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Technical specifications for a EU-wide baseline survey of antimicrobial resistance in bacteria from aquaculture animals

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Abstract

The European Commission requested scientific and technical assistance in the preparation of a EU-wide baseline survey of antimicrobial resistance (AMR) in bacteria from aquaculture animals. It is recommended that the survey would aim at estimating the occurrence of AMR in *Aeromonas* spp. isolated from Atlantic Salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*) and trout (*Salmo trutta, Salvelinus fontinalis, Oncorhynchus mykiss*) intended to consumption, at harvesting (at farm/slaughter), at the EU level and in addition, at estimating the occurrence and diversity of AMR of *Escherichia coli*, *Enterococcus faecium*, *Enterococcus faecalis*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in blue mussel (*Mytilus edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*) from production areas and at dispatch centres at the EU level. These technical specifications define the target populations, the sample size for the survey, sample collection requirements, the analytical methods (for isolation, identification, phenotypic susceptibility testing and further genotypic analysis of some of the bacteria targeted) and the data reporting requirements. The data to be reported by the EU Member States to support this baseline survey are presented in three data models. The results of the survey should be reported using the EFSA reporting system.

KEYWORDS

antimicrobial resistance, aquaculture, cross-sectional prevalence study, finfish, mussels

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1 | INTRODUCTION

1.1 | Background as provided by the requestor

In its Scientific Report on the technical specifications on harmonised monitoring of AMR in zoonotic and indicator bacteria from food-producing animals and food,[1] EFSA recommended to undertake complementary baseline surveys (BLSs) in addition to the routine testing and reporting of AMR in bovine animals, pigs and poultry as laid down in Commission Implementing Decision (EU) 2020/1729.[2] The purpose of these complementary BLSs is to assess specifically the epidemiological situation on certain AMR issues, such as prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in fattening pigs and prevalence of AMR in bacteria from aquaculture animals. In 2021, EFSA questioned the members of its network on zoonoses and AMR monitoring to collect their views regarding the scope, order of priority and timing of these BLSs. The outcome of this inquiry was that a BLS on AMR in bacteria isolated from aquaculture animals should be performed at a matter of second priority after a BLS on MRSA from fattening pigs. The BLS on MRSA from fattening pigs will start on 1 January 2025 further to the publication of EFSA technical specifications for a baseline survey on the prevalence of methicillin-resistant Staphylococcus aureus (MRSA) in pigs[3] and of Commission Implementing Decision (EU) 2023/1017[4]. However, EFSA technical specifications for a BLS on the prevalence of AMR in bacteria from aquaculture animals are still necessary before envisaging starting the collection and analysis of the relevant AMR data on aquaculture animals.

1.2 | Terms of Reference as provided by the requestor

In accordance with Article 31 of Regulation (EC) No 178/2002, the Commission requests EFSA to provide technical and scientific support for the development of a BLS on the prevalence of AMR in bacteria isolated from EU produced aquaculture animals,[5] considering the most recent scientific literature and technological developments, epidemiological trends, and relevance for public health. EFSA is notably asked to propose harmonised approaches for the collection and the analysis of AMR data from aquaculture animals by:

a. proposing priority combinations of aquaculture animals/target bacteria to be considered in the BLS;
b. proposing a complete sampling framework for the implementation of the BLS including the origins of bacterial isolates subject to AMR testing, the sampling design and the sample size;
c. proposing protocols for isolation and characterisation of bacteria;
d. proposing protocols for phenotypical antimicrobial susceptibility testing of bacterial isolates;
e. proposing protocols for the testing of bacterial isolates via molecular typing methods;
f. providing guidance for technical reporting of the BLS data collected by Member States to EFSA.

1.3 | Interpretation of the terms of reference

The aim of the BLS is to assess the occurrence of AMR in bacteria from the animal species involved in aquaculture productions (aquaculture animals). The legal definition of aquaculture animals should apply (see notably, article 4 (6) and 4 (7)1 of Regulation (EU) 2016/429 on transmissible animal diseases) to delineate the animal populations to be considered for the BLS. Therefore, animals deriving from on foot fishing/fishing from the shore have not been considered. It is also of note that AMR in bacterial pathogens for aquaculture animals and AMR in aquaculture animals imported from third countries do not belong to the remit of the mandate. The approach is, therefore, to provide first an overview of AMR in bacteria from the main aquaculture animals domestically produced before considering imported seafood.

2 | RATIONALE/PRINCIPLES OF THE TECHNICAL SPECIFICATIONS

2.1 | Rationale for a need for a baseline survey on AMR in aquaculture

Aquaculture is a growing food production sector in Europe, notably in value, and globally. Europe's varied culture systems include freshwater flow-through raceways or ponds, freshwater and marine cage culture, brackish water or marine ponds, land-based, indoor hatchery, nursery and aquaculture recirculating production systems. The use of antimicrobials

[1]EFSA Journal 2019;17(6):5709. https://doi.org/10.2903/j.efsa.2019.5709.
[2]OJ L 387, 19.11.2020, p. 8–21.
[3]EFSA Journal 2022;20(10):7620. https://doi.org/10.2903/j.efsa.2022.7620.
[4]OJEU L 136, 24.5.2023, p. 78–82.
[5]As defined in Article 4(7) of Regulation (EU) 2016/429 of the European Parliament and the Council of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health ('Animal Health Law').

in aquaculture in some cases is unavoidable to treat bacterial disease outbreaks. Several studies have established an association between the use of antimicrobials in aquaculture and an increase in AMR bacteria, as reviewed by Santos and Ramos (Santos & Ramos, 2018). Additionally, some studies support the hypothesis that the development of AMR in aquaculture environments could contribute to AMR of human pathogens and indeed many AMR genes such as, but not limited to, plasmid-mediated quinolone resistance genes, phenicol resistance genes and certain beta-lactamase-encoding genes were identified in aquatic bacteria prior to their detection and dissemination among human and animal pathogens (reviewed by Henriksson et al., 2018 and Santos & Ramos, 2018).

For a proper assessment of the risk posed by AMR in aquaculture to human and animal health, there is a need of comprehensive and comparable data on the occurrence of AMR. The literature search conducted for this work (see below) showed that, at present, there are limited data on the occurrence of AMR in aquaculture in Europe and such data are not comparable, as sampling and antimicrobial susceptibility testing (AST) were performed following different criteria and methods. Monitoring AMR in bacterial organisms from aquaculture poses challenges due to the diversity of aquaculture production in each EU MS/European country, the lack of harmonised AST methods for some bacteria and difficulties in identifying sporadic or intermittent source(s) of contamination, especially in open production system. A BLS on AMR in aquaculture productions in EU/EFTA MSs using statistically based sampling schemes and harmonised antimicrobial susceptibility testing methods will provide data to fill this gap.

These technical specifications have been designed to serve this purpose and to present a unique opportunity for MSs to carry out an EU-wide BLS of AMR in aquaculture animals. This BLS is considered a convenient starting point to initiate harmonised AMR monitoring in bacteria in aquaculture productions. This should optimally be complemented by the monitoring of AMR in pathogens in aquaculture.

2.2 | Aquatic species targeted

The technical specifications propose to consider the main aquaculture productions in Europe. The target aquaculture populations are fish from the aquatic species of interest in the EU from final production intended to consumption. The selection of aquatic animal species (and bacteria) to target within the framework of the BLS has been made on (a) the economic value of the commodity and (b) the aquaculture production data at the EU level, as well as (c) at the national level. The aquaculture production database (EUMOFA[6] database for the year 2021) and the report from the Scientific, Technical and Economic Committee for Fisheries (2023) for the year 2020 has been reviewed to identify the most important aquaculture productions in value and regarding their distributions among the EU/EFTA MSs (complemented with additional available national production data from EFTA countries), so that the highest number of EU/EFTA MSs are involved in the BLS.

It has been selected for marine finfish production: European seabass (*Dicentrarchus labrax*) (Mediterranean Sea) and salmon (*Salmo salar)* (Atlantic ocean) (3 MSs and Norway, Iceland), for freshwater finfish production, varying kinds of trout[7] (23 MSs and Norway) and for mollusc production, two kinds of mussel (*Mytilus edulis* and *M. galloprovincialis* from Atlantic ocean and from Mediterranean sea) (13 MSs). Statistics on production from more recent years will need to be reassessed before the implementation of the BLS. More detailed information about the distribution of the main aquaculture productions across the EU/EFTA is presented, in an indicative way, in Appendix B.

2.3 | Rationale for the bacteria targeted

The BLS focuses on bacteria from healthy aquaculture animals, instead of clinical isolates of pathogens in aquaculture animals. The aquatic animals' gut/gills/mucus is/are in continuum with its immediate environment. AMR findings from isolates from healthy aquatic animals will more likely be an indication of environmental risk factors associated with AMR introduction and spread than directly related to aquatic animal health. The AMR of bacteria from healthy aquatic animals is also likely to be influenced by recent or previous treatment with (where this has been done) and environmental exposure to antimicrobials. Such information remains important for understanding AMR along the aquaculture production chain and provides data for risk analyses and mitigation strategies for both human and animal populations.

2.3.1 | Rationale for including *Escherichia coli* as a target species

In aquaculture products, *Escherichia coli* are indicators of faecal contamination. In particular, filter-feeding shellfish naturally concentrate bacterial contaminants from the environment, EU rules[8] require and to regularly monitor *E. coli* levels in live bivalve molluscs to estimate risk of food-borne exposure to faecal pathogens and thereby protect EU consumers'

[6] https://eumofa.eu/.

[7] It is also of note that some sea trout are produced at sea and should therefore not be considered within the part of the BLS on freshwater finfish (e.g. in DK, SE, FI or FR) but may be considered within the framework of the sampling of marine finfish production, on a voluntary basis.

[8] Commission Regulation (EC) No 2073/2005 and Commission Implementing Regulation (EU) 2019/627.

safety. The occurrence of *E. coli* in seafood samples varies greatly according to study, which may be explained by environmental conditions, survival time in the water column and microbial quality of farms (Said et al., 2017).

E. coli may be a recipient of resistance genes from marine bacteria as shown in laboratory experiments in which tetracycline resistance from marine strains of *Photobacterium*, *Vibrio*, *Alteromonas* and *Pseudomonas* was transferred to *E. coli* by conjugation (Pepi & Focardi, 2021). Also, *E. coli* in seafood has been shown to carry genes conferring resistance to critically important antimicrobials for human medicine, with the compelling example of a bla_{VIM-1}-containing *E. coli* derived from a Venus clam, harvested in the Mediterranean Sea in Italy and purchased at a German retail market (Roschanski et al., 2017).

Review of the literature (Appendix C) showed that most studies dealing with AMR in *E. coli* from shellfish and finfish have the limitations of small sample size and diversity of laboratory methods, that compromise representativeness and comparability of results. Additionally, the number of studies dealing with samples collected at the farm level is limited, while various studies focus on samples collected at the processing industry and retail levels. However, the bacterial microbiota recovered from retail products probably represents the microbiota that shellfish and finfish are exposed to during processing (e.g. repeated handling and exposure to surfaces and water that may be contaminated) rather than the indigenous microbiota of shellfish and finfish or the environmental microbiota of the aquaculture site of origin (Noor Uddin et al., 2013).

The monitoring of AMR in *E. coli* from aquaculture products at production sites may give important indications regarding the circulation of resistant bacteria in the population residing in the basin of the area of origin (Albini et al., 2022), and determining the possible sources of AMR is important for controlling the occurrence and spread of AMR at shellfish and finfish farming facilities and for lowering the risk of AMR spread from the farms to surrounding environments and to humans.

2.3.2 | Rationale for including *K. pneumoniae* on a voluntary basis

Enteric bacteria can survive exposure to seawater but may subsequently lose the ability to form colonies on solid culture media (Rozen & Belkin, 2001). In freshwater, *E. coli* survival was higher in sediment than in the water column (Baker et al., 2021). Different species of Enterobacterales may have different properties in relation to environmental survival in sea and freshwater. *Klebsiella pneumoniae* and other Klebsiella species can frequently be detected in environments including surface water and have been the subject of previous studies of both marine and freshwaters (Podschun et al., 2001). On a voluntary basis, MSs may therefore include in the baseline survey, monitoring of *K. pneumoniae* in addition to monitoring of *E. coli*. In addition, like *E. coli*, *K. pneumoniae* may be a cause of bloodstream and urinary and respiratory tract infections in humans and is easily transmissible (ECDC, 2023). Inclusion of *K. pneumoniae* will be extremely useful in the baseline survey, if the recovery rate of *E. coli* is low, as *K. pneumoniae* is more frequently recovered from the aqueous milieu, including coastal waters.

2.3.3 | Rationale for including *enterococci*. as a target

Similar to *E. coli*, *Enterococcus* sp. may be used as indicators of faecal contamination in aquatic environments. Review of the literature (Appendix C) showed that the studies on AMR in *Enterococcus* sp. from aquaculture products suffer the same limitations of the studies on *E. coli*, i.e. limited representativeness and comparability of results due to small sample size and diversity of laboratory methods employed. Additionally, the number of studies on AMR in *Enterococcus* sp. from aquaculture products is limited, which shows that there is a need to collect such information for risk assessment.

Various *Enterococcus* species have been detected in aquaculture products, which may be a consequence of the contamination from faecal material from different sources. It has been suggested that the enterococcal composition is specific to host species, with the dominant species in cattle being *E. hirae*, *E. saccharolyticus* and *E. mundtii*, and the dominant species in pigs and birds being *E. faecalis* and *E. faecium*, respectively. In humans, *E. faecalis* and *E. faecium* are both present and are therefore the main enterococcal species for which AMR monitoring is relevant from a public health perspective (Tamai & Suzuki, 2023), and are therefore, proposed to be addressed in the BLS.

2.3.4 | Rationale for including *Aeromonas* spp. as a target genus

The genus Aeromonas belongs to the Aeromonadaceae family, which itself is part of the Aeromonadales order and Gammaproteobacteria class (Fernández-Bravo & Figueras, 2020). This genus is autochthonous of the aquatic environment and easily detected in all kind of water, from freshwater to marine water, including wastewater. *Aeromonas* populations were identified using 16S rRNA gene libraries among human, agriculture, aquaculture, drinking water, surface water and wastewater samples, supporting its use as indicator bacteria to study AMR (Jones et al., 2023; Lamy et al., 2022). Certain species of Aeromonas are causative agents of infections in fish (Austin, 2015). An important range of Aeromonas, including *A. allosaccharophila*, *A. bestiarum*, *A. caviae*, *A. hydrophila*, *A. jandaei*, *A. salmonicida*, *A. schubertii*, *A. sobria* biovar *sobria* and *A. veronii* biovar *sobria*, have been associated with disease of predominantly freshwater fish in a significant number of countries (Figueras & Baez-Higalgo, 2015) and primarily in Salmonids. Natural transformation is a general property of Aeromonas environmental isolates (Huddleston et al., 2013). Moreover, integrons and other genetic elements are frequently detected in Aeromonas. These properties have led to a greater interest in studying *Aeromonas* spp. at the genus level, as an indicator

of the dissemination of antimicrobial resistance in water (Usui et al., 2016; Varela et al., 2016) or in fish (Naviner et al., 2006, 2011). Aeromonas may also be an opportunist pathogen for humans (Chen et al., 2021; Lamy et al., 2009).

2.3.5 | Rationale for including *V. alginolyticus* and *V. parahaemolyticus* as target species

The Aquatic Animal Health Code published by the WOAH specifically identifies *V. parahaemolyticus* as one of the species that should be included in monitoring and surveillance programmes antibiotic susceptibilities of bacteria isolated from aquatic animals (WOAH, 2024).[9] *V. parahaemolyticus* is a ubiquitous Gram-negative bacterium found naturally in marine and estuarine waters (Baker-Austin et al., 2010) and is regularly isolated from aquaculture products (Yang et al., 2020). Infections of humans with *V. parahaemolyticus* are most frequently associated with the consumption of aquatic animals particularly those that are consumed without cooking. *V. parahaemolyticus* is not an invasive pathogen (Onohuean et al., 2022) and these infections normally result in a self-limiting gastroenteritis (Baker-Austin et al., 2010). *V. parahaemolyticus* infections have also been reported as the causal agents of diseases in aquatic animals. Of these, acute hepatopancreatic necrosis (AHPND) that primarily affects penaeid shrimp is probably the most economically significant (Kumar et al., 2020). In addition, Ina-Salwany et al. (2019) have reviewed reports of diseases that have been causally related to *V. parahaemolyticus* infections in prawns, tilapia, catfish and a variety of shellfish in a variety of Asian and African countries.

V. alginolyticus is considered as one of the most common pathogenic species for human (Baker-Austin et al., 2018). *V. alginolyticus* are most commonly responsible for ear and wound infection, and rarely sepsis, both related to exposure to seawater (Baker-Austin et al., 2017). Sporadic cases of *V. alginolyticus* infections have been reported in Europe: e.g. in Guernsey, as wound infection associated with seawater, and in North Sea, after bathing (Schets et al., 2006, 2011). *V. alginolyticus* has been isolated from mussels in central Adriatic Sea in Italy (Bacchiocchi et al., 2021): in 2018, 62% of samples of mussels tested positive for *V. alginolyticus* (50/81) (0% for *V. parahaemolyticus*), while in 2019, 40% of samples of mussels tested positive for *V. alginolyticus* (14/35). In North Sea, *V. alginolyticus* has been described as the dominant Vibrio species at Helgoland Roads, followed by *V. parahaemolyticus* (Oberbeckmann et al., 2011).

The EFSA Scientific Opinion on Vibrio has recommended as a key priority for future research to establish an EU-wide baseline survey for the relevant *Vibrio* spp. in relevant seafood products, including in particular, primary production (EFSA, 2024).

2.4 | Other technical aspects

The technical specifications follow harmonised AST methods within the framework of the BLS to provide representative, robust and reproducible data on the occurrence and spread of AMR and help identify emerging or specific resistance patterns.

The technical specifications are based on a robust randomised sampling procedure, mostly relying on stratified sampling approach with proportional allocation of the sample numbers per strata, as typically implemented within the framework of the routine monitoring of AMR in food-producing animals and food. The design has been developed and optimised to estimate the occurrence of AMR in bacteria at the EU level.

2.5 | Priority combinations of aquaculture animals/target bacteria in the BLS

In shellfish production (mussels), *E. coli* and *Enterococcus faecalis/E. faecium* appear as good candidates. Those bacteria inform about the faecal contamination of water and anthropogenic environmental pollution. Specific monitoring of extended-spectrum beta-lactamases (ESBL)/carbapenemase (CP)-producing *E. coli* can also be performed to compare with results obtained in terrestrial animals. On a voluntary basis, *E. coli* may be complemented with *Klebsiella pneumoniae*, in particular if the prevalence of *E. coli* in mussels is very low. Laboratory protocols developed by the EURL-AR and already implemented by the NRLs apply. *V. alginolyticus* and *V. parahaemolyticus* may be pathogenic species for human.

In finfish production, whether in marine water (salmon and seabass) or freshwater (trout), *Aeromonas* spp. (at the genus level) is retained. Monitoring of AMR in *Aeromonas* spp. (at the genus level) is easier to perform than in other bacteria for a number of technical reasons. The chance to detect *Aeromonas* spp. is higher than that of other bacteria and is evenly distributed all along the year, which allows to consider a possible seasonal effect.

