Shellfish as bioindicators for coastal antimicrobial resistance

Chief Scientist's Group report

October 2023

Version: SC220004/R

We are the Environment Agency. We protect and improve the environment.

We help people and wildlife adapt to climate change and reduce its impacts, including flooding, drought, sea level rise and coastal erosion.

We improve the quality of our water, land and air by tackling pollution. We work with businesses to help them comply with environmental regulations. A healthy and diverse environment enhances people's lives and contributes to economic growth.

We can't do this alone. We work as part of the Defra group (Department for Environment, Food & Rural Affairs), with the rest of government, local councils, businesses, civil society groups and local communities to create a better place for people and wildlife.

This report is the result if research jointly undertaken by the Environment Agency's Chief Scientist's Group and the Centre for Environment, Fisheries and Aquaculture Science (Cefas).

Published by:

Environment Agency
Horizon House, Deanery Road,
Bristol BS1 5AH

www.gov.uk/environment-agency

Further copies of this report are available from our publications catalogue: www.gov.uk/government/publications or our National Customer Contact Centre: 03708 506 506

Email: research@environment-agency.gov.uk

Author(s):
Wiebke Schmidt[1], Craig Baker-Austin[2], Jono Warren[1], Ben Maskrey[2], Nicola Coyle[2]
[1]Environment Agency, [2]Cefas

Keywords:
Shellfish, antimicrobial resistance (AMR), research, methods, surveillance

Environment Agency's Project Manager:
Dr Wiebke Schmidt

Project number: SC220004

Citation:
Environment Agency (2023) Shellfish as bioindicators for coastal Antimicrobial Resistance. Environment Agency, Bristol.

Research at the Environment Agency

Scientific research and analysis underpins everything the Environment Agency does. It helps us to understand and manage the environment effectively. Our own experts work with leading scientific organisations, universities and other parts of the Defra group to bring the best knowledge to bear on the environmental problems that we face now and in the future. Our scientific work is published as summaries and reports, freely available to all.

This report is the result of research jointly undertaken by the Environment Agency's Chief Scientist's Group and the Centre for Environment, Fisheries and Aquaculture Science (Cefas).

You can find out more about our current science programmes at https://www.gov.uk/government/organisations/environment-agency/about/research

If you have any comments or questions about this report or the Environment Agency's other scientific work, please contact research@environment-agency.gov.uk.

Dr Robert Bradburne
Chief Scientist

Contents

Acknowledgements

This work was carried out as part of ongoing Environment Agency research into Antimicrobial Resistance (AMR) in the environment and has been funded by the PATH-SAFE programme which has been funded by HM Treasury through the Shared Outcomes Fund.

We are thankful to the local authority sampling officers, who provided us with the samples for this pilot research project.

Executive summary

There is growing concern that aquatic environments, including coastal waters, represent hotspots for the evolution, retention and dissemination of antimicrobial resistance (AMR). However, the prevalence and monitoring data for AMR in these environments is currently limited. Filter-feeding shellfish present a potentially useful sentinel bioindicator model to help address this gap as they can concentrate microbial contamination from the environment and are already examined in many countries as part of microbiological and biotoxin monitoring programmes. The use of such samples for additional testing and AMR surveillance purposes was therefore the focus of this short study.

Here we utilised live bivalve shellfish from five sites in England (three potential 'impacted' and two 'reference' sites) from July 2022 to January 2023 to develop and implement a range of different testing approaches to better assess the utility of shellfish as sentinel species for routine AMR monitoring efforts. We developed a range of different testing approaches, which included the isolation and testing of bacterial indicator organisms (e.g., *E. coli*) for AMR, the testing of the same matrices for various pharmaceutical residues (e.g., antibiotics) using targeted and untargeted chemical analysis approaches and the detection of AMR genes and associated mobile genetic elements using a combination of metagenomic analysis and high throughput "SmartChip" qPCR arrays.

The shellfish samples proved suitable for the tested analytical methods and hence our study indicates that bivalves can be used as sentinels for monitoring AMR in the coastal environment and given further developmental work have the potential to be used more widely for environmental monitoring purposes.

1. Introduction

Antimicrobial resistance (AMR) is a true One Health challenge; microorganisms that are resistant to our antimicrobials are present in both built and natural environments and can produce illness in humans, animals and plants. While there are surveillance programmes for AMR in clinical and veterinary settings, there is a lack of data regarding AMR in the environment. This has been recognised internationally (WHO Global Action Plan (2016), UNEP, 2023) and nationally with the 20-year Vision for AMR and the 5-year National Action Plan (NAP), which lays out how the UK will address the AMR challenge. The NAP includes a specific section on the importance of better understanding the potential spread, transmission, and risk of AMR in the environment (UK Government, 2019). It also lists the exploration of an environmental surveillance component as part of ongoing efforts to tackle AMR.

Filter-feeding shellfish may represent a good bioindicator species to help address this current gap in environmental AMR surveillance (Grevskott, *et al*. 2017). Bivalve species have been used in environmental monitoring programmes since 1970s, such as the Mussel Watch Programme (Kimbrough et al., 2008). As filter or suspension feeders, shellfish use their gills to remove suspended particles from the water which is pumped through the mantle cavity (Gosling, 2003). Additionally, to these suspended particles various toxins, chemical or microbial contaminants (e.g., bacteria, viruses, etc.) can also been filtered and accumulated by the shellfish from their aquatic environment (Cefas, 2014, Hinder et al., 2011; Rupnik et al., 2021). Therefore, they are already examined regularly in Europe for bacterial faecal indicator organisms (*Escherichia coli*, *E. coli*) as part of various monitoring programmes (Walker *et al*. 2018).

Furthermore, shellfish samples, which are usually analysed monthly across the UK shellfish waters are typically discarded following their respective testing purposes. Thus, there is the potential for shellfish samples to be utilised more broadly, and judiciously to provide specific and valuable insights into the prevalence and dissemination of AMR in the coastal environment.

Shellfish as a food commodity also provide a link between environment and human health and there is growing concern regarding the presence of resistant microbes as well as antimicrobial residues in bivalve shellfish. Indeed, it was recently highlighted by the European Food Safety Authority (EFSA) that there is a need for utilising shellfish as part of wider environmental AMR studies (EFSA 2019).

This short proof-of-concept study sought to develop a suite of testing approaches to more fully understand if routine shellfish samples could be utilised in a broader context to better assess their potential role in environmental AMR.

The work described here involved a significant amount of developmental work to refine certain methods, in particular for chemical and nucleic acid extraction of various shellfish species. Briefly, this work included using the same bivalve shellfish samples obtained during routine testing but subsequently examined using various chemical, molecular (AMR gene and metagenomic analysis) and microbiological approaches (e.g., *E. coli* isolation and AMR testing), figure 1.

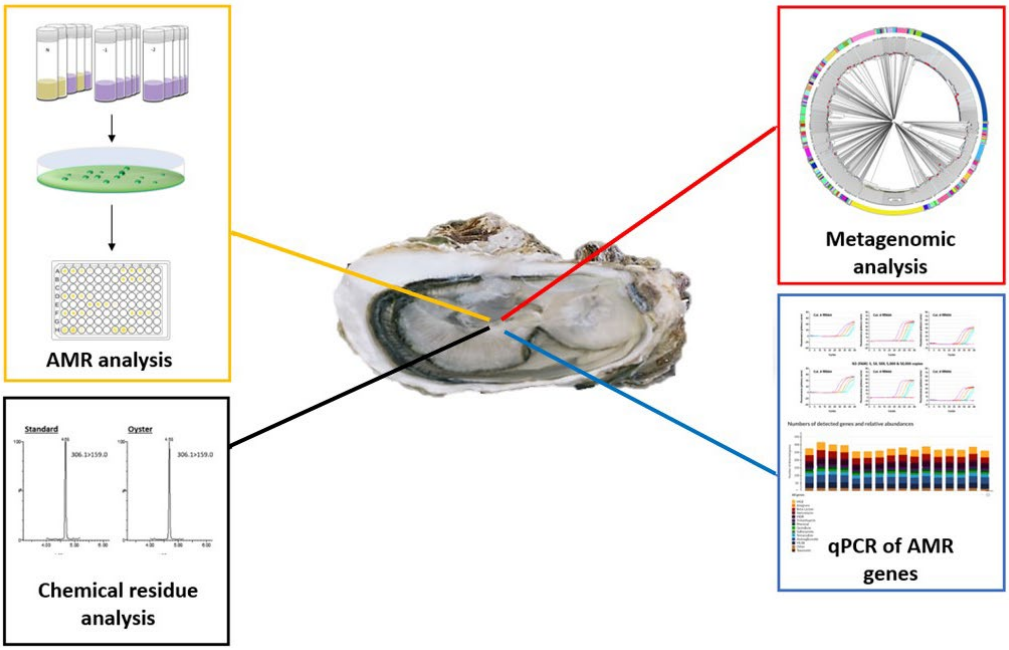


Figure 1. One sample = many tests. The broad array of tests developed in this project include isolation and testing of *E. coli* for AMR, and various molecular and chemical analyses to assess the use of shellfish as sentinels of environmental health.

This work forms a component to work stream 4 ‘Environmental surveillance methods in catchment scale pilots and a One Health data system’ of the HM Treasury funded PATH-SAFE programme (UK Government 2021). As part of this cross-governmental programme, work stream 4 aimed to identify appropriate methods suitable for monitoring a range of resistant organisms, resistant genes and antimicrobial substances in air, water and solids.

2. Methodology

2.1. Site selection & sampling of shellfish

Five shellfish production waters around England were selected to be sampled aligned to the ongoing shellfish classification work undertaken by Cefas on behalf of official controls monitoring. Consider

Study sites

For this study, shellfish production sites in England with satisfactory sampling compliance in official control monitoring in the previous sampling year were selected, as these reflect harvesting areas that are actively used. Briefly, several factors were considered for the selection of sample sites, based on the expected level of anthropogenic impacts on water quality, e.g., if sites are likely impacted with a high anthropogenic impact or if sites were unimpacted and more considers as reference sites. Firstly, shellfish classification data (https://www.food.gov.uk/business-guidance/shellfish-classification) was used, with sites ranked based on whether they were class A (80% of samples tested showed ≤ 230 *E. coli*/100g; all samples must be less than 700 *E. coli*/100g) or class B (80% of samples tested must be ≤ 4600 *E. coli*/100g; all samples must be less than 46,000 *E. coli*/100g).
Secondly, information on previous norovirus outbreaks, proximity to known sewage contamination sources and previous data on prevalence regarding norovirus was also taken into account for the choice of sites. In total, five sites were chosen, which encompassed three potential 'impacted' sites and two 'reference' sites (table 1).

Table 1. Sites of collected shellfish, their shellfish species and classification information.

Potential 'impacted' sites		**Reference 'non-impacted/ reference' sites**	
Name	*Relevant info*	*Name*	*Relevant info*
Site 1 (Blue mussels)	Class B area with history of viral contamination	Site 3 (Native oysters)	Class A area
Site 2 (Pacific oysters)	Class B area with some pollution history	Site 4 (Blue mussels)	Class A area
Site 5 (various species)	Class B area, with history of viral contamination		

Sampling

Various shellfish samples (e.g., blue mussels, pacific oysters, etc.) were collected from each study site in parallel with the collection of samples for the statutory classification monitoring programme. Typically, samples were taken at the start of each month from July 2022 until January 2023. Samples were dispatched to the laboratory under temperature-controlled conditions (<10°C). Upon arrival at the laboratory the shellfish samples were split into three portions which encompassed: a) five animals for molecular analysis (and extracted as described below), b) five animals for chemical analysis (homogenised and frozen -20°C) and c) five animals for phenotypic AMR analysis, which were processed as described below.

2.2. Microbiological and molecular analysis

The objective of this project is to trial and compare methods for the assessment of abundance and diversity of environmental AMR in shellfish. Therefore, specific tasks encompassed the following:

1. Development of an assay that enables the detection of a broad range molecular characterisation of AMR shellfish.
    a. Metagenomic sequencing of shellfish microbiome to identify both mobile genetic elements, AMR genes and detection of relevant bacterial species.

b. Detection of a wide array of antimicrobial associated genes by high throughput quantitative polymerase chain reaction (qPCR).

2. Development of an assay to enumerate and isolate antibiotic resistant *E. coli* from shellfish flesh, in a way that is complementary to both current routine monitoring for *E. coli* in shellfish, and the techniques used for the detection of AMR and ESBL-resistant *E. coli* (e.g., WHO tricycle recommendations (WHO 2021 and EFSA recommendations on AMR in environmental bacteria (EFSA 2019)). Further characterisation of resistant E. *coli* isolates to allow comparisons to broad range technique in task 1.

Detailed material and methods regarding these components are outlined below.

2.2.1 DNA extraction

Various DNA extraction procedures were trialled, including the Qiagen blood and Promega Wizard tissue, and Maxwell RSC tissue DNA extraction kits. Following a comparison of these methods, the Promega RSC method provided the best overall yield of DNA from stored samples and was used as the subsequent method for shellfish extraction purposes during this project.

Briefly, to extract DNA from shellfish tissues, 800 µl of Lifton's buffer was added to 25-80 mg tissue in a FastPrep Lysing Matrix A tube (MP Biomedicals). Samples were homogenised for 1 minute at 5 m/s using a FastPrep apparatus (MP Biomedicals, LLC, Solon, OH). Following tissue homogenisation, 20 µl of proteinase K (10 mg ml^{-1}) was added to the samples and they were subsequently incubated overnight at 55-56˚C. Sample homogenates were centrifuged at 10,000 x g for 2 minutes. Shellfish tissue DNA was subsequently extracted using the Maxwell extraction robot (Promega) by following instructions provided by the Maxwell RSC tissue DNA kit (Promega). The quality and quantity of extracted DNA was determined by a NanoDrop-1000 (Thermo Fisher Scientific). Samples that did not provide sufficient high-quality DNA (>10 ng/µl DNA) were re-extracted as appropriate. DNA samples were subsequently stored at -20°C until further analysis.

2.2.2 Detection of bacterial DNA in extracted shellfish samples

To further ensure bacterial content in extracted shellfish samples, five microliters of extracted shellfish DNA (extracted as outlined above) was amplified using conventional PCR using the 16S eubacterial primer pair 27F/1492R (Frank *et al*. 2008). Following PCR, 10 µl

of the total amplified products was separated using gel electrophoresis and ethidium bromide-stained 1% agarose gels were visualized under UV fluorescence. A 100-bp ladder (Promega) was used as the molecular size standard.

