	0	0	0	0	32	0	0.5	0	0	0	4	1	1
93	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	1
94	Seawater	winter	Al-Salam	0	0	0	32	16	0	128	32	0	64	32	4	32	32	4	32	32	16	4	8	0	32	4	0	0
95	Seawater	winter	Al-Salam	0	0	0	32	8	0	128	4	0	0	32	0	0	32	2	32	16	16	4	1	0	0	4	1	1

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins								Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	AMI	GENTOB		AMP	AZT	MERO	PIP	A/S2	P/T4	TIM2	FAZ	FEP	TANS	AXO	TAZ	FUR	FOX	POD	CIP	GAT	IMI	NIT	SXT	*intI1*	*GC*
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID #	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
96	Seawater	winter	Al-Salam	0	0	0	32	32	0	128	4	0	0	32	4	0	64	4	32	4	16	2	2	0	0	0	0	0
97	Seawater	winter	Al-Salam	0	0	0	8	0	0	128	0	0	0	0	0	0	0	0	0	4	0	4	2	0	0	0	0	1
98	Seawater	winter	Al-Salam	0	16	8	32	0	0	0	8	0	16	0	0	0	0	0	0	0	0	4	4	0	0	4	1	1
99	Seawater	winter	Al-Salam	0	0	0	16	0	0	0	4	0	0	4	0	0	0	0	4	4	0	0	0	0	0	4	1	1
100	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	32	32	0	0	0	0	8	16	8	0	0	0	0	4	1	1
101	Seawater	winter	Al-Salam	0	2	0	4	0	0	0	4	0	0	32	0	0	0	0	32	8	8	4	1	0	64	4	0	0
102	Seawater	winter	Al-Salam	8	0	0	32	0	0	64	8	0	16	0	0	0	0	0	4	4	0	0	0	0	0	4	1	0
103	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
104	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
105	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
106	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
107	Seawater	winter	Al-Salam	0	2	0	32	0	0	128	32	128	64	32	0	0	0	0	4	4	0	0	0	0	0	4	1	0
108	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
109	Seawater	winter	Al-Salam	0	16	4	32	0	0	64	32	0	64	8	0	0	0	0	0	4	0	0.5	0	0	0	4	1	0
110	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	8	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
111	Seawater	winter	Al-Salam	0	16	8	32	32	0	128	16	0	64	32	32	0	64	8	32	32	16	4	4	0	16	4	0	0
112	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	8	0	32	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
113	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	4	4	0	0	0	0	0	4	0	0
114	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
115	Seawater	winter	Al-Salam	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	0	0
116	Seawater	winter	Al-Salam	0	0	0	32	0	0	64	4	0	0	16	0	0	8	0	4	4	8	0	0	0	0	4	1	0
117	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	4	1	1
118	Seawater	winter	Al-Salam	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	1
119	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	64	0	0	0	0	0	4	4	0	4	8	0	0	0	0	1
120	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	1
121	Seawater	winter	Al-Salam	0	16	8	32	0	0	128	32	0	64	8	0	0	0	0	8	8	0	4	8	0	0	4	1	1
122	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0.5	0	0	0	4	1	0
123	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	8	0	0	0	0	0	0	0	0
124	Seawater	winter	Al-Salam	0	16	0	32	0	0	0	8	0	0	32	0	0	0	0	4	32	4	0	0	0	0	4	0	0
125	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
126	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	32	0	64	16	0	0	0	0	0	4	0	0.5	0	0	0	4	1	0
127	Seawater	winter	Al-Salam	0	8	0	32	0	1	64	32	0	64	8	0	0	0	0	0	0	0	0.5	1	0	0	4	1	1
128	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	8	0	0	4	1	0
129	Seawater	winter	Al-Salam	0	0	0	32	0	0	32	0	0	0	32	0	0	4	0	4	0	8	0	0	0	0	4	1	1
130	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	0	32	0	0	0	0	0	16	0	0	0	0	0	4	1	1
131	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	16	0	0	0	0	0	8	0	4	4	0	0	4	1	0
132	Seawater	winter	Al-Salam	0	2	0	32	32	1	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
133	Seawater	winter	Al-Salam	0	0	0	16	0	0	0	8	0	0	8	0	0	0	0	0	0	0	0	0	0	0	4	1	0
134	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	16	0	0	4	32	0	0	0	0	4	0	0	0	0	0	0	0	0
135	Seawater	winter	Al-Salam	0	0	0	16	0	0	0	4	0	0	32	0	0	0	0	16	32	2	0	0	0	0	0	0	1
136	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	32	0	0	4	0	4	0	8	0	0	0	0	4	1	0
137	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	0	4	0	4	4	0	0	4	1	0
138	Seawater	winter	Al-Salam	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	4	4	0	0	0	0	0
139	Seawater	winter	Al-Salam	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
140	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
141	Seawater	winter	Al-Salam	0	4	0	4	0	0	0	4	0	0	4	0	0	0	16	32	8	0	1	1	0	16	0	0	0
142	Seawater	winter	Al-Salam	0	16	0	32	0	0	128	4	0	0	16	0	0	0	0	4	16	2	4	4	0	0	4	0	0

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	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	*intI1*	*GC*
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID #	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
143	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
144	Seawater	winter	Al-Salam	8	0	0	8	0	0	0	0	0	0	16	0	0	1	0	16	32	8	0	0	0	0	4	1	1
145	Seawater	winter	Al-Salam	0	0	0	16	0	0	0	8	0	0	16	0	0	0	0	0	32	0	0	0	0	0	0	0	0
146	Seawater	winter	Al-Salam	0	0	0	32	32	0	128	4	0	0	32	32	0	64	2	32	0	16	0	0	0	0	4	0	0
147	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0
148	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0
149	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	32	4	0	0	0	0	4	4	0	4	8	0	0	4	0	0
150	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
151	Seawater	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	4	0	0	0	0	0	0	0	0
152	Seawater	winter	Al-Salam	0	0	0	32	0	0	128	16	0	0	32	0	0	8	1	32	0	16	0.5	8	0	16	4	0	0
153	Seawater	winter	Al-Salam	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	4	4	0	4	4	0	16	4	0	0
154	Seawater	winter	Al-Salam	0	0	0	32	0	0	16	32	0	0	16	0	0	1	0	0	4	0	0	0	0	0	0	0	0