Regarding AST, a plate of antimicrobial substances has been proposed recently to study AMR in Vibrio and Aeromonas (Baron et al., 2017; Baron et al., 2020; Smith et al., 2023). Table 1 below presents the proposed combinations of aquatic species and bacteria that should be targeted by the BLS.

[9] https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/.

TABLE 1 Combinations of bacterial organisms/aquatic animal species to be tested for antimicrobial susceptibility within the BLS in the EU.

	Marine finfish production: Salmon, seabass	Freshwater finfish production: Trout	Mollusc production: Mussels
Aeromonas spp.	☐	☐	NA
E. coli[a]	NA	NA	☐
ESBL-/CP-producing *E. coli*	NA	NA	☐
E. faecium and *E. faecalis*	NA	NA	☐
V. parahaemolyticus and *V. alginolyticus*	NA	NA	☐

Abbreviation: NA: not applicable.

[a]*E. coli* may be complemented on a voluntary basis with *Klebsiella pneumoniae*, in particular if the prevalence of *E. coli* in mussels is very low.

2.6 | Rationale for the year of implementation of the BLS

It is proposed to perform the BLS on AMR in aquaculture animals in 2027. From 2027 onwards, MSs will have to start reporting antimicrobial use data for the main finfish species, i.e. Atlantic salmon, trout, gilthead seabream, European seabass, common carp, to the European Medicine Agency, as per Article 15 1(d) of the Commission delegated regulation 2021/578. This would allow to compare data on AMR and antimicrobial consumption (AMC) at the country level, as a secondary objective. It would request a representative sampling at the national level, which would imply collecting complementary samples to those necessary to assess AMR in bacteria at the EU level.

3 | OBJECTIVES OF THE SURVEY

The primary objectives of the survey are:

- (Ia) To assess the prevalence of antimicrobial-resistant microorganisms and the occurrence and diversity of AMR in microorganisms from the main aquaculture productions in the EU,
- (Ib) To indirectly assess, through filter feeding molluscs produced within the EU, the degree of environmental anthropogenic contamination with resistant bacteria in European production waters.

The secondary objective of the survey is:

- To explore the link between AMC and AMR in finfish aquaculture.

4 | SURVEY DESIGN

This section describes the methodology used to design the BLS to estimate the occurrence of AMR in bacteria isolated from the EU-produced aquaculture animal species targeted. The methodological principles to determine the sample size (the required number of isolates to be tested for antimicrobial susceptibility and the related number of epidemiological units (batches of animals) to be sampled) are explained.[10] The focus is primarily on the primary objective, but some attention also goes to the secondary objective.

4.1 | General considerations on a representative and random sampling

Bacterial isolates tested for antimicrobial susceptibility should derive from a harmonised active sampling so that the determination of bacterial prevalence in the studied animal populations, as well as the occurrence of AMR and the prevalence of resistant bacteria can be estimated. Isolates should originate from healthy animals sampled from randomly selected epidemiological units. Randomised sampling strategies should be used, allowing for proper statistical data analysis, and reducing the effect of sampling bias. A random sample in each animal population targeted ensures the representativeness of the entire population, which requires a sufficiently large sample size per stratum, and also reflects variability in risk factors/markers and different country/regions.

[10]Detailed illustrations of the application of these principles are provided in Appendix G, based on a number of parameters and aquaculture production volumes for 2020 (JRC report).

4.2 | Definitions

The objective is to estimate the occurrence of AMR in bacteria isolated from EU-produced aquaculture animals for each combination (aquatic species/bacteria/antimicrobial substance, as enumerated in Section 2) in an effective way. The **occurrence**[11] **of AMR p_r** is defined as the proportion of resistance, i.e. the probability that a bacterial isolate from a particular aquatic species of EU-produced aquaculture animals is resistant to the antimicrobial substance of interest. The design is developed and optimised to estimate the **occurrence of AMR p_r at the EU level**.

Bacterial isolates tested for antimicrobial susceptibility originate from **production batches** (**PB**, see next section on sampling design and sampling frame) of the aquaculture species. The bacterial **prevalence π_{bp}** denotes the probability that a PB tested positive, i.e. it comprises at least one fish/mussel tested positive for the bacteria of interest. Given that a PB is positive, the proportion of positive fish within that positive PB is denoted by the **within PB prevalence π_{wbp}**. As testing all fish from a PB individually is not feasible, a small **sample batch SB** of fish/mussel of a particular size is randomly taken from the PB for testing. The SB will not result in isolated bacteria if the PB does not test positive. If the PB is positive for the bacteria of interest, there is a probability that the sample batch will lead to an isolate, depending on several factors including the magnitude of the within PB prevalence π_{wbp} and the size of the sample batch.

The estimation of the prevalence π_{bp} and the within PB prevalence π_{wbp} is not an objective of the survey per se, but their (unknown) values play a role in determining the required number of isolates to estimate the **AMR occurrence p_r** effectively.

4.3 | Sampling design and sampling frame

The specific characteristics of the sampling frame depend on the particular combination aquaculture animal/bacteria considered, but the general structure is generic, with the EU divided into MS-based subpopulations (**strata**), with production units (**PUs**) randomly sampled within each stratum and with **PB** randomly sampled within each **PU** (Table 2). The sampling procedure mostly relies on a stratified sampling[12] approach with proportional allocation of the sample numbers per strata. The general structure is presented below. An approximately equal distribution of the collected samples over the year enables the different seasons to be covered.[13] The design follows the generic proportionate stratified sampling approach already implemented for AMR monitoring, as presented in the technical specifications on harmonised AMR monitoring (EFSA, 2019).

The results of applying the generic approach of the sampling design to the characteristics of some illustrative combinations are illustrated in Appendix G, using available data. While preparing for the implementation of the BLS, sample size calculation will be adapted using more recent data. Further guidance will be provided by EFSA.

TABLE 2 Survey design: Stratified sampling with main strata at MS level, random sampling of production units at secondary level and sampling of batches (of minimal sizes).

	EU-strata	Production unit (PU)	Production batch (PB)	Minimal size of sample batch (SB)[a]
Shellfish	MS	Production area (PA)	At sampling point	≥ 15 mussel
Shellfish	MS	Dispatch centre (DC)	Packed & labelled	≥ 15 mussel
Marine finfish	(MS, Region)	Farm	Cage	≥ 5 fish
Freshwater finfish	(MS, Region)	Farm	Pond	≥ 5 fish

[a]The minimal size is based on the minimal biological material necessary for the testing.

The BLS is based on the representative and random collection of PBs at PUs. MSs or MS regions serve as strata[14] within the EU (referred to as EU strata).

The total number of PBs to be sampled within an EU stratum is based on stratified sampling with proportional allocation, using proportions reflecting the relative production volumes of the strata at the EU level. Deviations from standard proportional allocation might be applied by applying a minimum and maximum number of PBs for an EU stratum (see Section 5.1.2 for the minimum and maximum number of isolates). It is further proposed that four PBs be sampled from the same PU so that they can be approximately equally distributed over the four quarter of the year.

[11]The occurrence of AMR in isolated bacteria differs from the prevalence of AMR, which refers to the proportion of sample batches harbouring resistant bacteria.

[12]Stratified sampling is a method of sampling from a population which involves, in a first step, the division of a population into smaller groups known as strata. The strata are based on members' shared attributes or characteristics. In a second step, a random sample from each stratum is taken in a number proportional to the stratum's size when compared to the population. These subsets of the strata are then pooled to form a random sample. In practice, stratified random sampling is typically employed in large-scale surveys to reduce some of the logistical costs associated with collecting epidemiological information.

[13]Still, it is of note that in certain MSs, for the most part, slaughtering of fish is not done around the year equally, as some fish are under ice cover in the winter. These may limit the sampling possibilities all year round.

[14]Countries and regions are taken as strata, as seasonal effect, watershed and water quality (depending on the type of ground) can apply differently from a region to another.

The **epidemiological units** defining the sampling frame are the PBs, the production batches, defined as groups of fish at the harvesting, of the same age raised together under the same conditions and exposed to the same risk factors/markers.

5 | SAMPLE SIZES

The sample size (i.e. the number of isolates to be tested for susceptibility at each sampling time) should allow, within a predetermined accuracy, the calculation of the occurrence of AMR (proportion of antimicrobial resistance to a particular antimicrobial) for a given combination of bacteria/animal populations.

The first section describes the sample size calculation's starting point: the required number of isolates. In a later section, the required number of isolates is translated to the required number of PBs (production batches) to be sampled. The last section illustrates the application of the general principles, depending on the knowledge of unknown parameters and the chosen strategy.

5.1 | Standard calculation of the required number of isolates at the EU level

The required total number n_{iso} of bacterial isolates is determined to achieve an 'effective' estimation of the occurrence of AMR p_r, i.e. estimation with preassigned level of confidence and accuracy.

This required number n_{iso} depends on the target parameter p_r itself. The required sample sizes for a grid of values for p_r, for confidence levels 0.80 and 0.95, and for three values for accuracy[15] a are presented in Table D.1 in Appendix D. Note that the table of required numbers is symmetric around its maximum at $\boldsymbol{p_r = 0.5}$.[16] Several methods exist to compute the confidence interval for a proportion, leading to slightly varying numbers of sample sizes. The Wilson's generally recommended method was applied (Brown et al., 2001) with the R-function ssize.propCI() from the MKpower package.

5.1.1 | Accounting for possible missing data and loss during storage

As considered already in the technical specifications for harmonised AMR monitoring (EFSA, 2019), the required number of isolates to be tested should be further inflated by 5% to consider a 5% occurrence of possible missing data and by 2% to account for the possible loss of strains during storage, leading to the additional adjustments

$$n_{iso} \longleftarrow n_{iso} \times 1.05 \times 1.02.$$

The required numbers before and after adjustment are shown in Table 12 in Appendix D.

5.1.2 | Choices at the EU and the strata level

The standard choice of confidence level is 0.95. If the objective is to estimate the AMR occurrence at the MS level, a typical choice for the accuracy would be 0.1.[17] The objective here is to estimate the occurrence of AMR p_r at the EU level. Therefore, an accuracy of 0.05 is chosen, leading to **the required number of isolates 416** (last column of Table 12 in Appendix D) **at the EU level**. Proportional allocation will determine the required number n_{iso}^{str} of isolates for an EU stratum.

As the production volume distribution can be highly skewed between the MSs/strata, leading to highly varying proportions for the proportional allocation, the number n_{iso}^{str} of isolates for an EU stratum is truncated downwards by a minimum and upwards by a maximum.

- The maximum is determined by taking $p_r = 0.5$ and applying an accuracy of 0.1 and the confidence level to 0.95, leading to the max = 107.
- The minimum is determined by lowering the accuracy to 0.2 and the confidence level to 0.80. Although these are loose requirements, it implies at least min = 13 isolates for each EU stratum, guaranteeing a minimum number of resistance data for the second objective of the BLS (linking AMR to AMC).

Also, note that this minimum and maximum will still be further adjusted (increased) by the intra-PU correlation (see next section).

[15] Interval estimation with accuracy a corresponds to a confidence interval of width $2a$.

[16] In the absence of knowledge a priori about the magnitude of pr, it is conservative to consider pr = 0.5. Otherwise, if there is some available knowledge about $\boldsymbol{p_r}$, e.g. from the literature, this can be used to optimise the sample size calculation.

[17] See for example the technical specifications for the BLS on MRSA in pigs (EFSA, 2022).

5.1.3 | Multiple PB for the same PU

Resistance outcomes from multiple isolates from the same PU might not be independent but rather correlated, as they share similar conditions from the same PU (compared to PBs from different PUs). This correlation resulting from the hierarchical structure of the design needs to be taken into account by a so-called design effect $\left(1+\left(m_{PU}-1\right)\rho_{IPU}\right)$ with m_{PU} the number of isolates from the same PU and ρ_{IPU} **the intra-PU correlation**, resulting in the additional multiplicative adjustment factor:

$$n_{iso}^{str} \longleftarrow n_{iso}^{str} \times \min\left(\left(1+\left(m_{PU}-1\right)\rho_{IPU}\right)\right).$$

Note that this design effect has a substantial impact. For instance, for a moderate correlation of 0.2 (see example presented in Appendix F) and quarterly sampled PBs, the multiplicative adjustment factor equals $\left(1+\left(m_{PU}-1\right)\rho_{IPU}\right)=1.6$.

5.1.4 | Finite population correction factor

For a finite population size N, the sample size of the EU strata can be adjusted by the population correction factor (FPC)

$$n_{iso}^{str} \longleftarrow n_{iso}^{str} \times \frac{N}{N+n_{iso}^{str}}.$$

As the sample of isolates is small compared to the total population of isolated bacteria from batches (less than 5%), the FPC is approximately equal to 1, and applying an FPC is not useful.

5.2 | From the number of isolates to the number of production batches

This conversion can be applied uniformly across all EU strata or adapted to the specific knowledge available for specific EU strata. For notational simplicity, the superscript 'str' is omitted.

5.2.1 | From isolate to production batch

A sample batch will not always result in an isolate for susceptibility testing. Indeed, only a positive PB (production batch with at least one fish tested positive for the bacteria) may lead to an isolate. As **the prevalence** π_{bp} denotes the probability that a PB has at least one fish positive for the bacteria of interest, we have that $n_{iso}=n_b\times\pi_{bp}$ where n_b denotes the number of PBs, resulting in the number of production batches:

$$n_b=\frac{n_{iso}}{\pi_{bp}}.$$

However, even a positive PB will not necessarily result in isolated bacteria. It depends on the probability that bacteria are detected in the sampled batch SB from the PB. This **sample batch sensitivity** ***BSe*** depends on (i) the **within PB prevalence** π_{wbp} (proportion of positive fish within a positive batch, a proportion that might vary across PBs and PUs), (ii) the size of the production batch M, (iii) the size of the sample batch m, (iv) the sensitivity TSe of the test applied. The BSe increases with $\pi_{wbp}, m, Tse$and decreases with M and with heterogeneity across batches. We assume no false positives (specificity very high). This leads to the further adjusted required number of production batches:

$$n_b=\frac{n_{iso}}{\pi_{bp}BSe}.$$

Note that the denominator $\pi_{bp}BSe$ representes the probability that a PB is detected to be positive. For simplicity, we assume that M is very large and $TSe\approx 1$, and we apply the beta-binomial probability model to deal with heterogeneity. The beta-binomial is an extension of the binomial model and can accommodate heterogeneity (overdispersion) through the additional **intra-batch correlation parameter** ρ (see section 4.3.1 in Aerts et al., 2002). It is assumed that testing the batch as one pool as compared to testing the fish of the batch individually only reduces the BSe in a limited way (e.g. see Table 4 in EFSA, 2022). Table E.1 provides values for BSe for varying values of m, π_{wbp} and ρ (details and formulas producing this table are briefly discussed in Appendix F). It shows that the batch sensitivity BSe increases with π_{wbp} and decreases as intra-batch correlation increases. Note that for correlation $\rho=1$, the information in a batch of fish reduces to that of a single fish, and the BSe values are no longer varying with sample batch size m and are equal to the π_{wbp}.

Using the table for choosing an appropriate size m for the sample batch and for determining the required number of PBs assumes knowledge about the intra-batch correlation parameter ρ and the within PB prevalence π_{wbp}. Such knowledge might be obtainable from literature. For instance, suppose a particular stratum takes 25% of the total EU production;

suppose that four PBs of batch size 15 are sampled corresponding to four quarters and suppose that the intra-PU correlation can be taken as 0.2. This would lead to the required number of isolates for that stratum $n_{iso}^{str} = 416 \times 0.25 \times 1.6 = 166.4$. The analysis presented in Appendix F reveals that (i) π_{bp} is estimated as close to 0.9, (ii) π_{wbp} is estimated as close to 0.5 and (iii) ρ is estimated as close to 0.2. From Table E.1, we find that $BSe \approx 0.98$ such that $n_b^{str} = \frac{166.4}{0.9 \times 0.98} = 189$ PBs are needed, or four PBs need to be sampled quarterly from 48 PUs. See Appendix G for more elaborate illustrations.

If such knowledge is unavailable, two options can be taken: a conservative choice based on a maximum number of PBs (as based on the maximum number of isolates), or a sequential approach, in which knowledge over time is accumulated and the number $n_b$of PBs for the next period is based on analysing the data of all previous periods (see next section).

5.2.1.1 | *A sequential approach*

In case there is insufficient knowledge about the probability that a PB is detected to be positive, and if multiple BPs are collected over time (e.g. quarterly), the required number of PBs can be adjusted by a sequential procedure based on all data from previous periods. Such adjustments must be performed after each period and can go in both directions. If an MS opts for such a sequential procedure, it is desirable to document it by providing relevant data from the previous periods and a short report to EFSA with the calculations leading to the adjusted sample size. For an example of such a sequential approach, see section 4.3.4 in the technical specifications for a BLS on the prevalence of MRSA in pigs (EFSA, 2022).

6 | SAMPLING PLAN AND SAMPLE COLLECTION

6.1 | Sampling plan

The general characteristics of the proportional stratified sampling approach are summarised in Table 3. It illustrates stratified sampling concepts, such as strata, proportional allocation, epidemiological unit, to the sampling plans proposed. The BLS is based on the representative and random collection of PBs at PUs. MSs or MS regions serve as strata within the EU (referred to as EU strata). Deviations from standard proportional allocation might be applied by applying a minimum and maximum number of PBs for an EU stratum (see Section 5.1.2 for the minimum and maximum number of isolates). It is further proposed that four PBs be sampled from the same PU and approximately equally distributed over the year.

TABLE 3 General characteristics of the stratified sampling approach.

	Sampling of aquatic organisms			
Sampling concept	**Marine finfish production: Seabass/salmon**	**Freshwater finfish production: Trout**	**Mollusc production: Mussels**	
Target populations	EU/EFTA produced seabass/salmon[a]	EU/EFTA produced trout[a,b]	EU/EFTA produced mussels[a,c]	
Strata	(MS, Region)[a]	(MS, Region)[a]	MS	MS
Proportional allocation	Sample size (number of PBs) proportionate[d] to the stratum production, with a minimum and a maximum[e] number of PBs	Sample size (number of PBs) proportionate[d] to the stratum production, with a minimum and a maximum[e] number of PBs	Sample size (number of PBs) proportionate to the stratum production, with a minimum and a maximum[e] number of PBs	Sample size (number of PBs) proportionate to the stratum production, with a minimum and a maximum[e] number of PBs
Production Units	Random sampling of PUs (farms) per stratum[f,g]	Random sampling of PUs (farms) per stratum[f,g]	Random sampling of PUs (PA) per stratum[f,g]	Random sampling of PUs (DC) per stratum[f,g]
Epidemiological Units	PBs of seabass/salmon at slaughter	PBs of trout at slaughter	PBs at sampling point	PBs packed and labelled
Production Batches	Random sampling of 4 PBs per PU, approximately evenly distributed over the year	Random sampling of 4 PBs per PU, approximately evenly distributed over the year	Random sampling of 4 PBs per PU, approximately evenly distributed over the year	Random sampling of 4 PBs per PU, approximately evenly distributed over the year
Sampling	At farm/slaughter	At farm/slaughter	At sampling point[h]	At DC
Sample	Pooled sample of gills collected from 5 fish, randomly selected per PB	Pooled sample of gills collected from 5 fish, randomly selected per PB	Pooled sample of 15 mussels	Pooled sample of 15 mussels

Abbreviations: DC, dispatch centre; MS, member state; PA, production area; PB, production batch; PU, production unit.

[a]The source population of seabass/salmon and trout covers that domestically produced in the 'largest' regions accumulating at least 60% of the total production in the MS.