2.2.3 Whole-Metagenome sequencing (WMS)

Extracted DNA was subjected to library preparation using the Illumina Nextera XT DNA Library Preparation Kit (FC-131-1096, Illumina, CA, USA) according to the manufacturer's guidelines. Briefly, 1 ng DNA was subjected to tagmentation and amplification with Illumina® Nextera XT Indices, prior to library purification using Mag-Bind® TotalPure NGS beads (Omega Bio-tek, Inc., GA, USA), normalisation and pooling. The pooled sequencing libraries were loaded onto a NextSeq550 500/550 High-Output v2.5 (300 cycle) Flow Cell at a concentration of 1.8 pM according to the manufacturer's guidelines, to generate approximately 8 million read pairs per sample. Paired-end sequencing was completed at 2x 151 cycles with 8 bp index reads.

Metagenomic reads were quality assessed using FastQC (https://github.com/s-andrews/FastQC) and MultiQC (doi: 10.1093/bioinformatics/btw354). Fastp 0.23.2 (http://dx.doi.org/10.1093/bioinformatics/bty560) was used to trim and filter low quality reads using default settings. Reads were classified using Kraken2 (doi: 10.1186/s13059-019-1891-0). To detect antimicrobial resistance genes in these reads, Resfinder v4.0 with KMA (doi: 10.1099/mgen.0.000748, doi: 10.1186/s12859-018-2336-6) was used against the Resfinder database downloaded on the 4th of August 2022 (parameters: -l 0.8 -t 0.8 –acquired). RGI bwt v6.0.0 was also used to analyse some isolates using the Comprehensive Antibiotic Resistance Database CARD database v3.2.5 as a reference using standard parameters (http://dx.doi.org/10.1093/nar/gkac920). Outputs were filtered for hits with greater than 80% coverage of the gene length. No genes were detected using this method.

2.2.4 SmartChip qPCR analysis

The presence and abundance of antibiotic resistance genes (ARGs), integrons and mobile genetic elements (MGEs) and 16S rRNA gene in each shellfish sample were analysed using customised primer sets in the high throughput method SmartChip qPCR system (Resistomap, Helsinki, Finland). For the purpose of this study, we used a chip configuration with 248 genes, which consistent with chip arrays used in other work carried out for the Environment Agency as part of the PATHSAFE project (e.g., river surveillance pilots). Several primer sets were designed to target sequence diversity within the gene target to

more specifically assess the environmental resistome, therefore, each primer set was analysed independently. The threshold cycle (CT) of 27 was used as the detection limit (Zhu *et al*. 2013, Wang *et al*. 2014, Muziasari *et al*. 2016, Muziasari *et al*. 2017). Melting curve analysis and PCR efficiency were performed on all of the samples for each primer set. Amplicons with unspecific melting curves and multiple peaks based on the slope of melting profile were considered to be false positives and discarded from the analysis. Briefly, the SmartChip has 5184 reaction wells with a volume of 100 nl and filled using the SmartChip Multisample Nanodispenser. qPCR cycling conditions and initial data processing was done as previously described in (Wang *et al.* 2014). qPCR reagents recommended by the manufacturer were used. Mean CT of three technical replicates in each qPCR reaction was used to calculate the ΔCT values. Where the genes were detected in only one of the three technical replicates those results were removed. The 2−ΔCT method (where ΔCT = CT detected gene – CT 16S rRNA gene) was used to calculate the relative abundances of the detected gene in proportion to the 16S rRNA gene in each sample (Schmittgen & Livak 2008).

Statistical analysis of Resistomap and metagenomics results were conducted using R version 4.2.2 (R core team, 2013). Given the large number of values under the limit of detection, a permutational multivariate analysis of variance (PERMANOVA) was conducted, completed by a pairwise comparison between sites to assess differences in composition (diversity). An analysis of the dispersion (variance) was also conducted between sites using either a permutation-based test of multivariate homogeneity of site dispersions or an ANOVA, followed by a Tukey's pairwise test, to assess differences in dispersion between sites. Genes not found in any location are removed from the analysis (50 genes on 249). Control and 16S rRNA were removed from the analysis. Values below the limit of detection were replaced by an arbitrary value (9.536743^{e-07}).

2.2.5 *E. coli* isolation and AMR susceptibility analysis

Whole animal homogenates were prepared from the flesh and intravalvular fluid of five animals and assayed for *E. coli* using a standard, ISO 17025-accredited, most-probable-number (MPN) method (Walker *et al.* 2018). Briefly, the MPN standardized reference method for enumeration of *E. coli* in bivalves, with Minerals Modified Glutamate Broth (MMGB) (Oxoid, UK) as growth media combination with verification on Tryptone Bile with X-glucuronide (TBX) agar (Oxoid, UK) was used (Cefas, 2019). The presence of *E. coli* in all tubes showing acid production by subculture onto TBGA/TBX plates within 4 hours using a

10 µl sterile loop. A total of 38 bivalve samples (July 2022-January 2023) were collected and examined at the Cefas Weymouth Laboratory. Presumptive *E. coli* strains from culture-positive bivalve samples was grown to pure culture for further analysis. In some instances, no *E. coli* strains were obtained from specific samples (e.g., samples from 'clean' sample sites; data not shown). The bacterial isolates were susceptibility tested by broth microdilution on Mueller-Hinton broth (MHB; Oxoid,UK) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) using the Sensititre system from Trek Diagnostic Systems Ltd, UK. The minimum inhibitory concentration was determined for tested *E. coli* isolates against the 15 antimicrobials and ranges recommended by (European Food Safety Authority, 2012) for testing commensal *E. coli* (Sensititre EUCAST). These included amikacin, ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamycin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigeocycline and trimethoprim. Minimum Inhibitory Concentration (MIC) determination of the *E. coli* isolates was performed by broth microdilution European Committee on Antimicrobial Susceptibility Testing. EUCAST epidemiological cut-off values and Clinical breakpoints (where available) were used as interpretative criteria to define microbiological resistance (http://www.eucast.org/).

2.3. Chemical analysis

The chemical analysis of shellfish samples comprised a significant amount of developmental work as well as refinement of testing methods (in particular different extraction approaches) suitable for the detection and quantitation of key antimicrobial residues. An overview of this developmental work and the use of subsequent methods is outlined below.

2.3.1 Compound selection

A total of 19 compounds (table 2) were selected from an initial list of compounds to be included into an existing antibiotic mass spectrometric method in development at Cefas. These compounds included antibiotics, antifungals and covered a range of classes and compounds with wide ranging physico-chemical properties. Four of primary metabolites, such as amoxicilloic acid, N-desmethyl erythromycin, N-desmethyl azithromycin and 4 OH trimethoprim, were also included. The final targeted liquid-chromatography tandem mass spectrometry (LC-MS/MS) method included 35 compounds of interest (see table 3). Analytical standards were purchased from Sigma and LGC Standards.

Table 2. Selected antibiotics, antifungals, disinfectants and metabolites selected for inclusion in targeted method. Note: Log K_{ow} obtained from EPISuite database (via ChemSpider) and PubChem, where available.

Class	Compound	CAS Number	MW (monisotopic)	Formula	Log Kow
Penicillins & Derivatives	Amoxicillin	26787-78-0	365.1046	$C_{16}H_{19}N_3O_5S$	0.87
	Amoxicilloic acid	42947-63-7	383.1151	$C_{16}H_{21}N_3O_6S$	
	(Piv) mecillinam	32886-97-8	439.2141	$C_{21}H_{33}N_3O_5S$	3.23
β-lactamase inhibitors	Clavulanic acid	58001-44-8	199.0481	$C_8H_9NO_5$	-2.04
Tetracyclines	Oxytetracycline	79-57-2	460.1482	$C_{22}H_{24}N_2O_9$	-0.9
Aminoglycosides	Streptomycin	57-92-1	581.2657	$C_{21}H_{39}N_7O_{12}$	-7.53
Macrolides	Erythromycin	114-07-8	733.4612	$C_{37}H_{67}NO_{13}$	3.06
	N-desmethyl erythromycin	992-62-1	719.4456	$C_{36}H_{65}NO_{13}$	
	Azithromycin	83905-01-5	748.5085	$C_{38}H_{72}N_2O_{12}$	4.02
	N-desmethyl azithromycin	76801-85-9	734.4929	$C_{37}H_{70}N_2O_{12}$	
Cephalosporins	Ceftriaxone	73384-59-5	554.0461	$C_{18}H_{18}N_8O_7S_3$	
Carbapenems	Meropenem	119478-56-7	383.1515	$C_{17}H_{25}N_3O_5S$	-1.67
Quinolones	Trimethoprim	738-70-5	290.1379	$C_{14}H_{18}N_4O_3$	0.91
	4-hydroxy-trimethoprim	112678-48-5	276.1222	$C_{14}H_{18}N_4O_4$	
Antifungals	Clotrimazole	23593-75-1	344.1080	$C_{22}H_{17}ClN_2$	6.26
	Miconazole	22916-47-8	413.9860	$C_{18}H_{14}Cl_4N_2O$	6.25
	Tebuconazole	107534-96-3	307.1451	$C_{16}H_{22}ClN_3O$	3.7
	Imazalil	35554-44-0	296.0483	$C_{14}H_{14}Cl_2N_2O$	3.82
Disinfectants	Triclosan	3380-34-5	287.9512	$C_{12}H_7Cl_3O_2$	4.76

2.3.2 LC-MS/MS method development

Individual standards were prepared and diluted to a final concentration of 100 ng/ml prior to optimisation on the mass spectrometer. Standards were infused into the electrospray source of a Xevo TQ-S triple quadrupole mass spectrometer (Waters, UK) at a flow rate of 5 µl/min and combined into a flow rate of 50% aqueous acetonitrile containing 0.1% formic acid at a

flow rate of 0.4 ml/min. Parent ion and daughter ions were selected and individually optimised for instrument parameters including collision energy (eV) and cone voltage (V) as detailed in table 3. An example MS/MS spectra for trimethoprim is displayed in figure 2, showing the parent $[M+H]^+$ ion at *m/z* 291.2 with specific daughter ions at *m/z* 230.1 and 123.1. These parent/daughter ion transitions were used to develop a Multiple Reaction Monitoring (MRM) method specific for each compound. Where possible, a minimum of 2 MRM transitions were used, including quantitative and qualitative transitions (figure 3). Due to potential issues with charge switching and dwell times during polarity switching separate positive and negative polarity methods were developed.

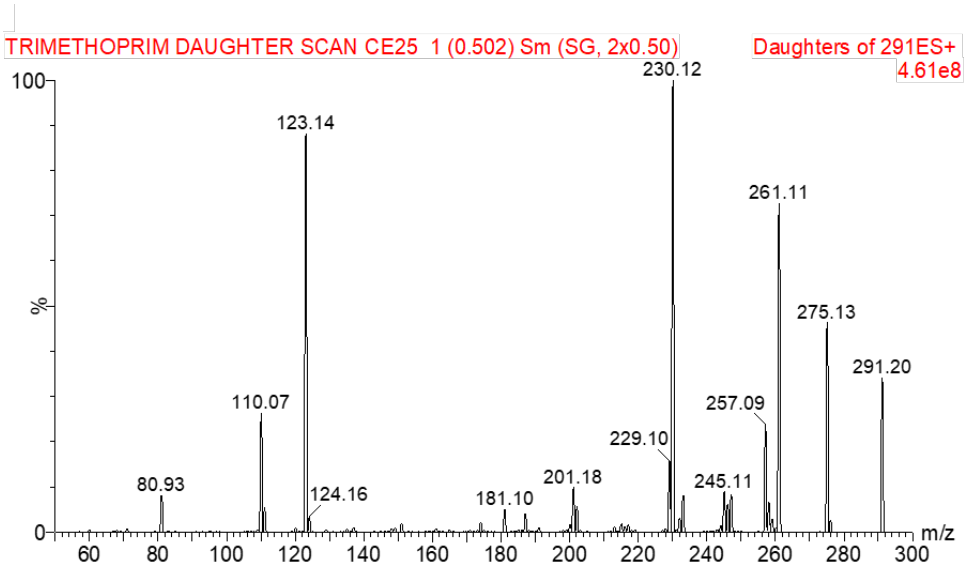


Figure 2. MS/MS spectra or trimethoprim standard.

Following development of the detection method, six different LC columns were tested to determine the most suitable column for a multi-compound, multi-class method. Columns tested were: 1. Waters HSS T3 Premier, 2. Waters BEH C18, 3. Waters Atlantis Premier BEH C18 AX, 4. Phenomenex Omega Polar, 5. Waters Phenyl and 6. Phenomenex F5. Whilst the majority of compounds exhibited acceptable chromatographic performance, a number of compounds were problematic. These included the highly hydrophilic compounds streptomycin and florfenicol amine which were not retained on any columns, along with the macrolides and quinolones which suffered peak tailing with certain columns. The optimum column which gave best performance was deemed the Waters BEH C18 (100 x 2.1 mm; 1.7 µm) which was used for all future work.