155	Seawater	winter	Al-Salam	0	0	0	32	0	0	0	16	0	0	32	0	16	8	2	32	32	16	0.5	0	0	0	4	1	0
1	Seawater	winter	Al-Ghazali	0	0	0	32	0	0	32	32	0	32	32	0	8	0	16	32	32	16	0	0	0	32	0	0	0
2	Seawater	winter	Al-Ghazali	0	0	0	16	0	0	0	8	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
3	Seawater	winter	Al-Ghazali	0	2	0	32	0	0	128	32	0	64	4	0	0	0	0	8	4	0	4	8	0	0	4	1	0
4	Seawater	winter	Al-Ghazali	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Seawater	winter	Al-Ghazali	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	0	0	4	0	1
6	Seawater	winter	Al-Ghazali	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	4	4	0	0	4	1	0
7	Seawater	winter	Al-Ghazali	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
8	Seawater	winter	Al-Ghazali	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	0	0
9	Seawater	winter	Al-Ghazali	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
10	Seawater	winter	Al-Ghazali	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
11	Seawater	winter	Al-Ghazali	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	1	0
12	Seawater	winter	Al-Ghazali	0	0	0	32	0	0	64	8	16	0	0	0	0	0	0	4	0	0	0	0	0	16	0	1	0
13	Seawater	winter	Al-Ghazali	0	16	4	32	0	0	64	4	0	0	0	0	0	0	0	8	4	0	0	0	0	0	4	0	0
14	Seawater	winter	Al-Ghazali	0	0	0	4	0	0	0	0	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	1	0
1	Seawater	winter	Khiran	64	16	8	32	32	1	128	32	128	64	32	4	32	64	32	32	4	16	4	8	0	128	4	0	0
2	Seawater	winter	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	4	8	0	0	0	0	0
3	Seawater	winter	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	0	0	0
4	Seawater	winter	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	0	0	0
5	Seawater	winter	Khiran	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	8	4	0	0	0	0	0	0	0	0
6	Seawater	winter	Khiran	0	8	0	16	0	0	0	0	0	0	16	0	0	0	0	0	8	0	4	4	0	0	4	0	0
7	Seawater	winter	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	4	4	0	0	0	0	0
8	Seawater	winter	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	0	0	0
9	Seawater	winter	Khiran	0	0	0	16	0	0	0	0	0	0	32	0	0	0	0	8	32	2	4	8	0	0	4	0	0
10	Seawater	winter	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	0	0	0
1	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0
2	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	0	0	0	0	0	0	4	1	0
3	Seawater	summer	Al-Salam	0	0	0	32	8	0	128	8	0	16	32	4	0	32	0	32	4	16	4	2	0	0	0	0	0
4	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	8	0	0	0	0	0	4	0	1	1	0	0	4	1	0
5	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
6	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	16	0	64	4	0	0	0	0	4	4	0	1	1	4	0	4	1	0
7	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	4	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
8	Seawater	summer	Al-Salam	0	0	0	32	0	0	32	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	Seawater	summer	Al-Salam	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
11	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	8	0	0	8	0	0	0	0	0	0	0	0	0	0	0	4	1	0

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins								Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	AMI	GENTOB		AMP	AZT	MERO	PIP	A/S2	P/T4	TIM2	FAZ	FEP	TANS	AXO	TAZ	FUR	FOX	POD	CIP	GAT	IMI	NIT	SXT	intI1	GC
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID ♦	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
12	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	8	0	0	0	0	16	2	0	0
13	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	2	0	4	0	0	0	0	0	4	1	0
14	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	4	8	0	4	8	0	0	4	1	0
15	Seawater	summer	Al-Salam	0	8	8	32	0	0	16	4	0	0	32	0	0	4	0	32	0	8	0	0	0	16	4	0	0
16	Seawater	summer	Al-Salam	0	0	0	32	0	0	0	4	0	0	4	0	0	0	0	0	4	0	0	0	0	0	0	0	0
17	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0
18	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	4	8	0	0	0	0	0	4	0	0
19	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	16	0	64	4	0	0	0	0	4	4	0	1	1	4	0	4	0	0
20	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	32	0	0	0	0	0	0	4	2	4	2	0	0	4	1	0
21	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	4	0	0	32	0	0	1	0	32	32	4	0	0	0	16	0.5	0	0
22	Seawater	summer	Al-Salam	0	0	0	32	8	0	16	4	0	0	32	0	0	8	1	32	0	0	0.5	1	0	16	4	0	0
23	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	0	0	4	2	0	16	4	1	0
24	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
25	Seawater	summer	Al-Salam	0	0	0	32	0	0	32	8	0	0	4	0	0	0	0	0	4	0	4	1	0	16	4	1	0
26	Seawater	summer	Al-Salam	0	0	0	32	32	0	128	8	0	0	32	32	0	64	4	32	4	16	0	0	0	0	0	0	0
27	Seawater	summer	Al-Salam	0	0	0	32	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0
28	Seawater	summer	Al-Salam	0	0	0	32	16	0	64	4	0	0	32	0	0	64	2	32	4	16	0	0	0	0	0	0	0
29	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	0	0	0	32	0	0	64	1	32	0	16	0	0	0	16	4	0	0
30	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
31	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	32	32	0	0	32	2	32	32	16	0	0	0	0	4	1	0
32	Seawater	summer	Al-Salam	0	16	4	32	32	0	128	8	0	16	32	32	0	64	4	32	0	16	4	4	0	0	4	1	0
33	Seawater	summer	Al-Salam	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35	Seawater	summer	Al-Salam	0	0	0	32	0	0	0	4	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	1	0
36	Seawater	summer	Al-Salam	0	8	4	32	0	0	0	4	0	0	0	0	0	0	0	0	4	0	4	2	0	0	4	0	1
37	Seawater	summer	Al-Salam	0	0	0	32	16	0	16	16	0	0	0	0	0	0	0	0	8	0	4	2	0	16	4	1	0
38	Seawater	summer	Al-Salam	0	0	0	32	0	0	0	8	0	16	4	0	0	0	0	4	8	0	4	8	0	16	0	0	0
39	Seawater	summer	Al-Salam	0	8	8	32	0	0	0	8	0	0	32	0	0	2	0	32	4	8	0	0	0	16	4	1	0
40	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0
41	Seawater	summer	Al-Salam	0	4	0	32	0	0	16	8	0	16	4	0	0	0	0	4	8	0	4	8	0	16	0	0	0