[b]Trout includes rainbow trout, brook trout and other kinds of trout (sea trout produced at sea excepted).

[c]Mussels includes blue mussels and Mediterranean mussels.

[d]The total number of PBs to be sampled within an EU stratum is based on stratified sampling with proportional allocation, using proportions reflecting the relative production volumes of the strata at the EU level.

[e]Deviations from standard proportional allocation might be applied by applying a minimum and maximum number of PBs for an EU stratum (see Section 5.1.2 for the minimum and maximum number of isolates).

[f]The PUs are randomly selected from the sampling frame (list of PUs) maintained by the Competent Authority of the MS.

[g]The number of PUs equals the number of PBs divided by 4.

[h]Within the production/harvesting area.

6.2 | Sampling and type of samples

6.2.1 | Sampling

Sampling should be planned at the required frequency at the selected locations (e.g. production units, dispatch centres and production areas). As far as possible, the locations should be visited on different days in the week and month over the period of the survey. Consideration should also be given to the requirement for initial processing of the sample by the laboratory to be performed within 72 h of taking the sample, and for samples to arrive at the laboratory during the working week. For example, sampling on Friday should be avoided without prior agreement with the laboratory.

6.2.2 | Samples from finfish

For each randomly selected epidemiological unit (production batch) of finfish, five marine finfish (seabass and salmon) or freshwater finfish (trout) are sampled, from which all gill arches are collected bilaterally and pooled. A grammage of at least 25 g per pooled sample of gills[18] deriving from the five finfish sampled[19] should be reached. Gills are relevant samples with respect to sampling standardisation, availability[20] and easiness to collect. Regarding the sampling stage, samples are taken at harvesting, i.e. either at post-harvest on the farm or after euthanasia at the slaughterhouse, depending on the size of the PUs (farm) and the structure of the production sector considered. The time elapsed between killing and sampling should be short. The time elapsed between sampling time and the starting of the isolation protocol should be harmonised at 72 h. Each sample should be labelled with a unique number which should be used from sampling to testing. The use of unique numbering system at the country level is recommended.

6.2.3 | Samples from mussels

The locations within a geographical region visited at the same time should be varied. Where more than one mussel species is present at the sample site, a sample of only one mussel species is required for the survey.

A sample of 15 live mussels should be taken and dispatched to the designated laboratory for pooled analysis. During sampling, precautions should be taken in order to avoid any activity that could affect the levels of bacterial contamination or result in a sample that is unsuitable for laboratory analysis. The mussels are to be placed in an intact food grade plastic bag or box (single use), securely packaged and dispatched to the laboratory.

Representative sampling points within production/harvesting areas: Where multiple representative sampling points[21] are present in the same production area, the representative sampling point, where mussel species are present, with the highest levels of *E. coli* contamination based on the routine monitoring performed under Regulation 2019/627 should be used to obtain the sample of mussels. It is important that the mussels sampled, have been growing in the selected production area for more than 28 days, in order to be representative of this area and not a previous one.

Approved dispatch centres: The sample should be taken from one batch of live mussels present on the premises at the time of visit. Fifteen mussels should be selected from the boxes on the packing line (representative of the sizes and grades of animals in the batch). The sample should not contain a mix of mussel species.

7 | LABORATORY METHODS

This chapter presents an overview of the laboratory methods for isolating and confirming the bacteria addressed in the BLS. For the sake of harmonisation, detailed protocols will be drafted and issued on the EURL-AR website while preparing for the implementation of the BLS.

7.1 | Laboratory methods for *E. coli* and *K. pneumoniae*

7.1.1 | Overview

Monitoring of AMR in indicator commensal *E. coli* is a pivotal element of the EU-wide monitoring and serve as a marker for the exposure of the population of *E. coli* to antimicrobial selection pressure. Thus, indicator commensal *E. coli* can

[18]Considering an expected prevalence of about 90% for *Aeromonas* spp. in finfish, it is considered sufficiently high grammage to detect contamination with a sufficiently high probability.

[19]Trout portion are the smallest finfish to sample, whereas trout for filleting are of a bigger size, as well as salmon and seabass. It implies to prioritise the sampling of bigger trout portion from the production batch.

[20]Gills are of a lesser economical value and removal has a limited impact on the commercial value of fish.

[21]Used for monitoring activities under Regulation 2019/627.

supplement the continuous evidence on trends of AMR in terrestrial food animals when introducing a baseline monitoring of in aquatic species and seafood.

7.1.2 | Isolation methods

It is proposed to continue with the methodology already applied by the MSs for the isolation of indicator commensal *E. coli* in food using 25 g of samples taken mixed with 225 mL of buffered peptone water (BPW) and incubated at 37°C ± 1°C for 18–22 h. Following incubation, one loopful (10 μL loop) of the pre-enrichment culture in BPW is applied by a single streak onto a MacConkey agar plate and incubated at 37°C ± 1°C for 18–22 h.

In cases where the MacConkey agar plate yields growth of mucoid colonies which may be *Klebsiella* spp. then on a voluntary basis, MSs may also subculture these colonies and presumptively identify them using standard biochemical tests (MALDI-TOF among others). As a further voluntary measure, the MacConkey plate may be supplemented with an additional Simmons citrate agar plate with inositol to target recovery of Klebsiella (Kregten et al., 1984).[22] Subsequent testing of *K. pneumoniae* should be run in parallel with that of *E. coli*.

7.1.3 | Confirmatory testing and typing

MSs can apply both conventional microbiological and biochemical approaches, as well as molecular and whole genome sequencing (WGS)-based methods, as there is no harmonised method for the identification of indicator commensal *E. coli* of the EU monitoring of AMR.

7.1.4 | Antimicrobial susceptibility testing

Standardised dilution methods give a semi-quantitative measurement of the susceptibility as an antimicrobial concentration (expressed in mg/L) that is reproducible between different laboratories with a biological variation (one dilution step). As the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website (https://www.eucast.org/) gives access to aggregated distributions of minimum inhibitory concentration (MIC) for these bacterial species, as well as defining epidemiological cut-off values (ECOFFs, Kahlmeter et al., 2003) and clinical breakpoints (CBPs) in human medicine, data obtained by making use of dilution methods can be interpreted for both epidemiological and clinical purposes, provided that the dilution range used frames both thresholds. It is proposed to continue with the previous recommendation for the use of standardised dilution methods for antimicrobial susceptibility testing (AST) of indicator commensal *E. coli* targeted by the harmonised monitoring (EFSA, 2019). Thus, the protocol of the Broth Micro Dilution (BMD) method according to the reference method ISO 20776-1:2019 and provided by the EURL for antimicrobial resistance (EURL-AR) shall be applied to test the susceptibility to the specified list of antimicrobials, with predefined appropriate dilution ranges and ECOFFs set out in Table 4. The existing quality assurance system using ATCC quality control strains should be also reinforced.

TABLE 4 Panel of antimicrobial substances to be included in AMR monitoring, EUCAST thresholds for resistance and concentration ranges to be tested in indicator commensal *E. coli*.

Antimicrobial	Class of antimicrobial	Interpretative thresholds of AMR (mg/L)[a]		Range of concentrations (mg/L) (No. of wells)
		ECOFF	CBP	
Amikacin	Aminoglycoside	> 8	> 16	4–128 (6)
Ampicillin	Penicillin	> 8	> 8	1–32 (6)
Azithromycin	Macrolide	NA	NA	2–64 (6)
Cefotaxime	Cephalosporin	> 0.25	> 2	0.25–4 (5)
Ceftazidime	Cephalosporin	> 0.5	> 4	0.25–8 (6)
Chloramphenicol	Phenicol	> 16	> 8	8–64 (4)
Ciprofloxacin	Fluoroquinolone	> 0.06	> 0.5	0.015–8 (10)
Colistin	Polymyxin	> 2	> 2	1–16 (5)
Gentamicin	Aminoglycoside	> 2	> 4	0.5–16 (6)
Meropenem	Carbapenem	> 0.125	> 8	0.03–16 (10)

[22]An optimised protocol, initially designed for human faeces has been validated for food items and is available at: https://www.protocols.io/view/isolation-of-klebsiella-strains-from-food-samples-4r3l282b4l1y/v1. This protocol is intended for isolation of Klebsiella strains from different food sources. It is derived from the initial description of the SCAi medium (van Kregten et al., 1984), and its validation across a diversity of Klebsiella strains (Passet & Brisse, 2015). The protocol entails enrichment using buffered peptone water (BPW), and plating on SCAi (Simmons Citrate with Inositol) agar.

TABLE 4 (Continued)

Antimicrobial	Class of antimicrobial	Interpretative thresholds of AMR (mg/L)[a]		Range of concentrations (mg/L) (No. of wells)
		ECOFF	CBP	
Nalidixic acid	Quinolone	> 8	NA	4–64 (5)
Sulfamethoxazole	Folate pathway antagonist	> 64	NA	8–512 (7)
Tetracycline	Tetracycline	> 8	NA	2–32 (5)
Tigecycline	Glycylcycline	> 0.5	> 0.5	0.25–8 (6)
Trimethoprim	Folate pathway antagonist	> 2	> 4	0.25–16 (7)

Abbreviations: CBP, clinical breakpoint; ECOFF, epidemiological cut-off value; NA, not available.

[a]EUCAST ECOFFs and CBPs available as in Decision (EU) 2020/1729. For the sake of consistency, the ECOFFs laid down in the legislation have been retained at this stage. Thresholds will be reviewed prior to the implementation of the BLS; further guidance will be provided by the EURL-AR and EFSA.

7.2 | Laboratory methods for ESBL-producing *E. coli*

7.2.1 | Overview

Enterobacterales producing extended-spectrum beta-lactamases (ESBLs), AmpC cephalosporinases and carbapenemases are of major public health significance. In 2021, it was decided to be mandatory to also monitor in the specific monitoring of ESBL- or AmpC- or CP-producing *E. coli* for carbapenemase producing *E. coli* including OXA-48 and OXA-48-like producers. Today, the selective isolation of ESBL/AmpC and/or carbapenemase-producing *E. coli* from the production of major terrestrial domestical food-producing animal populations and their derived meat serves as an indication of critical resistance and has demonstrated that it provides interesting complementary information to the monitoring of indicator commensal *E. coli* in particularly with the emerging occurrence of carbapenemase-producing *E. coli*.

7.2.2 | Isolation methods

It is proposed to continue with the isolation protocol provided by the EURL and already applied by the MSs for the isolation of ESBL- or AmpC- or CP-producing *E. coli* from seafood. The protocol is based on the principle of using a non-selective enrichment step without the supplement of neither a third-generation cephalosporin nor a carbapenem as the enrichment are also being used by the MSs for other parts of the monitoring. In brief, 25 g of samples should be mixed with 225 mL of buffered peptone water (BPW) and incubated at 37°C ± 1°C for 18–22 h. Following incubation, one loopful (10 µL loop) of the pre-enrichment culture in BPW is applied by a single streak onto a MacConkey agar plate containing 1 mg/L of cefotaxime (CTX) and incubated at 44°C ± 0.5°C for 18–22 h. Based on the colony morphology of presumptive ESBL- or AmpC- or CP-producing *E. coli* colonies, up to three colonies should be subcultured onto new MacConkey agar plate containing 1 mg/L of CTX in addition to a suitable selective agar(s) for isolation of CP-producing *E. coli*. All agar plates should be incubated at 37°C ± 1°C for 18–22 h. Subsequently, growth of one of these subcultures should be species identified.

7.2.3 | Confirmatory testing and typing

As there is no harmonised isolation method for the ESBL- or AmpC- or CP-producing *E. coli* of the EU monitoring of AMR, MSs can applied both conventional microbiological and biochemical approaches as well as molecular and whole genome sequencing (WGS)-based methods.

7.2.4 | Antimicrobial susceptibility testing

It is proposed to continue with the previous recommendation for the use of standardised dilution methods for testing the susceptibility of ESBL- or AmpC- or CP-producing *E. coli* similar to indicator commensal *E. coli*. Thus, the BMD method according to the reference method ISO 20776-1:2019 and provided by the EURL-AR[23] shall be applied to test the susceptibility of and confirm the presence of ESBL- or AmpC- or CP-producing *E. coli* to the specified list of antimicrobials, with predefined appropriate dilution ranges and ECOFFs set out in Tables 4 and 5. The interpretation of the confirmation shall be in accordance with the EFSA Journal 2019;17(6):5709. It is of note that the use of this second plate may be replaced by using WGS, as a number of MSs already do.

[23]https://www.eurl-ar.eu/protocols.aspx.

TABLE 5 Panel of antimicrobial substances, EUCAST epidemiological cut-off values (ECOFFs) and clinical resistance breakpoints and concentrations ranges to be used for testing *E. coli* isolates resistant to cefotaxime or ceftazidime or meropenem.

Antimicrobial	Class of antimicrobials	Interpretative thresholds of AMR[a] (mg/L)		Concentration range, mg/L (no. of w ells)
		EUCAST ECOFF	CBP	
Cefepime	Cephalosporin	> 0.125	> 4	0.06–32 (10)
Cefotaxime	Cephalosporin	> 0.25	> 2	0.25–64 (9)
Cefotaxime + clavulanic acid	Cephalosporin/beta-lactamase inhibitor combination	> 0.25	NA	0.06–64 (11)
Cefoxitin	Cephamycin	> 8	NA	0.5–64 (8)
Ceftazidime	Cephalosporin	> 0.5	> 4	0.25–128 (10)
Ceftazidime + clavulanic acid	Cephalosporin/beta-lactamase inhibitor combination	> 0.5	NA	0.125–128 (11)
Ertapenem	Carbapenem	NA	> 0.5	0.015–2 (8)
Imipenem	Carbapenem	> 0.5	> 4	0.12–16 (8)
Meropenem	Carbapenem	> 0.125	> 8	0.03–16 (10)
Temocillin	Penicillin	> 16	NA	0.5–128 (9)

Abbreviations: ECOFFs, epidemiological cut-off values; EUCAST, European Committee on Antimicrobial Susceptibility Testing; NA, not available.

[a]EUCAST ECOFFs and CBs as in Decision (EU) 2020/1729. For the sake of consistency, the thresholds laid down in the legislation have been retained at this stage. Thresholds will be reviewed prior to the implementation of the BLS; further guidance will be provided by the EURL-AR and EFSA.

7.3 | Laboratory methods for enterococci

7.3.1 | Overview

The enterococci fulfil a useful and unique function among the organisms which are monitored by representing a common or frequent Gram-positive indicator organism which is not subject to the pressures from targeted control measures. Monitoring AMR in enterococci as indicator bacteria representing Gram-positive organisms will complement the data from *E. coli* is for Gram-negative bacteria (Enterobacteriaceae).

7.3.2 | Isolation methods

In the EU monitoring of AMR of enterococci, just as for the isolation of indicator commensal *E. coli*, there is no harmonised isolation method. It is, however, proposed to continue with the methodology already applied by the MSs for the isolation of enterococci in food using 25 g of seafood mixed with 225 mL of buffered peptone water (BPW) and incubated at 37°C ± 1°C for 18–22 h. Following incubation, one loopful (10 µL loop) of the pre-enrichment culture in BPW is applied by a single streak onto a Slanetz–Bartley agar plate and incubated at 37°C ± 1°C for 48 h.

7.3.3 | Confirmatory testing and typing

MSs can applied both conventional microbiological and biochemical approaches as well as molecular, such as the EURL PCR protocol[24] and whole genome sequencing (WGS)-based methods.

7.3.4 | Antimicrobial susceptibility testing

It is proposed to continue with the previous recommendation for the use of standardised dilution methods for testing the susceptibility of enterococci similar to indicator commensal *E. coli*. Thus, the BMD method according to the reference method ISO 20776-1:2019 and provided by the EURL shall be applied to test the susceptibility of and confirm the presence of enterococci to the specified list of antimicrobials, with predefined appropriate dilution ranges and ECOFFs set out in Tables 5 and 6.

[24]https://www.eurl-ar.eu/protocols.aspx.

TABLE 6 Panel of antimicrobial substances to be included in AMR testing, EUCAST thresholds for resistance and concentration ranges to be tested in *E. faecalis* and *E. faecium*.

Antimicrobial	Class of antimicrobial	Species	Interpretative thresholds of AMR (mg/L)[a]		Range of concentrations (mg/L) (No of wells)
			ECOFF	CBP	
Ampicillin	Penicillin	*E. faecalis*	>4	>8	0.5–64 (8)
		E. faecium	>4	>8	
Chloramphenicol	Phenicol	*E. faecalis*	>32	NA	4–128 (6)
		E. faecium	>32	NA	
Ciprofloxacin	Fluoroquinolone	*E. faecalis*	>4	>4	0.12–16 (8)
		E. faecium	>4	>4	
Daptomycin	Lipopeptide	*E. faecalis*	>4	NA	0.25–32 (8)
		E. faecium	>8	NA	
Erythromycin	Macrolide	*E. faecalis*	>4	NA	1–128 (8)
		E. faecium	>4	NA	
Gentamicin	Aminoglycoside	*E. faecalis*	>32	NA	8–1024 (8)
		E. faecium	>32	NA	
Linezolid	Oxazolidinone	*E. faecalis*	>4	>4	0.5–64 (8)
		E. faecium	>4	>4	
Quinupristin/Dalfopristin	Streptogramin	*E. faecalis*	NA	NA	0.5–64 (8)
		E. faecium	NA	>4	
Teicoplanin	Glycopeptide	*E. faecalis*	>2	>2	0.5–64 (8)
		E. faecium	>2	>2	
Tetracycline	Tetracycline	*E. faecalis*	>4	NA	1–128 (8)
		E. faecium	>4	NA	
Tigecycline	Glycylcycline	*E. faecalis*	>0.5	>0.25	0.03–4 (8)
		E. faecium	>0.25	>0.25	
Vancomycin	Glycopeptide	*E. faecalis*	>4	>4	1–128 (8)
		E. faecium	>4	>4	

Abbreviations: CBP, clinical breakpoint; ECOFF, epidemiological cut-off value; NA, not available.

[a]EUCAST ECOFFs and CBPs available as in Decision (EU) 2020/1729. For the sake of consistency, thresholds laid down in the legislation have been retained at this stage. Thresholds will be reviewed prior to the implementation of the BLS; further guidance will be provided by the EURL-AR and EFSA.

7.4 | Laboratory methods for *Aeromonas* spp.

7.4.1 | Isolation methods

Streaking samples on glutamate starch phenol red agar (GSP) (FAO, NParks and SFA, 2023) and incubation during 48 h at 22°C. A first read could be done at 24 h, but the colonies could be small. Incubation at 22°C during 48 h.

Select yellow colonies and purification step on ChromAgar or Nutritive agar (Tryptone Soy Agar or Muller Hinton Agar).

7.4.2 | Confirmatory testing and typing

The commercial identification systems (e.g. API 20E, Vitek, BBL Crystal, MicroScan W/A, among others) based on phenotypical reactions are not accurate for Aeromonas identification and confusions with Vibrio can occur (Chacón et al., 2002; Lamy et al., 2010; Soler et al., 2003). MALDI-TOF is also useful, and likely more practical in certain circumstances, for identification at the genus level (Fernández-Bravo & Figueras, 2020). Still, identification at the species level may be problematic, as underpinning database may not include the species of *Aeromonas* recently described (Pérez-Sancho et al., 2018).