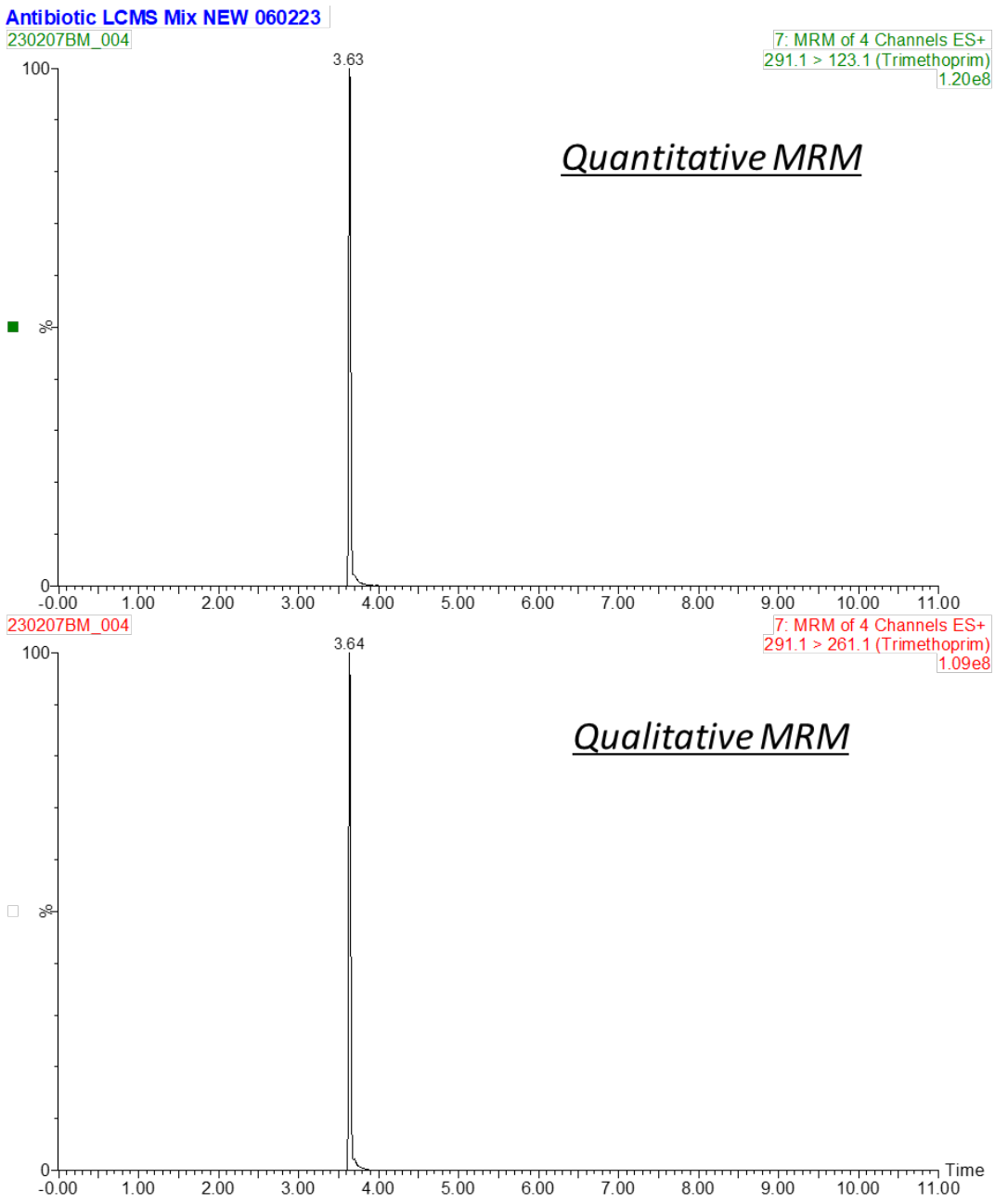


Figure 3. MRM chromatograms of trimethoprim standard showing quantitative and qualitative transitions of *m/z* 291.1>123.1 and 291.1>261.1 respectively.

Table 3. MRM transitions, MS/MS setting and retention times for all compounds (including primary metabolites). Bold denotes quantitative transition.

Compound	Polarity	Parent Ion	Daughter Ion	Collision Energy (eV)	Detention time (min)	Internal Standard
Nalidixic acid	Positive	233.2	**187.0**;215.1	28;28	6.01	Azithromycin d3
Sulfamethoxazole	Positive	254.1	**156.1**; 92.1;108.1	14;26;24	4.72	Azithromycin d3
Trimethoprim	Positive	291.1	**123.1**;261.1;230.1;275.1	26;22;20;22	3.63	Amoxicillin d4
Ciprofloxacin	Positive	332.2	**231.1**;288.1;245.1;203.1	24;16;22;36	3.76	Amoxicillin d4
Amoxicillin	Positive	**349.1**;366.1	**208.1**;114.0	14;8	2.60	Amoxicillin d4
Ampicillin	Positive	350.1	**106.1**;160.1;192.1;174.0	18;10;14;12	3.58	Amoxicillin d4
Cefoxitin	Positive	450.2	**345.0**;389.0;360.9;266.0	12;9;12;12	4.59	Clotrimazole d5
Oxytetracycline	Positive	461.2	**426.1**;201.1;98.1	20;38;44	3.78	Amoxicillin d4
Clarithromycin	Positive	748.5	**158.1**;83.1;590.2;558.2	28;38;16;22	6.61	Azithromycin d3
Azithromycin	Positive	749.5	**591.3**;158.1;83;116.1	24;34;38;32	4.71	Azithromycin d3
Florfenicol amine	Positive	248.1	**230.1**;130.1;151.1	10;22;22	1.24	Amoxicillin d4
Oxolinic acid	Positive	262.1	**160.0**;216.0;130.1;172.1	36;28;38;36	5.10	Amoxicillin d4
Flumequine	Positive	262.2	**201.9**;244.5	34;26	6.22	Azithromycin d3
Clotrimazole	Positive	277.2	**165.0**;242.0	24;22	6.77	Clotrimazole d5

Compound	Polarity	Parent Ion	Daughter Ion	Collision Energy (eV)	Detention time (min)	Internal Standard
Imazalil	Positive	297.1	**159.0**;69.1;201.1	24;16;14	6.23	Clotrimazole d5
4 OH Trimethoprim	Positive	307.1	**139.1**;97.1;181.1	24;34;18	3.54	Amoxicillin d4
Tebuconazole	Positive	308.2	**70.1**;151.1;125.1	18;26;38	8.49	Tebuconazole d6
Norfloxacin	Positive	320.2	**276.1**;233.1	18;24	3.68	Amoxicillin d4
Lomefloxacin	Positive	352.0	**265.0**;308.0	24;22	3.89	Amoxicillin d4
Danofloxacin	Positive	358.1	**340.0**;314.2	20;20	3.89	Amoxicillin d4
Enrofloxacin	Positive	360.3	**316.2**;245.1;203.1	20;26;36	4.00	Amoxicillin d4
Ofloxacin	Positive	362.0	**318.2**;261.1	18;28	3.70	Amoxicillin d4
Ampicilloic acid	Positive	368.2	**106.1**;307.1;175.1;151.1	28;14;18;14	3.22	Amoxicillin d4
Amoxycilloic acid	Positive	384.1	**189.1**;323	23;15	2.46	Amoxicillin d4
Meropenem	Positive	384.2	**141.0**;68.1;254.0	15;24;14	3.17	Amoxicillin d4
Sarafloxacin	Positive	385.9	**298.8**;367.8	28;23	4.29	Amoxicillin d4
Miconazole	Positive	**415.0**;417.0	**158.9**;161.0	24;24	7.94	Tebuconazole d6
Piv mecilinam	Positive	440.2	**167.1**;139.1	30;35	6.76	Tebuconazole d6
Streptomycin	Positive	582.3	**263.1**;246.0;221.0;176.0	36;44;46;40	0.56	Amoxicillin d4

Compound	Polarity	Parent Ion	Daughter Ion	Collision Energy (eV)	Detention time (min)	Internal Standard
Desmethyl erythromycin	Positive	720.5	**144.1**;82.8;562.3;102.0	32;52;20;50	5.74	Azithromycin d3
Erythromycin	Positive	734.5	**158.1**;83	28;34	5.74	Azithromycin d3
Desmethyl azithromycin	Positive	735.5	**82.8**;577.2;144.1	52;28;45	4.70	Azithromycin d3
Ceftriaxone	Positive	555.1	**241.0**;395.9;125.0	18;15;75	3.53	Amoxicillin d4
Triclosan	Negative	**287.0**;289.0	**287.0**;289.0;35;37	2;2;6;4	9.79	Tebuconazole d6
Florfenicol	Negative	356.1	**185.0**;336.1	18;10	4.80	Amoxicillin d4
Tebuconazole d6	Positive	314.2	**72.0**;125.0	20;34	8.47	na
Amoxicillin d4	Positive	**353.2**;370.2	**114.0**;212.0	16;8	2.60	na
Clotrimazole d5	Positive	282.2	**170.1**;247.1	24;22	6.76	na
Azithromycin d3	Positive	752.5	**594.3**; 158.1;116.1;82.9	24;36;36;38	4.72	na

Finally, a time segmented multi-channel MRM method was developed to allow optimum sensitivity and detection and MS/MS spectra and MRM chromatograms for all compounds were obtained. Due to poor sensitivity and performance, clavulanic acid was removed from the method. Samples were quantified by reference to a 9-point standard curve ranging from 0.01-100 ng/ml which contained all compounds and internal standards with r^2 values $\leq$0.98 deemed acceptable. Internal standards were used to normalise for extraction efficiency and matrix suppression. Details of relevant internal standards are documented in table 3.

2.3.3 Sample extraction

Due to the fact that the selected compounds covered a wide range of chemical properties, ranging from highly hydrophilic compounds, such as streptomycin, to the strongly hydrophobic compounds, such as triclosan and tebuconazole, three published multi-compound extraction methods which cover a range of compounds were used for all samples. Thirty-six homogenised oyster and mussel samples which had been stored at -20°C were provided. Prior to extraction, all samples were spiked with the deuterated internal standards tebuconazole d6, amoxicillin d4, clotrimazole d5 and azithromycin d3 at a concentration of 50 ng/g and left at room temperature for 10 min.

Extraction method A was based on (Maskrey *et al*., 2021). Briefly, following addition of the internal standards, 1 g tissue was extracted with 6 ml acidified acetonitrile (1% formic acid) at room temperature for 1 hr, with frequent vortexing. Samples were then centrifuged (4500 rpm, 10 min), with the supernatant removed and taken to dryness under vacuum and resuspended in 1 ml 75:25 (v/v) methanol:acetonitrile. 0.5 ml was then passed through an Oasis PRIME HLB 1cc (30 mg) SPE cartridge (Waters, UK), with the eluant collected in autosampler vials. Samples were stored at -20 °C until analysis with a 2 µl injection volume.

Extraction method B was adapted from (Guidi *et al*., 2017). Following addition of internal standards, 8 ml 5% TCA was added to 2 g homogenate and mixed for 10 min at room temperature, followed by centrifugation (12 min, 2400 rpm, 4°C). Samples were then filtered (0.45 µm) into autosampler vials and 10 µl injected onto the LC-MS/MS system.

Extraction method C was taken from Dasenaki and Thomaidis, 2015. A 1 g aliquot of homogenized sample was spiked with internal standards and allowed to stand for 10–15 min before proceeding. Two ml H2O containing 0.1% formic acid (v/v) and 0.1% EDTA (w/v) were added to the samples, followed by subsequent additions of 2 ml methanol and 2 ml acetonitrile. After the addition of each solvent the tube was vortex-mixed for 30 s. The sample set was placed in an ultrasonic bath at 40°C for 20 min to aid extraction. Samples were centrifuged at 4500 rpm for 10 min and the supernatant was decanted into a new polypropylene centrifuge tube and stored at −2°C for 12 h to precipitate lipids and remaining proteins. The samples were centrifuged, and the supernatant was defatted using 5 ml of hexane, vortexed for 1 min, and then centrifuged at 4000 rpm for 10 min. The hexane layer was taken to waste and the final extracts were evaporated to dryness under vacuum. The resulting residues were reconstituted in 1 ml of methanol/aqueous solution of formic acid, 0.05% (25:75 v/v) and then filtered through a 0.22-µm filter prior to LC-MS/MS analysis.

2.3.4 High-Resolution Mass Spectrometry (HRMS) Screen

HRMS screening was performed using a Orbitrap Exploris 120 mass spectrometer coupled to A Vanquish UPLC system, (ThermoFisher, Hemel Hempstead, UK). Compounds were chromatographically separated using a Waters HSS T3 column (100 x 2.1 mm; 1.7 µm) maintained at 40°C, with a gradient from 100% water + 0.1% formic acid to 95% acetonitrile +0.1% formic acid over 10 mins and an injection volume of 2 µl. Data was acquired in positive ionisation mode using a data dependent MS2 scan, whereby the 4 most intense ions from a full scan from 100-1000 amu at a resolution of 120,000 triggered MS2 scans to obtain maximum coverage. An exclusion list of background ions and an inclusion list of all compounds of interest (appendix A) was included. Data was acquired using Xcalibur and processed using Compound Discoverer v3.3.

3. Results and discussion

3.1 Sample availability

For this project, in total 38 shellfish samples were collected in parallel with the collection of regulatory samples and were received at the Cefas laboratory from July 2022 until January 2023. We noted some difficulties in receiving samples from site 4 ('reference' site) through this testing programme because of differing practical and logistical issues. A summary of shellfish samples is provided in appendix B. It should be noted that the homogenised samples provided for chemical analysis were very liquid and it is believed that these samples were not drained of extra fluid prior to homogenisation.

3.2 DNA extraction of shellfish and determination of bacterial DNA content

All stored samples yielded good quality DNA following at least one attempt at extraction using the Promega RSC tissue extraction robot. DNA yields varied from 18-2400 ng/µl (appendix B). PCR analysis of all extracted samples using the eubacterial PCR primer set 27f/1492r generated positive bands (data not shown) indicative of bacterial content in the extracted shellfish samples.

3.3 Metagenomics methods require further refinement to reduce host DNA

Metagenomic sequences for 37 samples were produced, with duplicates of four of these samples were included in this sequencing effort. While sequencing for four samples failed (less than 300,000 reads were sequenced) the remaining samples varied between 1.3 and 50.7 million reads. Classification of the reads produced revealed that approximately 1.26 % of the reads on average for each sample were of bacterial origin (appendix B). All reads in one sample (cefas id: 22-1693) were unclassified and thus have been omitted from subsequent data analysis as it indicated that the DNA extracted was predominantly host DNA. Of the bacteria reads that were sequenced, the most prevalent phyla detected were *Pseudomonadota*, *Bacillota* and *Bacteriodota* (figure 4). AMR genes were not recovered from these sequences. Since metagenomic sequencing is not as sensitive as approaches like PCR for detection of AMR genes, and to aid the metagenomic sequencing, PCR analysis for detection of AMR genes was used as this approach provides a more sensitive

by targeting and amplifying the DNA. Further refinement of methods is needed to reduce the presence host DNA and detect AMR genes in shellfish metagenomes, such as selective microbiome DNA enrichment kits (Feehery et al., 2013).