42	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	0	4	0	0	0	0	0	4	1	0
43	Seawater	summer	Al-Salam	0	16	8	32	32	0	128	16	0	32	32	32	0	64	8	32	8	16	4	8	0	16	4	1	0
44	Seawater	summer	Al-Salam	0	8	0	32	0	0	16	32	0	64	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
45	Seawater	summer	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
46	Seawater	summer	Al-Salam	0	0	0	32	0	0	32	8	0	16	0	0	0	0	0	0	4	0	0	0	0	0	4	1	0
47	Seawater	summer	Al-Salam	0	0	0	32	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0
48	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	8	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	1	0
49	Seawater	summer	Al-Salam	0	0	0	32	32	0	128	8	0	16	32	0	0	32	4	32	0	16	0	0	0	16	4	1	0
50	Seawater	summer	Al-Salam	0	0	0	32	32	0	128	16	0	32	32	32	0	64	1	32	8	16	0	0	0	0	4	0	1
51	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	8	0	16	4	0	0	0	0	0	4	0	4	2	0	0	4	0	0
52	Seawater	summer	Al-Salam	0	16	0	32	0	0	128	8	0	16	0	0	0	0	0	0	4	0	0	0	0	16	4	1	0
53	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
54	Seawater	summer	Al-Salam	0	0	0	32	8	0	32	0	0	0	32	0	0	16	0	16	4	16	0	0	0	0	0	0	0
55	Seawater	summer	Al-Salam	0	4	0	32	32	0	128	4	0	0	32	8	0	64	4	32	32	0	0	0	0	0	0	1	0
56	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
57	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	8	0	16	4	0	0	0	0	4	4	0	0	0	0	0	0	0	0
59	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamase	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	AMI	GEN	TOB	AMP	AZT	MERO	PIP	AIS2	PIT4	TIM2	FAZ	FEP	TANS	AXO	TAZ	FUR	FOX	POD	CIP	GAT	IMI	NIT	SXT	intI1	GC
Smple ID #	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
61	Seawater	summer	Al-Salam	0	8	8	32	0	0	128	4	0	0	0	0	0	0	0	0	4	0	4	4	0	0	4	1	0
62	Seawater	summer	Al-Salam	0	0	0	32	8	0	32	4	0	0	32	0	0	16	1	32	4	16	0	0	0	0	4	0	0
63	Seawater	summer	Al-Salam	0	16	4	32	0	0	128	32	0	16	4	0	0	0	0	0	0	0	0	0	0	0	4	1	0
64	Seawater	summer	Al-Salam	0	0	0	32	0	0	64	4	0	0	0	0	0	16	0	4	4	16	0	0	0	16	0	0	0
65	Seawater	summer	Al-Salam	0	0	0	32	32	0	128	16	0	64	32	32	0	64	32	32	8	16	0	0	0	16	0	0	0
66	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
67	Seawater	summer	Al-Salam	0	0	0	32	0	0	32	8	0	16	0	0	0	0	0	0	4	0	0	0	0	0	4	1	0
68	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	16	0	1	1	0	0	4	1	0
69	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	32	32	0	0	64	1	32	0	16	0	0	0	0	4	1	0
70	Seawater	summer	Al-Salam	0	0	0	32	0	0	16	8	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
71	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	0	4	4	0	4	4	0	0	0	0	0
72	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	16	0	0	0	0	4	32	0	0.5	0	0	0	4	1	0
73	Seawater	summer	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
74	Seawater	summer	Al-Salam	0	0	0	32	0	0	0	4	0	0	8	0	0	0	0	0	16	0	0	0	0	0	0	0	0
75	Seawater	summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
76	Seawater	summer	Al-Salam	0	0	0	32	32	0	128	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	1	1
77	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	4	0	0	0	0	0	4	1	0
78	Seawater	summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	8	0	0.5	0	0	0	4	1	0
1	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	0	0	0
2	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
3	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
4	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	0	1	0
5	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	4	0	16	32	0	0	64	4	32	4	16	0	0	0	0	0	0	0
6	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	16	0	0	8	0	0	32	0	32	2	1	8	32	4	4	8	0	128	4	1	0
7	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	16	0	16	0	0	0	0	0	8	4	0	0	0	0	0	4	1	0
8	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
9	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	0	4	0	0	0	0	0	0	0	4	8	0	4	4	0	16	0	1	0
10	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	32	0	64	16	0	0	0	0	4	8	0	4	8	0	32	4	0	0
11	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	16	0	0	0
12	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	4	0	0	32	0	0	32	4	32	0	16	0	0	0	0	0	1	0
13	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	16	0	32	4	0	0	0	0	4	4	0	0	0	0	0	4	1	0
14	Seawater	summer	Abu-Al-Hasaniya	0	0	0	4	0	0	0	4	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
15	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	32	32	0	64	0	0	0	0	0	0	0	0	4	0	0	32	4	0	0
16	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0	0
17	Seawater	summer	Abu-Al-Hasaniya	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	Seawater	summer	Abu-Al-Hasaniya	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	0	0	0	0
19	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	0	4	0	0	8	0	0	0	0	0	16	4	2	4	8	0	16	4	0	0
20	Seawater	summer	Abu-Al-Hasaniya	0	0	0	16	0	0	0	4	0	0	0	0	0	0	0	0	4	4	0	0	0	16	0	1	0
21	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	16	0	128	4	0	0	32	0	0	32	4	32	4	16	4	4	0	0	4	0	0
22	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	8	0	0	32	4	0	32	16	32	4	16	0	0	0	0	4	0	0
23	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	1	0
24	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	8	0	16	8	0	0	1	0	16	4	8	4	8	0	0	4	0	0
25	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	1	0
27	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	4	0	0
28	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
29	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	16	8	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0
30	Seawater	summer	Abu-Al-Hasaniya	0	0	4	32	0	0	128	8	0	32	0	0	0	0	0	0	0	16	0.5	1	0	32	4	0	0