Presumptive Aeromonas isolates identified during the BLS will be subjected to PCR assay, specific for the genus level. Gold standard method is based on the sequencing of housekeeping genes *gyrB* and *rpoD*. Confirmation of the identification at the genus level can be done by PCR targeting the conserved flanking regions of the gyrase B subunit (GyrB) gene (Khan et al., 2009) (Table 7).

TABLE 7 PCR assay.

Gene	Primer name	Primer sequence	Product size (bp)
gyrB	IA-Forward	CTG AAC CAG AAC AAG ACC CCG	130
	IA-Reverse	ATG TTG TTG GTG AAG CAG TA	

7.4.3 | Antimicrobial susceptibility testing

MIC tests against *Aeromonas* are performed at 28°C with incubation for 44–48 h according to the protocols provided in the CLSI guideline VET03 (CLSI, 2020a). The inocula are prepared by the colony suspension method recommended this guideline VET03 using cation adjusted Muller–Hinton broth (CAMHB). The MIC values are determined by the microdilution method using CAMHB that was not supplemented with NaCl.

Each laboratory should employ one or both of the quality control (QC) reference strains *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658 recommended by CLSI for this method (CLSI, 2020a). The CLSI document VET04 (CLSI, 2020b) provides acceptable ranges for these QC reference strains tested using the MIC protocol (Tables 8 and 9).

TABLE 8 Panel of antimicrobial substances to be included in AMR testing, interpretative thresholds for microbiological resistance and concentration ranges to be tested in *Aeromonas* spp.

Antimicrobial	Antimicrobial class	Interpretative thresholds of AMR (mg/L) (ref.)	Range of concentrations (mg/L) (No of wells)
Ampicillin	Penicillin	NA	0.015–16 (11)
Ceftazidime	Cephalosporin	0.5 (1)	0.002–16 (14)
Enrofloxacin	Fluoroquinolones	0.125 (1 and 2)	0.0005–0.25 (9)
Florfenicol	Phenicols	2 (2)	0.03–16 (10)
Gentamicin	Aminoglycosides	2 (2)	0.06–8 (8)
Meropenem	Carbapenem	NA	0.008–1 (8)
Oxolinic acid	Quinolones	0.131 (2)	0.002–1 (10)
Oxytetracycline	Tetracyclines	0.25 (1 and 2)	0.015–8 (7)
Trimethoprim-sulfamethoxazole	Folate pathway antagonist	0.25 (1 and 2)	0.008/0.15–1/19 (8)

Note: Ref.: (1) Lin et al. (2022); (2) Baron et al. (2017).
Abbreviation: NA, not available.

TABLE 9 Complementary information about quality control.

	Quality control	
Antimicrobial agent	*E. coli* (ATCC 25922) MIC (CAMHB)[a]	*Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658) MIC (CAMHB)[a]
Gentamicin	✓	✓
Trimethoprim-sulfamethoxazole	✓	✓
Ampicillin	✓	✓
Ceftazidime		
Meropenem	✓	✓
Florfenicol	✓	✓
Enrofloxacin	✓	✓
Oxolinic acid	✓	✓
Oxytetracycline	✓	✓

[a]QC values are available for Microbroth dilution in Cation Adjusted Muller Hinton Broth and condition of incubation 28 ± °C during 24–28 h – CLSI VetO4 (CLSI, 2020b) Table 4.

7.5 | Laboratory methods for *Vibrio parahaemolyticus* and *V. alginolyticus*

7.5.1 | Overview

To detect *Vibrio* spp. in a given sample, several successive analytical steps are needed. The first step is to isolate presumptive *Vibrio* spp. Screening and confirmation of *Vibrio* spp. are obtained by MALDI-TOF MS and PCR, respectively. Complementary analytical steps, antimicrobial susceptibility testing and WGS are performed to further characterise isolates.

7.5.2 | Isolation methods, confirmatory testing and typing

To ensure that the *V. parahaemolyticus* and *V. alginolyticus* prevalence assessed is comparable, the isolation procedure needs to be harmonised according to the ISO method. *V. parahaemolyticus* and *V. alginolyticus* are detected in four phases. The method recommended includes a pre-enrichment step followed by a second enrichment and an incubation on a chromogenic (CVA) and indicative agar plate (TCBS), followed by a confirmation:

Pre-enrichment increases the proportion of *Vibrio* in the samples (low numbers of *Vibrio* and abundant accompanying flora). Test samples (up to 25 g) are covered in 225 mL of alkaline peptone saline water (APSW) (preheated to 37°C ± 1°C before inoculation with the test sample). The initial suspension is homogenised and incubated at 37°C ± 1°C for 6 ± 1 h.

- A 10 µL loopful of pre-enrichment culture is spread on two solid selective media plates: TCBS medium and a chromogenic medium, such as Chrom agar *Vibrio*, incubated at 37°C ± 1°C for 24 ± 3 h.
- On TCBS plate, presumptive *V. parahaemolyticus* colonies appear as smooth, green (sucrose negative) and of 2–3 mm in diameter. Presumptive *V. alginolyticus* colonies appear as smooth, yellow (sucrose positive) and opaque. On Chrom agar *Vibrio* plate, presumptive *V. parahaemolyticus* colonies appear as mauve in colour, and presumptive *V. alginolyticus* colonies are colourless.
- As the agar is not ultimately specific, presumptive colonies need to be carefully assessed and typed. Only typical *Vibrio*-like colonies (according to the colours) should be used as mentioned. For confirmation, from each plate of each of the selective media, at least five colonies considered to be presumptive or similar to each of the vibrios being tested should be re-cultivated on either CVA or TCBS for purification and can be afterward subjected to MALDI-TOF MS for mass spectrometric analysis (screening). MALDI-TOF results will get better scores if freshly grown bacteria (16–26 h, 37°C). Only *Vibrio* species of interest, *V. parahaemolyticus* and/or *V. alginolyticus*, will be subjected to PCR confirmation using the designated PCRs.

It is of note that vibrios are very sensitive to cold and should not be stored at refrigeration temperatures. Otherwise, they should be kept there for a minimum period of time.

The workflow for isolation of *Vibrio* spp. is illustrated in Figure 1.

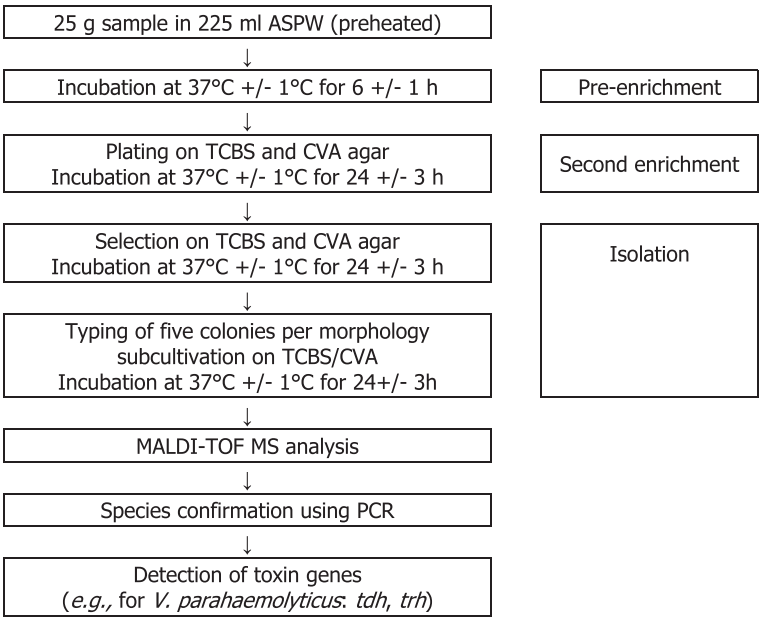

FIGURE 1 Flow chart for isolation and species confirmation of *V. alginolyticus* and *V. parahaemolyticus*.

7.5.3 | Antimicrobial susceptibility testing

The same methods and microplate as for *Aeromonas* spp. should be used (see Table 8).

7.6 | Further typing and whole genome sequencing

Whole genome sequencing (WGS) has already been implemented by several MSs for the specific monitoring of ESBL–/AmpC–/carbapenemase-producing *E. coli* due to the strong added value and the potential of re-analysis in need of translating the data into action. Considering the inherent advantages in the WGS technology, it is proposed to use WGS within the BLS using the protocols developed by the EURL-AR for DNA extraction, DNA quality and quantity assessment, library

preparation, library quality and quantity assessment, WGS and assembly.[25] It is noteworthy to mention that it is not that important how the genome is produced but rather that the genome is of a high quality needed for the down-stream analysis as described in the EURL protocol.

7.6.1 | Multi-locus sequence typing

Sequencing typing of selected *E. coli*, *K. pneumoniae* and *enterococci* recovered during the baseline survey will be performed by determination of multilocus sequence type (MLST). MLST will be performed on selected isolates by deriving the sequence type from the WGS data. This will allow an assessment to be made whether these bacteria belong to lineages which may be associated with humans or belong to important human clones, such as *E. coli* ST131 possessing the ESBL enzyme CTX-M-15. Sequencing typing will also assist in determining whether the occurrence of these bacteria might be linked to anthropogenic pollution of surface waters as well as aiding possible assessment of public health significance.

7.6.2 | Whole genome sequencing for the detection of AMR and virulence genes

WGS will be performed on a subset of *E. coli*, *K. pneumoniae*, *enterococci, Aeromonas*, *V. alginolyticus* and *V. parahaemolyticus* isolates, representing the geographic and genetic diversity within each country. It is proposed that a proportion of 15% of isolates should be sequenced, with the additional rule that, within each stratum, at least one isolate and a maximum of 20 isolates should be sequenced. The objective is to perform sequence typing, species prediction and to search for genetic antimicrobial resistance (AMR) determinants, host adaptation and virulence. It could also be relevant to sequence isolates that are resistant to certain antimicrobials such as those determined as medical important drug classes (3rd- and 4th-generation cephalosporins, carbapenems, quinolones, macrolides, polymyxins, oxazolidinones, glycopeptides among others) or carry certain virulence genes (e.g. encoding α-haemolysin (*hlyA*), *fim*H adhesin in *E. coli* and for vibrios, *tdh* and *trh* among others).

The protocols to be used specifically in the BLS will be proposed by the EURL-AR. Quality assurance should address a number of mandatory quality checks[26] (Tables 8 and 10).

TABLE 10 Quality checks.

Measure	Description
Mean insert size	Mean insert size of the number of base pairs sequenced from a DNA fragment. This element contains integer
Q30 rate	The percentage of bases with a quality score[a] of 30 or higher across the whole read length. This element contains decimal
Total bases	Absolute number of bases sequenced. This value should be calculated after trimming. This element contains integer
Assembly coverage (depth)	Sequence coverage (or depth) is the number of unique reads that include a given nucleotide in the reconstructed sequence. This should be calculated after mapping the sequencing raw reads against the assembly. This element contains decimal Expected to be more than 30X
Assembly N50	The sequence length of the shortest contig at 50% of the total genome length. This element contains integer For each species, it should be equal or more than: • *E. coli*: 80,000 • *Enterococcus faecalis*: 100,000 • *Enterococcus faecium*: 30,000 • *Klebsiella*: 80,000
Assembly total length	Absolute number of bases in the assembly. This element contains integer This is expected to close to the range for genome sizes observed in the species: • *E. coli*: 4.5–5.5 Mbp • *Enterococci*: 2.2–3.5 Mbp • *Klebsiella*: 5.1–5.6 Mbp
Assembly Number of contigs	Number of contigs of the assembly. Contigs are continuous stretches of sequence containing bases without gaps. This element contains integer The number of contigs for each species should be less than: • *E. coli*: 500 • *Enterococcus faecalis*: 750 • *Enterococcus faecium*: 650 • *Klebsiella*: 200 contigs

[a]A quality score is an estimate of the probability of a base being called wrongly by the sequencer and it is calculated as: $q = -10 \times \log_{10}(p)$.

[25]https://www.eurl-ar.eu/CustomerData/Files/Folders/34-wgs/628_protocol-for-wgs-v2-2.pdf.
[26]https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/sp.efsa.2022.EN-7413.

7.6.3 | Bioinformatics analysis

Analysing WGS data, several bioinformatic tools/pipelines are available to detect and characterise AMR traits, of which AMRFinderPlus, ResFinder and CARD (Comprehensive Antibiotic Resistance Database) are recommended by the EURL-AR. Various strategies can be used to perform genotypic determination of AMR genes and to predict AMR profiles as described in the EFSA Journal 17(6):5709 (EFSA, 2019).

To ensure a harmonised computational approach in the detection of AMR genes, its pivotal to search for AMR genes from the same AMR gene catalogue developed by the EURL-AR and EFSA. Thus, the MSs are encouraged to search several of the recommended bioinformatic tool(s)/pipeline(s) to detect the AMR genes listed in the AMR gene catalogue. It is important to note that not all the recommended bioinformatic tool(s)/pipeline(s) contain all AMR genes of the catalogue due to lack of curation; thus, the MSs need to ensure the AMR genes of the catalogue being present in the used bioinformatic tools/pipelines.

Bioinformatics analyses include determining a number of mandatory genotypic characteristics such as multilocus sequence typing (MLST), detection of AMR genes and chromosomal point mutations and detection of other genes associated with host adaptation and virulence (Table 11).

Complementary information will be provided by the EURL-AR to the MSs, as needed, while preparing for the implementation of the BLS so that the MSs can spread the testing all along the year of the survey.

TABLE 11 Genotypic characteristics of interest.

Measure		Description	Tool/database
MLST		–	**PubMLST,** Center for Genomic Epidemiology, Kleborate v2.3.2
AMR genes		–	ResFinder 4.1, AMRFinderPlus v3.12.8, CARD 3.2.9 or newer versions
E. coli		–	ResFinder 4.1, AMRFinderPlus v3.12.8, CARD 3.2.9 or newer versions
Klebsiella		–	Kleborate v2.3.2, ResFinder 4.1, AMRFinderPlus v3.12.8, CARD 3.2.9 or newer versions
Aeromonas		–	ResFinder 4.1, AMRFinderPlus v3.12.8, CARD 3.2.9 or newer versions
enteroccocci		–	ResFinder 4.1, AMRFinderPlus v3.12.8, CARD 3.2.9 or newer versions
tdh		Virulence factor genes encoding the thermostable direct haemolysin (*tdh*) in vibrios	Center for Genomic Epidemiology
trh		Virulence factor genes encoding the thermostable direct haemolysin-related haemolysin (*trh*) in vibrios	Center for Genomic Epidemiology
stx	Shiga toxin, Escherichia	Center for Genomic Epidemiology	
eae	Intimin, Escherichia	Center for Genomic Epidemiology	
cnf	Cytotoxic necrotising factor, Escherichia	Center for Genomic Epidemiology	
espC	Enterotoxin, Escherichia	Center for Genomic Epidemiology	
ehxA	Enterohaemolysin, Escherichia	Center for Genomic Epidemiology	
astA	Heat-stable enterotoxin, Escherichia	Center for Genomic Epidemiology	
ShET	Shigella enterotoxin, Escherichia	Center for Genomic Epidemiology	
entB	Enterobactin, Klebsiella	–	
rmpA	Regulator of mucoid phenotype, Klebsiella	Kleborate v2.3.2	
magA	Mucoviscosity-associated gene, Klebsiella	–	
fimH	Fimbrial adhesin, Klebsiella	–	
khe	Haemolysin, Klebsiella	–	
iucA	Aerobactin, Klebsiella	Kleborate v2.3.2	
iroB	Salmochelin, Klebsiella	Kleborate v2.3.2	
rmpA2	Hypermucoid phenotype, Klebsiella	Kleborate v2.3.2	
ybt	Yersiniabactin, Klebsiella	Kleborate v2.3.2	
clb	Genotoxin colibactin, Klebsiella	Kleborate v2.3.2	

(Continues)

TABLE 11 (Continued)

Measure		Description	Tool/database
esp	Surface protein, Enterococcus	Center for Genomic Epidemiology	
gelE	Gelatinase, Enterococcus	Center for Genomic Epidemiology	
hyl	Hyaluronidase, Enterococcus	Center for Genomic Epidemiology	
ace	Collagen binding adhesin, Enterococcus	Center for Genomic Epidemiology	
cyl	Cytolysin, Enterococcus	Center for Genomic Epidemiology	
asa1	Aggregation substance, Enterococcus	–	

7.7 | Storage of strains

Isolates verified as *E. coli*, *Aeromonas* spp., *V. parahaemolyticus*, *V. alginolyticus* and enterococci should be saved and stored under conditions not allowing changes in their properties at −80°C at least for 5 years. This is to allow, for instance, later testing for antimicrobial susceptibility or other types of characterisations if requested by the EFSA or for research or other purposes at a national or European level. Isolates sent to the EURL-AR will also be stored for a minimum of 5 years.

8 | DATA REPORTING

8.1 | Overall description on the implementation of the BLS

Three sets of data are to be reported to cover all information collected during sampling and analysis:

- Prevalence sample-based data model: It includes detailed analytical results of all samples taken (whether positive or negative) reported using the EFSA standard for reporting laboratory results: Standard Sample Description version 2 (SSD2).
- AMR isolate-based data model: It includes isolate-level quantitative antimicrobial resistance and WGS data reported according to the antimicrobial resistance (AMR) data model of EFSA.
- Population data model: Annual throughput of slaughterhouses involved in the survey and fish population size of the farms of origin.

The laboratory isolate code is to be used to link the data reported in the prevalence sample-based (SSD2) data model with those reported in the AMR isolate-based data model. The slaughterhouse identification code and the holding identification code will be used to link the analytical results of sampled to the population data model.

Potential slight alterations and supplements to the data reporting model may still occur while implementing it at EFSA and they will be communicated to the reporting countries considering the appropriate time for implementing them.

8.2 | Prevalence sample-based data

The EU MSs/participating countries can use the SSD2 data model to report sample-based zoonoses and zoonotic agent data to the data collection system of EFSA. Specific guidance to report information under the framework of Directive 2003/99/EC, Regulation (EU) 2017/625, Commission Implementing Regulation (EU) 2019/627 and of Commission Delegated Regulation (EU) 2018/772 is published annually by EFSA. Analytical results for BLS samples and some additional information will be requested in the context of the BLS will be reported to the EFSA's data collection system (Table 12).

The full list of data elements relevant for the BLS is presented below together with the default (fixed) value to be used in the context of the BLS, where applicable. The detailed description of all the data elements will be found in the prevalence sample-based guidance (EFSA, 2022).

The sampling unit for the BLS is the batch (fish/mussels/PA), and therefore, all elements at the sampling unit level (e.g. sampEventId) refer to a batch sampled at a given time in a given farm/slaughterhouse/dispatch centre.

The data will be reported at the level of the individual analytical results, e.g. if one pooled sample undergoes only one test for screening in the laboratory, one line will be reported for this sample, but if the screening test is positive and the samples undergo further testing, one additional line will be reported for each additional test. As a result, several lines will be reported per batch (at least one line per pooled sample) and possibly several lines will be reported per pooled sample. In this context, each analytical results can be reported with the exact date on which it was performed.

8.3 | AMR isolate-based data

MSs can use the AMR data model to report isolate-level quantitative antimicrobial resistance and WGS data to the data collection system of EFSA. Guidance to report such data under the framework of Directive 2003/99/EC and Commission Implementing Decision 2020/1729/EC is published annually. Isolate-level quantitative antimicrobial resistance results for bacteria targeted samples can already be reported to the EFSA's data collection system following the instructions provided there.