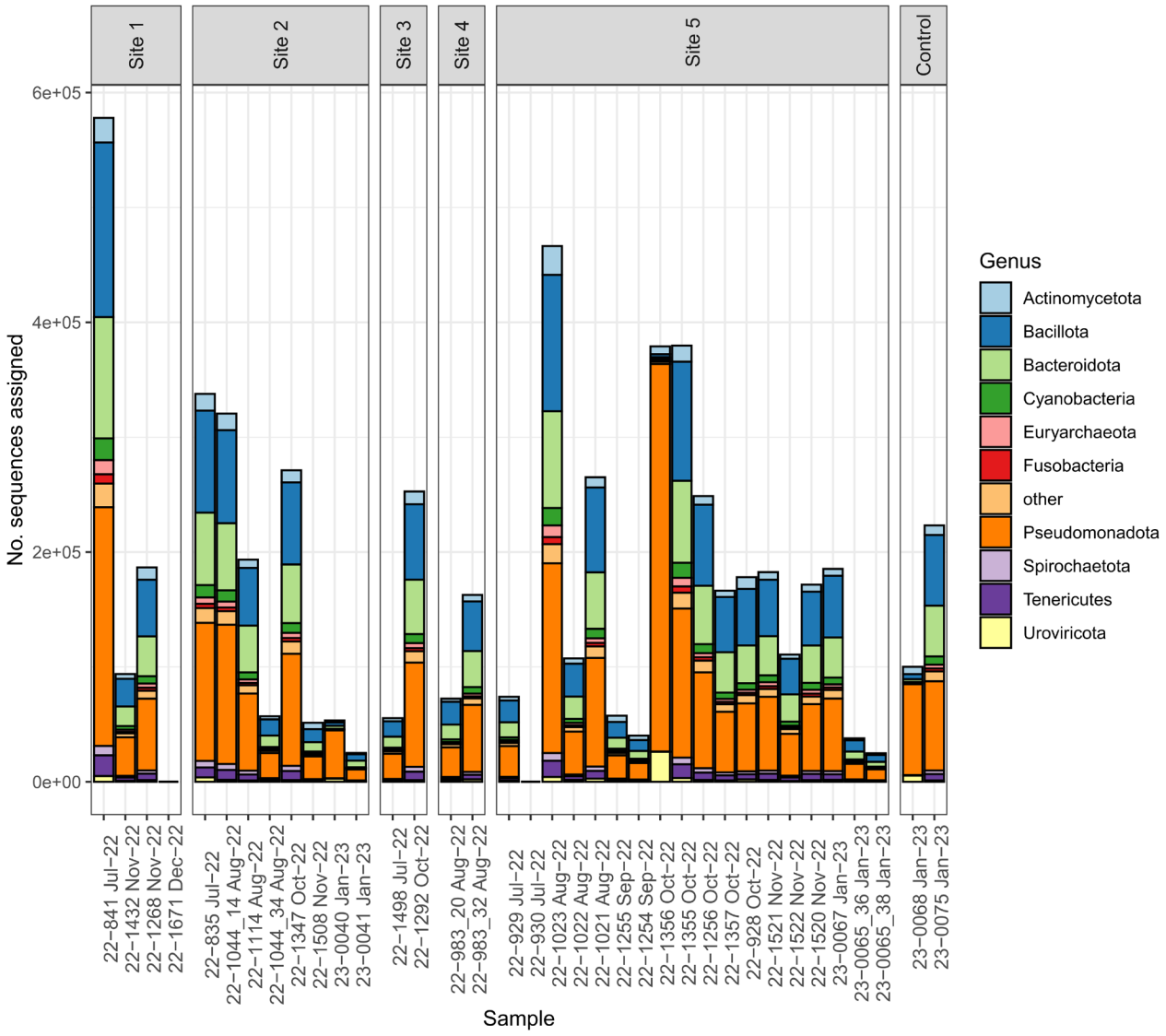


Figure 4. Number of sequences assigned to phyla detected in metagenomes. Unclassified reads and reads assigned to phylum *Chordata* are not shown. Top 15 phyla are shown while the remaining phyla are grouped into one category labelled "other". Samples are labelled as "cefas_id month-year". Duplicated samples are denoted as "cefas-id_sample_number month-year".

3.4 SmartChip qPCR

Samples were screened for a panel of 248 assays using SmartChip qPCR. It should be noted that due to potential quality control issues, the results for IntI1_1 were removed from the data analysis.

The number of assays detected in each sample was consistently high in the site 1 (potential ‘impacted’ site) across each time point (figure 5, appendix C). Samples from the site 2 and 5 (potential ‘impacted’ sites) varied in the number of AMR genes detected, where higher numbers of genes were identified from July until August, after which the number of genes detected fell. At site 5 the overall number of genes detected varied significantly within the summer months and then remained consistent at intermediate levels from September to November. Very few AMR genes were detected in one sample from site 4 (n=9).

While samples from the ‘reference’ site 3 carried very few AMR genes, a significant number (148) of AMR genes, including mobile genetic elements (MGE), were detected in one sample from January 23-001A. This sample ‘23-001’ was screened twice and very few genes were detected in the duplicated sample, with only one gene detection of the β-lactam group at the second screen (cefas id: 23-001B) (appendix C). The exact cause of the discrepancy between duplicate samples 23-001A and 23-001B is unclear.

Considering all sites, the models showed that there were statistically significant differences in composition (diversity) and dispersion (variance) between the sites. Specifically, site 1 significantly differed in composition from all the other sites (except site 5), and site 4 (‘reference’ site) displayed the most impact on dispersion. The latter could be due to the low number of samples, which may have biased the statistical analysis.

Finally, to investigate the overall effect of potential ‘impacted’ vs ‘reference’ sites, sites were pooled for their status. However, no difference was found, which suggests that the difference in diversity between sites seemed to be driven by the specific site 1 characteristics.

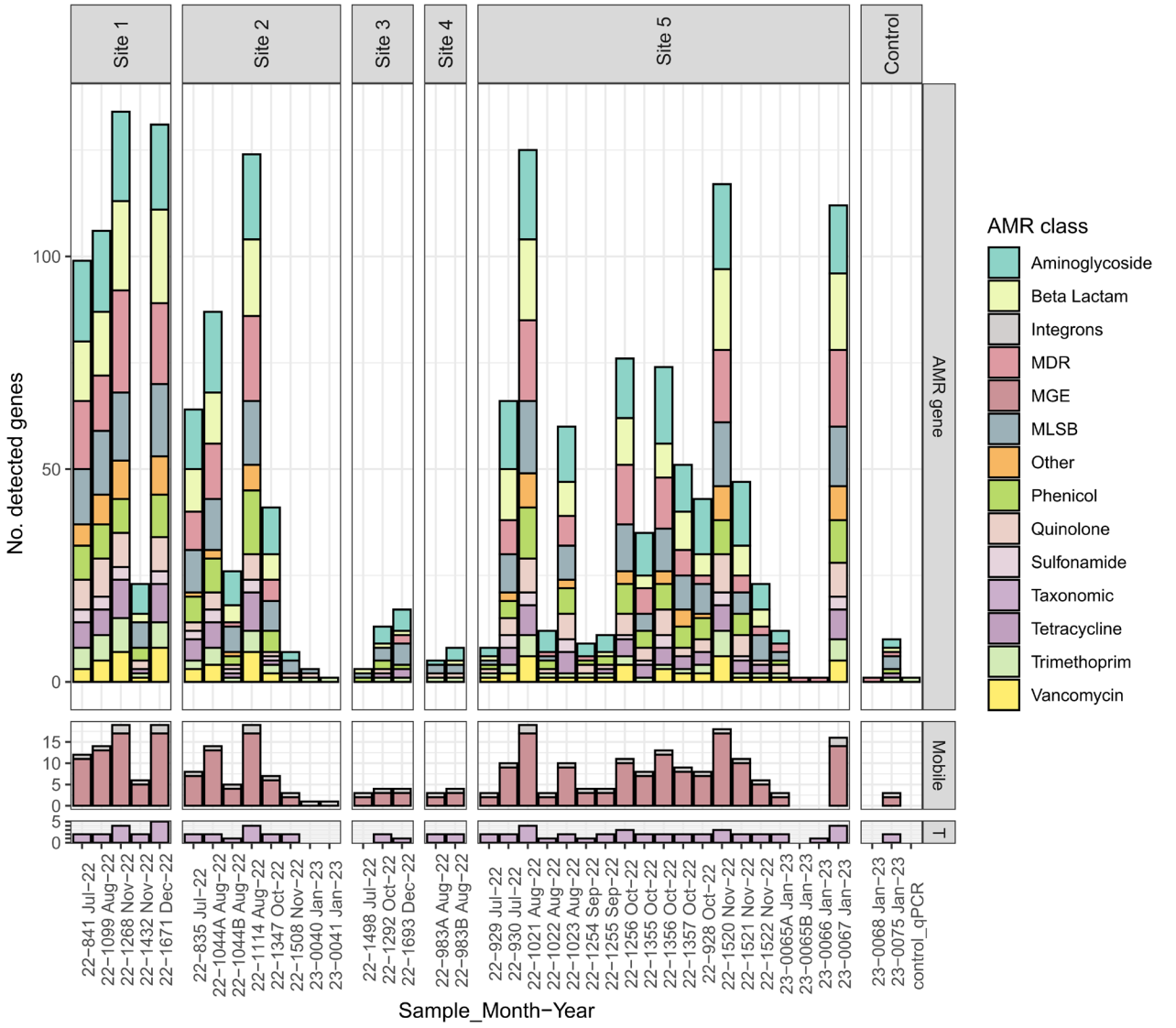


Figure 5. Numbers of genes detected in each sample from different sites between July 2022 and January 2023. Genes are grouped by AMR gene class, mobile genetic element (MGE), integrons and taxonomic are denoted on the y axis as "T". It is to note that one assay for intI1_1 was removed due to quality issues.

The summed relative abundances of each group of different AMR gene classes, mobile genes and taxonomic assays were also assessed (figure 6). This indicated that the abundances of genes found in site 1 (potential 'impacted' site) was relatively consistent across samples, although a higher abundance of genes of the 'beta lactams' group was reported in July 2022. In contrast to samples from site 1, many samples with high relative abundances across the remaining sites show significantly high abundances of individual groups of AMR genes. For example, MLSB (macrolide, lincosamide and streptogramin B) genes were highly prevalent in some samples from site 2 (potential 'impacted' site), site 3 ('reference' site) and site 5 (potential 'impacted' site). MLSB genes also show very high abundances in the singular sample taken from site 4 ('reference' site). Beta lactam genes

also showed very high relative abundances in a select number of samples from sites 2, 3 and 5. Trimethoprim resistance genes were found in high abundance in October in the site 2, 3 and 5.

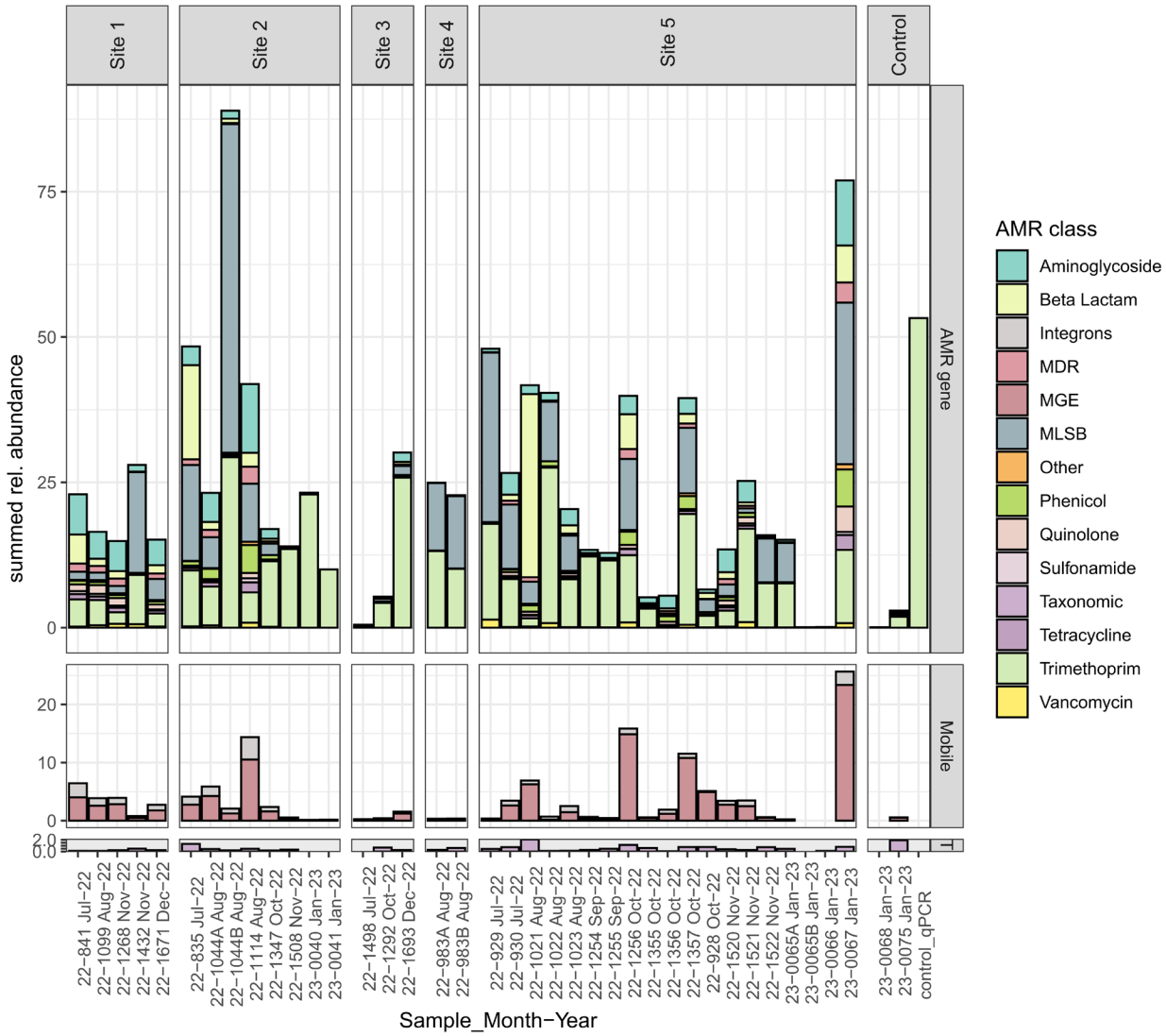


Figure 6. Summed relative abundance of genes detected. The relative abundance of genes detected in each sample summed is shown. Genes are grouped by AMR gene class mobile genetic element (MGE), and integrons and taxonomic genes are denoted on the Y axis as "T". It is to note that to quality issues with this assay.

While this data may allow for the detection of temporal and site-specific trends in AMR gene prevalence, it is recommended to undertake further work to determine the robustness of this data and its potential need for biological replicates. The AMR gene primer set used in this study was derived from clinical datasets and targets genes commonly found in the environment. Genes such as *OleC* which is commonly found in the soil bacteria *Streptomyces* spp. (Rodriguez et al., 1993). Others such as *dfrA17* are problematic in the clinic when associated with class-1 and 2 integrons (Sandalli et al., 2010) but are also

commonly found within bacterial chromosomes and plasmids, so presence alone may not always be relevant (Alcock et al., 2023). Both genes account for a large proportion of their respective resistance classes, and both are likely found in the upstream catchment and may or may not be result in phenotypically resistant bacteria or be transmissible to other species.