31	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
32	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
33	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	32	64	64	8	0	0	0	0	8	4	0	0	0	0	0	4	0	0
35	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	32	32	64	8	0	0	0	0	8	4	0	0	0	0	0	4	1	1

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integror	
	source	Season	Location	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	*intI1*	*GC*
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID ♦	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
36	Seawater	summer	Abu-Al-Hasaniya	0	0	8	32	0	0	0	8	0	0	32	0	0	2	0	32	4	8	0	0	0	16	4	0	0
37	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	64	32	0	64	4	0	0	0	0	4	4	0	0.5	0	0	16	4	1	0
39	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	1	0
40	Seawater	summer	Abu-Al-Hasaniya	0	8	4	32	32	0	128	32	128	64	32	8	32	64	32	32	32	16	4	8	0	0	4	0	0
41	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	8	0	128	16	0	32	32	0	0	64	2	32	4	16	0	0	0	16	4	0	0
42	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	8	0	16	32	32	0	64	32	16	0	16	4	8	2	128	4	1	0
43	Seawater	summer	Abu-Al-Hasaniya	0	0	0	16	8	0	128	8	0	0	32	0	0	2	2	16	4	16	0	0	0	0	0	0	0
44	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	16	8	0	0	0	0	0	0	0	0	4	0	0.5	0	0	0	4	1	0
45	Seawater	summer	Abu-Al-Hasaniya	0	0	4	32	16	0	128	8	0	16	32	8	0	64	4	32	4	16	4	4	0	0	4	1	0
46	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	64	8	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	0	0
47	Seawater	summer	Abu-Al-Hasaniya	0	0	0	4	0	0	0	0	0	0	4	0	0	0	0	4	8	0	0.5	0	0	0	0	0	0
48	Seawater	summer	Abu-Al-Hasaniya	0	0	0	4	0	0	0	8	0	0	4	0	0	0	1	4	0	0	4	0	0	0	2	0	0
49	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	0	8	0	0	0	0	0	0	0	0	4	0	0	0	0	0	1	0	0
50	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	16	0	128	32	0	64	32	0	0	32	2	32	4	16	4	4	0	16	4	0	1
51	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	32	16	64	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1
52	Seawater	summer	Abu-Al-Hasaniya	0	0	0	4	0	0	0	4	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
53	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	8	0	0	32	4	0	64	2	32	0	0	0	0	0	0	4	1	0
54	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
55	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	32	0	32	0	0	0	0	0	4	4	0	0	0	0	16	4	1	0
56	Seawater	summer	Abu-Al-Hasaniya	0	4	4	32	32	0	128	16	0	16	32	4	0	64	4	32	0	16	4	4	0	16	4	0	0
57	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	4	0	0	32	0	0	64	2	32	4	16	0	0	0	0	0	0	0
58	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	8	0	32	16	0	0	32	0	8	8	4	4	4	16	4	4	0	0	4	1	0
59	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	32	0	0	32	8	0	64	8	32	4	0	0	0	0	0	0	0	0
60	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	8	0	0	4	0	0	0	0	0	8	0	4	4	0	4	0	0	0	2	0	0
61	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	4	4	0	0	0	0	0	4	1	0
62	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	16	0	32	0	0	0	0	4	4	0	16	4	2	0	0	4	1	0
63	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	32	0	64	8	0	0	0	0	0	4	0	0	0	0	0	4	1	0
64	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	8	0	16	32	0	0	64	4	32	0	16	0	0	0	0	4	0	0
65	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
66	Seawater	summer	Abu-Al-Hasaniya	0	16	8	0	0	0	128	0	0	32	0	0	0	0	0	16	4	16	4	8	0	0	4	1	0
67	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	32	0	128	8	0	32	32	32	32	64	8	32	4	16	4	8	0	0	4	1	0
68	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	4	0	0	0	1	0
69	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	32	16	64	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
71	Seawater	summer	Abu-Al-Hasaniya	0	8	0	32	32	0	128	32	0	64	32	4	0	32	8	32	16	16	4	8	0	0	4	0	0
72	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	64	8	0	0	0	0	0	0	0	4	16	0	0	0	0	16	4	0	0
73	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
74	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	16	8	0	16	0	0	0	0	0	4	8	0	0	0	0	0	4	1	0
75	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	16	8	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0.5	0	0
76	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	4	1	0
77	Seawater	summer	Abu-Al-Hasaniya	0	16	0	32	0	0	0	16	0	32	4	0	0	0	0	4	16	0	4	8	0	32	4	0	0
78	Seawater	summer	Abu-Al-Hasaniya	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
79	Seawater	summer	Abu-Al-Hasaniya	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	4	8	0	0	0	0	0	0	1	0

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	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	*intI1*	*GC*
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID #	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
1	Seawater	summer	Al-Ghazali	0	0	0	32	16	0	128	16	0	32	32	8	0	64	4	32	4	16	0	0	0	0	0	1	0
2	Seawater	summer	Al-Ghazali	0	0	0	32	0	0	128	16	0	32	4	0	0	0	0	4	4	0	0	0	0	0	4	0	0
3	Seawater	summer	Al-Ghazali	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
4	Seawater	summer	Al-Ghazali	0	0	0	32	0	0	128	16	0	64	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
5	Seawater	summer	Al-Ghazali	0	0	0	32	0	0	0	16	0	0	0	0	0	0	0	4	32	0	0	0	0	16	4	0	0
6	Seawater	summer	Al-Ghazali	0	0	0	32	0	0	128	16	0	64	32	0	0	0	0	32	4	0	0	0	0	0	0	0	0
7	Seawater	summer	Al-Ghazali	0	0	0	32	0	0	0	4	0	0	0	0	0	0	0	0	4	0	0	0	0	32	0	1	0
8	Seawater	summer	Al-Ghazali	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	1	0
9	Seawater	summer	Al-Ghazali	0	0	0	32	16	0	0	32	0	64	32	0	0	0	0	32	0	16	0	0	0	0	4	0	0
1	Seawater	summer	Khiran	0	2	0	16	8	0	32	32	0	64	32	0	0	16	2	32	4	0	0	0	0	0	4	1	0