In the context of the BLS, the data model will be extended to receive WGS results of the targeted bacteria isolates. The full list of data elements is presented below together with the default (fixed) value to be used in the context of the BLS, where applicable (Table 13). The detailed description of all the data elements will be found in the guidance to be drafted for the purpose of the BLS.

8.4 | Population data

To improve the analysis that will be performed with the BLS data, MSs are requested to provide information about the fish population in the involved slaughterhouses/farms and in the farms of origin of the sampled slaughter batches. In particular, they should report the annual throughput of each slaughterhouse/farm and the number of animals in each PU of origin following the data model in the table below. This data model described below (Table 14) is a simplified version of a data model developed by EFSA to report animal population at the establishment level (EFSA, 2022).

TABLE 12 SSD2 data model for sample-based prevalence data collection.

Element code	Element/attribute label	Element/attribute name	Type	Constraint	Catalogue	Hierarchy
B.03	Sampling strategy	sampStrategy	xs:string (5)	Mandatory	SAMPSTR	zooSampstr
B.04	Programme type	progType	xs:string (5)	Mandatory	PRGTYP	zooSampContext
B.05	Sampling method	sampMethod	xs:string (5)	Madoatory	SAMPMD	
B.06	Sampler	sampler	xs:string (5)	Mandatory	SAMPLR	
B.07	Sampling point	sampPoint	xs:string (5)	Mandatory	SAMPNT	zooss
C.01	Sampling event identification code	sampEventId	xs:string (100)	Mandatory		
C.02	Sampling unit type	sampUnitType	xs:string (5)	Mandatory	SAMPUNTYP	
C.03	Sampling unit size	sampUnitSize	xs:double	Mandatory		
C.04	Sampling unit size unit	sampUnitSizeUnit	xs:string (5)	Mandatory	UNIT	
C.05	Other sampling unit identifications	sampUnitIds	CompoundType	Mandatory		
C.05	Slaughter batch identification code	sampUnitIds.batchId	xs:string (250)	Mandatory		
C.05	Slaughterhouse identification code	sampUnitIds.slaughterHouseId	xs:string (250)	Mndatory*	ESTABLISHMENTS	
C.05	Sampling holding identification code	sampUnitIds.sampHoldingId	xs:string (250)	Mndatory*	ESTABLISHMENTS	
C.05	Sampling farm identification code	sampUnitIds.sampSubUnitId	xs:string (250)	Mndatory*	ESTABLISHMENTS	
D.01	Sample taken identification code	sampId	xs:string (100)	Mandatory		
D.02	Reporting country	repCountry	xs:string (2)	Mandatory	COUNTRY	EUSRrepCountry
D.03	Country of sampling	sampCountry	xs:string (2)	Mandatory	COUNTRY	
D.04	Area of sampling	sampArea	xs:string (5)	Optional	NUTS	nuts2024
D.05	Reporting year	repYear	xs:integer (4)	Mandatory		
D.06	Year of sampling	sampY	xs:integer (4)	Mandatory		
D.07	Month of sampling	sampM	xs:integer (2)	Mandatory		
D.08	Day of sampling	sampD	xs:integer (2)	Mandatory		
D.09	Sample taken size	sampSize	xs:double	Mandatory		
D.10	Sample taken size unit	sampSizeUnit	xs:string (5)	Mandatory	UNIT	
E.01	Type of matrix	sampMatType	xs:string (5)	Mandatory	MTXTYP	
E.02	Coded description of the matrix of the sample taken	sampMatCode	CompoundType	Mandatory	FoodEx2	
E.04	Country of origin of the sample taken	origCountry	xs:string (2)	Mandatory	COUNTRY	

TABLE 12 (Continued)

Element code	Element/attribute label	Element/attribute name	Type	Constraint	Catalogue	Hierarchy
F.03	Year of analysis	analysisY	xs:integer(4)	Mandatory		
F.04	Month of analysis	analysisM	xs:integer (2)	Mandatory		
F.05	Day of analysis	analysisD	xs:integer (2)	Mandatory		
H.01	Sample analysed portion sequence	anPortSeq	xs:string (100)	Mandatory		
I.01	Isolate identification	Isolate identification	xs:integer (20)	Mandatory*		
J.01	Laboratory identification code	labId	xs:string (50)	Mandatory		
K.01	Type of parameter	paramType	xs:string (5)	Mandatory	PARAMTYP	
K.02	Coded description of the parameter	paramCode	xs:string (15)	Mandatory	PARAM	microParam
L.02	Analytical method reference code	anMethRefCode	xs:string (5)	Mandatory	ANLYREFMD	
L.03	Analytical method type	anMethType	xs:string (5)	Mandatory	ANLYTYP	
L.04	Analytical method code	anMethCode	xs:string(5)	Mandatory	ANLYMD	prvam
M.01	Result identification code	resId	xs:string (100)	Mandatory		
M.15	Result qualitative value	resQualValue	xs:string (3)	Mandatory	POSNEG	
M.16	Type of result	resType	xs:string (3)	Mandatory	VALTYP	
N.03	Type of limit for the result evaluation	evalLimitType	xs:string (5)	Mandatory	LMTTYP	
N.04	Evaluation of the result	evalCode	xs:string (5)	Mandatory		
N.06	Sample taken assessment	evalInfo.sampTkAsses	xs:string (5)	Mandatory	RESEVAL	
N.06	Sampling event assessment	evalInfo.sampEventAsses	xs:string (5)	Mandatory	RESEVAL	
	Amendment type	amType	xs:string (1)	Mandatory*		

Note: Mandatory* are optional in the schema (XSD file), but mandatory to be reported in certain circumstances (described in the business rules).

TABLE 13 EFSA data model for isolate-based antimicrobial resistance data reporting.

Element code	Element label	Element name (for XML transfer)	Type	Constraint	Catalogue	Hierarchy
AMR.01	Result code	resultCode	xs:string(100)	Mandatory		
AMR.02	Reporting year	repYear	xs:integer(4)	Mandatory		
AMR.03	Reporting country	repCountry	xs:string(2)	Mandatory	COUNTRY	EUSRrepCountry
AMR.05	Zoonotic agent	zoonosis	xs:string(4000)	Mandatory	PARAM	serovarsamr
AMR.06	Matrix	Matrix	xs:string(4000)	Mandatory	ZOO_CAT_MATRIX	
AMR.07	Total units tested	totUnitsTested	xs:integer(10)	Mandatory		
AMR.49	Total units positive	totUnitsPositive	xs:integer(10)	Mandatory		

(Continues)

TABLE 13 (Continued)

Element code	Element label	Element name (for XML transfer)	Type	Constraint	Catalogue	Hierarchy
AMR.45	Sampling unit type	sampUnitType	xs:string(5)	Mandatory	UNIT	amrsmpUn
AMR.08	Sampling stage	sampStage	xs:string(5)	Mandatory	SMPNT	zooss
AMR.46	Sample origin	sampOrig	xs:string(2)	Mandatory	COUNTRY	
AMR.09	Sample type	sampType	xs:string(5)	Mandatory	ZOO_CAT_SMPTYP	
AMR.10	Sampling context	sampContext	xs:string(5)	Mandatory	PRGTYP	zooSampContext
AMR.11	Sampler	sampler	xs:string(5)	Mandatory	SMPLR	
AMR.12	Programme code	progCode	xs:string(7)	Mandatory	AMRPROG	
AMR.13	Sampling strategy	progSampStrategy	xs:string(5)	Mandatory	SAMPSTR	
AMR.14	Sampling details	sampDetails	xs:string(2000)	Optional		
AMR.15	Area of sampling	sampArea	xs:string(5)	Optional	NUTS	nuts2024
AMR.16	Laboratory identification code	labCode	xs:string(100)	Mandatory		
AMR.17	Laboratory isolate code	labIsolCode	xs:string(20)	Mandatory		
AMR.19	Sampling year	sampY	xs:integer(4)	Mandatory		
AMR.20	Sampling month	sampM	xs:integer(2)	Mandatory		
AMR.21	Sampling day	sampD	xs:integer(2)	Mandatory		
AMR.22	Isolation year	isolY	xs:integer(4)	Mandatory		
AMR.23	Isolation month	isolM	xs:integer(2)	Mandatory		
AMR.24	Isolation day	isolD	xs:integer(2)	Mandatory		
AMR.25	Susceptibility test year	analysisY	xs:integer(4)	Mandatory		
AMR.26	Susceptibility test month	analysisM	xs:integer(2)	Mandatory		
AMR.27	Susceptibility test day	analysisD	xs:integer(2)	Mandatory		
AMR.56	Sequencing year	seqY	xs:integer(4)	Mandatory*		
AMR.57	Sequencing month	seqM	xs:integer(2)	Mandatory*		
AMR.58	Sequencing day	seqD	xs:integer(2)	Mandatory*		
AMR.28	Method	anMethCode	xs:string(5)	Mandatory	ANLYMD	amram
AMR.29	Antimicrobial substance	substance	xs:string(15)	Mandatory	PARAM	AMRSub
AMR.30	Cut-off value	cutoffValue	xs:double	Mandatory		
AMR.31	Lowest limit	lowest	xs:string(5)	Mandatory	ZOO_CAT_FIXMEAS	number
AMR.32	Highest limit	highest	xs:string(5)	Mandatory	ZOO_CAT_FIXMEAS	number
AMR.33	MIC value (mg/L)	MIC	xs:string(5)	Mandatory	ZOO_CAT_FIXMEAS	mic
AMR.58	Genotype	genotype	xs:string(4000)	Mandatory*		
AMR.48	Performed MLST characterisation	perMLST	xs:string(1)	Mandatory	YESNO	zoo
AMR.55	Sequencing technology used	seqTech	xs:string(2000)	Mandatory*	INSTRUM	

TABLE 13 (Continued)

Element code	Element label	Element name (for XML transfer)	Type	Constraint	Catalogue	Hierarchy
AMR.37	Comment	resComm	xs:string(2000)	Optional		
AMR.51	Amendment type	amType	xs:string(1)	Mandatory*		

Note: Mandatory* are optional in the schema (XSD file), but mandatory to be reported in certain circumstances (described in the business rules).

TABLE 14 EFSA data model for animal population data reporting.

Element code	Element label	Element name (for XML transfer)	Type	Constraint	Catalogue	Hierarchy
POP.00	Record unique identifier	*recordId*	xs:string(100)	Mandatory		
POP.01	Reporting country	repCountry	xs:string(2)	Mandatory	COUNTRY	EUSRrepCountry
POP.02	Year of extraction of census data	*recordCensusY*	xs:integer(4)	Mandatory		
POP.03	Month of extraction of census data	*recordCensusM*	xs:integer(2)	Mandatory		
POP.04	Day of extraction of census data	*recordCensusD*	xs:integer(2)	Optional		
POP.05	Establishment identification code	*establd*	xs:string(200)	Mandatory	ESTABLISHMENTS	
POP.06	Area of the establishment	*estabArea*	xs:string (5)	Optional	NUTS	nuts2024
POP.07	Type of establishment	*estabType*	xs:string(5)	Mandatory	SAMPNT	
POP.08	Subunit identification code	*subUnitId*	xs:integer(200)	Mandatory*		
POP.09	Area of the subunit	*subUnitArea*	xs:string (5)	Optional	NUTS	nuts2024
POP.10	Subunit species	*subUnitSpecies*	xs:string(5)	Mandatory		
POP.11	Type of farm from which the slaughter batch sampled comes from	*subUnitPurpType*	xs:integer(10)	Mandatory		
POP.12	Capacity of each farm	*subUnitCapacity*	xs: integer(200000)	Optional		
POP.13	Size of farms/slaughterhouses	*subUnitActualNumber*				
POP.14	Amendment type	amType	xs:string(1)	Mandatory*		

Note: Mandatory* are optional in the schema (XSD file), but mandatory to be reported in certain circumstances (described in the business rules).

ABBREVIATIONS

AMC	Antimicrobial consumption
AMR	Antimicrobial resistance
AST	Antimicrobial Susceptibility Testing
BLS	Baseline Surveys
BSe	Batch sensitivity
CBP	Clinical breakpoint
CP	Carbapenemase
DC	Dispatch Centre
DCF	Data Collection Framework
ECOFF	Epidemiological Cut-Off Value
EFTA	European Free Trade Association
ESBL	Extended-spectrum beta-lactamases
EURL-AR	EU Reference Laboratory for Antimicrobial Resistance
MALDI-TOF MS	Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry
MIC	Minimum inhibitory concentration
MS	Member States
MLST	multi-locus sequence type
PA	Production area
PB	Production batch
PU	Production unit
QC	Quality control
SSD2	Standard sample description version 2
TSe	Test sensitivity
TSp	Test specificity
WGS	Whole Genome Sequencing
WOAH	World Organisation for Animal Health

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

QUESTION NUMBER

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APPENDIX A

Protocol for designing technical specifications for a BLS on AMR in aquaculture animals

A.1 | INTRODUCTION

A.1.1 | Introduction and scope of this protocol

This document outlines the protocol developed for the assessment to be performed for the EFSA Scientific Report on the technical specifications for a BLS on AMR in aquaculture animals.

The aim of this document was to define the methods for collecting data, appraising the relevant evidence and analysing and integrating the evidence in the light of the identified uncertainties. It was developed following the principles and process defined in a project that aimed to further improve EFSA's scientific assessment processes and based on the recommendations for protocol development described in the draft framework for protocol development for EFSA's scientific assessments.

The protocol was drafted by the WG.

A.1.2 | Terms of Reference (TOR) as provided by the requestor

The European Commission requests scientific and technical assistance from EFSA to provide technical and scientific support for the development of a baseline survey as regards AMR in aquaculture animals considering the most recent scientific literature and technological developments, epidemiological trends and relevance for public health.

In particular, EFSA is asked to propose harmonised approaches for the collection and the analysis of AMR situation in aquaculture animals by:

- proposing priority combinations of aquaculture animals/target bacteria to be considered in the BLS;
- proposing a complete sampling framework for the implementation of the BLS including the origins of bacterial isolates subject to AMR testing, the sampling design and the sample size;
- proposing protocols for isolation and characterisation of bacteria;
- proposing protocols for phenotypical antimicrobial susceptibility testing of bacterial isolates;
- proposing protocols for the testing of bacterial isolates via molecular typing methods;
- providing guidance for technical reporting of the BLS data collected by Member States to EFSA.

A.2 | PROBLEM FORMULATION

A.2.1 | Assessment questions based on the interpretation of the mandate

Step 1 consists of the translation of the mandate into assessment question(s) (AQs) (step 1.1) and the definition of the subquestions (SQs) (step 1.2) of each assessment question and their relationship (conceptual model) where appropriate.
Table A.1 provides, for each of the ToR, the translation of the mandate into AQs as included in the second column (step 1.1). For the current mandate and protocol, as the AQs were already narrow, there was no need to further split them into SQs, as indicated in the third column (step 1.2).

The approach for each AQ, i.e. whether to apply a quantitative, qualitative or semi-quantitative approach, has been specified in the fourth column (step 1.3).

A.3 | METHODS FOR CONDUCTING THE ASSESSMENT

The second step includes the overall approach (step 2.1) as well as the evidence needs and the methods (step 2.1) for answering each AQ including uncertainty analysis (i.e. the use of a literature review, data from databases, expert judgement or primary data collection). Table A.1 provides this information in the fifth and sixth columns.

Step 2.2 (defining the methods for integrating evidence across subquestions) is not applicable for this protocol as no SQs have been defined.

TABLE A.1 Assessment of questions to propose harmonised approaches for the collection and the analysis of AMR data in bacteria from aquaculture animals.

ToRs	Step 1.1 Assessment questions (reflecting clarification of ToRs)	Step 1.2 Subquestions	Step 1.3 Approach to be followed	Step 2.1 Overview methods	Step 2.1 Evidence needs and methods
ToR1. Proposing priority combinations of **aquaculture animals/ target bacteria** to be considered in the BLS.	**AQ1a.** What shall be the **bacteria of interest**? **AQ1b.** What shall be the **aquaculture animals** of interest? **AQ1c.** What shall be the **combinations of interest**?	None	Qualitative (AQ1a to AQ1c)	**Literature review** and **expert judgement and experience**	**AQ1a. Literature review + expert judgement:** Based on: a) a review of the **ASK report**, used as starting point, followed by b) a **review of the literature** (see Appendix 1), to update the previous one, and complemented by c) the judgement and experience of experts, condensed into a series of scientific, technical and practical **criteria** (see Appendix 2), the WG will select the **bacteria of interest** **AQ1b.** Based on a review of the publicly available data on aquaculture production by animal species (e.g. EUMOFA database, STECF-22-17 report), the availability of antimicrobial consumption data in aquaculture species at EMA by 2027 and the judgement and experience of experts, the WG will consider the aquaculture species the most frequently produced within Europe and select the **aquaculture animals of interest** **AQ1c.** Based on considerations related to AQ1a and AQ1b, the WG will consider pros and cons for selecting **combinations of interest**
ToR2. Proposing a complete **sampling framework** for implementing the BLS, including the **origins of bacterial isolates** subject to AMR testing, the **sampling design** and the **sample size**	**AQ2a.** What shall be the **sampling stages**? **AQ2b.** What shall be the **sample types**? **AQ2c.** What shall be the **sampling design**? **AQ2d.** What shall be the **sample size**?	None	Qualitative (AQ1 and AQ3) Semiquantitative (AQ2) Quantitative (AQ4)	**Literature review** and **expert judgement and experience**	**AQ2a.** Based on a review of the literature and the judgement and experience of experts, the WG will consider pros and cons and defined the sampling stages for performing each part of the BLS either on the farm, at the slaughterhouse, at the production area or at the dispatch centre. **AQ2b.** Based on a review of the literature and the judgement and experience of experts, the WG will consider pros and cons for selecting the **sample types** to be collected for each part of the BLS. **AQ2c.** Based on a review of the literature, the judgement and experience of experts, and the experience of some baseline surveys previously performed (e.g. BLS on MRSA and BLS on norovirus) and practical constraints, the WG will define a representative **sampling design** of each part of BLS. The main objective is to assess the occurrence of AMR in the selected bacteria from batches of aquaculture species at the EU level, considering the hierarchical structure of the data. **AQ2d.** The WG will perform the calculation of the sample size needed to assess the occurrence of AMR with a given accuracy in considering the hierarchical structure of the data. Some parameters will be assessed based on some data sets

(Continues)

TABLE A.1 (Continued)

ToRs	Step 1.1 Assessment questions (reflecting clarification of ToRs)	Step 1.2 Subquestions	Step 1.3 Approach to be followed	Step 2.1 Overview methods	Step 2.1 Evidence needs and methods
ToR3. Proposing protocols for **isolation** and **characterisation of bacteria**	**AQ3.** Which is the harmonised protocols for isolation and characterisation of: – *E. coli*, – Aeromonas, … from aquaculture animals?	None	Qualitative	Literature review and expert judgement	**The methodology described below will be applied** **a. Eligibility criteria for study selection:** Aim: To find information on the most up to date methodology to be used for the isolation of bacterial isolates and for their further characterisation **b. Search strategy**: A literature search will be carried out in the Web of Science™ Core Collection PubMed, and/or Google Scholar to retrieve information Apart from the literature search, relevant documents (other published reports from other national and international agencies) will also be identified and reviewed, based on the knowledge and expertise of the WG on the subject **c. Methods for selecting studies for inclusion/exclusion**: The screening process will be undertaken in three subsequent steps: screening of (1) titles, (2) abstracts and (3) full-text documents to further identify records relevant to data needs in relation to the assessment **d. Methods for extracting data from included studies**. Selected full-text documents will be screened by the WG experts in charge of the different sections (appointed based on their expertise) to extract the relevant information needed **e. Methods for appraising evidence.** This will be done in a narrative way based on expertise of WG members **f. Sources of uncertainty and definition of the methods for prioritising them**. The uncertainty will mainly be linked to the quality and quantity of data retrieved from the literature search. Where possible, intrinsic characteristics (Sensitivity and Specificity) of the isolation methods will be assessed and compared **g. Methods for synthesising evidence**. The methods used for the synthesis will be qualitative **h. Methods for analysing uncertainties**. There is no need to plan beforehand