3.5 *E. coli* isolation and testing

Fifty-two *E. coli* strains were isolated from the tested shellfish samples and the vast majority 48/52 (92%) were susceptible (wild type; WT) to all antibiotics on the EUCAST panel (2022). Four isolates (table 4) demonstrated multi-drug resistance, with one strain from sample site 5 (cefas id: 22-2021, table 4) presenting resistance to 11 of the 15 tested antimicrobials (amikacin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, meropenem, nalidixic acid, sulfamethoxazole and tetracycline). This strain (22-12021) demonstrated sensitivity to just four drugs: ampicillin, gentamycin, tigecycline and trimethoprim (table 4). All of the multi-resistant strains isolated here originated from potential 'impacted' sites. In contrast, a related study (Grevskott *et al*., 2017), that tested *E. coli* isolates recovered from bivalves collected from Norwegian coastal waters by disc diffusion, demonstrated a much higher rate of AMR, with 75 of 199 (38%) isolates showed resistance to at least one antibacterial agent, while multidrug resistance was seen in nine (5%) of tested isolates. This strongly suggests that further temporospatial data on the presence and prevalence of AMR *E. coli* in UK reared bivalves is required to be able to properly assess potential risks.

Table 4. AMR capabilities of multi-drug resistant *E. coli* isolates.

Select antimicrobial capabilities of multi-drug resistant *E. coli* strains isolated from shellfish samples				
cefas id of sample	Site of isolation of tested strain	Month and year	Number of resistances	Resistance profile
22-1671A	Site 1	Dec 2022	4	Amp, Smx, Tet, Tmp
22-1671B	Site 1	Dec 2022	4	Amp, Smx, Tet, Tmp
22-1021	Site 5	August 2022	11	Amp, Fot, Cf, Taz,, Chl, Cip, Col, Mer, Nal, Smx and Tet

22-1355B	Site 5	October 2022	9	Ami, Fot, Taz, Cip, Col, Gen, Mer, Nal and Smx

[a]Abbreviations used: amikacin (AMI), ampicillin (AMP), azithromycin (AZI), cefotaxime (FOT), ceftazidime (TAZ), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), gentamycin (GEN), meropenem (MER), nalidixic acid (NAL), sulfamethoxazole (SMX), tetracycline (TET), tigeocycline (TGC) and trimethoprim (TMP).

3.6 Chemical analyses

3.6.1 LC-MS/MS optimisation

Of the compounds selected with the exception of clavulanic acid, which was excluded from further analysis, all compounds generated a strong parent ion. Both triclosan and florfenicol formed a $[M-H]^-$ ion in negative ionisation mode, while all other compounds formed strong $[M+H]^+$ ions under positive ionisation. In addition to the $[M+H]^+$ ion at *m/z* 349.1, amoxicillin also formed a $[M+NH_3]^+$ ion, consistent with the mass of an ammonium adduct at *m/z* 366.1. The chlorine containing compounds miconazole and triclosan formed parent ions 2 amu apart, consistent with a Cl_{35} and Cl_{37} isotopic pattern distribution. Upon Collision Activated Dissociation (CAD), all compounds generated a number of diagnostic ions that were selected for inclusion in the MRM method. Despite forming a strong $[M-H]^-$ ion, triclosan did not fragment well, and so was monitored as both the pseudo-molecular ion(s) and with daughter ions of *m/z* 35 and 37.

It should be noted that several of these compounds, particularly the penicillins, ampicillin and amoxicillin, were unstable and even when stored in the autosampler at 10°C it was found the compounds degraded in comparison to the stock solutions stored at -20°C. A number of LC columns were trialled, covering a range of differing stationary phases including C18, mixed-mode, phenyl and F5. Most compounds chromatographed well regardless of column type, although the fluoroquinolones and erythromycin and its metabolite were frequently found to demonstrate tailing. Even with the use of polar LC columns and starting the gradient at 100% water, no retention was observed for streptomycin which would result in inaccurate quantitation due to eluting in the void volume with salts. The majority of compounds eluted in the region from 40-60% organic, although triclosan, miconazole and tebuconazole were late eluting compounds. Due to time constraints, no additional solvents or modifiers were tested although may prove beneficial for future studies.

Whilst no formal analytical validation was performed as part of this method development, sensitivity was determined as the lowest standard level where the calculated concentration

was <25% of the actual concentration upon repeated injections. It should be noted that this test was performed using solvent based standards, analysed at the start and end of the analytical run, with shellfish samples in between and so does not provide the best representation of performance. As show in appendix D, sensitivity was good for most compounds, with LOQ values of 1 ng/ml or less and with good linear range. Sensitivity for some compounds such as clarithromycin and miconazole were so good that the linear range was reached at 10 ng/ml, with concentrations greater than this saturating the detector. Certain compounds, particularly fluoroquinolones such as norfloxacin and lomefloxacin demonstrated poorer sensitivity with LOQs of 10 ng/ml. Additionally, certain compounds such as azithromycin demonstrated non-linear performance at certain concentrations. The exact cause of this was not determined, but it is believed to be due to source conditions and matrix interference, and the use of matrix-matched standards for future studies is recommended.

3.6.2 Extraction development

Extraction of analytes from their matrix into a liquid solution is essential both to be able to inject the sample on the LC-MS/MS system as well as to reduce matrix effects and potentially concentrate samples. As part of this pilot study, three published multi-class methods using solvent extraction was tested. No catch-and-release solid phase extraction (SPE) methods were trialled due to time limitations. Furthermore, it was considered that due to the varying polarity of these compounds on LC columns, the development of a SPE method for all compounds of interest would not be feasible. No investigation into the potential of protein/lipid-bound conjugate forms of these compounds was explored and no formal method validation was performed on the tested samples.

Extraction efficiency as assessed by total recovery of the internal standards in all samples was calculated and detailed in table 5. As can be seen, recovery of internal standards was poor for all methods. Extraction method A performed the best for clotrimazole d5 and tebuconazole d6, although there was no extraction of amoxicillin d4, which is believed to be due to instability in the strong acidic conditions. Azithromycin d3 exhibited strong matrix enhancement effects and also strong variability as evidenced by high standard deviation (SD) when using solvent based standards as had been reported previously in (Maskrey et al., 2021). Extraction method B was poor for all compounds and was not assessed further. Whilst demonstrating poor recoveries, method C was the only method to demonstrate recovery of amoxicillin d4. Furthermore, azithromycin d3 demonstrated acceptable recovery at 69.2%, although it is noted that the variability is high, with a SD of 22.6%.

Table 5. Total recovery of internal standards (n=36, brackets denote Standard deviation (SD)).

	Total Recovery (%)			
Extraction Method	Clotrimazole d5	Tebuconazole d6	Amoxicillin d4	Azithromycin d3
A	43.9 (3.3)	36.4 (4.6)	0	327.6 (121.2)
B	2.3 (0.7)	11.0 (3.6)	0	0
C	13.0 (5.1)	13.6 (2.9)	8.5 (3.0)	69.2 (22.6)

The exact cause of the poor recoveries observed are unknown but believed to be attributed to a number of factors. These could be extraction efficiency from matrix, binding of analytes to plastic/metal, analyte instability/degradation and matrix suppression/enhancement. The high recovery of azithromycin d3 with extraction method A demonstrates significant matrix effects occurring in the source of the mass spectrometer. These appear to be removed with method C, which is possibly due to the hexane defatting step.

Further investigations are strongly recommended to determine the exact source of these poor recovery values. Initially it would be recommended to test matrix-matched standards to determine the extent of matrix effects. Catch-and-release SPE methods could also be trialled, but development of these would be complex and time-consuming. Samples would need an initial liquid extraction step as described above, before diluting to low organic before loading onto the cartridge. It would be recommended to use a low acid percentage to try and reduce degradation of the penicillin compounds.

3.6.3 Targeted Analysis of Environmental Samples

Despite the fact that poor recoveries were observed, the incorporation of fixed concentrations of internal standards prior to extraction allows normalisation of results in the samples, with the caveat of reduced sensitivities. Due to the poor performance of extraction method B, extracted samples using methods A and C were analysed.

Of the compounds of interest analysed for in the targeted method (35 compounds in total), the majority of these were not detected in any of the shellfish samples, independent of the applied extraction method. However, the antifungal clotrimazole and the antibiotics clarithromycin and trimethoprim were detected in samples extracted using both methods A

and C. Compounds were identified by the presence of chromatographic peaks present at matching retention times with authentic standards, and the absence of peaks in both solvent blank and a blank sample (appendix E, F and G, respectively). The presence of these compounds in both extraction methods give extra confidence in the samples. Tebuconazole and miconazole were also detected at very low levels with both extraction methods, but at levels <LOQ (data not shown).

Concentrations of the three compounds were calculated by reference to a solvent based standard curve, and all three of these compounds were normalised to the internal standard clotrimazole d5. The final concentrations for all the compounds, using both extraction methods, is presented in table 6.

Table 6. Concentrations of clarithromycin, clotrimazole and trimethoprim in bivalve samples, extracted using method A and C.

		Concentration (ng/g)					
		Clarithromycin		Clotrimazole		Trimethoprim	
Sample date	Sample ID	A	C	A	C	A	C
05.07.22	22-835 A	0.30	0.24	1.08	0.74	0.00	0.40
06.07.22	22-841 A	0.10	0.00	0.60	0.34	0.00	0.16
20.07.22	22-928 A	0.08	0.06	0.59	0.35	0.00	0.05
20.07.22	22-929 A	0.15	0.15	0.80	0.54	0.00	0.00
20.07.22	22-930 A	0.17	0.29	0.80	0.57	0.00	0.00
02.08.22	22-983 A	0.00	0.00	0.13	0.00	0.00	0.00
13.08.22	22-1021 A	0.10	0.06	0.73	0.39	0.02	0.00
13.08.22	22-1022 A	0.06	0.06	0.64	0.39	0.00	0.00
15.08.22	22-1023 A	0.11	0.13	0.94	0.40	0.01	0.06
16.08.22	22-1044 A	0.30	0.79	1.68	1.06	0.07	0.39
23.08.22	22-1099 A	0.06	0.00	0.72	0.34	0.01	0.00
05.09.22	22-1114 A	0.11	0.06	0.76	0.40	0.03	0.00
07.09.22	22-1125 A	0.26	0.61	0.61	0.25	0.00	0.00
07.09.22	22-1126 A	0.00	0.00	0.38	0.17	0.00	0.00
12.09.22	22-1156 A	0.00	0.00	0.10	0.00	0.03	0.12
28.09.22	22-1254 A	0.07	0.04	1.07	0.69	0.04	0.09
28.09.22	22-1255 A	0.08	0.00	1.23	0.79	0.03	0.11
28.09.22	22-1256 A	0.04	0.00	0.92	0.50	0.01	0.00
03.10.22	22-1268 A	0.00	0.00	0.26	0.14	0.01	0.08
06.10.22	22-1292 A	0.39	0.82	0.67	0.33	0.00	0.00
17.10.22	22-1347 A	0.12	0.12	1.03	0.47	0.08	0.28
19.10.22	22-1355 A	0.00	0.00	1.36	0.81	0.05	0.08
19.10.22	22-1356 A	0.00	0.00	1.23	0.77	0.06	0.10
19.10.22	22-1357 A	0.00	0.01	0.68	0.44	0.00	0.03
01.11.22	22-1432 A	0.04	0.00	0.68	0.50	0.40	0.66
14.11.22	22-1508 A	0.04	0.00	0.69	0.44	0.04	0.33
16.11.22	22-1520 A	0.05	0.00	1.27	1.09	0.16	0.34
16.11.22	22-1521 A	0.05	0.02	1.19	0.93	0.10	0.27
16.11.22	22-1522 A	0.06	0.00	0.89	0.66	0.00	0.00
06.12.22	22-1671 A	0.04	0.00	0.84	0.47	0.29	0.81
08.12.22	22-1693 A	0.22	0.73	0.50	0.30	0.06	0.27
06.01.23	23-0001 A	0.15	0.45	0.54	0.30	0.04	0.00
09.11.22	22-1498 A	0.13	0.62	0.45	0.26	0.01	0.00
17.01.23	23-0065 A	0.07	0.05	1.51	0.95	0.22	0.61
17.01.23	23-0066 A	0.08	0.03	1.87	1.36	0.23	0.74
17.01.23	23-0067 A	0.20	0.25	1.15	0.74	0.03	0.00

As can be seen, there is generally good correlation between concentrations calculated using the two different extraction methods which provides an extra degree of confidence in the results generated. This is confirmed by linear regression analysis of concentrations generated by the two extraction methods, resulting in r^2 values of 0.712 for clarithromycin, 0.919 for clotrimazole and 0.765 for trimethoprim (data not shown). It is believed that the higher level of correlation for clotrimazole between both extraction methods is due to the fact that clotrimazole d5 was used as the internal standard for normalisation.

Clotrimazole was the compound found at the highest concentrations, with a maximum concentration of 1.87 ng/g from site 5 (cefas id:23-0066) and the lowest concentration of 0.26 ng/g in a sample from site 1 (cefas id: 22-1268). Clarithromycin was present at the next lowest concentration, with a maximum concentration of 0.39 ng/g in one sample from site 3 (cefas id: 22-1292). Trimethoprim was detected at the lowest concentrations, with only a few detections with a maximum concentration of 0.23 ng/g.

Spatial variation of the detection of these compounds was observed, with highest concentrations of clarithromycin at sites 2, 3 and 5 (all potential 'impacted' sites; appendix H). A similar trend was observed for clotrimazole, with maximum concentrations recorded at site 2 and site 5 (both potential 'impacted' sites), although the differences were less pronounced (appendix I). Trimethoprim also revealed a similar trend, with maximum concentrations recorded at sites 2 and 5, although interestingly high levels were detected at site 1, in comparison to clotrimazole and clarithromycin (all impacted sites) (appendix J).

A potential seasonal trend was observed for trimethoprim, with maximum concentrations (0.81 ng/g) appearing to be present from November onwards, although more sampling points and consistency between sites would be required to confirm this observation.

3.6.4 HRMS non-targeted screening of environmental samples

Samples from extractions A and C were subjected to non-targeted HRMS screening. In total, nearly 10,000 hits were matched in the sample batches. Interrogation of the data using the compound target list for 76 compounds of interest (appendix A) identified the presence of trimethoprim in samples extracted with both methods A and C, with oxolinic acid and erythromycin also identified. The lower number of compounds identified is believed to be due to the reduced sensitivity of the non-targeted screening method in comparison to the targeted MRM method described above.