2	Seawater	summer	Khiran	0	0	0	32	16	0	128	32	32	32	32	0	16	0	4	32	32	16	0	0	0	0	4	1	0
3	Seawater	summer	Khiran	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	4	4	0	64	4	0	0
4	Seawater	summer	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
5	Seawater	summer	Khiran	0	0	0	32	32	0	128	16	0	32	16	4	0	64	8	32	4	16	0	0	0	16	4	0	0
6	Seawater	summer	Khiran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	16	2	4	4	2	2	1	0	0	4	0	0
2	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0.5	0	0
3	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	64	4	0	0	0	0	0	0	0	0	0	0	1	8	0	0	4	1	0
4	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	0	1
5	Bivalves	Summer	Al-Salam	0	0	0	4	0	0	0	0	0	0	16	0	0	0	0	0	0	2	0	0	0	0	4	0	1
6	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	Bivalves	Summer	Al-Salam	0	4	0	32	0	8	64	4	0	0	0	0	0	16	0	0	4	0	0	1	0	0	4	1	0
8	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	16	0	0	0	0	0	0	0	0	4	2	0	0	4	1	1
9	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	16	0	0	0	0	0	0	0	0	4	2	0	0	4	1	0
10	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	4	2	0	0	4	1	0
11	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	4	1	1
12	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
13	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
14	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	32	8	0	16	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
16	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	Bivalves	Summer	Al-Salam	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
18	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	8	0	0	0	0	1
19	Bivalves	Summer	Al-Salam	0	0	0	32	32	0	128	16	0	32	32	32	0	64	8	32	4	16	0	0	0	0	4	0	0
20	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	64	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1
21	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	0	0	4	0	0	0	0	0	4	0	1
22	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
23	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	Bivalves	Summer	Al-Salam	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	32	0	0	0
26	Bivalves	Summer	Al-Salam	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	32	0	0	0
27	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	16	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	32	0	0	0	0	0	0	0	0	16	0	0	0	0	0	32	0	1	0
31	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	16	8	0	32	0	0	0	0	0	4	0	0	4	2	0	32	4	1	0
32	Bivalves	Summer	Al-Salam	0	0	0	8	0	0	0	4	0	0	32	8	16	8	0	0	32	0	0	0	0	0	4	0	0
33	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	0	4	0	0	32	0	16	0	0	8	32	0	0	0	0	16	2	0	0

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	AMI	GEN	TOB	AMP	AZT	MERO	PIP	AIS2	P/T4	TIM2	FAZ	FEP	TANS	AXO	TAZ	FUR	FOX	POD	CIP	GAT	IMI	NIT	SXT	*intI1*	*GC*
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID #	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
34	Bivalves	Summer	Al-Salam	0	0	0	32	8	0	0	32	0	64	32	0	32	8	8	32	32	16	0	0	0	0	0	1	0
35	Bivalves	Summer	Al-Salam	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38	Bivalves	Summer	Al-Salam	0	0	0	16	16	0	64	0	0	0	32	0	0	32	2	32	0	16	4	2	0	0	4	0	1
39	Bivalves	Summer	Al-Salam	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	1	0
40	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	32	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
41	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
42	Bivalves	Summer	Al-Salam	0	0	0	32	32	0	128	4	0	16	32	4	0	64	16	32	0	16	0	0	0	16	4	0	0
43	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
44	Bivalves	Summer	Al-Salam	0	0	0	32	16	0	128	4	0	0	32	32	0	64	4	32	0	16	0.5	1	0	0	0	0	0
45	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
46	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	16	4	0	0
47	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
48	Bivalves	Summer	Al-Salam	0	0	0	32	16	0	64	4	0	0	32	0	0	64	4	32	0	16	0.5	1	0	0	0	0	0
49	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0
50	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	32	2	0	4	2	0	0	0	16	0	0	0
51	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	0	0	0	0	0	0	4	0	4	8	0	0	4	1	0
52	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	32	0	0	16	2	32	4	16	4	4	0	0	4	0	0
56	Bivalves	Summer	Al-Salam	0	0	0	32	8	0	64	4	0	0	32	0	0	8	2	16	4	16	0	0	0	16	0	0	0
57	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	64	4	0	0	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0
58	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
59	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	32	0	64	16	0	0	0	0	4	4	0	0	0	0	0	4	0	1
60	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	4	0	0	0	0	0	0	0	0	0	0	16	0	0	0
61	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	0	0	4	2	0	32	4	0	0
62	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	4	0	0	0	0	0	0	0	0
63	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	1	0
64	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	32	0	64	8	0	0	0	0	0	8	0	0	0	0	32	4	1	0
65	Bivalves	Summer	Al-Salam	0	0	0	32	32	0	128	16	128	16	16	0	0	4	4	32	4	0	0	0	0	0	4	0	0
66	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	64	8	0	16	8	0	0	0	0	0	4	0	0	0	0	0	0	0	0
67	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
68	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0
69	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	4	0	0	0	0	0	0	0	0
70	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
71	Bivalves	Summer	Al-Salam	0	4	4	32	0	0	0	4	0	0	0	0	0	0	0	8	8	0	0	0	0	0	2	1	0
72	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
73	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	0	0	0	0	0	8	8	0	0.5	0	0	64	0	0	0