TABLE A.1 (Continued)

ToRs	Step 1.1 Assessment questions (reflecting clarification of ToRs)	Step 1.2 Subquestions	Step 1.3 Approach to be followed	Step 2.1 Overview methods	Step 2.1 Evidence needs and methods
ToR4. Proposing protocols for phenotypical **antimicrobial susceptibility testing** of bacterial isolates	**AQ4.** Which is the harmonised protocol for antimicrobial susceptibility testing (**AST**) of **bacteria isolates**?	None	Qualitative	**Literature review** and **expert judgement**	**The methodology described above for AQ3 will be applied.** The search described for AQ2 will be also used to retrieve information reported in the literature on AST methodology. Additional searches will be done if considered necessary along the development of the Scientific Report. **AQ4.** The WG will gather information on the up-to-date methodology to perform AST in **bacteria selected** and the antimicrobials that should be tested. In particular, the previous EFSA report (EFSA, 2019) and other published reports from the EURL-AR and from other national and international agencies will also be identified and reviewed, based on the knowledge and expertise of the WG and EFSA staff experts on the subject
ToR5. Proposing protocols for the testing of bacterial isolates via **molecular typing methods**	**AQ5**	None	Qualitative	**Literature review** and **expert judgement**	**AQ5.** The WG will identify information on the up-to-date methodology to perform WGS of isolates of **bacteria selected** to provide information on selected targets
ToR6. Providing guidance for **technical reporting** of the BLS data collected by Member States to EFSA	**AQ5:** How shall the **data be reported** to EFSA?	None	Qualitative	Expert and EFSA knowledge	**AQ5**: The WG has defined the **reporting framework** and the **data elements** to be reported. This will be done considering **previous reports**, **expert knowledge** and the **existing EFSA data models** at the sample and isolate levels

APPENDIX B

Aquaculture production data of fishery products in Europe in 2021

Aquaculture involves the controlled cultivation of fish, molluscs and crustaceans. In 2021, an estimated 1.1 million tonnes of aquatic organisms were farmed in the EU,[27] valued at €4.2 billion. Four EU Member States accounted for more than two-thirds (68%) of the total production of farmed aquatic organisms in 2021: Spain 25%, France 17% and both Italy and Greece 13%. Aquaculture production within the EU was lower than that in Norway, where 1.6 million tonnes of aquatic organisms were produced, most of which was farmed salmon (Figures B.1–B.3).

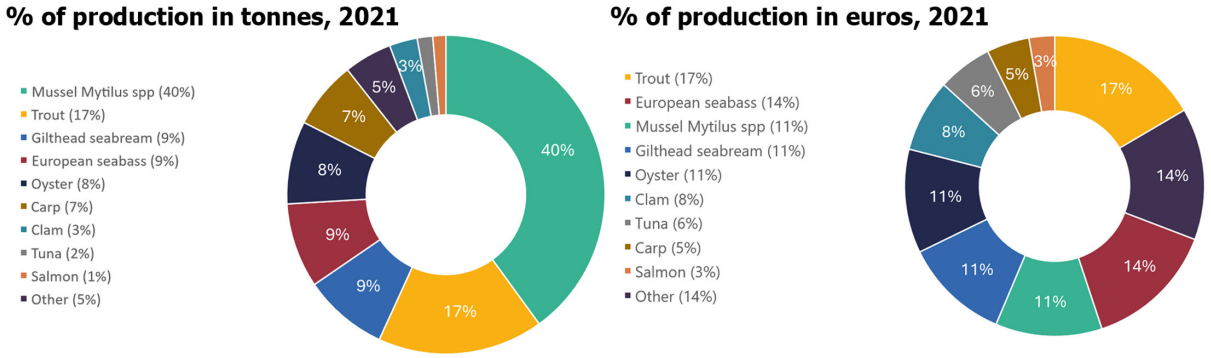

FIGURE B.1 Main species in aquaculture production in the EU: Percentages of production in tonnes and in euros, EUMOFA, 2021.

Production in the EU is focused primarily on finfish species (such as trout, seabream, seabass, carp, tuna and salmon) and molluscs (including mussels, oysters and clams), which together accounted for almost all the aquaculture production by weight in 2021. Different aquatic organisms command different prices. The production value of trout and seabass in 2021 was higher than other species in the EU (each accounting for a 14% share of the total value of the EU's aquatic farming in 2021).

Regarding shellfish aquaculture production, mussel is produced in 13 MSs, considering together the species blue mussel and Mediterranean mussel. Four MSs (ES, FR, IT, NL) produce nearly 90% of the EU production of mussels.

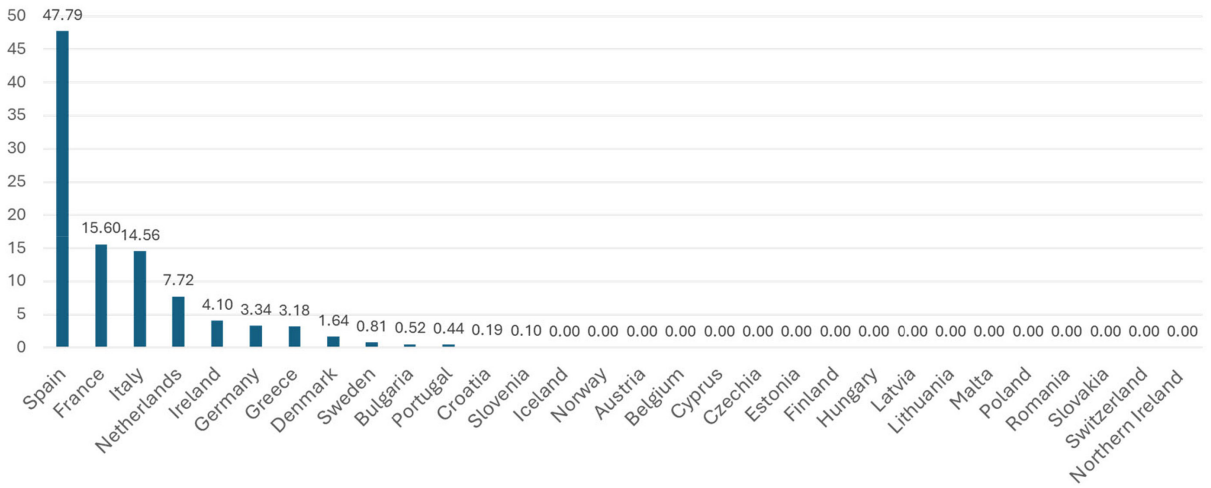

FIGURE B.2 Distribution of mussel production among EU/EFTA Member States (%), EUMOFA, 2021.
Northern Ireland: In accordance with the Agreement on the withdrawal of the United Kingdom of Great Britain and Northern Ireland from the European Union and the European Atomic Energy Community, and in particular Article 5(4) of the Windsor Framework in conjunction with Annex 2 to that Framework, for the purposes of this report, references to Member States include the United Kingdom in respect of Northern Ireland.

Regarding marine finfish production, the production of seabass is reported in eight MSs (HR, CY, EL, IT, MT, PT, SI, ES) and the production salmon is reported in three MSs (IE, FI, SE) and in NO. Norway is by far the main producer of salmon in Europe.

Regarding freshwater finfish production, the production of varying kinds of trout is reported in 23 MSs as well as in Norway and Iceland, considering together the rainbow trout, brook trout, sea trout. Norway is by far the main producer of trout in Europe. It is also of note that some sea trout are produced at sea and should therefore not be considered within the part of the BLS on freshwater finfish (e.g. in DK, SE, FI or FR) but may be considered within the framework of the sampling of marine finfish production, on a voluntary basis.

[27] Sources: Eurostat/EUMOFA for the year 2021.

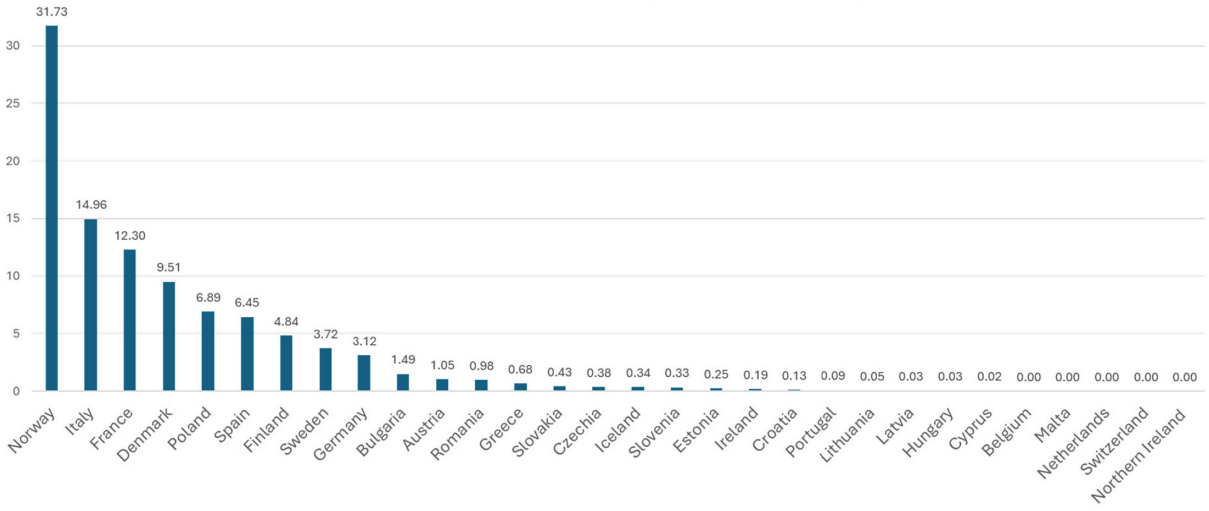

FIGURE B.3 Distribution of trout production among EU/EFTA Member States (%), EUMOFA 2021.
Northern Ireland: In accordance with the Agreement on the withdrawal of the United Kingdom of Great Britain and Northern Ireland from the European Union and the European Atomic Energy Community, and in particular Article 5(4) of the Windsor Framework in conjunction with Annex 2 to that Framework, for the purposes of this report, references to Member States include the United Kingdom in respect of Northern Ireland.

APPENDIX C

Outcome of the literature review on *E. coli* and enterococci

TABLE C.1 Studies investigating the occurrence of AMR in *Escherichia coli* in shellfish and finfish produced in Europe.

	Epidemiological information					Laboratory information				Microbiological information		
Reference	Seafood species	Sample type	Total number of samples	Country	Sample collection year	Isolation	AST methods (BMD, DD, Etest)	Criteria used for AST interpretation (EUCAST/CLSI; ECOFF/CB; year)	Cut-off	Number (%) of samples positive for *E. coli*	Number of isolates	Occurrence of resistance (%)
Vignaroli et al. (2016)	Clam (*Chamelea gallina)*	Flesh and intravalvular liquid	77	Italy	2013–2014	Homogenate obtained from 100 g of flesh and intravalvular liquid was used to enumerate *E. coli* using a most probable number (MPN) method, according to the EU reference method ISO TS 1664	DD	CLSI	Clinical Breakpoints	77 (100)	141	Ampicilllin, 17 Chloramphenicol, 2 Ciprofloxacin, 2.8 Gentamicin, 0.7 Nalidixic acid, 6 Streptomycin, 14 Sulfamethoxazole, 8 Tetracycline, 25
Grevskott et al. (2017)	Blue mussels (Mytilus edulis) Flat oysters (Ostrea edulis) Great scallops (Pecten maximus) Carpet shells (Mya arenaria) Northern horse mussels (Modiolus modiolus)	Flesh and intravalvular liquid	Blue mussels (Mytilus edulis), 447 Flat oysters (Ostrea edulis), 40 Great scallops (Pecten maximus), 39 Carpet shells (Mya arenaria), 12 Northern horse mussels (Modiolus modiolus), 11	Norway	2014–2015	A standardised most probable number (MPN) reference method for enumeration of *E. coli* in bivalves with minerals modified glutamate broth as growth media in combination with verification on Tryptone Bile with X-glucuronide agar	DD	EUCAST	Clinical breakpoints	Not reported (though 61% of samples was culture positive and *E. coli* were 180 out of 199 bacterial isolates)	180	A total of 75 (38%) of the 199 isolates showed resistance to at least one antibacterial agent. The majority were *E. coli* but precise numbers are not reported. Overall occurrence of resistance: Aminoglycosides, 16 Ampicillin, 48 Amoxicillin, 79 Amphenicols (5%), Extended-spectrum penicillins, 83 Nitrofurans, 5 Quinolones, 5 Sulfonamides, 11 Tetracyclines, 8 Third-generation cephalosporins, 7 Trimethoprim, 13

TABLE C.1 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	**Seafood species**	**Sample type**	**Total number of samples**	**Country**	**Sample collection year**	**Isolation**	**AST methods (BMD, DD, Etest)**	**Criteria used for AST interpretation (EUCAST/CLSI; ECOFF/CB; year)**	**Cut-off**	**Number (%) of samples positive for *E. coli***	**Number of isolates**	**Occurrence of resistance (%)**
Svanevik et al. (2023)	Blue mussels (Mytilus edulis) Flat oysters (Ostrea edulis) Great scallops (Pecten maximus) Northern horse mussels (Modiolus modiolus) Pacific oysters (Crassostrea gigas) Ocean quahogs (Arctica islandica) Soft-shell clams (Mya arenaria)	Flesh and intravalvular liquid	Blue mussels (Mytilus edulis), 312 Flat oysters (Ostrea edulis), 38 Great scallops (Pecten maximus), 26 Northern horse mussels (Modiolus modiolus), 6 Pacific oysters (Crassostrea gigas), 3 Ocean quahogs (Arctica islandica), 3 Soft-shell clams (Mya arenaria), 2	Norway	2016	All samples were quantitatively assessed for *E. coli* applying the standard Most Probable Number (MPN) method providing concentrations as MPN/100 g (ISO 16649-3, 2005). 25 g of bivalve soft tissue and mantel water (10–15 individuals) were homogenised (2 min) prior to the addition of 225 mL of BPW followed by a new round of homogenisation (30 s). The homogenate was enriched at 37 ± 1°C for 20 ± 2 h. After incubation, a loop-full (10 μL) from the enrichment broth was transferred to the following plates to detect: (1) *E. coli* on plain MacConkey agar, (2) ESC-resistant *E. coli* on MacConkey agar with 1 mL/L cefotaxime and MacConkey agar with 2 mg/L ceftazidime, (3) QREC on MacConkey agar with 0.06 mg/L ciprofloxacin. All plates were incubated at 44 ± 0.5°C for 20 ± 2 h. Additionally, to detect carbapenem-resistant *E. coli*, a loop-full (10 μL) from the enrichment was transferred to CHROMID CARBA and CHROMID OXA-48 and incubated at 37 ± 0.5°C for 20 ± 2 h	BMD	EUCAST	ECOFF	261 (67)	391	Non-selective screening: Full susceptibility, 92 Ampicillin, 5 Cephalosporins, 0 Quinolone, 0.8 Sulfamethoxazole, 3 Tetracycline, 6 ESBL-selective screening: ESBL-EC, 13 Quinolone selective screening: QR-EC, 12

(Continues)

TABLE C.1 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	Seafood species	Sample type	Total number of samples	Country	Sample collection year	Isolation	AST methods (BMD, DD, Etest)	Criteria used for AST interpretation (EUCAST/CLSI; ECOFF/CB; year)	Cut-off	Number (%) of samples positive for *E. coli*	Number of isolates	Occurrence of resistance (%)
Leoni et al. (2023)	Clam (Venus gallina)	Flesh and intravalvular liquid	308	Italy	2018–2019	Bivalve molluscs were externally cleaned with running potable water; then, the flesh and liquor of the bivalve molluscs were aseptically collected, diluted, homogenised, and further diluted in a 0.1% sterile peptone water to achieve a final suspension of 1:10. Subsequent decimal dilutions were prepared in a 0.1% sterile peptone solution. *E. coli* enumeration on bivalve molluscs was performed by a most probable number (MPN) method according to ISO 16649-3. ESBL-/AmpC-producing *E. coli* were isolated by streaking over the surface of MacConkey (MC) agar plates supplemented with 1 μg/mL of cefotaxime enriched broths of the initial shellfish suspension in double-strength mineral-modified glutamate (MMGB) broth from the *E. coli* enumeration method. Inoculated MC agar plates with cefotaxime were incubated at 37°C ± 1°C for 24 h ± 2 h in aerobic conditions	DD, BMD	EUCAST	Clinical Breakpoints (though CLSI breakpoints for nalidixic acid and tetracycline)	Samples positive for ESBL-producing *E. coli*, 10 (3)	11	Azithromycin, 15 Chloramphenicol, 27 Gentamicin, 18 Nalidixic acid, 54 Sulfamethoxazole, 82 Tetracycline, 54 Trimethoprim, 36 Colistin, ertapenem, imipenem, meropenem, tigecycline, temocillin: 0

TABLE C.1 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	Seafood species	Sample type	Total number of samples	Country	Sample collection year	Isolation	AST methods (BMD, DD, Etest)	Criteria used for AST interpretation (EUCAST/CLSI; ECOFF/CB; year)	Cut-off	Number (%) of samples positive for *E. coli*	Number of isolates	Occurrence of resistance (%)
Salgueiro et al. (2021)	Clams Japanese oysters Mussels	Bivalve molluscs	Clams, 2 Japanese oysters, 4 Mussels, 6	Portugal	2019	50 g of each sample were homogenised in BPW making a 1:10 dilution, and incubated for 12–18 h at 37°C. Each dilution was plated in selective media (MacConkey agar), containing specific concentrations of different antibiotics (100 mg/L of amoxicillin, 2 mg/L of cefotaxime, 20 mg/L of chloramphenicol, 0.5 mg/L of colistin, 50 mg/L of nalidixic acid and 8 mg/L of oxytetracycline.), and incubated for 18–20 h at 37°C	DD, BMD	EUCAST, CLSI	EUCAST (DD) CLSI (BMD)	Not specified	Not specified	Not specified (it only mentions that 8 *E. coli* were beta-lactamase and/or PMQR-positive strains)
Guedes et al. (2023)	Pacific oysters (*Magallana gigas*)	Oysters	4 samples each consisting of 4–5 oysters	Portugal	2021–2022	ISO 16649-2: 2001 (enumeration) and selective plates (i.e. plates with either cefotaxime (1 µg mL – 1), ciprofloxacin (0.125 µg mL – 1), or tetracycline (8 µg mL – 1), and incubated at 30°C for 24 h)	Not applicable as no *E. coli* grew on selective plates	Not applicable	Not applicable	*E. coli* counts < 10 CFU/g in all samples	Not applicable	Not applicable
Environment Agency (2023)	Blue mussels (Mytilus edulis) Pacific oysters (Crassostrea gigas) Other shellfish not specified	Flesh and intravalvular fluid	38	England	2022–2023	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 standardised reference method for enumeration of *E. coli* in bivalves, with Minerals Modified Glutamate Broth as growth media combination with verification on Tryptone Bile with X-glucuronide agar was used. The presence of *E. coli* in all tubes showing acid production by subculture onto TBGA/TBX plates within 4 h using a 10 µL sterile loop	BMD	EUCAST	ECOFF and clinical breakpoints	Not reported	52	Full susceptibility, 92 Amikacin, 2 Ampicillin, 6 Azithromycin, 0 Cefotaxime, 4 Ceftazidime, 4 Chloramphenicol, 2 Ciprofloxacin, 4 Colistin, 4 Gentamicin, 2 Meropenem, 2 Nalidixic acid, 4 Sulfamethoxazole, 8 Tetracycline, 6 Tigecycline, 0 Trimethoprim, 4