4. Conclusions and future work

To our knowledge this is the very first study of its kind utilising routine bivalve shellfish samples as part of a wider surveillance approach to study the environmental dimensions of AMR. Many key positives and firsts were delivered as part of this relatively short proof of concept project.

Firstly, we successfully set up a pilot study utilising routine testing samples for this purpose, encompassing potential impacted and control sites. We successfully isolated and tested a range of *E. coli* strains from diverse environmental samples (table 1), generating useful baseline data on the prevalence of AMR in these indicator organisms in England, UK.

Secondly, we developed a refined set of sample processing and extraction methods that can be used to isolate DNA for varied molecular purposes from a diverse range of live bivalve shellfish species (Pacific and native oysters and blue mussels etc). We were then able to detect a broad array of AMR genes associated with these samples using a SmartChip qPCR AMR array. Broadly speaking, a higher number of observed resistances appeared to be associated with sites of interest, with higher overall number of AMR genes found in potential 'impacted' sites compared to 'cleaner reference' sites, albeit not always with a statistically significant association. Site 1, for instance, which has the potential to be the most 'impacted' of all the analysed sites showed distinct differences and different composition in comparison to the other sites. While potential seasonal trends appear to occur in some sites, further sampling and validation of the SmartChip qPCR panel are needed to begin to explore the validity of these trends.

Finally, we have developed a sensitive and specific targeted mass spectrometric method for a range of antibiotics, antifungals and their metabolites and applied this to bivalve shellfish samples. Analysis of these samples identified the presence of the antibiotics, clarithromycin and trimethoprim, as well as the antifungal clotrimazole. These compounds appeared to show a trend to be at higher levels in samples from sites 1, 2, 3 and 5. Furthermore, trimethoprim appeared to show a seasonal trend, with highest levels detected from November onwards, although further investigations are needed to confirm this observation. Both clarithromycin and clotrimazole are relatively lipophilic and possess high log K_{ow} values and it would be expected to bioaccumulate in shellfish flesh. Whilst exhibiting lower sensitives, the use of HRMS screening is an additional extra tool which allows retrospective

screening of samples and the potential to look for correlation with other chemical contaminants as markers of sewage contamination such as antidepressants.

Some limitations to this work should be noted. Firstly, this was a short proof-of-concept project to assess the applicability and utility of routine shellfish testing as part of a wider AMR surveillance effort in the England, UK. Difficulties in obtaining regular shellfish samples from one of our ‘reference’ sites limited the comparability aspects of this project. However, if this work would be taken further and incorporated into the ongoing official routine testing, then the lack of samples in both space and time (as identified in this study) would probably represent a less pronounced issue.

In addition, we also observed limitations with regards to the metagenomic component of this project. Although the extracted shellfish samples generated strong DNA amplification using 16S eubacterial PCR, analysis of the metagenomic samples was disappointing. This is likely to be caused by a myriad of factors, including the predominance of host DNA coupled to the relative lack of sensitivity of metagenomic approaches e.g., compared to SmartChip. Methods to enrich the microbiome during library preparation to target the bacterial component of the sample are a potential avenue to overcome this constraint, however, it was beyond the scope of this project. Finally, while different extraction approaches are necessary to target a wide repertoire of chemicals, the outlined work represents a good first attempt to develop an analytical testing framework to cover a variety of different compounds, such as antibiotics and antifungals.

In summary, this study indicates that bivalve shellfish can be used to provide useful information on AMR in the coastal environment. Given that they are obtained and tested regularly as part of ongoing monitoring programmes, they represent a very useful additional matrix to investigate the emergence, spread and evolution of AMR from an environmental perspective.

5. Appendix

Appendix A. Inclusion list of all compounds of interest for High-Resolution Mass Spectrometry (HRMS) Screen

Compound	CAS	MW (monoisotopic)	Formula
Phenoxymethylpenicillin	87-08-1	350.093628	C16H18N2O5S
Benzylpenicillin	61-33-6	334.098724	C16H18N2O4S
Penicilloate	11039-68-2	352.109283	C16H20N2O5S
Amoxicillin	26787-78-0	365.104553	C16H19N3O5S
Amoxicilloic acid	42947-63-7	383.115112	C16H21N3O6S
Flucloxacillin	5250-39-5	453.056152	C19H17ClFN3O5S
Piperacillin	66258-76-2	535.173706	C23H29N5O8S
Pivmecillinam	32886-97-8	439.214081	C21H33N3O5S
Tazobactam	89786-04-9	300.052826	C10H12N4O5S
Clavulanic acid	58001-44-8	199.048065	C8H9NO5
Lymecycline	992-21-2	602.258789	C29H38N4O10
Oxytetracycline	79-57-2	460.148193	C22H24N2O9
Doxycycline	564-25-0	444.153259	C22H24N2O8
Minocycline	10118-90-8	457.184906	C23H27N3O7
Tetracycline	60-54-8	444.153259	C22H24N2O8
Streptomycin	57-92-1	581.265686	C21H39N7O12
Azithromycin	83905-01-5	748.508545	C38H72N2O12
N-desmethyl azithromycin	172617-84-4	734.492859	C37H70N2O12
Erythromycin	114-07-8	733.461243	C37H67NO13
N-desmethyl erythromycin A	992-62-1	719.445618	C36H65NO13
Clarithromycin	81103-11-9	747.476868	C38H69NO13
N-desmethyl clarithromycin	101666-68-6	733.461243	C37H67NO13
Ceftriaxone	73384-59-5	554.046082	C18H18N8O7S3
Meropenem	96036-03-2	383.151489	C17H25N3O5S
Ofloxacin	82419-36-1	361.143799	C18H20FN3O4
Ofloxacin N-oxide	104721-52-0	377.1386989	C18H20FN3O5
Desmethyl-ofloxacin	82419-52-1	347.1281342	C17H18FN3O4
Ciprofloxacin	85721-33-1	331.133209	C17H18FN3O3
Desethylene ciprofloxacin	528851-31-2	341.0942473	C15H17ClFN3O3
Trimethoprim	738-70-5	290.137878	C14H18N4O3
4-hydroxy-trimethoprim	112678-48-5	306.1328051	C14H18N4O4
Sulfamethoxazole	723-46-6	253.052109	C10H11N3O3S
N-acetyl sulfamethoxazole	21312-10-7	295.0626771	C12H13N3O4S
Metronidazole	443-48-1	171.064392	C6H9N3O3
Hydroxy-metronidazole	4812-40-2	187.0593058	C6H9N3O4
Amorolfine	78613-35-1	317.271851	C21H35NO
Clotrimazole	23593-75-1	344.108032	C22H17ClN2
Fluconazole	86386-73-4	306.104065	C13H12F2N6O
Flucytosine	2022-85-7	129.033844	C4H4FN3O

Compound	CAS	MW (monoisotopic)	Formula
Griseofulvin	126-07-8	352.071381	C17H17ClO6
Itraconazole	84625-61-6	704.239319	C35H38Cl2N8O4
Ketoconazole	65277-42-1	530.148743	C26H28Cl2N4O4
Deacetyl-ketoconazole	67914-61-8	488.138184	C24H26Cl2N4O3
Miconazole	22916-47-8	413.986023	C18H14Cl4N2O
Nystatin	1400-61-9	925.503479	C47H75NO17
Posaconazole	171228-49-2	700.329712	C37H42F2N8O4
Terbinafine	91161-71-6	291.1987	C21H25N
Voriconazole	137234-62-9	349.115051	C16H14F3N5O
Enilconazole	35554-44-0	296.048309	C14H14Cl2N2O
Myclobutanil	88671-89-0	288.114166	C15H17ClN4
Triticonazole	131983-72-7	317.129486	C17H20ClN3O
Climbazole	38083-17-9	292.097870	C15H17ClN2O2
Tebuconazole	107534-96-3	307.145142	C16H22ClN3O
Triclosan	3380-34-5	287.951172	C12H7Cl3O2
Chlorhexidine	55-56-1	504.203186	C22H30Cl2N10
Didecyldimethylammonium chloride	7173-51-5	361.347534	C22H48ClN
Benzyldimethyl ammonium chloride	8001-54-5	353.284943	C22H40ClN
Florfenicol	73231-34-2	357.000458	C12H14Cl2FNO4S
Ampicillin	69-53-4	349.109619	C16H19N3O4S
Ampicilloic acid	32746-94-4	367.120192	C16H21N3O5S
Gentamicin	1403-66-3	477.316254	C21H43N5O7
Imipenem	64221-86-9	299.093964	C12H17N3O4S
Cefoxitin	35607-66-0	427.050781	C16H17N3O7S2
Colistin	1066-17-7	1154.749878	C52H98N16O13
Florfenicol amine	76639-93-5	247.067841	C10H14FNO3S
Cefepime	88040-23-7	480.124969	C19H24N6O5S2
Cefotaxime	63527-52-6	455.056946	C16H17N5O7S2
Ceftazidime	72558-82-8	546.0991394	C22H22N6O7S2
Ertapenem	153832-46-3	475.141327	C22H25N3O7S
Daptomycin	103060-53-3	1619.710366	C72H101N17O26
Linezolid	165800-03-3	337.143799	C16H20FN3O4
Vancomycin	1404-90-6	1483.40686	C66H76Cl3N9O24
Clindamycin	18323-44-9	424.179871	C18H33ClN2O5S
Norfloxacin	70458-96-7	319.133209	C16H18FN3O3
Oxolinic Acid	14698-29-4	261.063721	C13H11NO5
Enrofloxacin	93106-60-6	359.16452	C19H22FN3O3

Appendix B. Samples obtained from project and associated data from molecular analysis.

Sample and date		Cefas number	Number of raw reads	%Classified reads	% Unclassified reads	% Microbial reads	% Bacterial reads
Control	Jan-23	23-0068	1,452,101	8.55%	91.50%	7%	6.55%
Control	Jan-23	23-0075	17,864,107	2.53%	97.50%	1.39%	1.28%
Site 1	Dec-22	22-1671	4	25%	75%	25%	25.00%
Site 1	Nov-22	22-1268	16,460,773	2.73%	97.30%	1.26%	1.15%
Site 1	Jul-22	22-841	48,869,769	3%	97%	1.35%	1.22%
Site 1	Nov-22	22-1432	8,317,427	2.48%	97.50%	1.29%	1.15%
Site 2	Nov-22	22-1508	5,631,549	1.97%	98%	1.06%	0.94%
Site 2	Oct-22	22-928	15,574,433	2.42%	97.60%	1.26%	1.15%
Site 2	Oct-22	22-1347	25,657,299	2.66%	97.30%	1.15%	1.06%
Site 2	Aug-22	22-1044*	30,127,985	2.58%	97.40%	1.21%	1.09%
Site 2	Jul-22	22-835	34,359,636	2.39%	97.60%	1.08%	0.99%
Site 2	Aug-22	22-1114	20,787,754	2.46%	97.50%	1.04%	0.94%
Site 2	Jan-23	23-0040	1,767,660	3.73%	96.30%	3.12%	2.88%
Site 2	Aug-22	22-1044*	6,058,145	2.16%	97.80%	1.09%	0.96%
Site 2	Jan-23	23-0041	3,530,819	1.75%	98.20%	0.89%	0.74%
Site 3	Jan-23	23-0001*	1,238,646	4.22%	95.80%	3.35%	3.04%
Site 3	Jul-22	22-1498	6,003,745	1.99%	98%	1.02%	0.93%
Site 3	Oct-22	22-1292	21,997,720	2.69%	97.30%	1.25%	1.15%
Site 3	Jan-23	23-0001*	267,409	37.80%	62.20%	24.10%	23.20%
Site 4	Aug-22	22-983*	6,791,047	2.07%	97.90%	1.17%	1.06%
Site 4	Aug-22	22-983*	12,159,017	2.59%	97.40%	1.44%	1.33%
Site 5	Sep-22	22-1255	5,590,072	2.17%	97.80%	1.17%	1.05%
Site 5	Sep-22	22-1254	3,974,730	2.15%	97.80%	1.17%	1.04%
Site 5	Oct-22	22-1356	1,776,865	21.70%	78.30%	21.40%	19.90%
Site 5	Oct-22	22-1355	29,560,199	2.88%	97.10%	1.40%	1.28%
Site 5	Aug-22	22-1023	36,661,161	3.02%	97%	1.45%	1.30%
Site 5	Aug-22	22-1022	9,078,809	2.54%	97.50%	1.32%	1.18%
Site 5	Jul-22	22-929	6,564,523	2.45%	97.60%	1.26%	1.13%
Site 5	Jul-22	22-930	3	66.70%	33.30%	66.70%	66.70%
Site 5	Aug-22	22-1021	23,175,924	2.41%	97.60%	1.25%	1.15%
Site 5	Nov-22	22-1521	14,714,832	2.83%	97.20%	1.38%	1.25%
Site 5	Nov-22	22-1522	8,913,092	2.37%	97.60%	1.34%	1.24%
Site 5	Oct-22	22-1256	19,862,636	2.59%	97.40%	1.41%	1.28%
Site 5	Oct-22	22-1357	13,550,961	2.48%	97.50%	1.34%	1.23%
Site 5	Nov-22	22-1520	14,195,996	2.82%	97.20%	1.34%	1.22%
Site 5	Jan-23	23-0067	15,653,184	2.54%	97.50%	1.33%	1.21%
Site 5	Jan-23	23-0065*	4,125,469	2.01%	98%	1.06%	0.94%
Site 5	Jan-23	23-0065*	3,288,772	1.80%	98.20%	0.88%	0.77%

Appendix C. Number of genes detected in each sample grouped by classes, with MDR = multi-drug resistance, MGE = mobile genetic element and MLSB = macrolides, lincosamide and streptogramin B.