74	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
75	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	32	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
76	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
77	Bivalves	Summer	Al-Salam	0	0	0	4	0	0	0	0	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	0	0
78	Bivalves	Summer	Al-Salam	0	16	8	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0
79	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	32	0	4	0	0	0	0	0	0	0	0
80	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	4	4	0	0	0	0	0	0	0	0
81	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	4	0	0	0	0	0	0	0	0
82	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	32	0	0	0
83	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
84	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
	source	Season	Location	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	*intI1*	*GC*
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive	positive
ID #	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative	negative
85	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	0	0	4	0	0	0	0	32	0	0	0
86	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	16	0	16	4	0	0	0	0	4	4	0	0	0	0	0	0	0	0
87	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
88	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	16	0	0	0	0	0	0.5	0	0
89	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	64	4	0	0	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0
90	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0	0
91	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
92	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0	0
93	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0
94	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
95	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
96	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
97	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	4	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4	0	0
98	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
99	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
100	Bivalves	Summer	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
101	Bivalves	Summer	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
102	Bivalves	Summer	Al-Salam	0	2	0	32	16	0	32	16	0	64	32	0	16	64	32	32	32	16	4	4	0	0	0	0	0
103	Bivalves	Summer	Al-Salam	0	0	0	32	32	0	128	16	0	64	32	0	32	64	32	32	32	16	4	4	0	0	0	0	0
1	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	32	0	0	32	0	32	0	2	16	32	4	0	0	0	0	0	0	0
2	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	4	0	0	0	0	0	4	1	1
3	Bivalves	winter	Al-Salam	0	4	0	0	32	8	16	0	0	0	32	32	32	64	0	4	32	4	0	2	0	16	4	1	1
4	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	1
5	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	16	0	0	32	0	32	4	4	8	32	0	0	0	0	0	0	0	1
6	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	32	0	16	0	0	32	32	0	0.5	1	0	0	0	0	1
7	Bivalves	winter	Al-Salam	0	0	0	32	32	0	128	8	0	0	32	16	8	64	8	32	32	16	4	4	0	0	0	0	1
8	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	1
9	Bivalves	winter	Al-Salam	0	0	0	32	8	0	0	16	0	64	32	0	16	8	0	8	32	16	0	1	0	0	0	0	1
10	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	1
11	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	1
12	Bivalves	winter	Al-Salam	0	0	0	32	32	0	128	32	0	0	32	32	32	64	8	32	32	16	4	4	0	0	0	0	1
13	Bivalves	winter	Al-Salam	0	0	0	8	0	0	0	8	0	0	32	0	8	1	0	32	32	16	0	0	0	128	0	0	1
14	Bivalves	winter	Al-Salam	0	0	0	32	16	0	128	4	0	0	32	16	0	64	4	32	4	16	0	0	0	0	0	0	1
15	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	16	0	32	0	0	0	0	0	0	4	0	0	0	0	0	4	1	1
16	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	4	0	0	0	0	0	4	0	0	0	0	0	4	1	1
17	Bivalves	winter	Al-Salam	0	0	0	32	16	0	128	0	0	0	32	32	0	64	4	32	8	16	0	0	0	0	0	0	1
18	Bivalves	winter	Al-Salam	0	0	0	32	8	0	32	0	0	0	32	0	0	64	1	32	4	16	0	0	0	0	0	0	1
19	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	0	0	0	32	0	0	16	1	32	4	16	0	0	0	0	0	0	1
20	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	32	0	8	0	0	32	32	2	0	0	0	0	0	0	1
21	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	16	0	0	0	0	0	32	0	0	0	0	0	0	0	0
22	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
23	Bivalves	winter	Al-Salam	0	0	0	32	8	0	32	4	0	0	32	0	0	32	2	32	32	16	0	0	0	0	4	0	1
24	Bivalves	winter	Al-Salam	0	0	0	4	0	0	0	0	0	0	32	0	0	0	0	4	32	0	0	0	0	0	4	1	1
25	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	0	0	0	32	0	0	32	2	32	32	16	0	0	0	0	0	1	1
26	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	0	1
27	Bivalves	winter	Al-Salam	0	0	0	32	8	0	32	8	0	0	32	0	0	32	1	32	32	16	0	0	0	0	4	1	1
28	Bivalves	winter	Al-Salam	0	0	0	16	0	0	32	0	0	0	8	0	0	0	0	0	16	16	0	0	0	64	0	0	1
29	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	32	0	0	32	0	32	16	1	32	32	16	0	0	0	0	0	0	1
30	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	1	1
31	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

				des			Beta-lactams				eta-lactamas	lactamase inhibitor		Cephalosporins								lones		pen em	olon e	reductas e	class 1 integron	
	source	Season	Location	AMI	GENTOB		AMP	AZT	MERO	PIP	A/S2	P/T4	TIM2	FAZ	FEP	TANS	AXO	TAZ	FUR	FOX	POD	CIP	GAT	IMI	NIT	SXT	intI1	GC
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID #	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
32	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	32	0	0	32	0	8	1	0	32	32	4	0.5	0	0	0	4	1	1
33	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	1
34	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	0	0	0	0	0	0	0	0	0	0	0	0	4	1	1