(Continues)

TABLE C.1 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	**Seafood species**	**Sample type**	**Total number of samples**	**Country**	**Sample collection year**	**Isolation**	**AST methods (BMD, DD, Etest)**	**Criteria used for AST interpretation (EUCAST/CLSI; ECOFF/CB; year)**	**Cut-off**	**Number (%) of samples positive for *E. coli***	**Number of isolates**	**Occurrence of resistance (%)**
Zacharias et al. (2021)	Mussels (*Corbicula* spp. and *Dreissena* spp.)	Mussels	22	Germany	Not reported	*E. coli*: CC agar supplemented with a selective supplement (Merck; 2.5 mg vancomycin and 2.5 mg cefsulodine per 500 mL media) to inhibit the growth of Pseudomonas spp., Aeromonas spp. and Gram-positive bacteria; incubation was performed at 37°C for 24 h AMR *E. coli*: CHROMagar ESBL, incubated for 24 h at 42°C	BMD	EUCAST	EUCAST, Version 9.0, 2019	23% with *E. coli* > LOD	1	4.5% of samples showed growth of presumptive ESBL-producing *E. coli*; Presumptive ESBL *E. coli* was confirmed to have resistance to cefotaxime and ceftazidime and also showed ciprofloxacin resistance. Other antimicrobials tested were meropenem, imipenem, piperacillin-tazobactam, for which the isolate was susceptible
Sousa et al. (2011)	Gilthead Seabream (Sparus aurata)	Faecal sample	118 (one sample per animal)	Portugal	2007	Levine agar supplemented with cefotaxime (2 mg/L)	DD	CLSI	CLSI 2011	Not applicable	Not applicable	4.2% of samples showed growth of presumptive ESBL-producing *E. coli* then confirmed by PCR
Antunes et al. (2018)	Rainbow trout	Muscle and viscera	31	Portugal	2010–2012	25 gr in 225 mL BPW, incubation at 37°C for 16–18 h; then 0.1 mL on MacConkey agar without antibiotics and supplemented with ciprofloxacin (0.125 Mg L – 1), ceftazidime (1 Mg L – 1) and cefotaxime (1 Mg L – 1) and incubated at 37°C for 24 h	DD	EUCAST and, if not available, CLSI	ECOFF	0	Not applicable	Not applicable
Boss et al. (2016)	Salmon (Not declared) Pangasius (*Pangasius hypophthalmus; Pangasius krempf*) Shrimp (*Penaeus monodon*; *Litopenaeus vannamei*; not declared) Oysters (Not declared)	Food	Salmon, 11 Pangasius, 12 Shrimp, 11 Oysters, 10	Denmark, Norway, Scotland (Salmon) Vietnam (Pangasius) Bangladesh, Ecuador, Indonesia, Thailand, Vietnam (Shrimp) France, Scotland (Oysters)	2014	TBX followed by subculture on BA	BMD	EUCAST	ECOFF	Salmon, 3 (27) Pangasius, 10 (83) Shrimp, 7 (64) Oysters, 4 (40)	60	Ampicillin, 8 Chloramphenicol, 5 Ciprofloxacin, 22 Nalidix acid, 12 Sulfamethoxazole, 13 Tetracycline, 17 Trimethoprim, 8 All other AM in EUSVEC, 0

TABLE C.1 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	**Seafood species**	**Sample type**	**Total number of samples**	**Country**	**Sample collection year**	**Isolation**	**AST methods (BMD, DD, Etest)**	**Criteria used for AST interpretation (EUCAST/CLSI; ECOFF/CB; year)**	**Cut-off**	**Number (%) of samples positive for *E. coli***	**Number of isolates**	**Occurrence of resistance (%)**
Silva et al. (2019)	Tuna (Thunnus thynnus) Snapper (Pagrus pagrus) Bramble shark (Echinorhinus brucus) Sea bass (Dicentrarchus labrax) Salmon (Salmo salar)	Food	Tuna, 30 Snapper, 30 Bramble shark, 30 Sea bass, 30 Salmon, 30	Portugal	2016–2017	25 g diluted with 200 mL of sterile BPW, homogenised in a stomacher, and 0.1 mL of this homogenate was spread onto Levine agar plates with and without cefotaxime (2 µg/mL) and incubated at 37°C for 24 h	DD	CLSI	Clinical breakpoints	Tuna, 12 (43) Snapper, 4 (13) Bramble shark, 5 (17) Sea bass, 9 (33) Salmon, 15 (53)	45	Ampicillin, 18 Chloramphenicol, 4 Streptomycin, 20 Sulfamethoxazole-trimethoprim, 22 Tetracycline, 35 ESBL, 2 No results are reported for some antimicrobials tested
Gross et al. (2022)	Atlantic herrings (Clupea harengus) Plaices (Pleuronectes platessa) Atlantic cods (Gadus morhua) Common dabs (Limanda limanda) European flounders (Platichthys flesus) Atlantic mackerels (Scomber scombrus) European smelts (Osmerus eperlanus) Solenette (Buglossidium luteum) European sea sturgeon (Acipenser sturio) Grey gurnard (Eutrigla gurnardus) European pilchard (Sardina pilchardus) Whiting (Merlangius merlangus)	Rectal or intestinal swabs	Atlantic herrings (Clupea harengus), 3 Plaices (Pleuronectes platessa), 7 Atlantic cods (Gadus morhua), 3 Common dabs (Limanda limanda), 9 European flounders (Platichthys flesus), 3 Atlantic mackerels (Scomber scombrus), 6 European smelts (Osmerus eperlanus), 4 Solenette (Buglossidium luteum), 1 European sea sturgeon (Acipenser sturio), 1 Grey gurnard (Eutrigla gurnardus), 1 European pilchard (Sardina pilchardus), 1 Whiting (Merlangius merlangus), 1	N/A	2017–2019	Selective plating on Chromocult, Gassner and MacConkey agar	Not applicable	Not applicable	Not applicable	0	0	0

(Continues)

TABLE C.1 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	Seafood species	Sample type	Total number of samples	Country	Sample collection year	Isolation	AST methods (BMD, DD, Etest)	Criteria used for AST interpretation (EUCAST/CLSI; ECOFF/CB; year)	Cut-off	Number (%) of samples positive for *E. coli*	Number of isolates	Occurrence of resistance (%)
Salgueiro et al. (2020)	Trout (*Sparus aurata*)	Gills, intestine, muscle and skin	Muscle, 5 Gills, 1 Intestine, 1 Skin,1	Portugal	2018	10 g homogenised in BPW, incubated for 12–18 h at 37°C and further diluted. Each dilution was plated in selective media (MacConkey agar for Enterobacteriaceae) and incubated for 18–20 h at 37°C	DD, BMD	EUCAST	Not reported	Not described but it seems that *E. coli* was isolated only from gills	Not described (most results are reported at Enterobacteriaceae level and they are unclear)	Not described but only 3 *E. coli* isolates are reported in the table with phenotypic profiles, and shows resistance to amoxicillin-calvulanic acid, florfenicol and chloramphenicol
Helsens et al. (2020)	Rainbow Trout Fillets (*Oncorhynchus mykiss*)	Fillet	56	France	2019	DNA extraction	Not applicable	Not applicable	Not applicable	All laboratory-processed fillets and many but not all factory-processed fillets had abundance of genus Escherichia	Not applicable	Not applicable (no association of AMR genes with genera)
Anagnostopoulos et al. (2022)	Carp (*Cyprinus carpio*)	Muscle and gut	Exact number not reported. It is reported 'at least nine individuals per area', for a total of three areas	Greece	2020	25 g of fish tissue or intestines was transferred aseptically to stomacher bags with 90 mL MRD (Maximum Recovery Diluent, 0.1% w/v peptone, 0.85% w/v NaCl) and homogenised for 2 min using a stomacher; then volumes (0.1 mL) of 10-fold serial dilutions were spread on the surface of dried media in Petri dishes for enumeration of *E. coli*/coliforms on *E. coli*/coliform chromogenic medium (HAL008) and incubated at 37°C for 24 h	DD (though not done on *E. coli*)	EUCAST	Clinical Breakpoint Tables v. 12.0	Not reported. Though mean and standard deviations of *E. coli* (in log CFU/g) were: For muscle: 2 ± 0 in all three areas For gut:, 5.35 ± 0.76	Not applicable	Not applicable
Ferri et al. (2023)	Cod (*Gadus macrocephalus; G. morhua*)	Fillet (three types based on processing: HPP, High-Pressure Procedure; SD, Salted and Seasoned; SP, Soaked)	450	Italy	Not reported	ISO 16654-1:2017 (Enterobacteriaceae)	VITEK® 2	CLSI	Clinical breakpoints	0 (0), G. macrocephalus and G. morhua HPP fillets; 2 (3), G. macrocephalus SP; 3 (4), G. macrocephalus SD; 5 (7), G. morhua SP; 8 (11), G. morhua SD	18	Amikacin, 0 Cefotaxime, 33 Ertapenem, 11 Gentamicin, 0 Meropenem, 0 Nitrofurantoin, 11 Sulfamethoxazole, 0 Note: the paper does not report results for all antimicrobials included in the panel

TABLE C.2 Studies investigating the occurrence of AMR in *Enterococcus* sp. in shellfish and finfish produced in Europe.

	Epidemiological information					Laboratory information				Microbiological information		
Reference	**Seafood species**	**Sample type**	**Total number of samples**	**Country**	**Sample collection year**	**Isolation**	**AST methods (BMD, DD, Etest)**	**Criteria used for AST interpretation (EUCAST/ CLSI; ECOFF/CB; year)**	**Cut-off**	**Number (%) of samples positive for *Enterococcus* sp.**	**Number of isolates**	**Occurrence of resistance (%)**
Wilson and McAfee (2002)	Cockels Mussels Oysters (Latin names not mentioned)	Flesh and intravalvular fluid	125	Ireland	1998	Method 1: 25 g was homogenised in a sterile food blender for 20 s, and then added to 225 mL BPW and incubated 37 ± 1°C for 18–24 h. One 10 µL loop of overnight culture was plated onto Slanetz and Bartley agar and Lewisham agar containing two levels of vancomycin (4 and 6 mg/L), and these were incubated at 37 ± 1°C for 48 h Method 2: 100 g of flesh and liquor collected into a stomacher bag. The bag was stomached for 1 min. 50 g of emulsion was weighed into a second stomacher bag and stomached for 2 min. The contents of the bag were poured into a honey jar containing 100 mL BPW and incubated at 37 ± 1°C for 24 h. After incubation, 10 µL loops were used to streak onto Slanetz and Bartley agar. Plates were incubated at 42°C for 24 h	Etest	Manufacturer's recommendations	Manufacturer's recommendations	98 (78) for enterococci reported at genus level (no species level information)	*E. faecalis*, 7 *E. faecium*, 5	Van-R *E. faecalis*, 28 Van-R *E. faecium*, 60 Note that this study employed selective isolation on vancomycin-containing plates
Guardabassi and Dalsgaard (2004)	Blue mussels	Not reported	Not reported	Denmark	2001–2002	Presumptive VRE were isolated by direct plating on Slanetz-Bartley agar supplemented with 20 µg of vancomycin/mL 10 g composite samples of marine sediment and blue mussels were mixed with 90 mL of physiological saline in a stomacher before inoculation onto Slanetz–Bartley agar (Method 1) and enrichment in azide dextrose broth supplemented with 20 µg of vancomycin/mL followed by plating on Slanetz–Bartley agar supplemented with 20 µg of vancomycin/ml. After 2–3 days of incubation at 37°C (Method 2)	DD	NA	NA	NA	Presumptive VRE, 4	Van-R *E. faecium*, 25 Note that this study employed selective isolation on vancomycin-containing plates

(Continues)

TABLE C.2 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	**Seafood species**	**Sample type**	**Total number of samples**	**Country**	**Sample collection year**	**Isolation**	**AST methods (BMD, DD, Etest)**	**Criteria used for AST interpretation (EUCAST/ CLSI; ECOFF/CB; year)**	**Cut-off**	**Number (%) of samples positive for *Enterococcus* sp.**	**Number of isolates**	**Occurrence of resistance (%)**
Heim et al. (2023)	Blue mussels (Mytilus edulis) Flat oysters (Ostrea edulis) Great scallops (Pecten maximus) Northern horse mussels (Modiolus modiolus) Ocean quahogs (Arctica islandica) Pullet carpet shells (Venerupis corrugata) Pacific oysters (Magallana gigas) Cockles (fam. Cardiidae)	Flesh and intravalvular fluid	Blue mussels (Mytilus edulis), 389 Flat oysters (Ostrea edulis), 44 Great scallops (Pecten maximus), 27 Northern horse mussels (Modiolus modiolus), 4 Ocean quahogs (Arctica islandica), 3 Pullet carpet shells (Venerupis corrugata), 2 Pacific oysters (Magallana gigas), 1 Cockles (fam. Cardiidae), 1	Norway	2016, 2019, 2020	Qualitative and quantitative analyses – thorough description of methods is reported	BMD	EUCAST	ECOFF	286 (61)	E. faecium, 247 E. faecalis, 66	*E. faecium*, 71% of isolates showed resistance to at least one antimicrobial (detailed AST results in the suppl. material)
Valenzuela et al. (2010)	Various kinds of finfish, raw fish fillets and frozen fillets, molluscs	Muscle	Not reported	Spain	Not reported	Samples obtained as above were placed in Stomacher bags and homogenised by gentle mixing by hand with sterile saline solution (~ 2 volumes by sample weight), settled for 10 min on ice, and spread on Slanetz & Bartley agar (Scharlab, Barcelona). After 48 h incubation at 37°C, typical dark red or maroon colonies were isolated and repurified by further spread on Slanetz & Bartley agar as above	Etest	NCCLS	NCCLS	Not reported. However, the following samples gave negative results on presumptive isolation or confirmation tests for enterococci: surmullet, trout, gilthead, codling, hake, seabass, sardine, pagel, prawns, squid, mussels, coquina clams, cockles, razor clams, oysters, frozen swordfish fillet, perch fillet, halibut fillet, salmon fillet, catfish fillet, smoked salmon, and smoked trout. Most *E. faecium* were isolated from fish fillets, and some were also found in clams and in fish intestine	24	Nitrofurantoin, 50 Erythromycin, 33 Rifampicin, 33 Quinupristin/dalfopristin, 12 None of the isolates tested demonstrated resistance to ampicillin, penicillin, tetracycline, chloramphenicol, vancomycin, teicoplanin, gentamicin or streptomycin

TABLE C.2 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	**Seafood species**	**Sample type**	**Total number of samples**	**Country**	**Sample collection year**	**Isolation**	**AST methods (BMD, DD, Etest)**	**Criteria used for AST interpretation (EUCAST/ CLSI; ECOFF/CB; year)**	**Cut-off**	**Number (%) of samples positive for *Enterococcus* sp.**	**Number of isolates**	**Occurrence of resistance (%)**
Barros et al. (2011)	Gilthead seabream (*Sparus aurata*)	Faecal samples	118	Portugal	2007	Peptone solution (0%–2%) was added in a proportion of 1:9 and the mixtures were homogenised using a Stomacher. Samples were seeded in Slanetz–Bartley agar plates and incubated for 48 h at 35°C	DD	CLSI	CLSI	73 (62)	*E. faecium*, 67 *E. faecalis*, 6	*E. faecalis* Erythromycin, 3 Tetracycline, 3 Quinupristin–dalfopristin, 6 Ampicillin, 0 Ciprofloxacin, 0 Streptomycin, 0 Kanamycin, 0 Gentamicin, 0 Chloramphenicol, 0 Vancomycin, 0 Teicoplanin, 0 E. faecium Erythromycin, 40 Tetracycline, 10 Quinupristin–dalfopristin, 4 Ampicillin, 3 Ciprofloxacin, 3 Streptomycin, 1 Kanamycin, 2 Gentamicin, 1) Chloramphenicol, 1 Vancomycin, 0 Teicoplanin, 0
Lauková et al. (2019)	Trout (*Salmo trutta*) Trout (*Salmo gairdneri*)	Intestinal content	50	Slovakia	2007, 2010, 2015	Standard microbial dilution method (ISO): intestinal content stirred (1: 9) in Ringer solution (pH 7.0), plated onto cultivation medium M-Enterococcus agar to count colonies of enterococci. Plates were cultivated at 37°C for 48 h	DD	CLSI	CLSI	Not reported	*E. faecium*, 3	Not reported specifically for *E. faecium* but merged for 7 isolates of 4 Enterococcus species
Novais et al. (2018)	Rainbow trout (*Oncorhynchus mykiss*)	Muscle and viscera	Trout from farm, 3 Trout from retail, 25	Portugal	Not reported	Samples were enriched (37°C for 16 h) in buffered peptone water (1:10 for solid samples) and 0.1 mL aliquot was plated onto Slanetz–Bartley agar plates without antimicrobial agents and supplemented with 8 mg/L of tetracycline, 1000 mg/L of streptomycin, 125 mg/L of gentamicin, 4 mg/L of vancomycin, 16 mg/L of ampicillin, 8 mg/L of chloramphenicol or 4 mg/L of ciprofloxacin. Plates were incubated at 37°C for 48 h	DD	CLSI	CLSI	*E. faecalis*, 11% *E. faecium*, 55%	Reported only for different sample types (water, feed, trout) merged	Reported only for different sample types (water, feed, trout) merged

(Continues)

TABLE C.2 (Continued)

	Epidemiological information					Laboratory information				Microbiological information		
Reference	**Seafood species**	**Sample type**	**Total number of samples**	**Country**	**Sample collection year**	**Isolation**	**AST methods (BMD, DD, Etest)**	**Criteria used for AST interpretation (EUCAST/ CLSI; ECOFF/CB; year)**	**Cut-off**	**Number (%) of samples positive for *Enterococcus* sp.**	**Number of isolates**	**Occurrence of resistance (%)**
Ferri et al. (2023)	Cod (*Gadus macrocephalus; G. morhua*)	Fillet	450	Italy	Not reported	ISO 7898-2:2000	VITEK® 2	CLSI	Clinical breakpoints	Not reported	44 *E. faecalis*; 7 *E. faecium* (17 *E. durans*)	*E. faecalis*: Clindamycin, 52 Cefotaxime, 0 Linezolid, 61 Oxacillin, 0 Tetracycline, 79 Vancomycin, 39 *E. faecium*: Clindamycin, 14 Cefotaxime, 0 Linezolid, 28 Oxacillin, 0 Tetracycline, 57 Vancomycin, 14 Note: the paper does not report results for all antimicrobials included in the panel

APPENDIX D

The required sample sizes (number of isolates) at the EU level

Table C.2 below provides the required sample sizes, i.e. the number of isolates to be tested for susceptibility at the EU level, for a grid of values for p_r, for confidence levels 0.80 and 0.95, and for three values for accuracy[28] a. The required numbers of isolates after adjustment, i.e. further inflated by 5% to consider a 5% occurrence of possible missing data and by 2% to account for the possible loss of strains during storage, are also shown in Table C.2 below.
For example, suppose a confidence level of 0.95 is chosen and suppose several literature studies across different MSs provide prevalence-estimates in the range of (0.05, 0.26). Using the value 0.3 (closest to the upper limit of the confidence interval, leading to a 'conservative' choice) and assuming this holds for the whole EU, we get the required numbers 25, 86 and 328 for accuracy 0.2, 0.1 and 0.05, respectively. For a confidence level of 0.95 and $\boldsymbol{p_r}=0.3$, the required adjusted numbers equal 27, 92 and 351 for accuracy 0.2, 0.1 and 0.05, respectively (Table D.1).