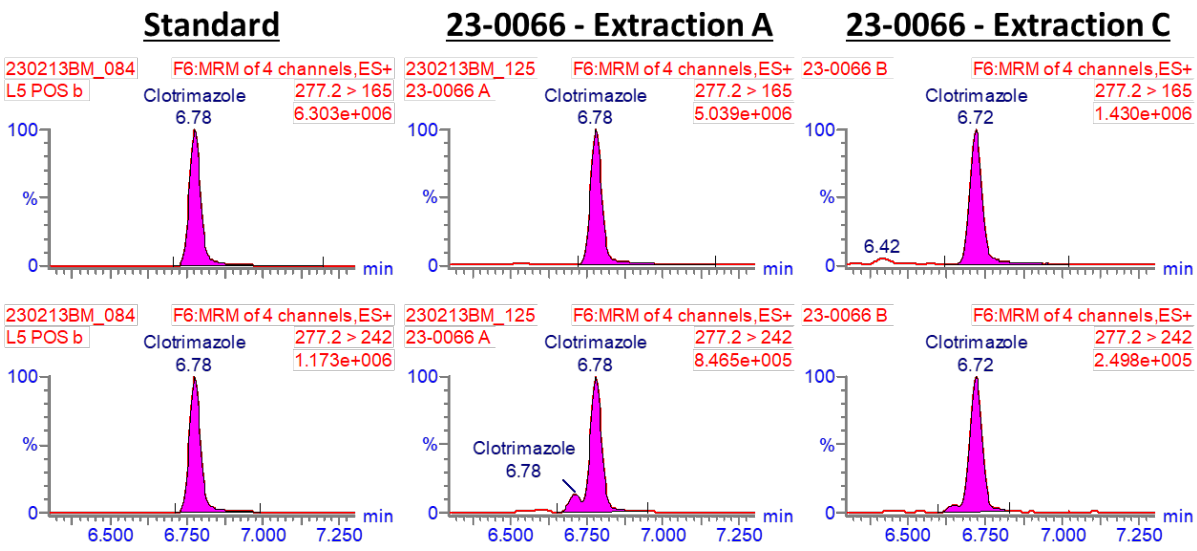
Cefas id of sample tested	Total number of detected	Beta lactam	Other	Vancomycin	MDR	Sulfonamide	Tetracycline	Trimethoprim	Integrons	Phenicol	Aminoglycosi	MGE	MLSB	Quinolone	Taxonomic
22-1508	**11**	0	0	0	0	0	0	0	1	0	2	2	3	1	2
22-1255	**16**	1	0	1	0	0	1	0	1	2	4	3	0	1	2
22-1254	**13**	0	0	1	1	0	0	0	1	1	3	3	0	2	1
23-001A	**148**	21	6	8	18	3	7	5	3	12	22	17	15	7	4
23-001B	**1**	1	0	0	0	0	0	0	0	0	0	0	0	0	0
22-1671	**154**	22	9	8	19	3	9	5	2	10	20	17	17	8	5
22-1268	**156**	21	9	7	24	3	9	7	2	8	21	17	16	8	4
22-1498	**6**	1	0	0	0	0	0	0	1	1	0	2	1	0	0
22-928	**53**	5	1	2	2	0	3	2	1	5	13	7	7	3	2
22-1292	**19**	1	0	0	0	0	1	1	1	2	4	3	3	1	2
22-1347	**50**	6	0	2	5	1	1	2	1	5	11	6	7	1	2
22-1356	**89**	8	3	3	12	3	4	1	1	6	18	12	10	6	2
22-1355	**45**	3	0	0	6	1	3	1	1	4	10	7	4	3	2
22-1099	**121**	15	7	5	13	3	6	5	1	8	19	13	15	9	2
22-1044	**103**	12	2	4	13	3	6	3	2	8	19	13	12	4	2
22-1023	**71**	8	2	1	7	3	5	0	1	6	13	9	8	6	2
22-841	**112**	14	5	3	16	3	6	4	1	8	19	11	13	78	2

22-1022	**15**	0	0	1	1	0	0	0	1	2	5	2	1	1	1
Cefas id of sample tested	**Total number of detected**	**Beta lactam**	**Other**	**Vancomycin**	**MDR**	**Sulfonamide**	**Tetracycline**	**Trimethoprim**	**Integrons**	**Phenicol**	**Aminoglycosi.**	**MGE**	**MLSB**	**Quinolone**	**Taxonomic**
22-835	**73**	10	1	3	9	2	5	1	1	6	14	7	10	2	2
22-929	**12**	1	0	1	0	0	0	0	1	1	2	2	1	1	2
22-983	**9**	0	0	0	0	0	0	0	1	0	1	2	2	1	2
22-930	**77**	12	2	2	8	3	4	1	1	4	16	9	9	4	2
22-1021	**148**	19	8	6	19	3	7	4	3	12	21	17	17	8	4
22-1521	**59**	7	0	1	4	1	3	0	1	5	15	10	5	5	2
22-1522	**30**	4	0	1	2	0	2	0	1	0	6	5	6	1	2
22-1256	**89**	11	3	4	14	1	4	1	1	7	14	10	11	5	3
22-114	**147**	18	6	7	20	3	9	4	3	15	20	17	15	6	4
23-0040	**3**	0	0	0	0	0	0	0	1	0	0	0	1	1	0
22-1693	**21**	1	0	0	2	0	2	0	1	1	5	3	5	0	1
22-1357	**61**	9	4	2	6	0	3	0	1	5	11	8	8	2	2

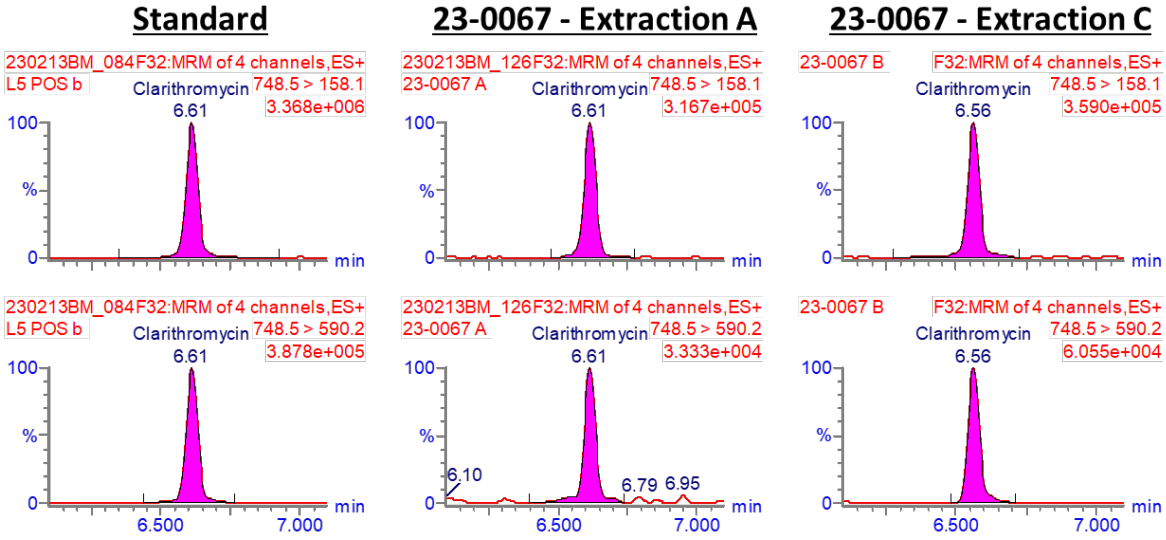
Appendix D. Limit of Quantification (LOQ) and linear range of compounds of tested.

Compound	LOQ (ng/ml)	Linear range (ng/ml)
Nalidixic acid	0.5	0.5 - 50
Sulfamethoxazole	0.1	0.1 - 50
Trimethoprim	0.5	0.5 - 50
Ciprofloxacin	5	5 - 50
Amoxicillin	0.1	0.1 - >100
Ampicillin	0.1	0.1 - 50
Cefoxitin	5	5 - >100
Oxytetracycline	0.5	0.5 - 50
Clarithromycin	0.05	0.05 - 10
Azithromycin	5	5 - 100
Florfenicol amine	1	1 - >100
Oxolinic acid	0.5	0.5 - >100
Flumequine	0.1	0.1 - 50
Clotrimazole	0.05	0.05 - 10
Imazalil	0.5	0.5 - >100
4 OH Trimethoprim	0.1	0.1 - 10
Tebuconazole	0.05	0.05 - 10
Norfloxacin	10	10 - >100
Lomefloxacin	10	10 - >100
Danofloxacin	10	10 - >100
Enrofloxacin	5	5 - >100
Ofloxacin	10	10 - >100

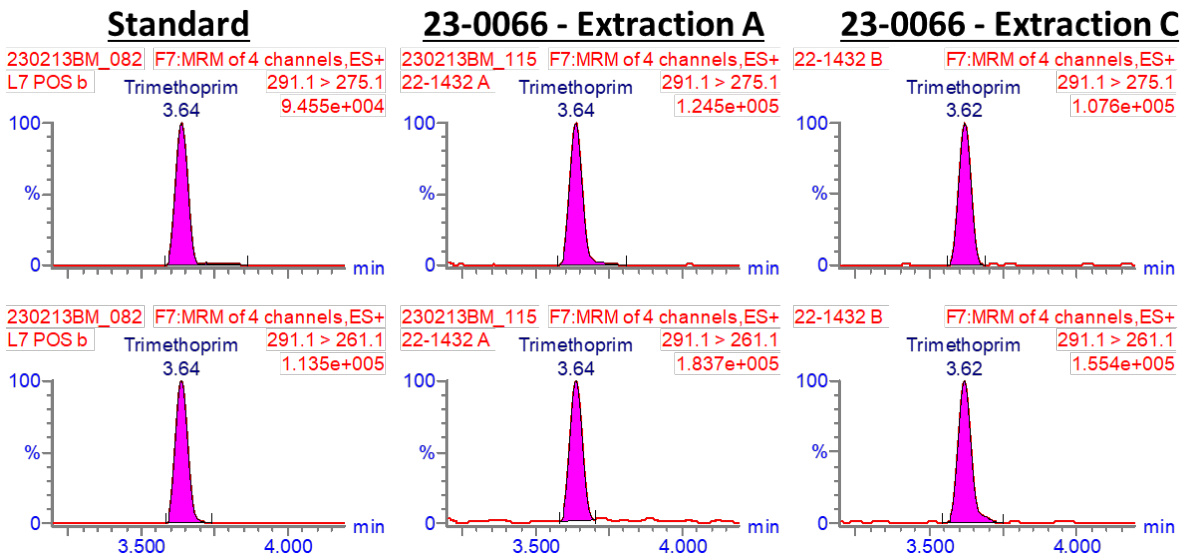
Compound	LOQ (ng/ml)	Linear range (ng/ml)
Ampicilloic acid	0.5	0.5 - >100
Amoxycilloic acid	0.5	0.5 - >100
Meropenem	0.1	0.1 - >100
Sarafloxacin	10	10 - >100
Miconazole	0.05	0.05 - 10
Piv mecilinam	0.5	0.5 - 10
Streptomycin	50	50 - >100
Desmethyl erythromycin	1	1 - >100
Erythromycin	0.1	0.1- >100
Desmethyl azithromycin	5	5 - >100
Ceftriaxone	0.5	0.5 - >100
Triclosan	5.0	5 - >100
Florfenicol	0.1	0.1- >100



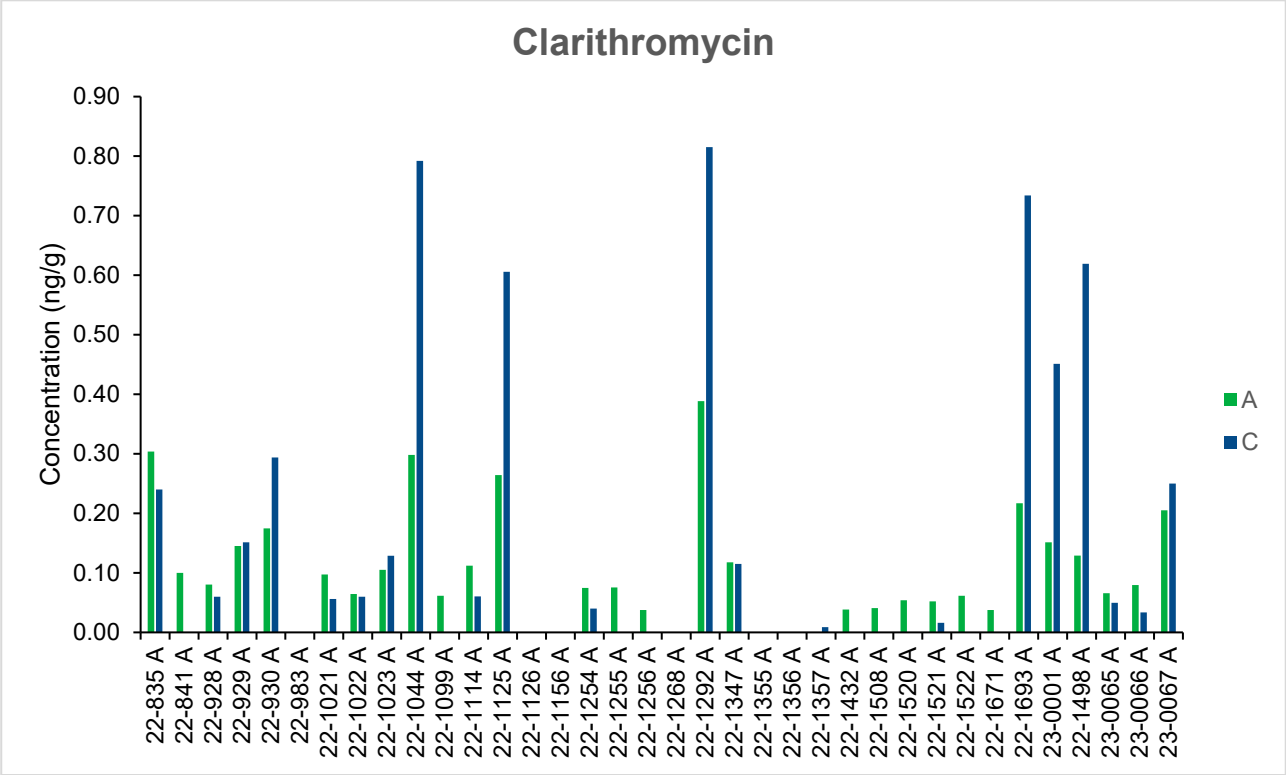
Appendix E. LC-MS/MS detection of clotrimazole using a quantitative MRM transition of *m/z* 277.2>165.0 (top traces) and a qualitative transition of *m/z* 277.2>242.0 (bottom traces) in an authentic standard (left traces), and sample 23-0066 extracted with method A (middle traces) and method C (right traces). Note: extraction method C was acquired in a different analytical run hence the slight shift in retention time.