35	Bivalves	winter	Al-Salam	0	16	0	32	0	0	0	8	0	0	32	0	0	0	0	0	32	0	0.5	0	0	0	4	1	1
36	Bivalves	winter	Al-Salam	0	0	0	4	0	0	0	0	0	0	32	0	0	0	0	16	32	0	0	0	0	0	0	0	1
37	Bivalves	winter	Al-Salam	0	0	0	16	0	1	0	16	0	0	32	0	0	1	0	16	32	16	0	0	0	64	0	0	1
38	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	4	0	0	4	0	0	1	0	0	0	0	0	0	0	32	4	1	1
39	Bivalves	winter	Al-Salam	0	0	0	32	8	0	128	32	0	64	32	0	0	64	2	32	32	16	0	0	0	0	4	1	1
40	Bivalves	winter	Al-Salam	0	4	0	32	0	0	128	8	0	16	32	0	8	4	0	32	32	8	0	0	0	0	0	0	1
41	Bivalves	winter	Al-Salam	0	4	4	32	0	0	16	0	0	0	32	4	32	4	0	32	32	8	0	0	0	0	0	1	1
42	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	0	0	4	1	1
43	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	32	0	64	32	0	0	16	2	32	32	16	0	0	0	0	4	1	1
44	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	32	0	0	0	0	0	4	4	0	4	4	0	0	4	0	1
45	Bivalves	winter	Al-Salam	0	0	0	32	0	1	0	16	0	0	32	0	16	2	0	16	32	16	0	0	0	128	0	1	1
46	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	0	16	0	0	0	0	8	4	0	0	0	0	0	4	0	1
47	Bivalves	winter	Al-Salam	0	16	4	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	4	4	0	0	4	1	1
48	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	4	1	1
49	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	0	8	0	0	0	0	0	32	0	0	0	0	0	4	1	1
50	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	16	0	16	32	0	16	8	1	8	32	2	0	0	0	0	4	1	1
51	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	0	0	0	0	0	0	0	0	4	8	0	0	4	1	1
52	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	4	0	0	8	0	0	0	0	0	4	4	0	0	0	0	4	1	1
53	Bivalves	winter	Al-Salam	0	16	8	32	0	0	32	8	0	0	32	0	0	0	0	4	32	0	4	8	0	0	4	1	1
54	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	8	0	0	32	0	0	0	0	4	32	2	4	1	0	16	4	1	1
55	Bivalves	winter	Al-Salam	0	0	0	8	0	0	0	0	0	0	32	0	0	0	1	4	32	0	0	0	0	0	0	0	1
56	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	32	0	16	0	0	8	32	8	0	0	0	0	4	0	1
57	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	32	0	32	0	0	4	32	2	0	0	0	16	0	0	1
58	Bivalves	winter	Al-Salam	0	0	0	8	0	0	0	4	0	0	32	0	0	0	0	8	32	2	0	0	0	16	0	0	1
59	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	32	0	32	0	0	8	32	2	0	0	0	16	0	0	1
61	Bivalves	winter	Al-Salam	0	0	0	16	0	0	0	8	0	0	32	0	0	0	0	8	32	4	0	0	0	16	0	0	1
62	Bivalves	winter	Al-Salam	0	0	0	32	8	0	16	32	0	64	32	0	32	8	4	32	32	16	0.5	0	0	16	0	0	1
63	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	32	0	0	4	0	32	32	16	0	0	0	16	4	0	1
64	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	4	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	0	1
65	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	4	4	2	0	0	0	0	0	0	1
66	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	0	1
67	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	32	0	0	0	4	8	32	0	0	0	0	0	0	1	1
68	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	32	0	0	0	0	32	32	4	4	4	0	0	0	0	1
69	Bivalves	winter	Al-Salam	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	1
70	Bivalves	winter	Al-Salam	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
71	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	4	0	0	0	0	0	0	0	0	1
72	Bivalves	winter	Al-Salam	0	0	0	16	0	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0	0	0	1
73	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	4	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	1
74	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integror	
	source	Season	Location	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	*intI1*	*GC*
Smple	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
ID #	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
75	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	0	0	0	0	0	0	0	0	0	0	0	0	32	4	1	1
76	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
77	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	8	0	0	0	0	0	0	0	4	8	0	0	0	0	1
78	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	16	32	0	16	1	0	0	32	0	0	0	0	16	4	1	1
79	Bivalves	winter	Al-Salam	0	4	0	8	0	0	0	0	0	0	8	0	0	0	0	8	0	0	0	0	0	0	0	0	1
80	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1
81	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	8	0	16	8	0	0	0	0	0	4	0	0	0	0	0	0	1	1
82	Bivalves	winter	Al-Salam	0	0	0	16	0	0	0	0	0	0	32	0	0	0	0	0	32	0	4	2	0	0	0	0	1
83	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	16	32	0	0	0	0	4	32	16	0	0	0	0	4	0	1
84	Bivalves	winter	Al-Salam	0	0	4	16	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	64	0	0	1
85	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	32	0	32	0	0	0	32	0	4	1	0	64	4	0	1
86	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	64	4	0	1
87	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	32	0	64	32	0	0	0	0	0	32	0	0	0	0	0	4	0	1
88	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	32	0	64	32	0	32	1	0	32	32	16	0	0	0	0	4	1	0
89	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	16	16	64	32	0	0	0	0	0	32	0	0	0	0	0	4	1	1
90	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	32	0	32	32	0	16	0	0	0	32	0	2	1	0	0	4	0	0
91	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0	16	0	0	0	0	64	4	1	1
92	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	64	0	0	0
93	Bivalves	winter	Al-Salam	0	0	0	4	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
94	Bivalves	winter	Al-Salam	0	0	0	16	0	0	0	0	0	0	32	0	0	0	0	0	32	0	0	0	0	0	0	0	1
95	Bivalves	winter	Al-Salam	0	0	0	16	0	0	0	4	0	0	32	0	0	0	0	8	32	0	0	0	0	16	0	0	1
96	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	4	0	0	16	0	0	0	0	0	0	0	0	0	0	0	4	1	0
97	Bivalves	winter	Al-Salam	0	16	0	16	0	1	0	4	0	0	32	0	0	0	0	4	32	0	0	0	0	16	0.5	0	1
98	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	32	0	64	32	0	16	2	8	32	32	16	0	0	0	16	4	1	1