TABLE D.1 The required number of isolates $\boldsymbol{n_{iso}}$, corresponding to different choices of accuracy $\boldsymbol{a}$, confidence, and for different values of the unknown occurrence of AMR $\boldsymbol{p_r}$. Left number: Before loss adjustment, right number, with loss adjustment (i.e. +5% for missing data and +2% for loss of strain during storage).

Accuracy	$\boldsymbol{a}=0.2$		$\boldsymbol{a}=0.1$		$\boldsymbol{a}=0.05$	
Confidence	**80%**	**95%**	**80%**	**95%**	**80%**	**95%**
$\boldsymbol{p_r}=0.1$	8, 8	17, 19	19, 21	45, 48	64, 69	149, 160
$\boldsymbol{p_r}=0.2$	10, 10	22, 23	29, 31	68, 73	108, 116	252, 270
$\boldsymbol{p_r}=0.3$	11, 12	25, 27	37, 40	86, 92	140, 150	328, 351
$\boldsymbol{p_r}=0.4$	12, 13	28, 29	42, 45	97, 104	160, 171	373, 400
$\boldsymbol{p_r}=0.5$	12, 13	28, 30	43, 46	100, 107	166, 178	388, 416
$\boldsymbol{p_r}=0.6$	12, 13	28, 29	42, 45	97, 104	160, 171	373, 400
$\boldsymbol{p_r}=0.7$	11, 12	25, 27	37, 40	86, 92	140, 150	328, 351
$\boldsymbol{p_r}=0.8$	10, 10	22, 23	29, 31	68, 73	108, 116	252, 270
$\boldsymbol{p_r}=0.9$	8, 8	17, 19	19, 21	45, 48	64, 69	149, 160

[28]Interval estimation with accuracy a corresponds to a confidence interval of width $2a$.

APPENDIX E

Sample batch sensitivity

TABLE E.1 Sample batch sensitivity *BSe* as a function of the intra-batch correlation parameter ρ,the size of the sample batch *m*, the within PB prevalence π_{wbp}.

		π_{wbp}								
ρ	*m*	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
0	25	0.93	1	1	1	1	1	1	1	1
	20	0.88	0.99	1	1	1	1	1	1	1
	15	0.79	0.96	1	1	1	1	1	1	1
	10	0.65	0.89	0.97	0.99	1	1	1	1	1
	5	0.41	0.67	0.83	0.92	0.97	0.99	1	1	1
0.1	25	0.72	0.93	0.98	1	1	1	1	1	1
	20	0.67	0.9	0.97	0.99	1	1	1	1	1
	15	0.61	0.86	0.95	0.99	1	1	1	1	1
	10	0.52	0.78	0.91	0.96	0.99	1	1	1	1
	5	0.35	0.6	0.76	0.87	0.93	0.97	0.99	1	1
0.2	25	0.58	0.83	0.93	0.98	0.99	1	1	1	1
	20	0.54	0.8	0.92	0.97	0.99	1	1	1	1
	15	0.5	0.76	0.89	0.95	0.98	0.99	1	1	1
	10	0.43	0.68	0.83	0.92	0.96	0.98	0.99	1	1
	5	0.31	0.54	0.7	0.82	0.89	0.94	0.97	0.99	1
0.3	25	0.47	0.73	0.87	0.94	0.97	0.99	1	1	1
	20	0.45	0.7	0.85	0.92	0.96	0.98	0.99	1	1
	15	0.41	0.66	0.81	0.9	0.95	0.98	0.99	1	1
	10	0.36	0.6	0.76	0.86	0.92	0.96	0.98	0.99	1
	5	0.27	0.48	0.64	0.76	0.85	0.91	0.95	0.98	0.99
0.4	25	0.39	0.64	0.79	0.88	0.94	0.97	0.98	0.99	1
	20	0.37	0.61	0.77	0.87	0.93	0.96	0.98	0.99	1
	15	0.34	0.58	0.74	0.84	0.91	0.95	0.97	0.99	1
	10	0.31	0.53	0.69	0.8	0.88	0.93	0.96	0.98	0.99
	5	0.24	0.43	0.59	0.71	0.8	0.87	0.92	0.96	0.99
0.5	25	0.32	0.55	0.71	0.82	0.89	0.93	0.97	0.98	0.99
	20	0.31	0.53	0.69	0.8	0.87	0.93	0.96	0.98	0.99
	15	0.29	0.5	0.66	0.77	0.86	0.91	0.95	0.98	0.99
	10	0.26	0.46	0.62	0.74	0.82	0.89	0.93	0.97	0.99
	5	0.21	0.39	0.53	0.66	0.75	0.83	0.89	0.94	0.98
0.6	25	0.27	0.47	0.63	0.74	0.83	0.89	0.93	0.97	0.99
	20	0.26	0.45	0.61	0.73	0.81	0.88	0.93	0.96	0.98
	15	0.24	0.43	0.59	0.7	0.8	0.86	0.92	0.95	0.98
	10	0.22	0.4	0.55	0.67	0.77	0.84	0.9	0.94	0.98
	5	0.18	0.35	0.48	0.6	0.7	0.79	0.86	0.92	0.96
0.7	25	0.22	0.4	0.54	0.66	0.76	0.83	0.89	0.94	0.97
	20	0.21	0.38	0.53	0.65	0.74	0.82	0.88	0.93	0.97
	15	0.2	0.37	0.51	0.63	0.73	0.81	0.87	0.93	0.97
	10	0.19	0.35	0.49	0.6	0.7	0.79	0.86	0.92	0.96
	5	0.16	0.31	0.44	0.55	0.65	0.74	0.82	0.89	0.95
0.8	25	0.17	0.33	0.46	0.58	0.68	0.76	0.84	0.9	0.96
	20	0.17	0.32	0.45	0.57	0.67	0.76	0.83	0.9	0.95
	15	0.16	0.31	0.44	0.55	0.66	0.75	0.82	0.89	0.95
	10	0.15	0.3	0.42	0.54	0.64	0.73	0.81	0.88	0.94
	5	0.14	0.27	0.39	0.5	0.6	0.7	0.78	0.86	0.93

TABLE E.1 (Continued)

		π_{wbp}								
ρ	m	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
0.9	25	0.14	0.26	0.38	0.49	0.59	0.69	0.77	0.86	0.93
	20	0.13	0.26	0.38	0.48	0.59	0.68	0.77	0.85	0.93
	15	0.13	0.25	0.37	0.48	0.58	0.68	0.77	0.85	0.93
	10	0.13	0.25	0.36	0.47	0.57	0.67	0.76	0.84	0.92
	5	0.12	0.23	0.34	0.45	0.55	0.65	0.74	0.83	0.92
1	25	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
	20	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
	15	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
	10	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
	5	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9

APPENDIX F

Estimation of prevalence π_{bp}, within PB prevalence π_{wbp}, intra-batch correlation parameter ρ and batch sensitivity *BSe*

F.1 | ESTIMATION OF PREVALENCE π_{bp}, WITHIN PB PREVALENCE π_{wbp}, INTRA-BATCH CORRELATION PARAMETER ρ

The data concern batch samples of gills of freshwater trout (Salmonidae) from France, tested for *Aeromonas* spp. More precisely, 20 batches of size 10 were tested for *Aeromonas* spp. and resulted in the following total number of positive fish:

4, 4, 5, 4, 5, 1, 4, 8, 1, 2, 8, 8, 9, 8, 8, 3, 2, 5, 4, 1.

As all batches have at least one fish tested positive, the observed prevalence π_{bp} equals 1. A Wilson 95% confidence interval for the proportion 20/20 is given by (0.84, 1). We suggest using the midpoint 0.92 as estimate for the prevalence π_{bp} (instead of the boundary value 1).

The beta-binomial model was fitted to the 20 proportions, leading to the following estimates (rho refers to the intra-batch correlation and pi to the within PB prevalence).

Additional estimates

Label	Estimate	Standard error	DF	*t* value	Pr > |*t*|	Alpha	Lower	Upper
rho	0.1753	0.06608	20	2.65	0.0153	0.05	0.03748	0.3132
pi	0.4712	0.05637	20	8.36	< 0.0001	0.05	0.3536	0.5888

The PB prevalence π_{wbp} is estimated as 0.47 with 95% CI (0.35, 0.59) and the intra-batch correlation parameter ρ as 0.18 with 95% CI (0.04, 0.31).

The results were generated using SAS software (code in *Proc nlmixed*). Copyright © 2020 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

F.2 | ESTIMATION OF BATCH SENSITIVITY *BSE*

As an example, consider a batch of size 5. We assume test sensitivity and specificity to be very high (and taken as 100%). Based on the beta-binomial distribution for clustered binary data, the batch sensitivity *BSe* equals (with $y_i = 0$ denoting that the I -th fish is tested negative).

$$BSe = 1 - P(y_1 = 0, y_2 = 0, y_3 = 0, y_4 = 0, y_5 = 0).$$

$$= 1 - \left(1 - \pi)(1 - \pi + \pi\rho)\left(1 - \pi + \frac{2\pi\rho}{1+\rho}\right)\left(1 - \pi + \frac{3\pi\rho}{1+2\rho}\right)(1 - \pi + \frac{4\pi\rho}{1+3\rho}\right),$$

with π denoting the within PB prevalence and ρ the intra-batch correlation. A similar extended formula holds for a batch of size 15.

Taking $\pi = 0.5$ and $\rho = 0.2$ the batch sensitivity for a batch of size 15 equals $BSe = 0.98$, as shown in Table D.1. For $\pi = 0.47$ and $\rho = 0.18$, the batch sensitivity for a batch of size 5 equals $BSe = 0.88$, as used in Appendix G.

APPENDIX G

Illustration for combinations: Freshwater trout and Atlantic salmon with *Aeromonas* spp.

G.1 | PRINCIPLES

An overview of general principles and choices for unknown parameters, applied to both cases of trout and salmon is presented below.

- **Stratified sampling** with EU/EFTA Member States/regions as strata
- **Proportional allocation** using relative production volumes of strata
- **Production batches** are the epidemiological units to be sampled
- For the estimation of the **occurrence of resistance at the EU level**, the **required number of isolates** is based on the following parameters:
    - Accuracy 0.05 (leading to a confidence interval of length at most 0.1)
    - Confidence level 0.95
    - Taking the unknown occurrence of AMR as 0.5[29]
    - Extra 5% for missing observations
    - Extra 2% for losses[30]
    - Quarterly[31] measurements on the same production unit
    - Correlation 0.2[32] of observations from the same production unit.
- The conversion to the **required number of production batches** (PBs) is based on:
    - Batch of size ≥ 5[33]
    - Batch prevalence 0.92, within-batch prevalence 0.47, intra-batch correlation 0.18 (see Appendix F)
    - Test sensitivity and test specificity were assumed to be very high (and taken as 1), resulting in a batch sensitivity $BSe = 0.88$.
    - These values were applied **to all strata**. If different estimates are available for different strata, they could be used for each stratum separately.
    - As the required number of production batches (PBs), or the required number of production units (PUs) (being the number of PBs divided by 4, given 4 quarterly measurements from the same PU) can be extremely small (say 1 PU) or extremely large for some strata, depending on the relative production volumes, a truncation with a **minimum** and **maximum number of PUs** for any stratum (MS or region) was applied, based on:
        - For the **minimum**: Estimating the AMR occurrence with an accuracy of only 0.2 and a confidence level of only 0.80, and zero intra-PU and intra-batch correlation (leading to the smallest sample sizes). This leads to a **minimum** of **4 PUs** or **16 PBs per MS**.
        - For the **maximum**: Estimating the AMR occurrence with an accuracy of 0.1 and a confidence level of 0.95, and intra-PU and intra-batch correlation as applied during the sample size calculations. This leads to a **maximum** of **53 PUs** or **212 PBs per MS**.

G.2 | FRESHWATER TROUT: MS AND REGIONS WITHIN FRANCE AS STRATA

Using the total production volumes (last column in the table below) and including Iceland, Norway and Switzerland (source: STECF-22-17 report).

Country	Rainbow trout	Brook trout	Trout unspecified	Trout
Austria	1539.18	588.51	0	2127.42
Belgium	0	0	0	0
Bulgaria	4114.32	0	0	4114.32
Croatia	0	0	0	0
Cyprus	0	0	0	0
Czechia	602.49	0	0	602.49

[29]Taking an occurrence of AMR of 0.6 is the worst case leading to the widest interval.
[30]Such as loss of strains during storage.
[31]Approximately every 3 months.
[32]We assume a small but still impactful value of 0.2, based on the example presented in the Appendix F.
[33]Because 5 fish are sampled and tested per production batches.

(Continued)

Country	Rainbow trout	Brook trout	Trout unspecified	Trout
Denmark	46,525.14	0	0	46,525.14
Estonia	772.8	0	0	772.8
Finland	1128.28	0	0	1128.28
France	35,732.25	0	0	35,732.25
Germany	6739.5	0	0	6739.5
Greece	8250.3	0	0	8250.3
Hungary	0	0	0	0
Ireland	754.7	0	0	754.7
Italy	0	0	33,448.52	33,448.52
Latvia	74.88	0	0	74.88
Lithuania	134.31	0	0	134.31
Malta	0	0	0	0
Netherlands	0	0	0	0
Poland	18,126	0	954	19,080
Portugal	0	0	0	0
Romania	0	0	6588.34	6588.34
Slovakia	780.64	0	0	780.64
Slovenia	0	0	0	0
Spain	16,593.72	0	0	16,593.72
Sweden	9105.04	0	0	9105.04
Iceland	295	0	0	295
Norway	96,633	0	0	96,633
Switzerland	1200	0	0	1200

Stratum 'France' was split further in regional strata according to the production volume column in the table below (Agreste).

Table Aquaculture production for consumption of freshwater salmonids in France in 2020, by region (of the seat of the undertaking), hatchery/nursery excepted (source: Agreste).

Region	Salmonid-producing undertakings (no. entreprises)	Production volume (in tonnes)
Auvergne-Rhone-Alpes	41	4286
Bourgogne-Franche-Comté	20	594
Bretagne	26	8304
Centre-Val de Loire + Pays de la Loire	17	89
Grand Est	29	1033
Hauts-de-France	24	8255
Île de France	4	125
Normandie	24	1525
Nouvelle-Aquitaine	64	10,121
Occitanie	47	2895
Provence-Alpes-Côte d'Azur + Corse	11	269

To exclude very small regions in France. Only the largest regions accumulating at least 60% of the total production of France were included as strata.

Table G.1 below shows in the different columns:

(i) The EU/EFTA Member States and regions for France (the strata);
(ii) The relative production percentages;
(iii) The required number of isolates: when unadjusted: 388, when adjusted for missingness and losses: 416, when additionally adjusted for multiple isolates from the same PU (production unit): 665, with rounding to integers (across the strata): 677;
(iv) The required number of production units: the number of batches as multiples of 4 divided by 4;
(v) The required number of PBs (production batches): adjusting the number of isolates by dividing by the batch prevalence 0.92 and the batch sensitivity 0.88 (factor 1.23); next turned into multiples of 4. For example, for Italy, the number of PBs is $74/(0.92 \times 0.88) = 92$ (rounded up) and the number of production units is $92/4 = 23$;

(vi) The required number of PUs (production units) with minimum 4 and maximum 53;
(vii) The required number of PBs (production batches) with minimum 16 and maximum 212;
(viii) The totals in the last row.

TABLE G.1 Sample size calculations for freshwater trout.

Country	Country relative production	No. isolates	No. production units	No. production batches	Truncated No. production units	Truncated No. production batches
Austria	0.007	5	2	8	**4**	**16**
Bulgaria	0.014	10	4	16	**4**	**16**
Czechia	0.002	2	1	4	**4**	**16**
Denmark	0.154	103	32	128	**32**	**128**
Estonia	0.003	2	1	4	**4**	**16**
Finland	0.04	27	9	36	**9**	**36**
FR Bretagne	*0.037*	*25*	*8*	*32*	***8***	***32***
FR Hauts-de-France	*0.037*	*25*	*8*	*32*	***8***	***32***
FR Nouvelle-Aquitaine	*0.045*	*30*	*10*	*40*	***10***	***40***
Germany	0.022	15	5	20	**5**	**20**
Greece	0.027	19	6	24	**6**	**24**
Iceland	0.001	1	1	4	**4**	**16**
Ireland	0.003	2	1	4	**4**	**16**
Italy	0.111	74	23	92	**23**	**92**
Latvia	0	1	1	4	**4**	**16**
Lithuania	0	1	1	4	**4**	**16**
Norway	0.321	214	66	264	**53**	**212**
Poland	0.063	43	14	56	**14**	**56**
Romania	0.022	15	5	20	**5**	**20**
Slovakia	0.003	2	1	4	**4**	**16**
Spain	0.055	37	12	48	**12**	**48**
Sweden	0.03	21	7	28	**7**	**28**
Switzerland	0.004	3	1	4	**4**	**16**
Total	1	677	219	876	**232**	**928**

G.3 | ATLANTIC SALMON: MS AS STRATA

The design for Atlantic salmon is illustrated below, using the same parameter values as for freshwater trout, and taking into account very skewed production volumes, as 98% of the European production of Atlantic Salmon is produced in Norway. Note how the untruncated required number of 804 production batches for Norway is truncated to its maximum of 212 batches. Consequently, the required accuracy of 0.05 is not guaranteed at the EU level, but the required accuracy of 0.1 at the MS level is also guaranteed at the EU level.

Country	Atlantic salmon
Finland[a]	131.08
Ireland	13,207.25
Sweden	128.24
Iceland	13,448
Norway	1,377,185
Switzerland	240

[a] Sea trout.

Table G.2 shows the results. All sample size numbers were truncated due to the extremely skewed production volume distribution. Indeed, Norway exceeds, with a number of 201 required PUs (column 3), STECF-22-17 the maximum number of 53 PU by far and is truncated on the maximum of 53 in column 5. All other countries have very low required numbers of PUs, varying from 1 to 3 and are all truncated upwards to 4 (in column 5). So, the truncation has a huge effect in this very extreme case. The initial total of 840 production batches has been reduced to 292.

TABLE G.2 Sample size calculations for Atlantic salmon.

Country	Country relative production	No. isolates	No. production units	No. production batches	Truncated no. production units	Truncated no. production batches
Finland	0	1	1	4	**4**	**16**
Ireland	0.009	7	3	12	**4**	**16**
Sweden	0	1	1	4	**4**	**16**
Iceland	0.01	7	3	12	**4**	**16**
Norway	0.981	652	201	804	**53**	**212**
Switzerland	0	1	1	4	**4**	**16**
Total	1	669	210	840	**73**	**292**