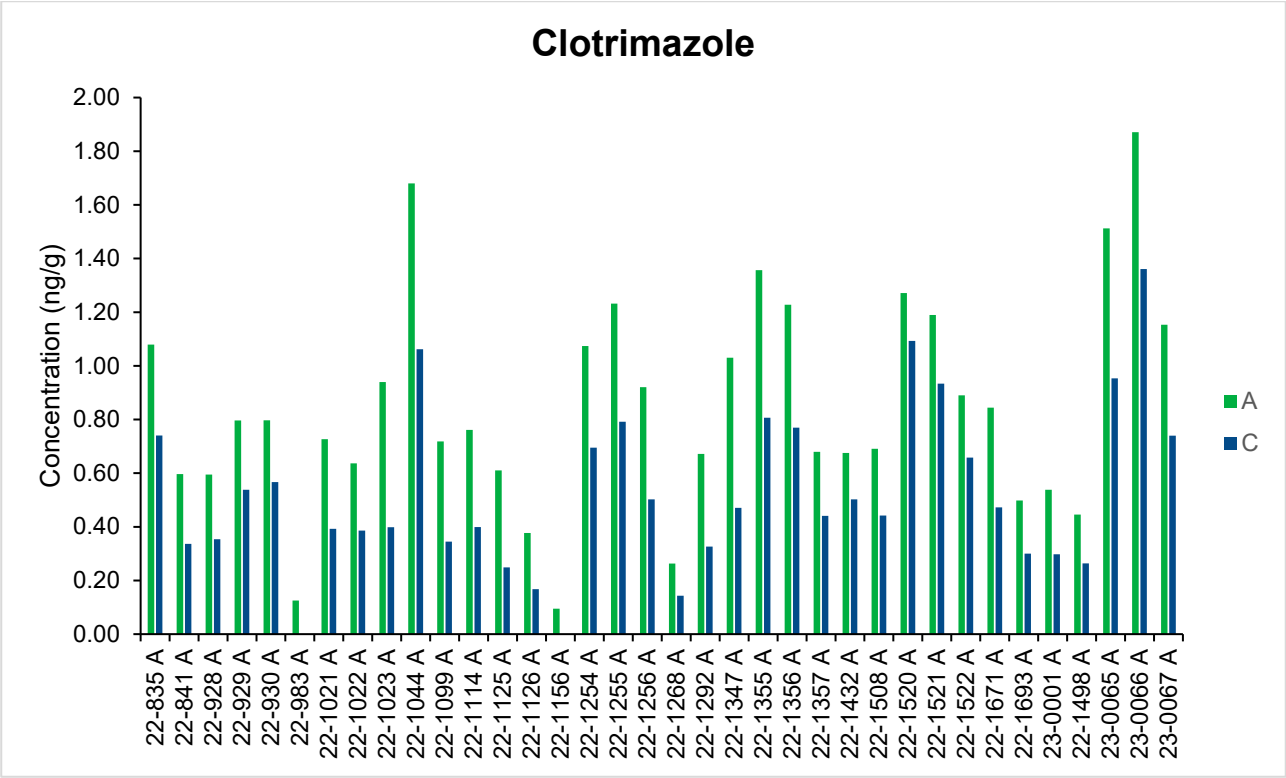
Appendix F. LC-MS/MS detection of clarithromycin using a quantitative MRM transition of *m/z* 748.5>158.1 (top traces) and a qualitative transition of *m/z* 748.5>590.2 (bottom traces) in an authentic standard (left traces), and sample 23-0067 extracted with method A (middle traces) and method C (right traces). Note: extraction method C was acquired in a different analytical run hence the slight shift in retention time.



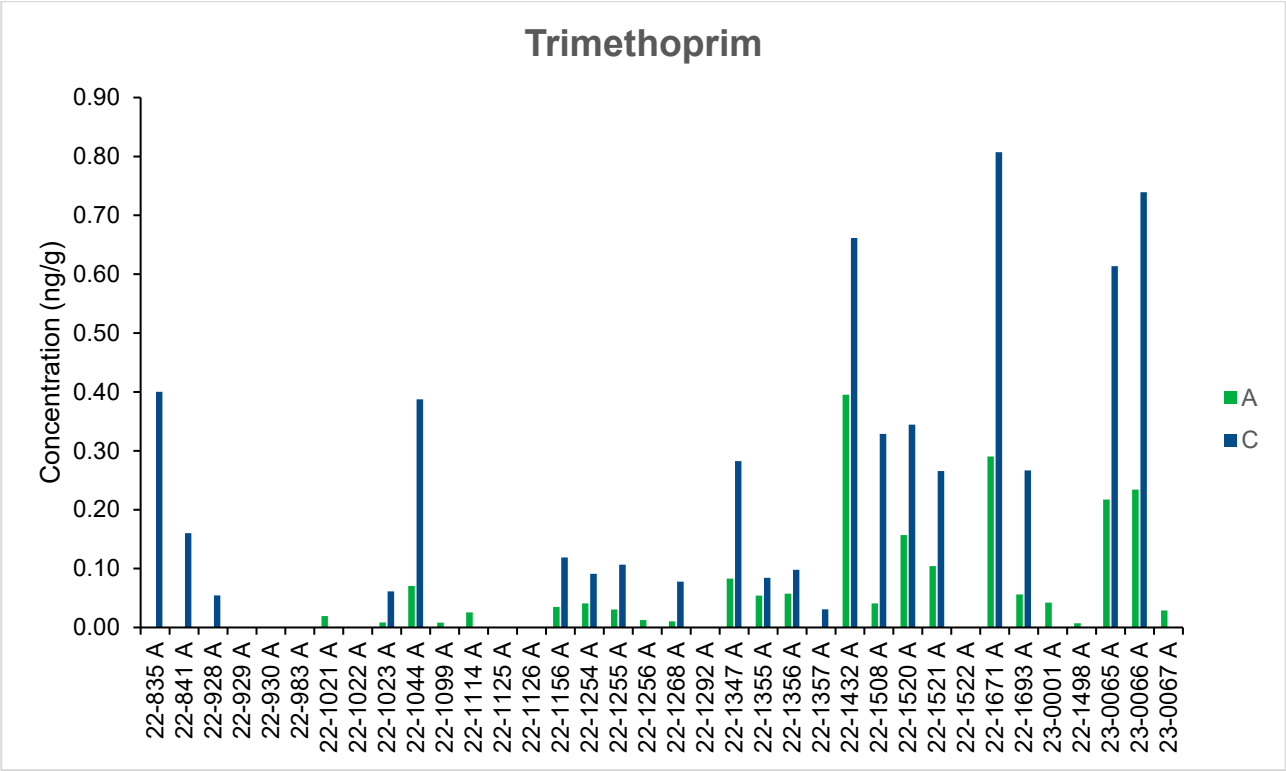
Appendix G. LC-MS/MS detection of trimethoprim using a quantitative MRM transition of *m/z* 291.1>275.1 (top traces) and a qualitative transition of *m/z* 291.1>261.1 (bottom traces) in an authentic standard (left traces), and sample 22-1432 extracted with method A (middle traces) and method C (right traces). Note: extraction method C was acquired in a different analytical run hence the slight shift in retention time.



Appendix H. Concentrations of clarithromycin in bivalve samples. Green bars annotated with 'A' show results for extraction method A and blue, annotated by 'C' for extraction method C.



Appendix I. Concentrations of clotrimazole in bivalve samples. Green bars annotated with 'A' show results for extraction method A and blue, annotated by 'C' for extraction method C.



Appendix J. Concentrations of trimethoprim in bivalve samples. Green bars annotated with 'A' show results for extraction method A and blue, annotated by 'C' for extraction method C.

6. References

Alcock B.P, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, Edalatmand A, Petkau A, Syed SA, Tsang KK, Baker SJC, Dave M, McCarthy MC, Mukiri KM, Nasir JA, Golbon B, Imtiaz H, Jiang X, Kaur K, Kwong M, Liang ZC, Niu KC, Shan P, Yang JYJ, Gray KL, Hoad GR, Jia B, Bhando T, Carfrae LA, Farha MA, French S, Gordzevich R, Rachwalski K, Tu MM, Bordeleau E, Dooley D, Griffiths E, Zubyk HL, Brown ED, Maguire F, Beiko RG, Hsiao WWL, Brinkman FSL, Van Domselaar G, McArthur AG. 2023. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. Nucleic Acids Res. 51 (D1): D690-D699

Cefas, 2014. A critical review of the current evidence for the potential use of indicator species to classify UK shellfish production areas. FS51206 https://www.food.gov.uk/sites/default/files/media/document/865-1-1607_FS512006_VMcFarlane.pdf

Cefas 2019. Most probable number (MPN) *E. coli* assay. https://www.cefas.co.uk/bacteriological-contamination-of-bivalves/methods/

Dasenaki, M.E., Thomaidis, N.S., 2015. Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta. https://doi.org/10.1016/j.aca.2015.04.013

European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0, 2022. http://www.eucast.org

EFSA. 2019. Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food | EFSA (europa.eu)

Feehery, G.R., Yigit, E., Oyola, S.O., Langhorst, B.W., Schmidt, V.T., Stewart, F.J., Dimalanta, E.T., Amaral-Zettler, L.A., Davis, T., Quail, M.A., Pradhan, S. 2013. A method for selectively enriching microbial DNA from contaminating vertebrate host DNA. PLoS One 28, 8(10): e76096

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., and Olsen, G. J. 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl. Environ. Microbiol. 74, 2461–2470. doi: 10.1128/AEM.02272-07

Grevskott, D. H., Svanevik, C. S., Sunde, M., Wester, A. L., & Lunestad, B. T. (2017). Marine bivalve mollusks as possible indicators of multidrug-resistant escherichia coli and other species of the enterobacteriaceae family. Frontiers in Microbiology, 8(JAN), 1–10. https://doi.org/10.3389/fmicb.2017.00024

Gosling, E.M., 2003. Bivalve Molluscs: Biology, Ecology and Culture. Blackwell Publishing Ltd., Oxford, UK.

Guidi, L.R., Santos, F.A., Ribeiro, A.C.S.R., Fernandes, C., Silva, L.H.M., Gloria, M.B.A., 2017. A simple, fast and sensitive screening LC-ESI-MS/MS method for antibiotics in fish. Talanta. https://doi.org/10.1016/j.talanta.2016.10.089

Hinder SL, Hays GC, Brooks CJ, Davies AP, Edwards M, Walne AW, Gravenor MB. 2011. Toxic marine microalgae and shellfish poisoning in the British isles: history, review of epidemiology, and future implications. Environ Health. 10:54.

Kimbrough, K.L., Johnson, W.E., Lauenstein, G.G., Christensen, J.D., Apeti, D.A., 2008. An assessment of Two Decades of Contaminant Monitoring in the Nation's Coastal Zone, in: Silver Spring, M.D. (Ed.), NOAA Technical Memoandum, pp. 1-105.

Maskrey, B.H., Dean, K., Morrell, N., Turner, A.D., 2021. A Simple and Rapid Ultra–High-Performance Liquid Chromatography–Tandem Mass Spectrometry Method for the Quantitation of Pharmaceuticals and Related Compounds in Mussels and Oysters. Environ. Toxicol. Chem. https://doi.org/10.1002/etc.5046

Maxwell RSC tissue DNA extraction kit https://www.promega.co.uk/products/nucleic-acid-extraction/genomic-dna/maxwell-rsc-tissue-dna-kit/?catNum=AS1610

Muziasari WI et al. 2016. Aquaculture changes the profile of antibiotic resistance and mobile genetic element associated genes in Baltic Sea sediments. FEMS Microbiol Ecol 92(4): fiw052.

Muziasari WI et al. 2017. The resistome of farmed fish feces contributes to the enrichment of antibiotic resistance genes in sediments below baltic sea fish farms. Front Microbiol 7: 2137.

Promega Wizard tissue DNA extraction kit: https://www.promega.co.uk/products/nucleic-acid-extraction/genomic-dna/wizard-genomic-dna-purification-kit/?catNum=A1120

Qiagen blood and tissue DNA extraction kit: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.8

Rodriguez, A., Olano, C., Vilches, C., Méndez, C. and Salas, J.A. (1993). *Streptomyces antibioticus* contains at least three oleandomycin-resistance determinants, one of which shows similarity with proteins of the ABC-transporter superfamily. Molecular Microbiology, 8: 571-582

Rupnik A, Doré W, Devilly L, Fahy J, Fitzpatrick A, Schmidt W, Hunt K, Butler F, Keaveney S. 2021. Evaluation of Norovirus Reduction in Environmentally Contaminated Pacific Oysters During Laboratory Controlled and Commercial Depuration. Food Environ Virol. 13(2):229-240.

Sandalli, C., Buruk, C.K., Sancaktar, M. and Ozgumus, O.B. (2010), Prevalence of integrons and a new dfrA17 variant in Gram-negative bacilli which cause community-acquired infections. Microbiology and Immunology, 54: 164-169

Schmittgen TD & Livak JK. 2008. Analyzing real-time PCR data by the comparative Ct method. Nat Protoc 3:1101- 8

UK government 2019. UK 20-year vision for antimicrobial resistance - GOV.UK (www.gov.uk)

UK government 2021. PathSafe programme: https://food.blog.gov.uk/2021/11/23/path-safe-tracking-foodborne-pathogens-and-antimicrobial-resistant-microbes/

United Nation Environment Programme (2023) Bracing for Superbugs: Strengthening environmental action in the One Health response to antimicrobial resistance. Geneva https://www.unep.org/resources/superbugs/environmental-action

Walker, D., A., Younger, L. Stockley, and C. Baker-Austin. Escherichia coli testing and enumeration in live bivalve shellfish – present methods and future directions. Food Microbiology, Volume 73, August 2018, Pages 29-38 (2017).

Wang FH et al. 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Environ Sci Technol 48:9079-85

World Health Organization (2016) Global Action Plan on AMR: https://www.who.int/publications/i/item/9789241509763

World Health Organisation (WHO), 2021. WHO integrated global surveillance on ESBL-producing E. coli using a “One Health” approach: implementation and opportunities. Geneva: WHO; 2021

Zhu YG et al. 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. P Natl Acad Sci 110:3435-40

Would you like to find out more about us or your environment?

Then call us on

03708 506 506 (Monday to Friday, 8am to 6pm)

Email: enquiries@environment-agency.gov.uk

Or visit our website

www.gov.uk/environment-agency

incident hotline

0800 807060 **(24 hours)**

floodline

0345 988 1188 **(24 hours)**

Find out about call charges (https://www.gov.uk/call-charges)

Environment first

Are you viewing this onscreen? Please consider the environment and only print if absolutely necessary. If you are reading a paper copy, please don't forget to reuse and recycle.