99	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	8	32	0	4	2	0	0	0	0	1
100	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	8	0	16	32	0	0	0	0	0	32	0	0	0	0	16	0	1	0
101	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	32	0	32	0	0	4	32	0	0	0	0	0	4	1	1
102	Bivalves	winter	Al-Salam	0	0	0	32	0	0	32	8	0	0	16	0	0	8	1	32	0	0	0	4	0	16	0	0	0
103	Bivalves	winter	Al-Salam	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	16	0	0	1
104	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	32	0	16	2	16	32	32	16	0	0	0	0	4	1	0
105	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	32	0	64	32	0	32	2	1	4	32	8	4	8	0	16	4	1	0
106	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
107	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0	4	1	1
108	Bivalves	winter	Al-Salam	0	0	0	4	0	0	0	0	0	0	32	0	0	0	0	0	32	0	0	0	0	16	0	0	1
109	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	16	0	16	32	0	0	0	0	0	32	0	0	0	0	0	4	1	1
110	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	16	32	0	16	0	0	4	32	8	4	4	0	0	4	1	1
111	Bivalves	winter	Al-Salam	0	0	0	32	32	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	1	1
112	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	16	0	0	32	0	32	0	0	32	32	0	0	0	0	16	4	0	1
113	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	16	4	0	0	0	0	0	4	0	4	2	0	0	4	1	1
114	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	16	0	64	32	0	32	0	0	8	32	4	4	4	0	16	4	1	1
115	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	32	128	64	32	0	32	8	4	32	32	16	0	0	0	16	4	1	1
116	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	16	0	16	32	0	0	0	0	8	32	2	0	0	0	0	4	1	1
117	Bivalves	winter	Al-Salam	0	0	0	32	0	0	16	8	0	16	32	0	0	4	0	32	4	8	0	0	0	0	0	1	1
118	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	1
119	Bivalves	winter	Al-Salam	0	0	0	32	32	0	0	4	0	64	32	32	0	32	32	32	32	16	0	0	0	0	4	0	1
120	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	1	1

				Aminoglycosides			Beta-lactams				Aminoglycoside/beta-lactamas	Beta-lactam / beta-lactamase inhibitor		Cephalosporins							Fluoroquinolones		Imipenem	Quinolone	Dihydrofolate reductase	class 1 integron	
Smple ID #	source	Season	Location	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	*intI1*	*GC*
	Resistant MIC break points (µg/L)			≥32	≥8	≥8	≥32	≥32	≥8	≥128	≥32	≥128	≥64	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥8	≥4	≥8	≥16	≥128	≥4	positive 1	positive 1
	Sensitive MIC break points (µg/L)			≤8	≤4	≤4	≤8	≤8	≤4	≤16	≤8	≤16	≤16	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤2	≤1	≤2	≤4	≤32	≤2	negative 0	negative 0
121	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	4	0	0	8	0	0	0	0	0	16	0	0	0	0	0	2	0	1
122	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	16	0	0	0	0	0	0	0	0	0	0	0	0	4	1	1
123	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	0	32	0	0	0	0	4	4	0	0	0	0	0	4	1	1
124	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	1	1
125	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	8	0	0	0	0	0	0	0	0	0	0	4	4	0	0	4	0	1
126	Bivalves	winter	Al-Salam	0	2	8	16	0	0	0	8	0	0	32	0	0	2	8	8	8	0	0	0	0	0	4	1	1
127	Bivalves	winter	Al-Salam	0	0	0	32	0	0	64	0	0	0	32	0	0	64	0	32	0	8	0	0	0	0	0	0	1
128	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	8	0	0	32	0	0	0	0	4	32	2	0	0	0	16	0	0	1
129	Bivalves	winter	Al-Salam	0	0	0	4	0	0	0	0	0	0	4	0	0	0	0	4	0	0	0	0	0	0	0	0	1
130	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0	32	16	0	0	0	0	4	1	1
131	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	32	0	0	0	0	4	32	0	0	0	0	0	4	1	1
132	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
133	Bivalves	winter	Al-Salam	8	0	0	32	0	0	0	16	0	0	32	0	16	0	0	4	32	2	0	0	0	16	0	0	0
134	Bivalves	winter	Al-Salam	0	0	0	32	0	0	0	16	0	16	32	0	0	0	0	0	32	0	0	0	0	0	0	0	0
135	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
136	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
137	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	8	0	0	32	0	0	0	0	0	32	0	4	2	0	0	4	1	1
138	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	0	0	0	0	0	0	0	0	0.5	1	0	0	4	1	0
139	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
140	Bivalves	winter	Al-Salam	0	0	0	16	0	0	128	16	0	16	4	0	0	0	0	0	4	0	0	0	0	0	4	1	0
141	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
142	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	4	0	0	4	0	0	0	0	0	0	0	2	2	0	0	4	1	0
143	Bivalves	winter	Al-Salam	0	0	0	32	0	0	128	32	0	64	8	0	0	0	0	0	0	0	4	2	0	0	4	1	0
144	Bivalves	winter	Al-Salam	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	32	0	0	0	0	0	0	0	1

Appendices 2 to 4

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Appendix 2 Al-Sarawi, H. A., Jha, A. N., Al-Sarawi, M. A. and Lyons, B. P. (2015) 'Historic and contemporary contamination in the marine environment of Kuwait: An overview', *Marine Pollution Bulletin,* 100(2), pp. 621-8.DOI: 10.1016/j.marpolbul.2015.07.052

Appendix 3 Al-Zaidan, A. S., Al-Sarawi, H. A., Massoud, M. S., Al-Enezi, M., Smith, A. J., Bignell, J. P., Green, M. J., Askem, C., Bolam, T. P., Barber, J. L., Bersuder, P. and Lyons, B. P. (2015) 'Histopathology and contaminant concentrations in fish from Kuwait's marine environment', *Marine Pollution Bulletin,* 100(2), pp. 637-45. DOI: 10.1016/j.marpolbul.2015.07.030

Appendix 4 Al-Sarawi, H. A., Jha, A. N., Baker-Austin, C., Al-Sarawi, M. A. and Lyons, B. P. (2017) 'Baseline screening for the presence of antimicrobial resistance in *E. coli* isolated from Kuwait's marine environment' *Marine Pollution Bulletin, in press.* DOI:*10.1016/j.marpolbul.2017.10.044*

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